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Characterisation of PRRSV1 infection in bone marrow-derived dendritic cells

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Tesi doctoral

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IRTA RECERCA I TECNOLOGIA AGROALIMENTÀRIES

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Declara

Que la memòria titulada: **"Characterization of PRRSV1 infection in bone marrow-derived dendritic cells"**, presentada per l'**Yanli Li** per a l'obtenció del grau de Doctor en Veterinària, s'ha realitzat sota la meva direcció en el programa de doctorat de Medicina i Sanitat Animals, del Departament de Sanitat i d'Anatomia Animals, opció Sanitat Animal.

I per a que consti als efectes oportuns, signo la present declaració a Bellaterra, 18 de setembre del 2017:

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SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is one of the most devastating diseases for the swine industry worldwide. PRRS virus (PRRSV) can establish long-term infections in pigs, partially due to the slow development of protective immune responses. Besides this, secondary infections are common and add serious economic losses to the primary impact of PRRSV infection. PRRSV targets macrophages but also may infect dendritic cells (DC). The main characteristics of the infection in macrophages have been more or less resolved but much less is known about the interaction between PRRSV and DC. The present thesis aims to characterize some aspects during PRRSV infection in DC, including the attachment, replication and the induction of apoptosis. The first study of the present thesis was directed to evaluate the attachment and replication of PRRSV1 in BMDC. Three PRRSV1 isolates (3249, 3262 and 3267) with known distinct immunobiological properties were used. Pig bone marrow hematopoietic cells were stimulated with recombinant porcine GM-CSF *in vitro* for 8 days to produce what was defined as immature (i) bone marrow-derived DC (iBMDC). iBMDC were further stimulated with LPS overnight to produce mature (m) BMDC. Replication of the three PRRSV1 isolates was evaluated using a multiplicity of infection (MOI) of 0.1. Cell culture supernatants were collected at 12, 24 and 48 hours post-infection (hpi) and titrated in porcine alveolar macrophages (PAM). For two isolates (3249 and 3262), replication peaked earlier in iBMDC (24 h) compared to mBMDC (48 h) while for the other (3267) peak was reached at 24 h for both types of BMDC. Moreover, the viral yield at 12 and 24 hpi was significantly higher in iBMDC than in mBMDC. These results indicated that iBMDC were more efficient than mBMDC in supporting viral replication. Given that the proportion of

$CD163^+$ cells was higher in mBMDC than in iBMDC (as determined by flow cytometry) and that no detectable IFN-α in the cell culture supernatants of both iBMDC and mBMDC, the most plausible explanation would be that maturation resulted in the decreased efficiency for the entry, decapsidation or efficiency of replication. In addition, the replication efficiency was strain-dependent. Isolate 3262 showed the lowest titres in both cell types at 12 and 24 hpi. When examined by confocal microscopy and flow cytometry, it became evident that the proportion of cells infected by 3262 at earlier times was significantly lower compared to the other isolates. This pointed towards a lower attachment of this isolate. Then, the attachment of the three isolates was compared by confocal microscopy and flow cytometry and isolate 3262 apparently presented a lower binding rate. The attachment and replication of the three isolates was further studied in association with the expression of PoSn, CD163 and heparan sulphate. A three-colour confocal microscopy staining (PoSn, CD163 and PRRSV) on iBMDC showed that, besides double positive $PoSn^+/CD163^+$ cells, isolates 3249 and 3267 were also seen attached onto the single positive subsets $PoSn^+/CD163^-$ and $PoSn^-/CD163^+$ and, more interestingly, on the double negative subset $PoSn^-/CD163^-$. After removal of heparan sulphate by treatment with heparinase, attachment could still be observed on the four subsets defined by PoSn and CD163. These results indicated that the attachment on BMDC may occur beyond the intervention of heparan sulphate, PoSn and CD163. For examining replication with regards to these receptors, a two-colour confocal microscopy staining (CD163 and PRRSV or PoSn and PRRSV) was performed. Replication was observed in both PoSn-positive and negative cells. Also, replication of isolates 3249 and 3267 happened in apparently $CD163^-$ iBMDC. When this was further examined by flow cytometry with isolate 3267, 8.4 ± 0.5% of apparently $CD163^-$ cells were labelled as positive for PRRSV N protein. To further clarify this, a

sorting experiment was designed. Sorting of cells was done in two different ways based on the variable amounts of CD163 expressed ($CD163^{-}$, $CD163^{lo}$ and $CD163^{hi}$). The first sorting focused on "beyond doubt" $CD163^{-}$ cells, namely $CD163^{-}$ cells selected in the left side of the histogram without $CD163^{lo}$ contamination. The second sorting grouped $CD163^{-}$ cells together with $CD163^{lo}$. Unsorted iBMDC were used as controls. Then, cells were infected with isolate 3267 and the course of the infection was examined by a two-colour flow cytometry. In unsorted iBMDC, approximately 5.7 ± 0.2% of apparently $CD163^{-}$ cells were detected as PRRSV-positive by 40 hpi. In contrast, the "beyond doubt" pure $CD163^{-}$ were not infected by 40 hpi. When $CD163^{-}$ were sorted together with $CD163^{lo}$ cells, the infected $CD163^{-}$ population (0.6 ± 0.07%) appeared again by 40 hpi and when the incubation was extended to 60 hpi, the percentage of infected $CD163^{-}$ cells increased more (1.6% ± 0.08%). Interestingly, the proportion of infected cells (and the absolute numbers) infected at 60 hpi was higher than the initial number of $CD163^{+}$ cells. One explanation would be the generation of new $CD163^{lo}$ during the culture that would be infected as soon as they expressed this molecule, even at levels beyond the detection capability of the cytometer. An alternative hypothesis would be the milieu created by the infection of $CD163^{+}$ subsets induced the emergence of that $CD163^{-}$ susceptible population. It cannot be excluded that these negative cells developed alternative receptors yet unknown or a receptor-independent antigen uptake occurred. The second study aimed to characterize the apoptosis induced by the same three PRRSV1 isolates (3249, 3262 and 3267). Using confocal microscopy, in PAM, positive signals for cleaved caspase-3 were observed in both infected and bystander cells for all three isolates, despite of their different replication kinetics. In contrast, in BMDC the cleaved caspase-3 labelling was mainly found in bystander cells. This finding suggests that in PAM apoptosis was basically the result of the

activation of the intrinsic and extrinsic pathways while in BMDC the extrinsic pathway was more relevant. Besides this, at MOI 0.1, the caspase 3 signal in BMDC peaked at 48 hpi, namely, later than in PAM (24 hpi). This slower development of apoptosis may allow more cycles of viral replication, resulting in higher viral yields in BMDC. Further examination of inoculated BMDC cultures for apoptosis/necrosis showed significant differences between isolates. Whereas 3249 and 3267 apparently induced apoptosis and necrosis, cells infected by 3262 only had minor changes. Neutralization of IL-10 induced by 3262 resulted in the occurrence of apoptotic cells, but this did not happen with a second IL-10 inducing isolate (designated as 2988). Therefore, it is still unclear the role of IL-10 plays in PRRSV-induced apoptosis. The above-mentioned results in terms of PRRSV1 attachment, replication and induced apoptosis in BMDC, will be useful to understand the role of DC in PRRSV pathogenesis.

RESUM

La síndrome reproductiva i respiratòria porcina (PRRS) és una de les malalties de major impacte econòmic per al sector porcí a tot el món. El virus del PRRS (vPRRS) pot produir infeccions cròniques en porcs, en part com a conseqüència del lent desenvolupament de la resposta immune protectora. D'altra banda, les infeccions secundàries són freqüents i afegeixen serioses pèrdues econòmiques a l'impacte primari de la infecció per vPRRS. El vPRRS infecta els macròfags però també pot infectar les cèl·lules dendrítiques (DC). S'ha acumulat prou coneixement sobre les característiques de la infecció en els macròfags però se sap molt menys sobre la interacció entre vPRRS i DC. Aquesta tesi té com a objecte caracteritzar alguns aspectes de la infecció per vPRRS en DC, incloent l'adhesió, la replicació i la inducció d'apoptosi. El primer estudi de la tesi es va dirigir a avaluar l'adhesió i la replicació de PRRSV1 en DC derivades de medul·la òssia (BMDC). Per a això, es van usar 3 aïllats de PRRSV1 (3249, 3262 i 3267) amb propietats inmunobiològiques diferents conegudes amb anterioritat. Per a la producció de BMDC, les cèl·lules hematopoètiques de medul·la òssia de porc es van estimular *in vitro* amb GM-CSF porcí recombinant durant 8 dies per produir el que es va definir com DC immadures (i) derivades de medul·la òssia (iBMDC). Les iBMDC es van estimular posteriorment amb LPS durant una nit per produir BMDC madures (m). La replicació dels tres aïllats de PRRSV1 es va avaluar utilitzant una multiplicitat d'infecció de 0,1. Els sobrenedants del cultiu cel·lular es van recollir a les 12, 24 i 48 hores post-infecció (hpi) i es van titular en macròfags alveolars porcins (MAP). Per a dos aïllaments (3249 i 3262), la replicació va tenir un pic abans a les iBMDC (24 h) que en les mBMDC (48 h) mentre que per a l'altre (3267) el pic va ser a les 24 h en ambdós tipus de BMDC. A més, la productivitat a 12 i 24 hpi va ser significativament més gran en iBMDC que en mBMDC. Aquests resultats indicaven que les iBMDC eren més eficaces que les mBMDC pel que fa a la seva permissivitat i a la productivitat

de virus. Atès que la proporció de cèl·lules $CD163^{+}$ era més gran en les mBMDC que en les iBMDC (segons es va determinar per citometria de flux) i que no havia IFN-α detectable en els sobrenedants de cultius cel·lulars tant de iBMDC com de mBMDC, l'explicació més plausible per aquestes observacions seria que la maduració va resultar en una disminució de l'eficiència per a l'entrada, decapsidació o capacitat de mantenir la replicació. A més, l'eficàcia de replicació depenia de la soca usada. El aïllat 3262 va mostrar els títols més baixos en els dos tipus de cèl·lules a les 12, 24 i 48 hpi. Quan es van examinar els cultius mitjançant microscòpia confocal i citometria de flux, va resultar evident que a temps propers al de infecció la proporció de cèl·lules infectades per 3262 era menor en comparació amb els altres aïllaments. Aquesta observació apuntava cap a una menor adhesió d'aquest aïllat a les cèl·lules. A continuació, l'adhesió dels tres aïllaments es va comparar mitjançant microscòpia confocal i citometria de flux i la soca 3262 aparentment va presentar una adhesió menor. Posteriorment, es va estudiar l'adhesió i replicació dels tres virus en iBMDC amb relació a l'expressió de sialoadhesina porcina (PoSn), CD163 i sulfat de heparán. Per aquest objectiu es va realitzar un marcatge en tres colors (PoSn, CD163 i PRRSV) que es va examinar per microscòpia confocal. A més de les cèl·lules doblement positives $PoSn^{+}/CD163^{+}$, les soques 3249 i 3267 també es van unir a cèl·lules dels altres fenotips possibles d'acord amb els marcadors examinats: $PoSn^{+}/CD163^{-}$ i $PoSn^{-}/CD163^{+}$ i, més sorprenentment, a cèl·lules doblement negatives $PoSn^{-}/CD163^{-}$. A més, la replicació dels aïllaments 3249 i 3267 també es va observar a iBMDC aparentment $CD163^{-}$. Quan aquest aspecte es va examinar més a fons mitjançant citometria de flux emprant l'aïllat 3267, un 8,4 ± 0,5% de cèl·lules aparentment $CD163^{-}$ es van marcar com a positives per la proteïna N de PRRSV. Per aclarir això, es va dissenyar un experiment de separació per citometria de flux. Aquesta separació es va realitzar de dues formes diferents basant-se en les quantitats variables de CD163 expressat ($CD163^{-}$, $CD163^{lo}$ i $CD163^{hi}$). La primera separació es va centrar en aquelles cèl·lules $CD163^{-}$ la classificació de les quals estava "més enllà del

