Characterization of the attachment and infection by *Porcine reproductive and respiratory syndrome virus* 1 isolates in bone marrow-derived dendritic cells.

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is known to infect porcine dendritic cells (DC). Previous studies indicated that different PRRSV1 isolates regulated differently the cytokine profiles and phenotype of DC. However, the characterisation of the infection is lacking. The current study aimed to characterise the replication and attachment of different PRRSV1 isolates in bone marrow-derived DC (BMDC). For this purpose, immature (i) and mature (m) BMDC were infected with three PRRSV1 isolates. The replication kinetics showed that titres in iBMDC were significantly ($p<0.05$) higher than in mBMDC by 24 hpi, and for two isolates titres peaked earlier in iBMDC, suggesting that iBMDC were more efficient in supporting PRRSV1 replication than mBMDC. The attachment was revealed by a three-color confocal microscopy staining. All three isolates were seen attached to iBMDC even in cells lacking CD163 -the essential receptor for PRRSV- or porcine sialoadhesin (PoSn). The attachment was not fully avoided after removal of heparan sulphate by heparinase I. Furthermore, the infection was examined with regards to CD163 expression. By flow cytometry and confocal microscopy, positive signals of PRRSV1 nucleocapsid could be observed in $CD163^-$ iBMDC. Additional sorting experiment demonstrated that $CD163^-$ iBMDC were infected only when $CD163^{lo/hi}$ cells were present. This can be interpreted in different ways: susceptible $CD163^-$ cells arose as result of milieu created by $CD163^+$ infected BMDC; $CD163^-$ cells were infected by receptor independent mechanisms (i.e. exosomes) or, some cells expressed CD163 at levels beyond the technical sensitivity.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the costliest diseases of pigs and many efforts are being directed to its control. The causative agents are PRRS viruses (PRRSV1 and 2), enveloped, positive-strand RNA viruses belonging to the Genus *Porartevirus*[1], Family *Arteriviridae*, within the Order *Nidovirales* (Cavanagh, 1997). The PRRS virion consists of a nucleocapsid (protein N), with several membrane proteins embedded, including major envelope proteins GP5 and M, minor proteins GP2a, E, GP3 and GP4 and a recently discovered ORF5a protein (Snijder et al., 2013; Kappes and Faaberg, 2015).

In vivo, PRRSV has a narrow tropism for cells of the monocyte/macrophage lineage, preferentially highly differentiated macrophages located in lungs, lymphoid tissues and placenta (Duan et al., 1997a, 1997b). This restricted cell tropism is partially the result of receptors required for completing viral replication cycle. At present CD163 is thought to be essential for the infection of macrophages (Calvert et al., 2007; Whitworth et al., 2015; Burkard et al., 2017). Besides this, other receptors such as porcine sialoadhesin (PoSn) or heparan sulphate (HS) have been identified (Delputte et al., 2002; Vanderheijden et al., 2003).

The process of viral entry involves the interaction of PoSn with M/GP5 heterodimers that triggers endocytosis (Nauwynck et al., 1999; Vanderheijden et al., 2003; Delputte et al., 2007). In early endosomes, CD163 interacts with the GP2-GP3-GP4 trimers resulting in the release of genome and the initiation of replication (van Gorp et al., 2009; Das et al., 2010; Van Breedam et al., 2010). HS also plays a role in the attachment (Delputte et al., 2002, 2005)

In several *in vitro* experiments, PRRSV productively infected bone marrow-derived (BMDC) and monocyte-derived DCs (MoDC). The infection of those cells further compromised their effector capabilities by inducing apoptosis or by regulating the expression of CD11b/c, CD80/86 and SLA-I/II among other molecules involved in the

[1] Approved by the International Committee on Taxonomy of Viruses in August 2016 in Budapest (Hungary), classification available at https://talk.ictvonline.org/taxonomy/

immune response (Wang et al., 2007; Chang et al., 2008; Flores-Mendoza et al., 2008; Park et al., 2008; Peng et al., 2009; Gimeno et al., 2011).