dubte", en seleccionar les situades a l'extrem esquerre de l'histograma, el que permetia eliminar cèl·lules $CD163^{lo}$ o amb alta autofluorescència. La segona separació es va enfocar en el grup de cèl·lules $CD163^{-}$ juntament amb $CD163^{lo}$. Com a controls es van emprar iBMDC sense separar. A continuació, les cèl·lules es van infectar amb l'aïllat 3267 i el curs de la infecció es va examinar mitjançant una citometria de flux de dos colors. A les iBMDC no separades, es van detectar com a positives a PRRSV a 40 hpi aproximadament 5,7 ± 0,2% de cèl·lules aparentment $CD163^{-}$. En les cèl·lules CD163- "més enllà del dubte" no es va observar infecció. Quan les cèl·lules $CD163^{-}$ es van separar juntament amb cèl·lules $CD163^{lo}$, la població $CD163^{-}$ infectada va ser de 0,6 ± 0,07% a les 40 hpi augmentant a 1,6% ± 0,08% a les 60 hpi. Curiosament, la proporció de cèl·lules infectades (i la quantitat absoluta) a 60 hpi va ser més gran que el nombre inicial de cèl·lules $CD163^{+}$. Una explicació a aquest fet seria la generació de noves cèl·lules $CD163^{lo}$ durant el cultiu que s'infectarien tan aviat com expressessin aquesta molècula, probablement en quantitats per sota de la capacitat de detecció del citòmetre. Una hipòtesi alternativa seria que el medi creat per la infecció de les cèl·lules $CD163^{+}$ va induir l'aparició d'aquesta població $CD163^{-}$ susceptible. No es pot excloure que aquestes cèl·lules negatives desenvoluparan receptors alternatius encara desconeguts o que es produís una captació d'antigen independent del receptor. El segon estudi va tenir com a objectiu caracteritzar l'apoptosi induïda pels mateixos tres aïllats de PRRSV1 (3249, 3262 i 3267). Utilitzant microscòpia confocal amb PAM, per als tres aïllaments es va observar un marcatge positiu per a la caspasa-3 activada tant en cèl·lules infectades com en cèl·lules no infectades, malgrat la seva diferent cinètica de replicació. Per contra, en BMDC el marcatge de caspasa-3 activada es va localitzar principalment en cèl·lules no infectades. Aquesta troballa suggereix que, en PAM, l'apoptosi va ser bàsicament el resultat de l'activació de les vies intrínseca i extrínseca, mentre que a BMDC la via extrínseca va ser més rellevant. A més d'això, a multiplicitats d'infecció de 0,1, el senyal de caspasa 3 en BMDC va assolir un màxim a les 48

hpi, és a dir, més tard que a PAM (24 hpi). Aquest desenvolupament més lent de l'apoptosi podria permetre més cicles de replicació vírica, resultant en majors rendiments vírics en BMDC. Un examen posterior per apoptosi/necrosi de cultius de BMDC inoculats va mostrar diferències significatives entre aïllaments. Mentre que els aïllats 3249 i 3267 aparentment van induir apoptosi i necrosi, les cèl·lules infectades per 3262 només van tenir canvis menors. La neutralització de la IL-10 induïda pel aïllat 3262 va donar lloc a l'aparició de cèl·lules apoptòtiques, però aquest efecte no va ocórrer amb un segon aïllat que induïa la producció d'IL-10 (soca 2988). Per tant, encara no està clar el paper d'IL-10 juga en l'apoptosi induïda per PRRSV. Els resultats d'aquesta tesi poden ser útils per comprendre el paper de DC en la patogènesi de PRRSV.

RESUMEN

El síndrome reproductivo y respiratorio porcino (PRRS) es una de las enfermedades de mayor impacto económico para el sector porcino en todo el mundo. El virus del PRRS (VPRRS) puede producir infecciones crónicas en cerdos, en parte como consecuencia del lento desarrollo de la respuesta inmune protectora. Por otra parte, las infecciones secundarias son frecuentes y agregan serias pérdidas económicas al impacto primario de la infección por VPRRS. El VPRRS infecta a los macrófagos pero también puede infectar las células dendríticas (DC). Se ha acumulado bastante conocimiento sobre las características de la infección en los macrófagos pero se sabe mucho menos sobre la interacción entre VPRRS y DC. Esta tesis tiene como objeto caracterizar algunos aspectos de la infección por VPRRS en DC, incluyendo la adhesión, la replicación y la inducción de apoptosis. El primer estudio de la tesis se dirigió a evaluar la adhesión y la replicación de PRRSV1 en DC derivadas de médula ósea (BMDC). Para ello, se usaron tres aislados de PRRSV1 (3249, 3262 y 3267) con propiedades inmunobiológicas distintas conocidas con anterioridad. Para la producción de BMDC, las células hematopoyéticas de médula ósea de cerdo se estimularon *in vitro* con GM-CSF porcino recombinante durante 8 días para producir lo que se definió como DC inmaduras (i) derivadas de médula ósea (iBMDC). Las iBMDC se estimularon posteriormente con LPS durante una noche para producir BMDC maduras (m). La replicación de los tres aislados de PRRSV1 se evaluó utilizando una multiplicidad de infección de 0,1. Los sobrenadantes del cultivo celular se recogieron a las 12, 24 y 48 horas post-infección (hpi) y se titularon en macrófagos alveolares porcinos (MAP). Para dos aislamientos (3249 y 3262), la replicación alcanzó el pico antes en las iBMDC (24 h) que en las mBMDC (48 h) mientras que para el otro (3267) el pico se alcanzó a las 24 h en ambos tipos de BMDC. Además, la productividad a 12 y 24 hpi fue significativamente mayor en iBMDC que en mBMDC. Estos resultados indicaban que las

iBMDC eran más eficaces que las mBMDC con respecto a su permisividad y a la productividad de virus. Dado que la proporción de células $CD163^+$ era mayor en las mBMDC que en las iBMDC (según se determinó por citometría de flujo) y que no había IFN-α detectable en los sobrenadantes de cultivos celulares tanto de iBMDC como de mBMDC, la explicación más plausible para estas observaciones sería que la maduración resultó en una disminución de la eficiencia para la entrada, decapsidación o eficiencia de replicación. Además, la eficacia de replicación dependía de la cepa usada. El aislado 3262 mostró los títulos más bajos en ambos tipos de células a las 12, 24 y 48 hpi. Cuando se examinaron los cultivos mediante microscopía confocal y citometría de flujo, resultó evidente que a tiempos cercanos al de infección la proporción de células infectadas por 3262 era menor en comparación con los otros aislamientos. Esta observación apuntaba hacia una menor adhesión de este aislado a las células. A continuación, la adhesión de los tres aislamientos se comparó mediante microscopía confocal y citometría de flujo y la cepa 3262 aparentemente presentó una adhesión menor. Posteriormente, se estudió la adhesión y replicación de los tres virus en iBMDC con relación a la expresión de sialoadhesina porcina (PoSn), CD163 y sulfato de heparán. Para ello se realizó un marcaje en tres colores (PoSn, CD163 y PRRSV) que se examinó por microscopía confocal. Además de las células doblemente positivas $PoSn^+/CD163^+$, las cepas 3249 y 3267 también se unieron a células de los otros fenotipos posibles de acuerdo a los marcadores examinados: $PoSn^+/CD163^-$ y $PoSn^-/CD163^+$ y, más sorprendentemente, a células doblemente negativas $PoSn^-/CD163^-$. Además, la replicación de los aislamientos 3249 y 3267 también ocurrió en iBMDC aparentemente $CD163^-$. Cuando este aspecto se examinó más a fondo mediante citometría de flujo empleando el aislado 3267, un 8,4 ± 0,5% de células aparentemente $CD163^-$ se marcaron como positivas para la proteína N de PRRSV. Para aclarar esto, se diseñó un experimento de separación por citometría de flujo. Esta separación se realizó de dos formas diferentes basándose en las cantidades variables de CD163 expresado ($CD163^-$, $CD163^{lo}$ y

$CD163^{hi}$). La primera separación se centró en aquellas células $CD163^{-}$ cuya clasificación estaba "más allá de la duda", al seleccionar las situadas en el extremo izquierdo del histograma, lo que permitía eliminar células $CD163^{lo}$ o con alta autofluorescencia. La segunda separación se enfocó en el grupo de células $CD163^{-}$ junto con $CD163^{lo}$. Como controles se emplearon iBMDC sin separar. A continuación, las células se infectaron con el aislado 3267 y el curso de la infección se examinó mediante una citometría de flujo de dos colores. En las iBMDC no separadas, se detectaron como positivas a PRRSV en 40 hpi aproximadamente 5,7 ± 0,2% de células aparentemente $CD163^{-}$. En contraste, en el grupo de $CD163^{-}$ "más allá de la duda" no se observó infección. Cuando las células $CD163^{-}$ se clasificaron junto con células $CD163^{lo}$, la población $CD163^{-}$ infectada fue de 0,6 ± 0,07% a las 40 hpi aumentando a 1,6% ± 0,08% a las 60 hpi. Curiosamente, la proporción de células infectadas (y la cantidad absoluta) a 60 hpi fue mayor que el número inicial de células $CD163^{+}$. Una explicación a este hecho sería la generación de nuevas células $CD163^{lo}$ durante el cultivo que se infectarían tan pronto como expresaran esta molécula, probablemente en cantidades por debajo de la capacidad de detección del citómetro. Una hipótesis alternativa sería que el medio creado por la infección de las células $CD163^{+}$ indujo la aparición de esa población $CD163^{-}$ susceptible. No se puede excluir que estas células negativas desarrollaran receptores alternativos aún desconocidos o que se produjese una captación de antígeno independiente del receptor. El segundo estudio tuvo como objetivo caracterizar la apoptosis inducida por los mismos tres aislados de PRRSV1 (3249, 3262 y 3267). Utilizando microscopía confocal, en PAM, para los tres aislamientos se observó un marcaje positivo para la caspasa-3 activada tanto en células infectadas como en células no infectadas, a pesar de su diferente cinética de replicación. Por el contrario, en BMDC el marcaje de caspasa-3 activada se localizó principalmente en células no infectadas. Este hallazgo sugiere que, en PAM, la apoptosis fue básicamente el resultado de la activación de las vías intrínseca y extrínseca, mientras que en BMDC la vía extrínseca fue más relevante. Además de esto, a

multiplicidades de infección de 0,1, la señal de caspasa 3 en BMDC alcanzó un máximo a las 48 hpi, es decir, más tarde que en PAM (24 hpi). Este desarrollo más lento de la apoptosis podría permitir más ciclos de replicación vírica, resultando en mayores rendimientos víricos en BMDC. Un examen posterior para apoptosis/necrosis de cultivos de BMDC inoculados mostró diferencias significativas entre aislamientos. Mientras que los aislados 3249 y 3267 aparentemente indujeron apoptosis y necrosis, las células infectadas por 3262 sólo tuvieron cambios menores. La neutralización de la IL-10 inducida por el aislado 3262 dio lugar a la aparición de células apoptóticas, pero este efecto no ocurrió con un segundo aislado que inducía la producción de IL-10 (cepa 2988). Por lo tanto, todavía no está claro el papel de IL-10 juega en la apoptosis inducida por PRRSV. Los resultados de esta tesis pueden ser útiles para comprender el papel de DC en la patogénesis de PRRSV.

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List of abbreviations

ADE	antibody-dependent enhancement
BMDC	bone marrow-derived dendritic cells
BMHC	bone marrow hematopoietic cells
cDC	classical or conventional dendritic cells
CDP	common dendritic cells precursor
CSF2	colony stimulating factor 2
CTL	cytotoxic T cells
DC	dendritic cells
dpi	days post-inoculation
dsRNA	double-stranded RNA
DV	dengue virus
EAV	Equine arteritis virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FasL	Fas ligand
Flt3L	fms-related tyrosine kinase 3 ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	glycoprotein
HCV	hepatitis C virus
HIV	immunodeficiency virus
HP-PRRSV	highly pathogenic PRRSV
hpi	hours post-inoculation
HS	heparan sulphate
IF	intermediate filament
IFA	immunofluorescence assay
IFN	interferon
IFN-γ-SC	interferon-γ secreting cells
IgM/IgG	immunoglobulin M/G
IHC	immunohistochemistry
IL	interleukin
IPMA	immunoperoxidase monolayer assay

IRF-7	interferon regulatory factor 7
ISH	in situ hybridization
JAK–STAT	cytokine-activated Janus kinase (JAK)–signal transducer and activator of transcription (STAT)
JNK	Jun-N-terminal kinase
LC	Langerhans cells
LDV	lactate dehydrogenase-elevating virus
M	matrix protein
MAVS	mitochondrial antiviral-signalling protein
MHC	major histocompatibility complex
MIIC	MHC class II-rich compartments
MoDC	monocyte-derived dendritic cells
N	nucleocapsid protein
NA	neutralizing antibody
NF-κB	nuclear factor-κB
NIPC	natural IFN producing cells
nsp	non-structural proteins
ORF	open reading frame
PAM	porcine alveolar macrophages
PBMC	peripheral blood mononuclear cells
PCD	programmed cell death
pDC	plasmacytoid dendritic cells
pDC-SIGN	dendritic cell porcine dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
PI	post-inoculation
PoSn	porcine sialoadhesin
PRF	programmed ribosomal frameshift
PRR	pattern recognition receptors
PRRSV	porcine reproductive and respiratory syndrome virus
RIG	retinoic acid inducible gene
RLR	RIG-I-like receptors
RT-PCR	reverse transcription-polymerase chain reaction
SAMS	sow abortion and mortality syndrome

sgRNA	subgenomic ribonucleic acid
SPF	specific-pathogen-free
SRCR	scavenger receptor cysteine-rich
TAP	transporter associated with antigen processing
TGF-β	Transforming growth factor beta
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptors
TNF-α	tumour necrosis factor-α
TRAILR	tumour necrosis factor-related apoptosis-inducing ligand receptor
Tregs	regulatory T cells
TRS	transcription-regulating sequence
UTR	untranslated regions
VNT	virus neutralization test
WPDV	wobbly possum disease virus

PART I: Introduction and objectives

Introduction

Hypotheses and objectives

Introduction

Porcine reproductive and respiratory syndrome virus

1.1 History of PRRS

In the mid-decade of 1980, a series of disease outbreaks characterised by reproductive failure and post-weaning pneumonia appeared in the United States (Keffaber, 1989; Loula, 1991). At that moment, the outbreaks could not be related to any known agent and thus, the name “Mystery Swine Disease" (MSD) was coined to design the cases (Hill, 1990). In Europe, similar outbreaks were reported initially in 1990 in Germany (OIE., 1992) that spread rapidly throughout the country. By May 1991, more than 3,000 outbreaks had been documented in Germany (OIE., 1992). In the next four years, most countries in the continent became infected (Baron *et al.*, 1992; Botner *et al.*, 1994; Edwards *et al.*, 1992; Pejsak and Markowska-Daniel, 1996; Plana Duran *et al.*, 1992; Valicek *et al.*, 1997). In Asia, outbreaks were reported in Japan in 1988 (Hirose *et al.*, 1995) and in Taiwan in 1991 (Chang *et al.*, 1993). The disease was later called “porcine reproductive and respiratory syndrome (PRRS)”, a name that was widely adopted and is the official name nowadays.