Given the heterogeneity of DC (Summerfield et al., 2015) and the genetic diversity of PRRSV isolates, it was hypothesized that different PRRSV isolates and cells in different stages of maturity would show different patterns of infection. In this study, the binding and replication of three PRRSV1 isolates were evaluated and compared in immature (iBMDC) and mature (mBMDC) BMDCs.

2. Materials and methods

2.1. Isolation of porcine alveolar macrophages (PAM) and production of bone marrow-derived dendritic cells.

Porcine alveolar macrophages (PAM) were obtained from 4-week-old piglets from PRRSV-free high health status farms by bronchoalveolar lung lavage. PAM were tested and found free of PRRSV, *porcine circovirus type* 2 (PCV2), *Mycoplasma hyopneumoniae*, and *Torque teno sus virus* (TTSuV) 1 and 2 by PCR as described before (Mattsson et al., 1995; Quintana et al., 2002; Segalés et al., 2009).

Bone marrow hematopoietic cells (BMHC) were aseptically isolated from the femora and humeri of 4-week-old pigs. Briefly, bones were cut into 1cm^2 pieces and agitated in PBS at room temperature (RT) for 60 min. Then, the cell suspension was filtered through a 40 µm strainer after being depleted of erythrocytes by 0.15 M NH4Cl lysis. Cells were washed and frozen in liquid nitrogen until used. The BMHCs were tested as done for PAM. Bone marrow-derived dendritic cells (BMDC) were derived according to the method previously described by Carrasco et al. (2001) using 100 ng/ml of recombinant porcine granulocyte-monocyte colony stimulating factor (rpGM-CSF) (R&D Systems, Minneapolis, USA). To produce mature BMDC (mBMDC), immature BMDC (iBMDC) were treated overnight with 1µg/ml LPS (Invitrogen, Madrid, Spain) at day 8 of the process explained before. Any given experiment was performed with all cells coming from the same animal.

2.2. Viruses.

Three PRRSV1 isolates designated as 3249, 3262 and 3267 were used. These isolates have been previously used in different experiments, presenting different patterns of IL-10/TNF-a induction in BMDC (Gimeno et al., 2011), and TLR regulation in PAM (Kuzemtseva et al., 2014). Two of the isolates, 3262 and 3267, were tested *in vivo*, displaying different outcomes in virology and immunology (Díaz et al., 2012). The genomic sequences are accessible in Genbank (accession nº JF276433, JF276431, JF276435). Viral stocks of the three isolates were produced as a fifth passage in PAM. Each viral stock was produced in an amount enough to be used in all the experiments for at least a given technique.

Viruses were concentrated by precipitating from PAM supernatants with PEG Virus Precipitation Kit (Abcam, Cambridge, UK). To test whether trace amount of PEG would have an impact on virus infectivity, PEG was removed by adding solid KCl and spinning at 12,000 g. After removal of PEG, viral concentrates were titrated again and no differences in titres were seen compared to those with trace PEG, accordingly the centrifugation step was omitted afterwards. The PEG-concentrated virus was used only in the attachment experiment to reach a high MOI, while the original viral stock was used for the infection.

2.3. Replication kinetics of PRRSV1 in PAM, iBMDC and mBMDC cultures.

PAM, iBMDCs or mBMDCs were seeded in triplicates in plates (48-well, 2.5×105 cells/well) and inoculated with PRRSV1 isolates 3249, 3262 or 3267 at a MOI 0.1. After incubation for 1.5 h at 37 ℃, unbound virus was washed away and fresh medium with 10% foetal calf serum (FCS) was added. An uninfected macrophage culture supernatant was used as mock infection material. Cell cultures were collected at 0, 12, 24 and 48 h post-infection (hpi) and supernatants were titrated in PAM cultures in 96-well plates after centrifugation. Briefly, supernatants were diluted from10^{-1} to 10^{-6} and inoculated (50 µL) in PAM cultures. The titre of the virus in the supernatants was calculated according to the Reed-Muench method (Reed and Muench, 1938) after revealing the infection at day 5 post-inoculation by means of immunofluorescence staining with mAb anti-PRRSV1 N protein 1CH5 (Ingenasa, madrid, Spain) and a secondary fluorescein-labelled goat-anti mouse IgG (H+L) (Jackson ImmunoResearch, Madrid, Spain).