In 1991, Wensvoort *et al.* (1991) identified the etiological agent of the disease in Netherlands. The agent was shown to be a previously unknown enveloped single-stranded RNA virus that was named after the place of the laboratory: Lelystad virus. Shortly thereafter, the virus was isolated in the United States being designated VR-2332 (Collins *et al.*, 1992).

From the very beginning, it became evident that the European and the American viruses, although closely related, were different (Meng *et al.*, 1995). Accordingly, two PRRS virus

(PRRSV) types were recognized within a single species in the Genus *Arterivirus* (Cavanagh, 1997): the European (type 1) and the North American (type 2) PRRSV.

The origin of PRRSV remains obscure. As shown by retrospective serologic studies, PRRSV infection in North America can be traced back to 1979 (Carman *et al.*, 1995). In Western Europe, the first evidences of the virus were found in samples of 1987 from the former German Democratic Republic (Ohlinger *et al.*, 2000). Similarly, in other countries of Europe and Asia evidences of the virus can be dated back to the mid or late years of the 1980 decade (Grebennikova *et al.*, 2004; Shin *et al.*, 1993).

The evolutionary origin of PRRSV is also a mystery. The genetic differences between European and American PRRSV make extremely unlikely that one derives from the other and the concept of parallel evolution in two continents is the most widely accepted one (Shi *et al.*, 2010a). Plagemann (2003) hypothesized that PRRSV might derive from a closely related artervirus, the *Lactate dehydrogenase-elevating virus* (LDV) of rodents. This LDV-like ancestor would have later adapted to wild boars which served as the intermediated host to reach domestic pigs. However, ultimate evidences such as viral isolation from rodents or the finding of diverse PRRSV-related viruses in wild boars are lacking and this weakens this hypothesis of interspecies transmission (Reiner *et al.*, 2009; Shi *et al*., 2010a). Nevertheless, given the high diversity of PRRSV in Eastern Europe it cannot be discarded that such intermediate viruses could be found there.

Since the original outbreaks in the 1980 decade, PRRSV has undergone a rapid evolution. In 1996 an acute PRRS virus emerged in Iowa (Meng *et al.*, 1996a); in 2001 a virulent variant appeared in Minnesota (Han *et al.*, 2006) and in 2006 a highly pathogenic PRRSV (HP-PRRSV) caused severe outbreaks in China (Tian *et al.*, 2007). In Europe, a highly virulent

subtype 3 isolate designated as Lena was retrieved from PRRS cases in Belarus in 2007 (Karniychuk *et al.*, 2010).

Nowadays, PRRSV has become endemic in most swine-producing countries, causing huge economic losses worldwide. Only in U.S., the total cost of productivity losses due to PRRSV was estimated at US$560 million in 2005 and at US$664 million in 2013 (Holtkamp *et al.*, 2013; Neumann *et al.*, 2005). The cost of the disease in Europe has not been fully assessed but it is estimated that an outbreak in a sow farm costs on average 126€/sow (Nieuwenhuis *et al.*, 2012).

1.2 Etiological agent

1.2.1 Taxonomy

PRRSV is an enveloped RNA virus with a single-stranded, positive-sense genome. At the 10th International Congress of Virology (ICV) (Pringle, 1996), PRRSV, together with LDV, *Equine arteritis virus* (EAV), and *Simian hemorrhagic fever virus* (SHFV) were classified in the family *Arteriviridae* within the newly created Order *Nidovirales*. This Order grouped *Arteriviridae, Coronaviridae, Roniviridae* and *Toroviridae* (Pringle, 1996). The name *Nidovirales* derives from a Latin word meaning nest, reflecting a common characteristic of these viruses, namely that a 3' co-terminal nested set of subgenomic mRNAs is generated during gene expression (Snijder and Meulenberg, 1998). Later on the *Arteriviridae* included the newly found wobbly possum disease virus (WPDV) (Dunowska *et al.*, 2012).

In 2016, the International Committee on Taxonomy of Viruses proposed a new classification of the family *Arteriviridae* based on a modification of the proposal by Bailey *et al*. (Adams *et al.*, 2016). The new organization of the family included five genera: *Diparterivirus*,

Equarterivirus, *Nesarterivirus*, *Simarterivirus* and *Porarterivirus*. PRRSV was divided in two species: PRRSV1 and PRRSV2 (the former type 1 and 2) within the Genus *Porarterivirus*.

1.2.2 Physical-Chemical properties

The buoyant density of PRRSV particles in sucrose is 1.13-1.15 g/ml and the sedimentation coefficient ranges from 214S to 230S. PRRSV is stable for years at -70°C (Hermann *et al.*, 2007) but the viability rapidly declines with temperature. Thus, Bloemraad *et al*. (Bloemraad *et al.*, 1994) showed that infectivity of the virus disappeared after 140 h at 4°C, 3h at 37°C or 6 min at 56°C. Besides that, the virus is sensitive to ultraviolet light exposure, pH changes (<6.0 and >7.7) and, low- or non-ionic detergent containing solutions (Bloemraad *et al*., 1994). Solvents such as chloroform efficiently disrupt the lipid envelope and destroy viral infectivity (Benfield *et al.*, 1992).

1.2.3 Genomic organization and virion structure

The genome of PRRSV ranges from 14.9 kb to 15.5 kb in length (Meulenberg *et al.*, 1993) with a 3'-polyadenylated end and a 5'-capped initiation. Two untranslated regions (UTR), 5'UTR and 3'UTR, flank the protein-coding regions. The coding region comprises at least 10 open reading frames (ORFs). Downstream of 5' UTR there is the large overlapping ORF1a/1b comprising about 80% of the viral genome and encoding the non-structural proteins (nsp). Further downstream, the genome contains another eight smaller ORFs with both 5' and 3'-terminal sequences overlapping between neighbouring ORFs. These later ORFs 2a, 2b, and 3 to 7 encode structural proteins, glycoprotein 2a (GP2a), non-glycosylated protein E, GP3, GP4, GP5, the matrix protein (M), and the nucleocapsid protein (N), respectively. (Johnson *et al.*, 2011; Snijder and Kikkert, 2013). Recently, a novel non-glycosylated protein ORF5a was discovered. This protein is encoded by ORF5a which overlaps with the 5'-end of ORF5 (Firth *et al.*, 2011; Johnson *et al*., 2011).

PRRSV virions are round to egg-shaped particles with a diameter ranging from 50 to 65 nm (Spilman *et al.*, 2009). When observed in cryo-electron microscopy and tomographic reconstruction, the particles display a double-layered, hollow core and a roughly smooth outer surface with the membrane proteins embedded in it (Spilman *et al*., 2009). In the nucleocapsid core, two layers of N proteins interact with the viral RNA, forming a helical chain which is bundled into a hollow ball (Spilman *et al*., 2009). N proteins exist as disulphide-linked homodimers, being the only structural protein on PRRSV virion that lacks the transmembrane domain (Doan and Dokland, 2003; Wootton and Yoo, 2003). The outer surface is smooth, likely due to the short ectodomains of major envelope proteins GP5/M (Spilman *et al*., 2009). GP5 and M form disulphide-bound heterodimers representing more than 50% of the envelope protein mass (Mardassi *et al.*, 1996). The minor proteins GP2, E, GP3 and GP4 form tetramers incorporated into the lipid envelope (Lee and Yoo, 2006). As for the recently identified protein ORF5a, it is essential for virus viability, but its position on the lipid and the interaction with other proteins remains unknown (Firth *et al*., 2011; Johnson *et al*., 2011).

1.2.4 Genetic diversity

The current recognition of two PRRSV species reflects the genetic distance between PRRSV1 and PPRSV2 that are separated by >45% dissimilarity, an indirect evidence for their separate evolution over a long time.

The genetic diversity of PRRSV has been expanding continuously and rapidly since its emergence. The ultimate causes behind this is the high substitution rate, facilitated by the lack of 3’ proofreading capabilities of the viral polymerase, and the contribution of recombination. Intra-species similarities are as low as 70% within PRRSV1 and 78% within PRRSV2 (Snijder and Kikkert, 2013).

At the subspecies level, PRRSV1 can be classified into four major subtypes based on ORF5 and ORF7. Subtype 1 isolates are present almost worldwide (except for Latin America) and are the predominant endemic subtype in Central and Western Europe. Subtypes 2, 3 and the putative subtype 4 have only been found in Eastern Europe (eastwards of the Poland borders) (Stadejek *et al.*, 2008). This geographic diversification of PRRSV1 is thought to reflect the movement of animals and the historical circumstances in Europe after World War II. Subtype 1 was supposed to be exported from the former Soviet Union to the former German Democratic Republic and from there to the former Federal German Republic, while subtypes 2, 3 and 4 remained within the border of the Soviet Union (Stadejek *et al.*, 2006).

For PRRSV2 no subtypes have been accepted yet but nine lineages have been reported, of which two are Asiatic. These two Asian lineages are paraphyletic and are thought to have been introduced from North America in the late 1980s (Shi *et al.*, 2010b).

1.3 PRRSV replication

1.3.1 Attachment and entry

1.3.1.1 Cell tropism

PRRSV has a narrow cell tropism. *In vivo*, the replication is restricted to cells of the monocyte/macrophage lineage, preferentially highly differentiated macrophages in lungs, lymphoid tissues and in the placenta (Duan *et al.*, 1997a; Duan *et al.*, 1997b). *In vitro*, the immortalized cell line MA-104 (epithelial monkey kidney cells) and its derivatives, MARC-145, and CL-2621, also support PRRSV replication (Kim *et al.*, 1993). Those cell lines are routinely used for the *in vitro* propagation of PRRSV, especially for PRRSV2 strains, and for large-scale production of vaccine strains. Although precursors of macrophages (for example, bone marrow cells and peripheral blood monocytes) are believed to be resistant to PRRSV

infection, dendritic cells (DC) derived from them –the so-called bone marrow-derived DC (BMDC) and monocyte-derived DC (MoDC)- are susceptible (Chang *et al.*, 2008; Flores-Mendoza *et al.*, 2008; Gimeno *et al.*, 2011; Park *et al.*, 2008; Peng *et al.*, 2009; Wang *et al.*, 2007).

The tropism of the virus is thought to be determined by ORFs 2-4. Replacing ORF2-4 of PRRSV by its counterparts from EAV resulted in the loss of infectivity on macrophages but in turn acquired a tropism similar to that of EAV (Tian *et al.*, 2012).

1.3.1.2 viral entry and receptors involved

The entry and initiation of the replication cycle of PRRSV has been partially elucidated (reviewed by Zhang and Yoo (2015)). Using porcine alveolar macrophages (PAM) as a model, Nauwynck *et al.* (1999) showed that viral entry occurred via clathrin-mediated endocytosis, a fact that suggested that the viral receptors should have endocytic abilities. Early work also indicated that the virus may bind heparin and that treatment of susceptible MARC-145 cells with heparinase reduced the susceptibility of these cells (Jusa *et al.*, 1997). Later on, it was shown that the initial attachment to macrophages involved the interaction of the GP5/M heterodimer on PRRSV virions with heparan sulphate (HS) (Delputte *et al.*, 2002; Jusa *et al.*, 1997; Vanderheijden *et al.*, 2001). HS is a proteoglycan that can be found on the surface of mammalian cells and may bind several proteins (Prydz and Dalen, 2000).

The interaction of HS with the GP5/M heterodimer is unstable and in PAM the treatment with heparinase does not fully avoid the infection (Delputte *et al.*, 2005) indicating that HS is a non-essential attachment receptor. Moreover, the presence of HS on non-permissive cells is not enough to permit internalization of PRRSV virions (Delputte *et al.*, 2005). Thus, the most likely role of HS is to bring virions into close proximity of the specific receptors (Van Breedam *et al.*, 2010a). Additional research (Guo *et al.*, 2017) indicated that in late stages of viral

replication, PRRSV activates NF-κB and cathepsin L, resulting in upregulation of heparinase and cleavage of HS. This will be helpful to understand the whole replication cycle of PRRSV.