The replication in iBMDC at 12 hpi and 24 hpi was also assessed by flow cytometry. Briefly, cells were collected and fixed/permeabilized with methanol:ethanol 75:25 for 30 min at -20°C. Then they were labelled by mAb 1C5H (Ingenasa) with anti-mouse Alexa Fluor 610-R-phycoerythrin (RPE) (A20980, ThermoFisher, Madrid, Spain) as the secondary antibody. Cells were analysed on a FACSCalibur cytometer (BD Biosciences).

To test whether the differences in replication between iBMDC and mBMDC were caused by IFNα produced during the infection, the cell culture supernatants collected at 0, 12, 24 and 48 hpi were tested by capture ELISA using K9 mAb (R&D Systems) and biotinylated F17 mAb (R&D Systems; Thermofisher) for capture and detection, respectively. Streptavidin-HRP (ThermoFisher) was used to reveal the reaction. A standard curve ranging from 3.9 to 250 pg/ml was generated by serial dilutions of recombinant porcine IFN-α protein (R&D Systems). Optical density (OD) of the mock-inoculated cultures were used to assess the background. Samples were examined in triplicate.

For the comparison of CD163 expression in iBMDC, mBMDC and PAM, a flow cytometry assay was performed. Cells were stained with mAb anti-pig CD163 (clone 2A10/11, Bio-Rad, Oxford, UK) followed by a secondary Alexa Fluor 488 conjugated goat anti-mouse IgG1 (A21121, ThermoFisher). Cells were finally examined on a FACSCalibur cytometer (BD Biosciences).

2.4. Attachment of different PRRSV1 isolates to BMDC.

The attachment of three PRRSV1 isolates to iBMDC and mBMDC was initially analysed by confocal microscopy using a double labelling for PRRSV and PoSn or CD163. With this aim, the produced iBMDC were dispensed into a 96-well V-bottomed plate at a density of 2×10^5 cells/well. Cells were cooled down for 10 min on ice and then isolates 3249, 3262 and 3267 were added at MOI 1 in cold PBS containing 2% FCS (Sigma) reaching a volume of 50 µL. After 90 min of incubation on ice, cells were washed twice with cold PBS containing 2% FCS (Sigma) and then transferred onto microscope slides (ThermoFisher). The slides were dried under an air flow and cells were then fixed with 2% paraformaldehyde at RT for 10 min. For double staining (PRRSV/CD163 or PRRSV/PoSn) the primary antibodies used were: mAbs anti-PRRSV (Ingenasa), anti-PoSn (clone 3B11/11, Bio-Rad) and anti-CD163 (Bio-Rad). Then goat anti-mouse IgG (H + L) conjugated with Alexa Fluor 610-RPE and goat anti-mouse IgG1 conjugated with Alexa Fluor 488 (both from Thermofisher) were added as secondary antibodies. Non-

specific binding of secondary antibodies was reduced with a horse serum (10% in PBS) blocking. Samples without virus and samples where primary antibodies were omitted were used as negative control. Cells PK-15 were used as the irrelevant control for PoSn and CD163 staining. In the final step, ProlongGold Antifade mounting with DAPI (ThermoFisher) was used. Images were captured using a Leica TCS SP5 confocal microscopy (Wetzlar, Germany). Channel merging and image processing was performed with ImageJ (Schneider et al., 2012).

Since attachment of PRRSV1 was observed on either CD163 or PoSn negative cells, a three-color immunofluorescence labelling (plus DAPI nuclear staining) with MOI 3 was developed to further assess whether the CD163/PoSn double negative cells permitted viral attachment. In this case, only iBMDC were tested and mAb anti-CD163:RPE (Bio-Rad) in combination with mAb for PRRSV (Ingenasa) and PoSn (Bio-Rad) were used. Secondary antibodies (ThermoFisher) conjugated with Alexa Fluor 633 (A21052) or Alexa Fluor 488 were subsequently added. Mounting and examination of the slides was done as above.

2.5. Removal of cell surface heparan sulphate using Heparinase I.

Heparinase I (Sigma, Alcobendas, Spain) was reconstituted in the dilution buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 4 mM CaCl2, and 0.01% Bovine serum albumin (BSA) (Sigma, Madrid, Spain) and adjusted at 10 U/ml. iBMDCs were washed three times with RPMI1640 containing 0.2% BSA, and then were treated with heparinase I for 60 min at 37°C followed by washing with PBS containing 0.2% BSA. Plain medium plus BSA was used as the negative control. Cell viability was checked by trypan blue staining before further use. Then, cells were subjected to the CD163/PoSn/PRRSV staining as stated above.

2.6. Visualisation of PRRSV1 infection and CD163 expression in BMDC by confocal microscopy.

The inoculation of iBMDC and mBMDC with PRRSV1 isolates 3249, 3262 and 3267 was done as described above (see 2.3). Cells collected at 12, 24 and 48 hpi were transferred onto microscope slides (ThermoFisher), and were fixed/permeabilized. An indirect staining for PRRSV and CD163 was performed as described above (see 2.4).

2.7. Flow cytometry analysis of PRRSV1 infection and CD163 expression in BMDC.

The target cells were sorted with a BD FACSJazz cell sorter (BD Biosciences). Briefly, BMDC were treated with 10% pig serum in PBS for 15 min to block Fc receptors. Cells were then incubated with mAb anti-CD163 (BioRad) in 10% FCS in PBS for 45 min on ice, and labelled with goat anti-mouse IgG1 conjugated with Alexa Fluor 647 (Invitrogen). Three washes in 2% FBS in PBS were performed to remove unbound antibodies. Unstained cells and background from Alexa Fluor 647 were used as gating reference. Irrelevant isotype-matched mAb labelled with Alexa 647 was used to evaluate the unspecific staining. Since the autofluorescence of BMDC was high, the fluorescence channel adjacent to that of Alexa Fluor 647 was examined at the same time to further discriminate between signals from labelling and autofluorescence. The staining divided BMDC into three populations based on the expression of CD163, namely $CD163^{-}$, $CD163^{lo}$ and $CD163^{hi}$. Accordingly, two different approaches for sorting were performed. The first sorting assay focused strictly on $CD163^{-}$ cells, leaving $CD163^{lo}$ and $CD163^{hi}$ cells together. After sorting, the purity of $CD163^{-}$ subset was 99.0 ± 0.2% and of $CD163^{lo\ plus\ hi}$ subset was 96.5% ± 0.3%. The contamination by $CD163^{lo\ plus\ hi}$ in the $CD163^{-}$ sorted cells was on average less than 0.4%. The second sorting assay grouped together $CD163^{-}$ and $CD163^{lo}$ cells, leaving $CD163^{hi}$ cells as the other population. The purities after sorting were 96.1% ± 0.1% and 96.2% ± 0.2%, respectively. Again contamination by $CD163^{hi}$ in the $CD163^{-\ plus\ lo}$ was on average less than 0.2%.