As mentioned above, endocytic receptors may contribute to the susceptibility of cells to PRRSV infection. By comparing the expression of cell surface antigens on PAM and on peripheral monocytes -permissive and non-permissive cells, respectively- a new receptor was discovered: porcine sialoadhesin (PoSn, Siglec-1) (Duan *et al.*, 1998a; Duan *et al.*, 1998b). Using immunoprecipitation assays it was confirmed that PoSn interacts with the PRRSV M/GP5 complex (Van Breedam *et al.*, 2010b) and this receptor-ligand interaction is related to the sialic acid-binding capabilities of PoSn (Van Breedam *et al*., 2010b). Vanderheijden *et al.* (2003) observed the colocalization of PoSn and PRRSV on the cell surface and beneath the plasma membrane of macrophages. The functional region of PoSn is at the N-terminal V-set domain of PoSn, between the residues 17-150 (An *et al.*, 2010). In pigs, similarly to in humans and mice, arginine 116 is the critical residue to bind sialic acid on the virus (Delputte *et al.*, 2007). Attachment to PoSn triggers viral internalisation via clathrin-mediated endocytosis which could be blocked by a mAb anti-PoSn (Delputte and Nauwynck, 2004). Further evidences of the role of PoSn was provided by transfecting the non-permissive cell line PK-15 with PoSn. This transfection allowed the internalization of PRRSV, but not the disassembly of the nucleocapsid (Vanderheijden *et al*., 2003). This experiment demonstrated that although PoSn may initiate the process of internalization, another molecule was needed to complete the release of the viral genome into the cytoplasm.

Thus, for PRRSV1 HS would probably act as unspecific attachment receptor that helps to increase the amount of virus on the cell surface and then PoSn starts the internalization process (Delputte *et al*., 2005). Besides this, the interaction of PRRSV1 with PoSn impairs the phagocytosis of PAM, a mechanism that possibly contributes to the development of secondary bacterial infections (De Baere *et al.*, 2012).

However, it is worth mentioning that the role of PoSn in the attachment and internalization of the virus has been questioned, at least for PRRSV2. Removal of N-sialic acid from the virus (the ligand for PoSn) did not result in the abolition of infectivity (Li and Murtaugh, 2015) and therefore, PoSn would not be essential for PRRSV2. In addition, the profiling of glycans in PRRSV GP5 showed that not only sialic acid but N-acetyllactosamine and N-acetylglucosamine could be involved in the binding (Li *et al.*, 2015a). Recently, Xie *et al.* (2017) identified another porcine Siglec member, Siglec-10, that was involved in the endocytosis of PRRSV.

The next step in the viral cycle involves the formation of clathrin-coated vesicles that deliver PRRS virions to early endosomes. Then, the virus fuses with the endosome and the genome is released into the cytoplasm. The endosomal stage of the cycle only involves early endosomes and does not continue to late endosomes and lysosomes, which probably helps the virus escaping deep degradation (Van Gorp *et al.*, 2009). Uncoating is related to the activity of cathepsin E (Misinzo *et al.*, 2008). In this process, CD163 is required together with a protease activity and a pH drop (pH 6.0–6.5) (reviewed by Van Breedam *et al*. (2010a)). Non-muscle myosin heavy chain 9 (MYH9) probably cooperates with CD163 for genome release (Gao *et al.*, 2016). Table 1 summarizes this process.

Table 1. Summary of the steps involved in the attachment and internalization of PRRSV virions into susceptible cells.

Step	Action	Result
1	Viral GP5/M interacts with heparan sulphate	Accumulation of virions in the cell surface
2	Sialic acid residues in the viral proteins (GP5, others?) interact with porcine sialoadhesin	The internalization process starts via the formation of an endocytic vesicle
3	CD163 interacts with the GP2a-GP3-GP4 trimer	Internalization completed, viral genome released

CD163 is a member of the scavenger receptor cysteine-rich (SRCR) superfamily, containing a nine-tandem repeat of SRCR domain (SRCR1-9) (Kristiansen *et al.*, 2001; Sanchez *et al.*, 1999; Schaer *et al.*, 2006; Van den Heuvel *et al.*, 1999). It is expressed on the cell surface and within the endosomal vesicles of PAM, being considered as the essential receptor to initiate PRRSV infection (Van Gorp *et al.*, 2008). Actually, a mAb anti-CD163 significantly reduced the infection of PAM and transfection of non-permissive cell lines CHO-K1, BHK-21 and PK-15 with recombinant CD163 conferred cells susceptibility (Calvert *et al.*, 2007; Van Gorp *et al*., 2008), although the combined participation of PoSn and CD163 is more efficient than CD163 alone (Van Gorp *et al*., 2008).

CD163 interacts with GP2a-GP3-GP4 complexes on PRRSV virions through the functional region SRCR5 (Das *et al.*, 2010; Tian *et al*., 2012; Van Gorp *et al.*, 2010). Since no surface bound or internalized virus particles were detected in CD163-transfected PK-15 cells, CD163 was assumed not to mediate PRRSV binding and internalisation (Van Gorp *et al*., 2008).

In vivo, CD163-defective pigs did not develop viremia after inoculation with a PRRSV2 strain or when exposed to infected animals; in contrast PoSn-knockouts got infected as did the controls (Whitworth *et al.*, 2016). With a similar approach, genome-edited pigs lacking CD163 SRCR5 domain were resistant to a panel of PRRSV 1 and 2 strains (Burkard *et al.*, 2017; Wells *et al.*, 2017) and substitution of the SRCR5 domain with a humane homolog impaired susceptibility of pigs against PRRSV (Wells *et al.*, 2017). Burkard *et al*. (2017) further evaluated the physiological impact on pigs after SRCR5 knocking out, but found no side effects on growth rates and blood counts.

Although CD163 is thus widely accepted as the essential receptor for PRRSV infection, there are some evidences supporting the possibility of the existence of other receptors. For example, in nasal explants the highly virulent PRRSV1 subtype 3 (Lena) was found in $PoSn^-$ or/and $CD163^-$ cells (Frydas *et al.*, 2013). Sang *et al.* (2014) described PRRSV infection in polarized porcine monocytic cells that are $CD163^-$. In another experiment, an increasing proportion of $CD163^-$ PAM were found susceptible to PRRSV at the later incubation stages (Doeschl-Wilson *et al.*, 2016).

In studies using MARC-145, another two molecules, simian vimentin and CD151, were described as putative receptors for the virus (De Baere *et al.*, 2012). Vimentin, a type III intermediate filament (IF) protein, is distributed on the cell surface and in the cytoplasm of MARC-145 cells. Expression of recombinant simian vimentin conferred susceptibility to PRRSV infection to non-susceptible cells BHK-21 and CRFK. Conversely, anti-vimentin antibodies blocked PRRSV infection of MARC-145 cells. Due to its association with other cytoskeletal filaments, vimentin was also supposed to be involved in the transportation of PRRSV inside MARC-145 cells (Kim *et al.*, 2006).

Regarding CD151, this molecule interacts with the viral 3' UTR RNA in MARC-145 cells. BHK-21 cells became susceptible upon CD151 transfection. In MARC-145 cells, transfection of CD151-targeted siRNA or overexpression of miRNA significantly impaired PRRSV replication and the infection was completely blocked by anti-CD151 antibodies. However, since no direct interaction between CD151 and PRRSV proteins has been proved yet, the exact role of CD151 remains undetermined (De Baere *et al*., 2012).

Dendritic cells (DC) derived *in vitro* are susceptible to PRRSV infection as well. But whether PRRSV and DC interact through specific receptors is largely unknown. Huang *et al.* (2009) demonstrated that porcine DC-SIGN (pDC-SIGN, CD209) mediated PRRSV binding. BHK cells expressing pDC-SIGN enhanced PRRSV transmission to target cells in trans, which occurred likely through the interaction with certain N-glycans on structural proteins of the virus. But the interaction of PRRSV with pDC-SIGN on BHK cells was not able to trigger the entry process. Blocking this interaction on MoDC with an anti-pDC-SIGN monoclonal antibody regulated specific proinflammatory gene expressions.

In summary, at present CD163 has been shown to be the only essential receptor of PRRSV. However, the evidences for other potential receptors existing could have some relevance.

1.3.2 Replication mechanism

The replication cycle of PRRSV is presumed to occur entirely in the cytoplasm (Snijder and Kikkert, 2013). Once the genome is released into the cytoplasm, ORF1a/1b are translated to produce two large polyproteins: pp1a and pp1ab. While pp1a is translated in the +1 frame; pp1ab is expressed by a mechanism of -1 programmed ribosomal frameshift (-1 PRF) just before ORF1a translation is terminated (Brierley *et al.*, 1989; Kappes and Faaberg, 2015). Then, pp1a and pp1ab undergo a post-translational processing, being cleaved into 14 nsp by the action of 4 self-encoded proteases: papain-like cysteine proteases 1α (PLP1α), PLP1β,

PLP2 and a serine protease (SP). These protease activity resides in nsp1α, nsp1β, nsp2 and nsp4, respectively (Han *et al.*, 2009; King *et al.*, 2011; Snijder *et al.*, 2013). Of these 14 nsp, 10 are encoded in ORF1a (nsp1α, nsp1β, nsp2 to nsp6, nsp7α, nsp7β and nsp8) and 4 are encoded in ORF1b (nsp9 to nsp12). Non-structural proteins 9-11 contain the domains of the replication enzymes. Thus, nsp9 contains the RNA polymerase activity; nsp10 acts as a zinc-binding domain and as a RNA helicase and, nsp11 acts as an endoribonuclease (Ulferts and Ziebuhr, 2011). Besides these, two additional nsp -nsp2TF and nsp2N, respectively- are synthesized by a -2 PRF and a - 1 PRF in the nsp2-encoding region (Fang *et al.*, 2012; Kappes and Faaberg, 2015; Li *et al.*, 2015b; Li *et al.*, 2014; Nelsen *et al.*, 1999).

The replication follows a pattern common to nidoviruses (Figure 1). This pattern includes the production of a nested set of subgenomic (sg) RNAs (sgRNAs) via a discontinuous minus-strand RNA transcription strategy (Pasternak *et al.*, 2006; Sawicki and Sawicki, 1995).

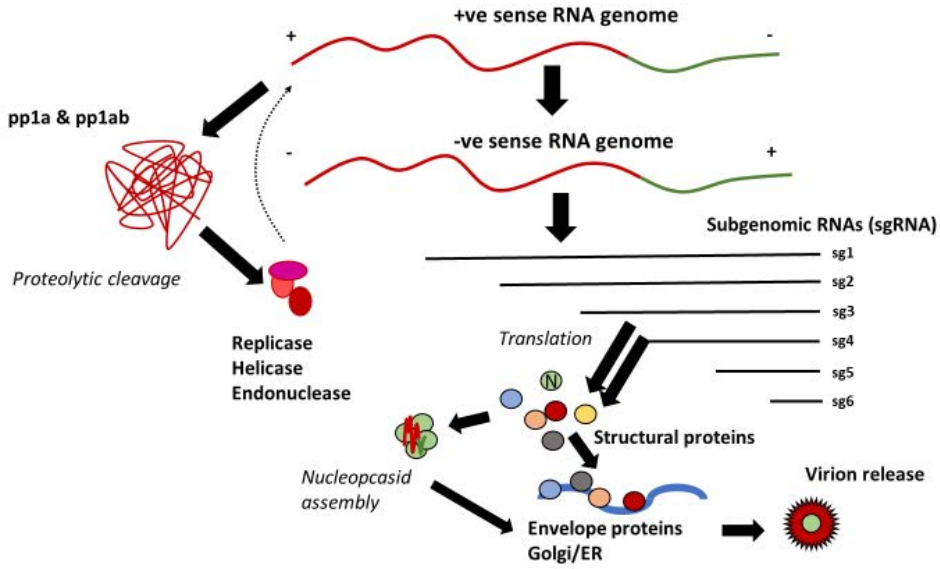

Figure 1. Schematic representation of the replication cycle of PRRSV. Initially, the viral genome segment encoding the non-structural proteins is translated into two polyproteins pp1a and pp1ab that contain all the machinery needed for the synthesis of the structural proteins. After a proteolytic cleavage of pp1ab by enzymes encoded in pp1a, the viral encoded RNA-polymerase (encoded in nsp9) produces a negative-sense copy of the viral genome. This negative-sense copy serves a template for a set of nested subgenomic RNAs that encode the structural proteins. After translation of these, a nucleopcasid complex containing a copy of the viral RNA is formed and the envelope proteins are further acquired by budding from the endoplasmic reticulum and/or Golgi apparatus.

For PRRSV, six sgRNAs (sgRNA2-7) are generated (Conzelmann *et al.*, 1993; Meng *et al.*, 1996b). Their synthesis start from a negative-sense copy of the genome that decouples from the positive-sense template at given points, designated as transcription-regulating sequence (TRS). These TRS are located near the 5'-terminal of each structural protein encoding region and form a loop with a conserved TRS (UUAACC) located at the 3'-terminal of the 5'UTR. Then the negative-strand sgRNAs are generated (van Marle *et al.*, 1999). These non-contiguous negative-sense sgRNAs are the templates for the synthesis of the positive-strand sgRNA. The positive sense sgRNA will be subsequently translated into the structural proteins (Kappes and Faaberg, 2015; Snijder *et al.*, 2013; Yun and Lee, 2013).

Finally, eight structural proteins are synthesized. GP2 and protein E are synthesized from sgRNA2; GP3 and GP4 are translated from sgRNAs 3-4; GP5 and ORF5a are produced from sgRNA5; M protein is produced from sgRNA6 and N protein is generated from sgRNA7 (Firth *et al*., 2011; Johnson *et al*., 2011; Meulenberg and Petersen-den Besten, 1996; Meulenberg *et al.*, 1995; Snijder *et al.*, 1999; van Nieuwstadt *et al.*, 1996; Wu *et al.*, 2001).