The sorted BMDC (24-well, 5×10^{5} cells/well) were inoculated with isolate 3267 at MOI 0.1 as described in 2.3. Culture supernatants from uninfected macrophages were used as mock-infection negative controls. The experiment was run in triplicate cultures. In parallel, unsorted cells were also infected. Cells were collected at 24, 40 or 60 hpi and subjected to a two-colour flow cytometry staining for CD163 and PRRSV1. Briefly, Fc receptors were blocked and CD163 was labelled as described in the cell sorting section. For detecting the virus, cells were permeabilised with methanol (100%) for 15 min at -20°C. Then, cells were incubated with mAb 1C5H (Ingenasa) followed by a secondary antibody conjugated with Alexa 488 (Invitrogen) (45 min at 4 ℃). All antibodies included were previously titrated for optimal staining performance. Cells were washed for three times between each step. Finally, cells were analysed on a FACScalibur cytometer (BD

Biosciences). At least 20,000 events were acquired. Negative controls included unstained cells, background from the secondary antibody and irrelevant isotype-matched mAb labelled with secondary antibody. Gating and compensation were based on the single-stained cells. The flow cytometry readings were analysed using FCS Express 6 (De Novo Software).

2.8. Statistical analysis.

Comparison of means in CPE assay, cytokine measurement and flow cytometry were analysed by means of Kruskal–Wallis test with StatsDirect (v4.0). A p value < 0.05 was considered as statistically significant.

3. Results

3.1. PRRSV1 titres increase faster in iBMDC than in mBMDC.

The replication kinetics of three PRRSV1 isolates in iBMDC and mBMDC were assessed by titrating the cell culture supernatants in PAM (Figures 1A and 1B). At time 0, no residual virus was detected in the supernatants. Thereafter for all three isolates, titres at 12 hpi were significantly ($p<0.05$) higher in iBMDC than in mBMDC (Figure 1A). This difference tended to narrow at 24 hpi, but was still statistically significant. In iBMDC (Figure 1B), all three isolates reached peak titres at 24 hpi. In contrast, in mBMDC (Figure 1B) viral titres peaked at 48 hpi for two isolates (3249 and 3262) and at 24 hpi for only one (3267). In both cell types, isolate 3262 showed the lowest titres at 12 and 24 hpi, as performed in PAM (Figure 1C). This is consistent with the result examined by flow cytometry that the proportion of infected cells for isolate 3262 was always lower ($p<0.05$) than for the other isolates (Figure 1D).

The differences in replication between iBMDC and mBMDC were not related with IFNα induced during the infection, as no detectable levels of IFN-α were obtained in any of the cultures. We also examined the expression of CD163 in different cell types by flow cytometry. Before infection, 66% of the mBMDC were shown as $CD163^+$ while 57% of the iBMDC were $CD163^+$.

3.2. Attachment to BMDC depends on the PRRSV1 isolate examined.

The examination of different PRRSV1 isolates attaching to iBMDC (Figure 2A) showed that while isolates 3249 and 3267 produced clear signals considering the MOI used, for isolate 3262, attachment was scarcely seen. For the purpose of comparison, mBMDC were produced and the attachment was assessed as explained above. In this case, no differences were seen compared to the results obtained in iBMDC except for isolate 3262, that showed a somewhat higher attachment to mBMDC (data not shown). In PAM, the attachment of isolate 3262 was also lower compared to the other two isolates, but apparently not so low as in iBMDC (data not shown).

3.3. Attachment of PRRSV1 to BMDC with regards to CD163/PoSn expression.

The relationship of the attachment with the expression of CD163/PoSn was assessed by a two/three-color confocal microscopy staining. In the two-color staining, PRRSV1 attachment to $CD163^-$ or $PoSn^-$ subsets could be observed in both iBMDC and mBMDC. A further three-color labelling on iBMDC showed that besides double positive $CD163^+/PoSn^+$ cells, all three isolates were also seen attached to single positive subsets ($CD163^-/PoSn^+$ and $CD163^+/PoSn^-$) and, more interestingly, to the double negative subset $CD163^-/PoSn^-$ (Figure 2A).

To see the effect of removal of heparan sulphate on the attachment, iBMDC were treated with heparinase I. The confocal microscopy images showed that there was apparent reduction of the attachment by isolate 3267, but that was not abolished. The attachment to the double negative subset $CD163^-/PoSn^-$ was still seen (Figure 2B). This reduction was not so evident for isolate 3249. As regards isolate 3262, attachment to the double negative cells could also be observed.

3.4. Infection of BMDC by PRRSV1 with regards to CD163 expression.

To examine the infection of BMDC with relationship of CD163 expression, cultures of iBMDC and mBMDC were subjected to a two-color confocal microscopy labelling at 12, 24 and 48 hours after inoculation. The result showed that all three isolates were able to infect iBMDC or mBMDC that lacked CD163 expression, although in a low proportion (Figure 3).

The infection of iBMDC by isolate 3267 was further analysed by a two-color flow cytometry labelling. In unsorted iBMDC, 8.4% ± 0.5% of the PRRSV1 N positive cells were labelled as $CD163^-$ by 40 hpi (3.3% ± 0.1% by 24 hpi) (Figure 4A). However, when pure $CD163^-$ cells were sorted, no infection could be detected in this population by 40 hpi (Figure 4C). When $CD163^-$ were sorted together with $CD163^{lo}$ cells, 0.6% ± 0.1% of $CD163^-$ cells were labelled by PRRSV1 N protein at 40 hpi (Figure 4B), and when the incubation was extended to 60 hpi, the percentage of infected $CD163^-$ cells increased to 1.6% ± 0.1% (Figure 4B).

4. Discussion

Classically, PAM have been considered the main target for either PRRSV 1 or 2 (Duan et al., 1997a). Nevertheless, previous works (Wang et al., 2007; Chang et al., 2008; Flores-Mendoza et al., 2008; Park et al., 2008; Peng et al., 2009; Gimeno et al., 2011) indicated that DC could also be infected.

The results of the present work suggest that iBMDC are not only infected by PRRSV1 but the infection is as productive, if not more, as the infection of PAM. According to the titres attained in the culture supernatants, iBMDC appeared to be more efficient in supporting PRRSV1 replication than mBMDC. A similar finding was reported with MoDC that PRRSV1 subtype 3 Lena replicated more efficiently in immature MoDC (Singleton et al., 2016). Also, several studies have shown the transition to mature DC was delayed or avoided by PRRSV1 or 2 infection (Wang et al., 2007; Flores-Mendoza et al., 2008; Park et al., 2008). Since it was not related with the proportion of $CD163^+$ cells nor with the level of anti-viral cytokine IFN-α, the higher or lower productivity should reside in the intrinsic features of each cell type . The most plausible reason can be that maturation resulted in the impaired antigen uptake ability of BMDC (Lambotin et al., 2010; Platt et al., 2010). As has been demonstrated by Platt et al. (2010), mature DC down-regulated the uptake via constitutive micropinocytosis and phagocytosis while retained the receptor-mediated endocytosis. Apart from the uptake process, some post-entry blocking during replication or lack of efficient transmission to neighbouring susceptible cells can also play some roles (Canque et al., 1999; Ryu, 2017).

Besides, the replication efficiency was strain-dependent. Isolate 3262 showed the lowest titres in both iBMDC and mBMDC, especially at earlier times after infection. This is

304 consistent with its lower virulence in pigs (Díaz et al., 2012) and could be related to a
305 lower attachment capability as later confirmed by confocal microscopy.

306 During PRRSV entry into target cells, HS is assumed to act as an unspecific attachment
307 receptor, while virus interacting with PoSn initiates the internalization process. CD163
308 plays a role in the subsequent viral genome release. In our study, the attachment showed
309 better correlation with PoSn rather than CD163. This is in agreement with the previous
310 works showing that PoSn was the main receptor mediated PRRSV attachment (Delputte
311 et al., 2005; Van Gorp et al., 2008). Furthermore, when iBMDC were treated with
312 heparinase, the attachment was not fully prevented, not even to the CD163/PoSn double-
313 negative cell subset. This indicates that in BMDC, PRRSV entry may happen without the
314 participation of these molecules. Other authors, working with transfected BHK-21 cells
315 suggested that DC-SIGN (CD209), a C-type lectin that is expressed on the surface of DC,
316 could act as a potential receptor (Huang et al., 2009). Similarly, CD151 has been
317 suggested as a potential PRRSV receptor (Shanmukhappa et al., 2007) and can also be
318 expressed in some types of DC of humans (Sincock et al., 1997). Besides, the possibility
319 of virus attaching on some non-DC cells cannot be ruled out due to the high heterogeneity
320 of BMDC (Therrien et al., 2000). Nevertheless, virus uptake is not always equivalent to
321 viral replication, as the subsequent analysis showed that purely sorted $CD163^-$ cells were
322 refractory to PRRSV infection.