The last stage of the viral replication is virion assembly. Initially, several N proteins embedded into a newly synthesized RNA genome form a nucleocapsid complex that is a somewhat loose structure (Tijms *et al.*, 2002). Then, the nucleocapsid acquires its envelope by budding into the lumen of the smooth endoplasmic reticulum and/or Golgi apparatus, where the envelope proteins are retained. (Snijder and Kikkert, 2013; Snijder *et al*., 2013; Yun and Lee, 2013). After budding, virions are transported to the cell membrane and released into the extracellular space by exocytosis (Dea *et al.*, 1995).

1.4 Clinical signs, pathogenesis and diagnosis

1.4.1 Clinical signs

The main features of PRRSV infection are reproductive failure in sows and respiratory disease in weaners and growers. Abortions occur mainly after 90 days of gestation and, at a herd level, they are usually accompanied by an increase in the proportion of stillbirths, mummies, premature or delayed farrowing and weak-born piglets with elevated mortality rates in maternities (Kranker *et al.*, 1998; Mengeling *et al.*, 1994; Rossow, 1998). In weaned and grower pigs, the signs of the acute infection may include anorexia, lethargy, hyperemia, tachypnea/dyspnea, rough hair coats and variable reduction in daily gains (Moore *et al.*, 1990; White, 1992). By contrast, in finishing pigs, boars and non-pregnant sows, infection appears

only as a transient period of fever and occasional anorexia (Done and Paton, 1995; Stevenson *et al.*, 1993).

Gross and microscopic lesions are mainly observed in lungs and lymph nodes, where most of the viral replication takes place. In lungs, interstitial pneumonia is the predominant lesion. Affected lungs appear mottled, red-tanned and do not collapse after being removed from the thoracic cavity. Usually, the cranioventral lobes are most affected. It is worth noting that PRRSV2 isolates are more pneumovirulent than PRRSV1, resulting in less apparent lesions for PRRSV1 (Martinez-Lobo *et al.*, 2011a). Lymph nodes are moderately to severely enlarged, firm and tan in colour (Done and Paton, 1995; Halbur *et al.*, 1996a).

In the field, co-infections with bacteria like Bordetella bronchiseptica, Mycoplasma hyopneumoniae, Mycoplasma hyorhinis and Haemophilus parasuis (Brockmeier *et al.*, 2000; Thacker *et al.*, 1999), or other viruses like swine influenza virus and porcine respiratory coronavirus (Van Reeth *et al.*, 1996), complicate the disease. Experimentally, PRRSV has been claimed to favour Streptococcus suis and Haemophilus parasuis infections although the mechanism for these synergies have not been elucidated (Feng *et al.*, 2001; Galina L *et al.*, 1994; Solano *et al.*, 1998; Thanawongnuwech *et al.*, 2000a).

In 2006, a highly virulent isolate of PRRSV2 was reported in China. In that case, the disease was characterized by high fever (>41°C), high morbidity and mortality (up to 100%), and lesions never seen before in PRRS cases: lung haemorrhage and oedema, splenic infarcts, bladder dilatation with haemorrhagic urine, multifocal necrosis in kidneys, and haemorrhagic spots in lymph nodes and even in the encephala (Li *et al.*, 2007; Tian *et al*., 2007; Zhou *et al.*, 2008). The disease soon spread to Vietnam (Metwally *et al.*, 2010); Thailand, Cambodia (Jantafong *et al.*, 2015) and other countries of Asia.

In addition, in the past few years it has been shown that PRRSV1 isolates belonging to subtypes 2 and 3 may exhibit higher virulence compared to the common subtype 1 isolates. Upon infection with subtype 3 isolate Lena animals developed high fever up to 42°C and suffered higher mortality (Karniychuk *et al*., 2010; Weesendorp *et al.*, 2014), either because of the infection itself or because of the complicating agents. Later, Stadejek *et al.* (2017) showed that subtype 2 PRRSV1 also displayed increased virulence.

As a matter of fact, PRRSV2 isolates are generally thought to be more virulent than PRRSV1, at least for piglets (Martinez-Lobo *et al*., 2011a). In the case of the reproductive disease, the reports are not conclusive enough; but it is worth noting that the first highly virulent PRRS outbreak was reported with a PRRSV2 isolate in the USA causing the so-called sow abortion and mortality syndrome (SAMS) in 1996-1997 (Epperson and Holler, 1997; Halbur and Bush, 1997; Zimmerman *et al.*, 1997).

In the field, the impact of PRRSV infection is influenced by different elements including the presence of concurrent pathogens, the virulence of the acting strain, the host genetic background and the immune status of the animals. Younger pigs (4–8 weeks of age) generally suffer a severer disease compared to older ones (Thanawongnuwech *et al.*, 1998; van der Linden *et al.*, 2003) and some breeds such as the large White are thought to be more susceptible to the infection (Vincent *et al.*, 2005; Vincent *et al.*, 2006). The genetic background thus seems to be important, particularly regarding the duration of the viremia and some QTL have been identified (Boddicker *et al.*, 2014; Dekkers *et al.*, 2017; Kim *et al.*, 2013). Moreover, the farm management, including the housing style and the pig flow affect the final impact of PRRS in the herd (Goldberg *et al.*, 2000).

1.4.2 Pathogenesis

PRRS is a multisystemic disease. After entry of the virus into the organism, viremia develops as early as 12 hours post-inoculation (hpi) (Rossow *et al.*, 1995) and soon thereafter, the virus can be found in different organs where differentiated macrophages reside. Peripheral blood mononuclear cells (PBMC) and monocytes have been shown to be refractory to the infection (Duan *et al.*, 1997a; Duan *et al.*, 1997b). After some weeks, viremia disappears but the animal remains infected in the lymphoid tissue for weeks or months before finally clearing the virus from the body. Figure 2, summarizes the virologic course with its correspondence with the clinical and immunological events.

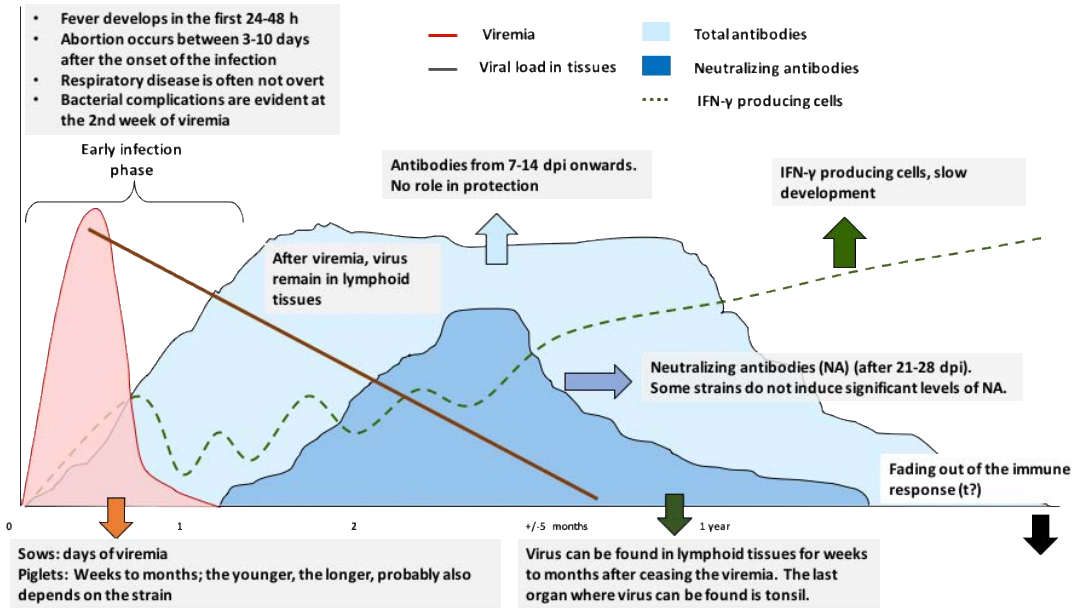

Figure 2. Schematic representation of the main events during PRRSV infection.

The main sources of virus are the lymphoid tissues and the lung. PRRSV is found in the lung during the viremic phase of the infection and sometimes after the cease of the viremia. These target cells are $CD163^{+}$ pulmonary alveolar macrophages and pulmonary intravascular macrophages (Duan *et al.*, 1997a; Thanawongnuwech *et al.*, 2000b). Macrophages in lymph nodes, spleen and tonsils are reported to be infected as well (Duan *et al.*, 1997a; Lawson *et al.*, 1997; Thanawongnuwech *et al.*, 2000b). Tonsil is the tissue where the virus persists for the

longest time (Lamontagne *et al.*, 2003). The virus can also be detected in semen up to 92 days post-inoculation (dpi) (Christopher-Hennings *et al.*, 1995a; Christopher-Hennings *et al.*, 1995b).

The typical course of PRRSV viremia is about 3-4 weeks in the piglets and 1-2 weeks in older pigs or adults. However, several factors may influence the length of this viremia, for instance the age of the animal, the strain used and the individual variation (Cho *et al.*, 2006; Diaz *et al.*, 2012; Klinge *et al.*, 2009; van der Linden *et al.*, 2003). Thus, a viremia up to three months could be found in very young pigs infected with PRRSV2 (Allende *et al.*, 2000) and viral RNA can be detected up to 251 dpi in 35-day-old pigs, although virus could be isolated only until day 56 PI (Wills *et al.*, 2003).

The infected animals can finally eliminate the circulating virus in blood, but the mechanisms leading to this viral clearance are not yet fully determined. Neutralizing antibodies (NA) have been correlated with protection in homologous models of passive transfer (Lopez *et al.*, 2007; Osorio *et al.*, 2002), but clearance of viremia has been observed in animals with or without NA (Batista *et al.*, 2004; Diaz *et al.*, 2005).

As stated above, after clearance of the viremia, the virus remains in lymphoid tissues for a long time, with the potential to transmit and produce new infections in susceptible animals. Bilodeau *et al.* (1994) found that newly introduced specific-pathogen-free (SPF) pigs were infected after contacting with previously PRRSV-infected pigs which clinical signs had disappeared more than 4 months ago (Bilodeau *et al.*, 1994). In other experiments, Allende *et al.* (2000) showed that at 150 dpi, tonsil tissue of 2/5 pigs was infectious for susceptible pigs. Similarly, non-viremic sows transmitted the infection by direct contact at 49, 56, and 86 dpi (Bierk *et al.*, 2001) and non-viremic pigs transmitted the virus to naïve sentinels up to 62 dpi (Wills *et al.*, 2002).

The clinical signs and lesions observed during the infection are thought to be the result of three different mechanisms: a) the destruction of target or bystander cells by either necrosis or apoptosis; b) the induction of inflammatory responses and, c) the interaction with the immune system (Figure 3).

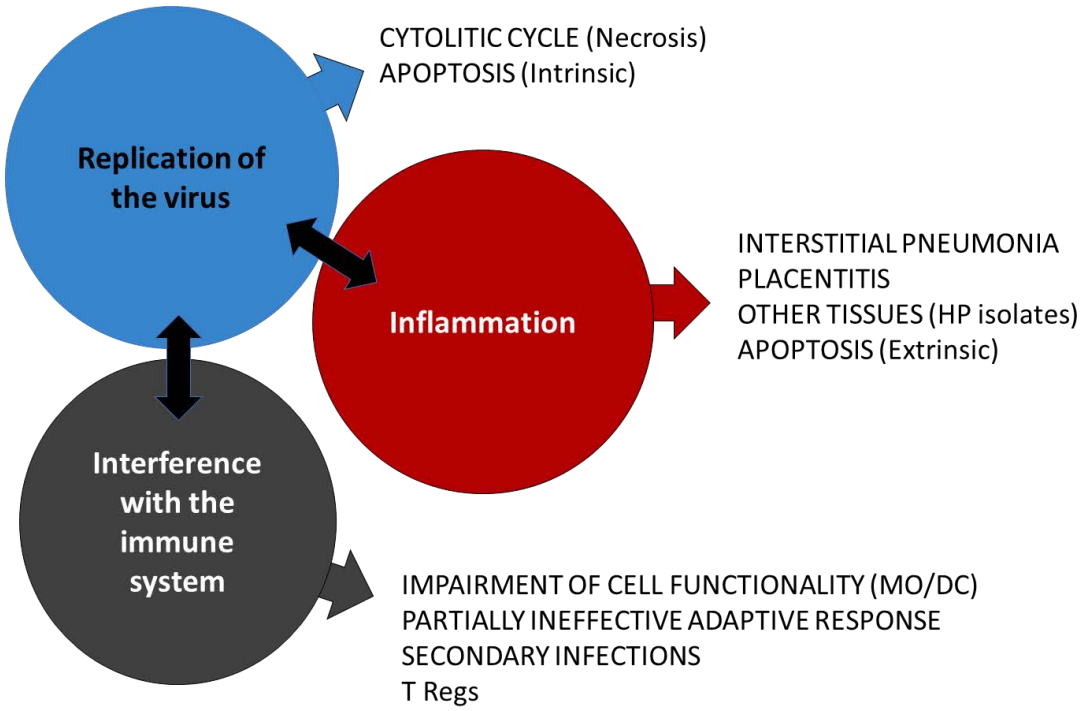

Figure 3. Schematic representation of the main mechanisms of PRRSV-associated damage to the host.

As reviewed above, by targeting PAM, PRRSV may impair the defence mechanism of the lung, favouring secondary bacterial infections. In addition, the induction of inflammatory responses, mediated by pro-inflammatory cytokines, are responsible for the development of the typical interstitial pneumonia, but also, can cause placentitis (Gomez-Laguna *et al.*, 2010; Liu *et al.*, 2010; Thanawongnuwech *et al.*, 2004; Van Gucht *et al.*, 2003). For the highly pathogenic PRRSV2 Asian strains and for the subtype 3 highly virulent PRRSV1 isolates Lena or Su1-Bel, inflammatory responses have been shown to be responsible for most of the signs and lesions observed (Han *et al.*, 2015; Liu *et al.*, 2010; Morgan *et al.*, 2013; Weesendorp *et al.*, 2014; Weesendorp *et al.*, 2016; Zhang *et al.*, 2013). Regarding the interaction of PRRS with

cells and mechanisms of the immune system, this will be reviewed in other sections of the present introduction.

1.4.3 Diagnosis

The presumptive diagnosis of PRRS is relatively easy in a previously free herd: the disease appears as a reproductive outbreak characterized by late-term abortion storms, pre-term or delayed farrowing, birth of weak-born piglets and mummified foetuses and more rarely, death of sows. When the infection moves to nurseries, respiratory disease manifests, either because of the primary action of the virus or because of the bacterial complicating agents. Interstitial pneumonia and enlarged lymph nodes are often observed (Done and Paton, 1995; Halbur *et al.*, 1996b).

In endemic phases, the clinical suspicion of PRRS is not that clear. On one hand, reproductive problems may be obscured by other circumstances and in the nurseries, several pathogens commonly act concomitantly, complicating the diagnosis. In general, the endemic phases are associated with decreased productive efficiencies as revealed by farm records.

Confirmation of the clinical suspicions must be done in the laboratory. In the epidemic phases, when the rapid identification of the etiological agent is crucial, the detection of PRRSV is most often done by RT-PCR and, less commonly, by serology or viral isolation. Nowadays, there are several RT-PCR kits available and easy-to-use for PRRSV diagnosis. The skills required for using those kits are within the common technical knowledge of most laboratory technicians of animal health laboratories. Beyond this, some of the commercial kits offer the possibility of adaptation to high throughput robotic systems that make possible the processing of hundreds of samples per day. In contrast, viral isolation requires adequate facilities for cell culture, relatively highly skilled personnel and, more critical, requires the use of PAM, since many

PRRSV isolates cannot be adapted to MARC-145, particularly with PRRSV1 strains. As a result, isolation is less used and is limited to a handful of laboratories.

For the reproductive form, RT-PCR can be used to determine viremia the day of the abortion, to detect the virus in the aborted foetuses or in the new-borns. Each approach has pros and cons. Testing the serum of aborted sows is an efficient way to confirm the infection of the female, but it must be taken into account that the abortion happens between the 3rd and the 20th day PI, most often around days 7-10, after the onset of the infection (Christianson *et al.*, 1992; Terpstra *et al.*, 1991). Since viremia in sows lasts 1-2 weeks (Christianson *et al.*, 1993; Dewey *et al.*, 1999; Lager *et al.*, 1997), for a given sow, it is technically possible that the animal was not viremic anymore when sampled after the abortion. Detection of the virus by RT-PCR in the aborted foetuses usually yields a negative result even in PRRS cases (Maldonado *et al.*, 2005). This can be explained because only a proportion of the aborted foetus get infected (about 22-36%) (Rowland, 2010) and because of the rapid destruction of viral RNA in the autolyzed foetuses. A good alternative for the diagnosis of PRRSV when an outbreak happens is to examine the weak-born piglets before taking colostrum. This can be done easily by collecting and examining umbilical cords or by bleeding piglets.

In the respiratory form, RT-PCR can also be used for identifying viremic piglets or detecting the virus post-mortem. In the latter case, lungs or lymph nodes are the reference samples. However, the interpretation of the RT-PCR positive results as a PRRSV outbreak should be cautious. In an unstable farm (Holtkamp *et al.*, 2011), there is always a flow of viremic piglets from maternities to nurseries and accordingly, transmission will happen among weaners. Since viremia in piglets can last several weeks, any other condition happening in the nurseries will overlap somewhat with the circulation of PRRSV. Thus, an accurate diagnostic approach is needed to precisely define the case, and to combine the epidemiological data, the pathological

data, the virologic data and the chronology of events to give a correct meaning to the identification of PRRSV.

As showed by Martinez-Lobo *et al*. (2011a), PRRSV1 seems to be less pneumovirulent than PRRSV2 strains. However, in the field, the complicating action of secondary pathogens, often bacteria, may obscure the diagnosis, particularly when it is based on the necropsy findings. In these cases, the combination of immunohistochemistry (IHC)/ in situ hybridization (ISH)/PCR and histopathology is a good way to see the relationship between viral detection and tissue lesions (Sur *et al.*, 1996).

For monitoring purposes, RT-PCR is adequate to trace the viral circulation in nurseries, either by examining sera of weaners or by analysing oral fluids collected in different pens (De Regge and Cay, 2016; Decorte *et al.*, 2015; Gibert *et al.*, 2017; Kittawornrat *et al.*, 2010; Kuiek *et al.*, 2015; Prickett *et al.*, 2008; Ramirez *et al.*, 2012). Compared with blood sampling, oral fluid collection is less intrusive and if pens are sampled results can be interpreted at a group level whereby it is gaining increasing popularity.

Serology is another diagnostic approach commonly used in the laboratory, mainly by using ELISA. Antibodies appear as soon as 7 dpi and almost all animals are seropositive by day 14 PI (Labarque *et al.*, 2000; Meier *et al.*, 2003; Nelson *et al.*, 1994; Yoon *et al.*, 1992; Yoon *et al.*, 1994). In this initial phase, the antibodies are directed mainly against the N protein. Most available ELISAs are made based on this protein although some also include GP5. Since N protein contains epitopes that are shared by PRRSV1 and PRRSV2, most of these ELISAs are universal. However, there are differential ELISAs for PRRSV1 or PRRSV2 (Seuberlich *et al.*, 2002; Sorensen *et al.*, 1998). This is useful when both viruses co-exist on farms in one area.

Besides these, other techniques such as the immunoperoxidase monolayer assay (IPMA), immunofluorescence assay (IFA) or virus neutralization test (VNT) are available as well, but mainly for research purposes (Benfield *et al.*, 2000; Rovira *et al.*, 2007; Snijder *et al*., 2013).

1.5 Immune response against PRRSV

1.5.1 Innate immune response

1.5.1.1 Interference of TLR- and RIG-mediated signalling

In mammals, pathogens are sensed by cell membrane or intracellular pattern recognition receptors (PRRs). Host PRRs recognizing RNA viruses include Toll-like receptors (TLRs) and RIG -(retinoic acid inducible gene) I- like receptors (RLRs). TLRs that can sense viral RNA are TLR3 and TLR7. Both are endosomal TLRs respectively recognizing double and single stranded RNA (Bowie and Unterholzner, 2008; Sun *et al.*, 2012; Thompson *et al.*, 2011). RIG-I is an intracellular receptor for viral dsRNA (Bowie and Unterholzner, 2008; Gantier and Williams, 2007).

PRRSV mainly interferes with the TLR3 signalling pathway. Sang *et al.* (2008) and Miller *et al.* (2009) reported that activation of TLR3 by using chemical or exogenous dsRNA decreased the replication of PRRSV, while the same treatment on TIR (Toll/interleukin-1 receptor)-domain truncated TLR3 did not. In PRRSV-infected pigs, Miguel *et al.* (2010) and Liu *et al.* (2009) detected the upregulation of TLR3 in discrete brain areas and the lymphoid tissues. In another study, Chaung *et al.* (2010) presented a contradictory result, showing that *in vitro* infection of PAMs and immature DC resulted in a transient inhibition of TLR3. Moreover, according to Kuzemtseva *et al.* (2014), TLR3 expression increased in PAM infected with PRRSV1 isolate 3262 but much less with isolate 3267, indicating the regulation of TLR3 may be strain-dependent. By contrast, TLR7 and TLR9 were not affected by PRRSV infection.

There are also evidences that PRRSV may interact with RIG-I. Luo *et al.* (2008) and Song *et al.* (2010) showed that PRRSV nsp1α inactivated RIG-I in the IFNβ induction pathway. Huang *et al.* (2014) reported that nsp4 antagonized RIG-I-mediated NF-κB activation. And Sun *et al.* (2016) showed nsp11 interferes with transcription and translation of two critical factors, MAVS (mitochondrial antiviral-signalling protein) and RIG-I, in the RLR-mediated pathway.

1.5.1.2 Inhibition of type I interferons (IFN)

Production of type I IFNs represents the most effective innate antiviral immune response, limiting viral replication and spread (Baum and Garcia-Sastre, 2010). PRRSV appears to inhibit the production of type I IFN in PAM and in myeloid DC. In an early experiment, Albina *et al.* (1998a) showed that PAM exposed to PRRSV did not produce any detectable amount of IFN-α. When PAM were further superinfected with the gastroenteritis transmissible virus -a potent inducer of type I IFN- PRRSV abolished IFN-α production as well.

Buddaert *et al.* (1998) also studied the *in vitro* and *in vivo* expression of IFN-α after PRRSV infection. Their results indicated that induction of IFN-α by PRRSV *in vitro* was very low but seemed not to be affected *in vivo*, since the cytokine could be detected in the lung of infected animals. In any case, treatment of PAM with recombinant type I IFN significantly blocked PRRSV replication. Royaee *et al.* (2004) provided evidence that exogenous addition of IFN-α during PRRSV vaccination increased the Th1 response. This observation partly explains the poor cell-mediated response generated after vaccination.

The inhibition of IFN depends to some extent on the strains as evidenced by Lee *et al.* (2004). Those authors examined IFN-α responses of macrophages and observed that different isolates -and even some plaque-purified variants- have different sensitivities to IFN-α and induced different levels of this cytokine.

When plasmacytoid DC (pDC) were examined, results were more controversial. While Calzada-Nova *et al.* (2011) indicated that PRRSV2 inhibited the release of type I IFN in pDC regardless of the virus viability, Baumann *et al.* (2013) showed that all examined PRRSV1 and most PRRSV2 strains had no or very weak suppression of IFN-α in enriched pDC. Most likely, only some PRRSV isolates had the capability of inhibiting pDC functionality, among them were several highly pathogenic PRRSV2 strains (Baumann *et al*., 2013). The effects of PRRSV on different types of DC are revised in depth in another section of this introduction.

Regarding the possible mechanisms involved in the inhibition of type I IFNs, Miller and Fox (2004) showed that, blocking of the transcription in MARC-145 cells, was the most likely cause. However, many other studies suggested a post-transcriptional regulatory mechanism by showing abundant IFNα/β gene transcripts but negligible amounts of the cytokine protein in PRRSV-infected macrophages, MoDC and, DC collected from lungs (Lee *et al*., 2004; Loving *et al.*, 2007; Zhang *et al.*, 2012).

Until now, five PRRSV proteins have been identified as IFN antagonists, including four non-structural proteins: nsp1, nsp2, nsp4 and nsp11, and the N protein. Among these antagonists, nsp1 is considered the most potent inhibitor. Nsp1 contains two sub-units designated as nsp1α and nsp1β (Kappes and Faaberg, 2015). Non-structural protein 1β inhibits dsRNA-mediated IRF3 phosphorylation and nuclear translocation, and inhibits STAT1 translocation in JAK–STAT signalling pathway, resulting in the inhibition of both IFN synthesis and signalling (Chen *et al.*, 2010; Song *et al*., 2010).

Non-structural protein 2, the biggest nsp of the virus, contains a cysteine protease domain (belonging to the ovarian tumor protease family, OTU) that possesses ubiquitin-deconjugating activity. *In vitro*, PRRSV infected cells suffer a blocking of IRF3 phosphorylation and nuclear

translocation (Li *et al.*, 2010), and the inhibition of NF-κB activation by the OTU domain (Sun *et al.*, 2010).

For nsp4, it has been shown that this protein suppresses IFNβ transcription by blocking NF-κB (Huang *et al.*, 2014) and, nsp11 inhibits Poly(I:C)-induced IFN*β* production through the endoribonuclease activity (Shi *et al.*, 2011). N protein, similarly to nsp1, localizes in both cytoplasm and nucleus. Its ability to suppress type I IFN induction has been verified, but whether the nuclear translocation is involved remains unknown (Huang *et al.*, 2015).

1.5.1.3 TNF-α, IL-10 and other cytokines

Tumor necrosis factor-α (TNF-α) is one of the central pro-inflammatory cytokines. It can be produced as a part of the innate immunity by macrophages, DC and NK cells, and in the development of acquired responses by T-lymphocytes.