323 The fact that one of the isolates examined (3262) had a remarkably lower attachment to
324 iBMDC but not to PAM could be compatible with the notion that different receptors exist
325 in different cell types and that some isolates could preferentially use one or the other. For
326 example, Li et al. (2015) showed that PRRSV2 GP5 harbour different sugars such as N-
327 acetylglucosamine and N-acetyllactosamine that could bind receptors present in PAM.
328 Recently, Xie et al. (2017) showed that siglec-10 was an efficient receptor for PRRSV2
329 but less capable of supporting the infection by PRRSV1. Apart from the affinity of
330 different isolates to different receptors, alternative entry pathways not driven by the
331 receptor-ligand interaction might exist.

332 Since CD163 has been closely related to viral uncoating rather to viral attachment (Van
333 Breedam et al., 2010), we subsequently took an in-depth look on its role in PRRSV1
334 replication. The infection was found in cells negative for CD163 as examined by both
335 confocal microscopy and flow cytometry. The cell sorting experiment further showed that

this PRRSV-infected $CD163^{-}$ population appeared only when $CD163^{lo}$ or $CD163^{hi}$ were present in the culture. To analyse the origin of these infected $CD163^{-}$ cells, phenotype transition was firstly considered. That seemed to be unlikely because the sorted $CD163^{hi}$ and $CD163^{lo\ plus\ hi}$ did not show an evident decrease in the percentage of $CD163^{+}$ cells after infection and, in fact, $CD163^{+}$ cells increased by approximately 10% during the course of infection in unsorted BMDC (Figure 4A). In our opinion, the subset of $CD163^{-}$ cells supporting replication arose during the incubation, either as a result of the milieu created by the infection or in a non-receptor mediated way; for instance by exosomes carrying the viral genome (Wang et al., 2017), or through intercellular nanotube connections whereby PRRSV transported the infectious materials to neighbouring cells (Guo et al., 2016). Anyway, the present observations did not permit to conclude beyond any doubt that $CD163^{-}$ BMDC were susceptible to PRRSV. It is still possible that the infected $CD163^{-}$ cells were indeed cells with an extremely low expression of CD163 (beyond the sensitivity of confocal microscopy and flow cytometry) but enough to permit the infection or; which harbour intracellular CD163, for instance those expressed on endosomal vesicles, that might mediate the infection but was not revealed by the surface staining.

Our results are consistent with the work of Doeschl-Wilson et al. (2016) who observed an increasing proportion of PRRSV-infected $CD163^{-}$ PAM appearing at the later incubation stages. Frydas et al. (2013) also found that in nasal mucosa explants some PRRSV1 isolates may replicate in $CD163^{-}$/$PoSn^{-}$ double negative cells. And in another work by Singleton et al. (2016), MoDC with negligible expression of CD163 and PoSn, were permissive to PRRSV1 infection.

To some extent, these observations could be in conflict with the notion that CD163 is the only essential receptor of PRRSV. *In vivo*, CD163-defective pigs or genome-edited pigs lacking CD163 SRCR5 domain were resistant to PRRSV infection (Whitworth et al., 2015; Burkard et al., 2017; Wells et al., 2017). Besides, substitution of SRCR5 domain with a homolog humane counterpart could impair pigs' susceptibility (Wells et al., 2017). However, it can be argued firstly that the gene-editing of a particular CD163 pathway affects all maturity stages of the affected cells (Doeschl-Wilson et al., 2016) and this could be different from what would happen in a very heterogeneous population such as BMDC. Secondly, the fact that PAM were affected by substitutions in CD163 does not

368 preclude that in any other cell types a different mechanism or receptor for entry could
369 exist.