The role of TNF-α in PRRSV infection has been thoroughly studied. Early works reported contradictory results that some authors found an inhibition of this cytokine (Lopez-Fuertes *et al.*, 2000; Thanawongnuwech *et al.*, 2001; Van Reeth *et al.*, 1999), while others observed an induction (Ait-Ali *et al.*, 2007; Chen *et al.*, 2010; Choi *et al.*, 2002). Darwich *et al.* (2011) clarified this issue by showing that the induction of TNF-α was strain-dependent. Chen *et al.* (Chen *et al.*, 2010) indicated that the production of TNF-α in PRRSV-stimulated (or infected) cells may result from the response to a variable part of nsp2.

IL-1 and IL-8 are also considered as important pro-inflammatory cytokines in PRRS and, in general, have been related to the development of inflammatory responses during the infection, for example, development of interstitial pneumonia (Aasted *et al.*, 2002; Labarque *et al.*, 2003; Thanawongnuwech *et al.*, 2001; Van Gucht *et al.*, 2003; Van Reeth *et al.*, 1999).

IL-10, however, is a crucial immune-regulatory cytokine that can inhibit the production of inflammatory cytokines and counteract adaptive immunity. A variety of cells, including monocytes/macrophages, DC, T and B cells, can be induced to produce IL-10. Several studies (Diaz *et al*., 2005; Diaz *et al.*, 2006; Peng *et al*., 2009; Silva-Campa *et al.*, 2009; Suradhat and Thanawongnuwech, 2003; Suradhat *et al.*, 2003) indicated that *in vitro* infection with PRRSV resulted in an increase of IL-10 levels. However, other studies suggested a minor role of this cytokine (Silva-Campa *et al.*, 2010; Subramaniam *et al.*, 2011). Later, this discrepancy was explained by the observation that different PRRSV isolates showed different profiles in IL-10 induction (Darwich *et al*., 2011; Silva-Campa *et al*., 2010).

1.5.2 Adaptive immune response

1.5.2.1 Humoral immunity

The antibody response develops rapidly during PRRSV infection, with most animals seroconverting by 14 dpi (Labarque *et al*., 2000; Meier *et al*., 2003; Nelson *et al*., 1994; Yoon *et al*., 1992; Yoon *et al*., 1994). Early antibodies mostly belong to the IgM class, with a later switch to IgG, that become predominant by day 21 PI. Those early antibodies are mostly directed against the 15kDa N protein, followed by the 19kDa M protein and then the 26kDa GP5 (Loemba *et al.*, 1996; Nelson *et al*., 1994). The antibodies generated can last several months. For example, according to Lager *et al*. (1997) sows can be seropositive until 604 dpi. Antibodies against epitopes in the nsp are also found early after the onset of the infection, particularly anti-nsp2 antibodies which kinetic is similar to that of anti-N antibodies (de Lima *et al.*, 2006; Oleksiewicz *et al.*, 2001).

The early antibodies are non-neutralizing and are not associated with protection. Conversely, they may contribute to the antibody-dependent enhancement (ADE) of viral replication. This phenomenon is thought to be based on the formation of virus-antibody complexes that bind to

the Fc receptor of macrophages, facilitating thus the viral binding and uptake. The phenomenon of ADE is still controversial with different studies reporting somewhat conflicting results regarding the actual occurrence and importance in PRRSV infection (Delputte *et al.*, 2004; Yoon *et al.*, 1996).

Neutralizing antibodies (NA) usually appear after the fourth week of infection for both PRRSV1 and PRRSV2, being rarely detected before (Diaz *et al*., 2005; Yoon *et al*., 1994). This delayed development is thought to be the result of several circumstances including the shielding of envelope proteins by glycosylations (Ansari *et al.*, 2006; Faaberg *et al.*, 2006), the existence of decoy epitopes close to the neutralization epitopes (NE) (Fang *et al.*, 2006; Ostrowski *et al.*, 2002) and factors that may deter the antigen presentation (Ansari *et al*., 2006). At present, NE have been described or suspected in GP5, GP4, GP3, GP2 and M proteins (Cancel-Tirado *et al.*, 2004; Costers *et al.*, 2010; E *et al.*, 1999; Gonin *et al.*, 1999; Kim and Yoon, 2008; Kwang *et al.*, 1999a; Kwang *et al.*, 1999b; Meulenberg *et al.*, 1997; Pirzadeh and Dea, 1997; Plagemann *et al.*, 2002; Yang *et al.*, 2000). For many years it was assumed that GP5 contained the main neutralization epitope (E *et al*., 1999; Gonin *et al*., 1999; Ostrowski *et al*., 2002; Pirzadeh and Dea, 1997; Plagemann *et al*., 2002; Wissink *et al.*, 2003; Yang *et al*., 2000). Nevertheless, some studies questioned the role of GP5 for the induction of neutralizing antibodies (Li and Murtaugh, 2012).

NA play a critical role in the control of viral infections, and are believed to be important for PRRSV control as well (Lopez and Osorio, 2004). Passive transfer of NA can block viremia, protect sows from reproductive failure and prevent transplacental infection in a titre-dependent manner (Lopez *et al*., 2007; Osorio *et al*., 2002). However, in experimentally infected animals viremia could be found in presence of NA (Vezina *et al.*, 1996). Also, viremia can be resolved before the development of NA (Diaz *et al*., 2006). These data reflect that probably NA are

important to prevent infection if already present but the role of humoral immunity in the clearance of the natural infection is more controversial.

Another point that add difficulties to the understanding of the role of NA is the not well explained basis for their cross-reactivity. While the homologous NA have certainly a protective capacity as explained above, at present, the cross reactivity cannot be forecasted with precision. As shown by (Martinez-Lobo *et al.*, 2011b) in an experiment of cross-neutralization, different isolates had different susceptibility to neutralization by hyperimmune sera raised against other isolates. They reported that no correlation could be found between the sequence of known NE or the number of N-linked glycosylation sites in different proteins and the neutralization phenotype of the isolates. Robinson *et al.* (2015) reported that broadly NA -even between PRRSV1 and PRRSv2- although rare, may indeed occur under field circumstances and therefore, a biological basis for this phenomenon must exist. Trible *et al.* (2015), showed that in PRRSV2, Tyr-10 in the M protein could be critical for conferring susceptibility to heterologous neutralization since its deletion rendered the isolate susceptible only to homologous antisera. Popescu *et al.* (2017) suggested that for GP5, susceptibility to broadly reactive NA, may be blocked by non-neutralizing antibodies adjacent to the neutralization epitope as well as by conformational changes in the GP5-M heterodimer.

1.5.2.2 Cell-mediated immunity (CMI)

In most viral infections, the cell-mediated adaptive response is considered crucial to eliminate infected cells by means of the cytotoxic activity. However, until now, the development of cytotoxic CD8 lymphocytes have not been proved for PRRSV in functional experiments (Costers *et al.*, 2009).

Early studies used the lymphoproliferation assay to elucidate the cell-mediated immune response to PRRSV (Bautista and Molitor, 1997). Proliferative responses could be detected at

the 4th week of infection and began to decline by the 11th week PI. $CD8^+$ T-cells increase after the first month of infection (Albina *et al.*, 1998b; Dwivedi *et al.*, 2012; Kawashima *et al.*, 1999; Shimizu *et al.*, 1996), but their precise role in the clearance of PRRSV is not yet known. $CD4^+$ T-cells are supposed to contribute to recall responses (Lopez Fuertes *et al.*, 1999; Loving *et al.*, 2015).

Most often, the determination of PRRSV-specific IFN-γ secreting cells (IFN-γ-SC) have been used as an indicator of the development of cell-mediated immunity (Diaz and Mateu, 2005; Meier *et al*., 2003). PRRSV-specific IFN-γ-SC appear around the second week of infection; then, they increase gradually and reach a plateau several weeks later (Diaz *et al*., 2006; Klinge *et al*., 2009; Meier *et al*., 2003). These IFN-γ-SC are mainly composed of double positive CD4/CD8 cells, with a small percentage of $CD4^-/CD8\alpha\beta^+$ T-cells (Silva-Campa *et al*., 2010). Nevertheless, the frequency of IFN-γ-SC after infection or vaccination is 3-4 times lower than the frequencies reached with pseudorabies virus (Meier *et al*., 2003) indicating that the cell-mediated response to PRRSV is impaired for some reasons. The significance of IFN-γ in protective immunity against PRRSV is uncertain although high levels of these cells seem to be related to protection (Diaz *et al*., 2006).

One hypothesis to explain the causes of delayed and relatively low IFN-γ responses could be the development of regulatory T cells (Tregs). Wongyanin *et al.* (2010) demonstrated that transfection of MoDC with the nucleocapsid protein of PRRSV2 resulted in the generation of TGF-β-producing Tregs. Silva-Campa *et al.* (2012) showed that during the infection with a PRRSV2 strain, pigs also developed $CD4^+CD8^+CD25^+Foxp3^+$ Tregs. However, some apparently contradictory observations have been published. For example, in the work of Silva-Campa *et al*. (2009), PRRSV1 upregulated IL-10 production of DC but did not generate Tregs. Rodriguez-Gomez *et al.* (2015) did not observe the proliferation of Tregs with either PRRSV1 or PRRSV2. These inconsistencies may result from different strains used.

1.5.3 Apoptosis

Cell death is a critical event that maintains tissue homeostasis in multicellular organisms. It can result from non-physiological processes (for example, injury) or can happen as a part of a regulated process, often called programmed cell death (PCD). Apoptosis is a type of PCD aimed to remove redundant cells and maintain tissue homeostasis in a non-immunogenic manner (Ravichandran and Lorenz, 2007; van Delft *et al.*, 2010). It restrains harmful molecules in the intact dead cells that will then be rapidly recognized and cleared by the phagocytes.

The term apoptosis was first described by Kerr *et al.* (1972) to describe a particular type of cell death characterized by cell shrinkage, nuclear condensation, and plasma membrane blebbing. Usually, apoptosis involves the exposure of phosphatidylserine residues on the outer leaflet of plasma membrane (earlier phases), a massive activation of the caspases cascade and finally the fragmentation of nuclear DNA (later stages). All of these phenomena are used to assess apoptosis.

Triggering of apoptosis may occur by what are known as the intrinsic or the extrinsic pathway (Elmore, 2007). The intrinsic pathway is activated by sensing of stress signals (DNA damage, unfolding of the endoplasmic reticulum, hypoxia, etc.) that activate BH3-only proteins leading to a series of events that will end with the permeabilization of the outer mitochondria membrane. This permeabilization allows the release of cytochrome c to the cytosol. Cytochrome c subsequently binds to its adaptor Apaf-1 (apoptotic protease activating factor-1), causing the autocleavage of caspase-9 and finally activating the executioner caspases (caspase-3 and caspase-7) (Chinnaiyan, 1999; Hill *et al.*, 2004; Saelens *et al.*, 2004). In the extrinsic pathway, the triggering event is the binding of substances such as TNF-α and Fas ligand (FasL) to the so-called death receptors in the cell membrane (for example the tumour necrosis factor-related apoptosis-inducing ligand receptor (TRAILR))(Locksley *et al.*, 2001).

This binding activates pro-caspase8 and pro-caspse10 and will finally end with activation of caspase-3 and caspase-7 (Kischkel *et al.*, 1995). Crosstalk between the two pathways occurs through caspase-8 cleavage of Bid to truncated Bid (tBid) which translocates to the mitochondrion activating intrinsic pathway (Tait and Green, 2010). (Figure 4 summarizes these pathways)

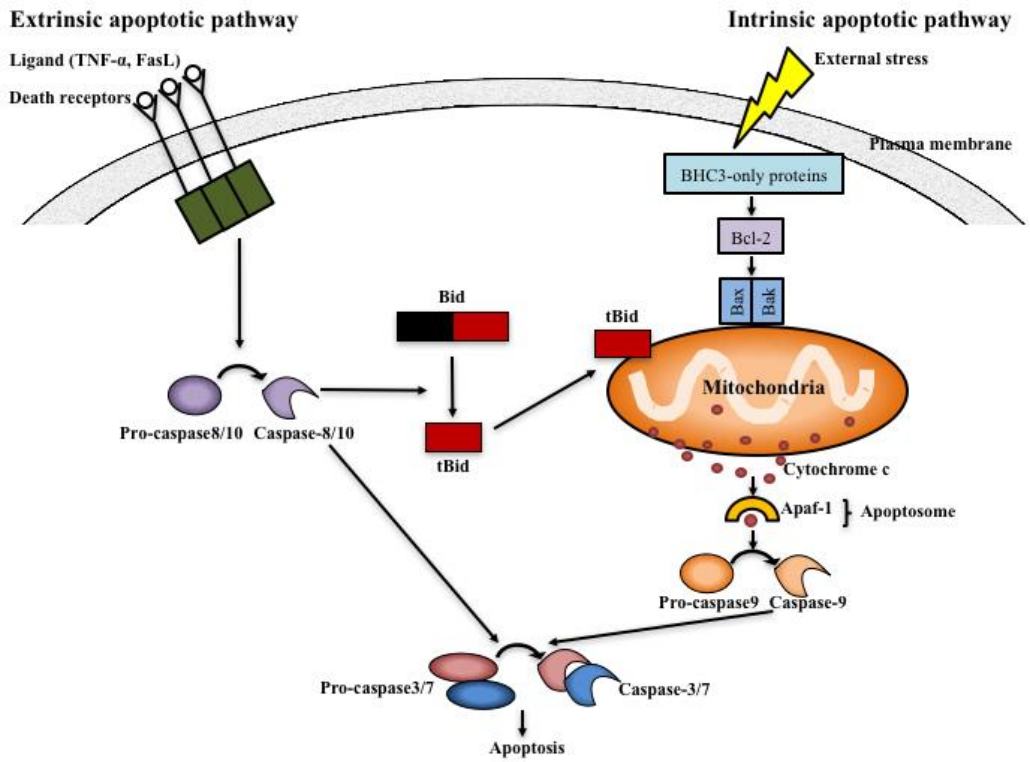

Figure 4. Extrinsic and intrinsic apoptotic signalling pathways.