370 BMDC together with MoDC can be considered as *in vitro* representatives of the non Flt3-
371 derived DCs. In studies performed with lung DC and macrophages, it was shown that
372 $CD163^{lo}$ cells, similar to what can be found in BMDC, were fully functional DC and
373 presented the characteristics of MoDC while $CD163^{hi}$ were mostly macrophage-like cells
374 (Maisonnasse et al., 2016). Other authors, using the mice model showed that BMDC are
375 comprised of conventional DC and macrophages (Helft et al., 2015). Thus, the knowledge
376 on the characteristics of the infection by PRRSV in BMDC would have significance in
377 elucidating the viral invasion mechanism.

378 In summary, we showed that iBMDC are relevant targets for PRRSV, at least *in vitro*;
379 and the infection of $CD163^{-}$ cells occurred only when $CD163^{lo/hi}$ subsets were co-
380 cultured. In addition, different PRRSV1 isolates seem to interact with the cell membrane
381 receptors on BMDC in a different way. Our results emphasize the need for a more in-
382 depth examination of the role of DC infection in PRRSV immunopathogenesis.

383 Competing interests

384 The authors declare that they have no competing interests.

385 Authors' contributions

386 All three authors contributed to the design of the experiments, writing of the paper and
387 the scientific discussion of the results. Yan-li Li performed the experimental work in the
388 laboratory.

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Figure 1. Titres of different PRRSV1 isolates in cell culture supernatants of immature (i) and mature (m) bone marrow-derived dendritic cells (BMDC). The figure shows the titres attained in cell culture supernatants of iBMDC and mBMDC as determined by titration in PAM. **A** Comparison of the replication in iBMDC and mBMDC according to different isolates (3249, 3262 or 3267). **B** Comparison of the replication rate of three isolates in **both** iBMDC and mBMDC and **C in** PAM. **D** Proportion of virus-infected iBMDC at different times. The graph shows the average proportion of infected cells of the three isolates after 12 or 24 hours of culture (triplicates) as determined by flow cytometry. Error bars account for standard deviations of three replicas. Different letters indicate significant differences ($p<0.05$).

Figure 2. Attachment of three PRRSV1 isolates (3249, 3262 and 3267) to different subsets of immature bone marrow-derived dendritic cells (iBMDC) determined by confocal microscopy. A The viral attachment was shown by the staining of PRRSV N (red) to subsets defined by PoSn (blue) and CD163 (green). Nuclei (grey) were stained with DAPI. The upper row shows uninfected cells. The scale bar represents 5 μm. **B** Effect of heparan sulphate removal by heparinise I on the attachment of isolate 3267. The staining was as described above. The scale bar represents 5 μm.

Figure 3. Infection of immature bone marrow-derived dendritic cells (iBMDC) determined by confocal microscopy. The picture shows the infection observed in $CD163^-$ iBMDC by isolate 3249 at 24 hours post-infection (hpi), as indicated with red arrows. The infection was stained in red for PRRSV N; CD163 in green; and nuclei in blue. The upper row shows the uninfected cells; the lower row, infected cells. The scale bar represents 10 μm.

Figure 4. Flow cytometry analysis of the infection of immature bone marrow-derived dendritic cells (iBMDC) by isolate 3267 with regards to CD163 expression. A Infection of unsorted iBMDC by 0, 24 and 40 hours post-infection (hpi). **B** Infection of sorted $CD163^{-\ plus\ lo}$ by 40 and 60 hpi. **C** Overlaid histograms of the infection of sorted $CD163^{-}$ (red empty) by 40 hpi. The isotype (grey shadowed) and the mock-infected samples (black empty) were used as negative controls. The infection of subset $CD163^{lo\ plus\ hi}$ was used as positive control.