Besides apoptosis, virus infection can also activate necrosis, a caspase-independent form of programmed cell death (Ravichandran and Lorenz, 2007; van Delft *et al*., 2010). It will result in plasma membrane rupture, pro-inflammatory molecule release and collateral tissue damage.

In the case of PRRSV infection, apoptotic cells have been observed both *in vivo* and *in vitro*. According to Sur *et al*. (Sur *et al.*, 1997; Sur *et al.*, 1998), PRRSV-induced apoptosis was widely distributed within infected tissues, including lungs, testes and lymph nodes. Upon

performing dual-labelling experiment, most apoptotic cells were shown to be non-infected bystanders (Labarque *et al*., 2003; Sirinarumitr *et al.*, 1998; Sur *et al*., 1998).

However, *in vitro* studies with MARC-145 cells, Kim *et al.* (2002) provided evidences of apoptosis in the infected cells. The virus-mediated apoptosis happened in the late infection stage. Consistently, another study carried out using macrophages further outlined that apoptosis may be modulated by PRRSV replication (Costers *et al.*, 2008). Thus, at early stages of infection, it was shown an evident anti-apoptotic activity, but from 12 hpi onwards, the infected macrophages started to die in a caspase-dependent mechanism highly indicative that they underwent apoptosis. Both studies (Costers *et al*., 2008; Kim *et al*., 2002) agreed that many cells showed the characteristics of necrosis-like cell death.

Miller and Fox (2004) evaluated 26 apoptosis-related genes in PRRSV-infected MARC-145 cells, and found that they were unaltered during the first 24 h of infection. Thus, they proposed that PRRSV did not induce apoptosis; instead, necrosis was shown as the main contributor to cell death. Given the genetic and immunobiological diversity of PRRSV isolates, the use of different strains might explain some of the abovementioned discrepancies.

Generally, viral proteins can interfere with the development of apoptosis by directly interacting with the elements in the apoptotic signalling pathways or, by regulating pro- and anti-apoptotic genes in transcription. Until now, the precise mechanism by which PRRSV induces apoptosis is still undetermined, and some conflicting evidence makes the picture more confused.

PRRSV GP5 was firstly reported to induce apoptosis (Suarez *et al.*, 1996). But this was not confirmed in the work performed by Lee *et al*. (2004). Later on, nsp4 was identified as the apoptotic inducer (Ma *et al.*, 2013), while GP2a and nsp11 were identified as inhibitors of apoptosis (Sun *et al*., 2012).

A study conducted by Yin *et al.* (2012) demonstrated that activation of the Jun-N-terminal kinase (JNK) signalling pathway is a key event to induce apoptosis upon PRRSV infection. Huo *et al.* (2013) further showed that p53 and Akt at the early stage of infection were also activated, negatively regulating JNK activation to counteract PRRSV-mediated apoptosis.

Moreover, apoptosis may contribute to the pathogenesis in another way. According to Barker *et al.* (2002), after phagocytosis of apoptotic cells, macrophages became ineffective to stimulate T cell proliferation, and inversely secreted inhibitory cytokines like TGF-β or IL-10 to suppress inflammation. This is consistent with low inflammation of lungs in pigs after PRRSV infection.

In any case, it is still unclear to what extent cell death occurs in infected, bystander cells or both after PRRSV infection and, if different isolates may have a different impact on the development of apoptosis.

Dendritic cells (DC)

2.1 DC Ontogeny

Dendritic cells have been identified based on the morphology, phenotype, function and cytokine profiles; however, the ontogeny of these cells remained somewhat obscure until recently. Since DC could be derived from monocytes, for many years it was considered that DC shared a common ontogeny with macrophages. Nevertheless, ontogenic studies of the murine immune system have identified a common DC precursor (CDP) in bone marrow (Merad *et al.*, 2013). Actually, DC differentiated from CDP are considered as the only bona fide DC subsets. In contrast, monocyte-derived DC (MoDC), have a common monocyte precursor in bone marrow and later move to the blood for seeding both lymphoid tissue and non-lymphoid tissue as resident DC in steady-state conditions (Summerfield *et al.*, 2015). This trafficking is accelerated under inflammation. In addition to the above-mentioned types of DC, there are Langerhans cells (LCs), the first DC subset discovered in the epidermis by Paul Langerhans in 1868 (Langerhans, 1868). LC arise from embryonic foetal liver monocytes, independently of bone marrow derived precursors (Hoeffel *et al.*, 2012).

According to the studies in mice and humans, bona fide DC include classical or conventional DC (cDC), and plasmacytoid DC (pDC). cDC are specialized in antigen presentation and primary T-cell activation, including two lineages: a) cDC1 accounting for $CD8\alpha^+$ ($CD103^+$) DC and, b) cDC2 accounting for $CD11b^+$ DC (Guilliams *et al.*, 2014). CD8a ($CD103^+$) DC are characterized by cross-presenting of endogenous and exogenous antigens to $CD8^+$ T cells and promoting Th1 immune responses at mucosal surfaces, while $CD11b^+$ DC prime both $CD4^+$ and $CD8^+$ T-cells and are more proinflammatory (Schlitzer and Ginhoux, 2014). By contrast, pDC are considered to be the anti-viral DC since they are the most potent producer of type I

IFNs. For this reason they are often called natural IFN producing cells (NIPC) (Summerfield *et al*., 2015).

In domestic animals although cDC1 and cDC2 can still be recognized, the distribution of cell surface markers can be different. Summerfield *et al*. (2015) have recently reviewed this issue. Figure 5 shows the molecular markers on four porcine DC subsets.

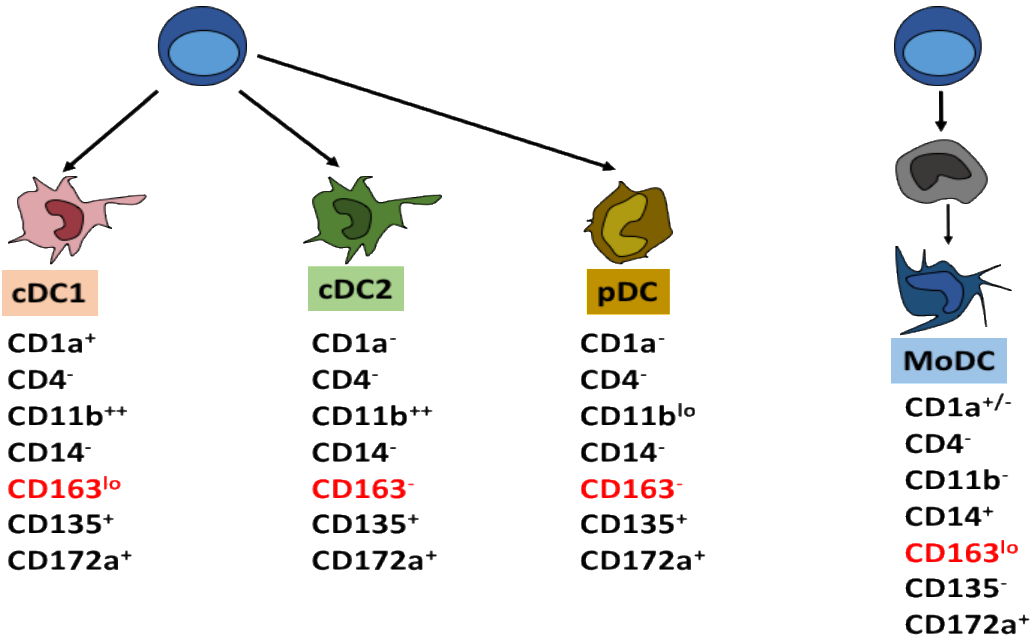

Figure 5. Proposed classification of porcine DC subsets and markers identified on each subset. This figure is adapted from Summerfield *et al*. (2015).

2.2 DC maturation, trafficking and antigen presentation

DC are considered as immunological sentinels specialized in pathogen-sensing and pathogen-presentation (Lambotin *et al.*, 2010). In accordance with this function, they are present in almost all tissues, especially in those that are constantly exposed to the environment and the microorganisms.

In the tissues, DC are considered to exist in two different functional states: immature and mature DC. This is a somewhat misleading classification since maturation may be regarded either at the phenotypic level or at the functional level. Phenotypically, maturation means that the DC express high levels of ligands such as CD80/86, along with the MHC class II molecule, that enable them for an efficient antigen presentation. Functionally, the mature DC are highly efficient in releasing cytokines that are adequate for the environmental context; that is, favouring inflammatory or regulatory responses depending on the nature of the pathogen encountered (reviewed by Dudek *et al.* (2013)).

In contrast, immature DC are very efficient in antigen capture: a) they can take up particles and microbes by phagocytosis (Inaba *et al.*, 1993), 2) they can form pinocytic vesicles to catch extracellular fluid and solutes in a process known as macropinocytosis (Sallusto and Lanzavecchia, 1994) and, 3) they express a series of pathogen recognition receptors that mediate endocytosis (Sallusto *et al.*, 1995). DC-SIGN (CD209) is one of the most relevant endocytic receptors. It mediates the binding of viruses like human immunodeficiency virus (HIV), hepatitis C virus (HCV), SARS coronavirus, human herpesviruses, Ebola virus and dengue virus (DV) (Freer and Matteucci, 2009).

Once the immature DC encounter the pathogen, they undergo several phenotypic and functional changes that will lead to it transformation to semi-mature DC with full antigen-presenting capabilities. These changes include at least: a) down-regulation of the endocytic/phagocytic receptors; b) upregulation of antigen-loaded MHC-II along with the costimulatory molecules CD80, CD86, CD40 and CD40L; c) shift in lysosomal compartments with up-regulation of DC–lysosome-associated membrane proteins; and d) morphological changes and the acquisition of high cellular motility (Banchereau *et al.*, 2000; Byrne and Halliday, 2002).

The now-maturing DC migrate from peripheral tissues to secondary lymphoid organs via afferent lymphatic vessels. This migration involves a coordinated action of several chemokines. DC down-regulate CCR6 (receptor for the immature DC specific chemokine MIP-3α) (Charbonnier *et al.*, 1999), and up-regulate CCR7 (receptor for secondary lymphoid tissue chemokine MIP-3β and 6Ckine) (Yanagihara *et al.*, 1998), enabling them to leave the inflamed tissues and home to draining lymph nodes.

In the lymph node, DC are driven into the paracortical area in response to MIP-3b and/or 6Ckine, which are produced by cells over the T cell areas (Dieu-Nosjean *et al.*, 1999). This is critical for antigen-bearing DC to encounter T cells.

Antigen presentation by DC, may develop in different ways depending on the localization of the antigens. Soluble and particulate antigens captured by immature DC are always targeted towards MHC-II-rich compartments (MIICs), namely, the lysosome-related intracellular compartments, where MHC class II-associated invariant chain (Ii) constantly accumulate. In the case of viruses, viral proteins are degraded into small peptides in the MIICs. Upon maturation, the invariant MHC-II chain is cleaved by cathepsin S. Then, the peptide-loaded MHC-II are exported to the cell surface to be recognized by $CD4^+$ T helper cells. T helper cells will later assist B cells to proliferate and mature into antibody-producing cells (Neefjes *et al.*, 2011).

When the antigen (for example, a virus) is found in the cytosolic compartment of the DC, the endogenous MHC-I pathway of presentation happens. In this case, the multi-subunit proteasome complex in the cytosol degrades the viral proteins. Then, the generated peptides are translocated into the endoplasmic reticulum (ER) lumen with the aid of the transporter associated with antigen processing (TAP). In the ER, MHC-I molecules are folded and assembled. After peptide loading, the MHC-I/peptide complexes dissociate from TAP and

traffic through the Golgi apparatus to the plasma membrane (Hewitt, 2003). There, they can be recognized by virus-specific $CD8^+$ cytotoxic T cells (CTLs).

Sometimes DC can also acquire exogenous antigens from the infectious agent and present them on MHC-I molecules. This is known as cross-presentation. This process can happen through 'cytosolic' and 'vacuolar' pathways. Viral proteins in the 'cytosolic' pathway are supposed to be degraded by the proteasome in the cytosol. The generated peptides are then loaded as in the classical MHC-I presentation pathway. By contrast, cross-presentation through the 'vacuolar' pathway is sensitive to lysosomal proteolysis inhibitors, implying the occurrence of antigen processing and loading on MHC-I molecules is in the endocytic compartments (Joffre *et al.*, 2012).

2.3 *In vitro* studies of the host-pathogen interaction using DC

Due to the central role of DC -sensing pathogens and acting as the bridge between the innate and the adaptive immunity- they have become a major target for studying the interaction between pathogens and the host and for understanding the early events that afterwards drive the immune response.

In the tissues of mammals, DC are a heterogeneous population deriving from different precursors and in different stages of maturation and activation. Accordingly, they are different in terms of phenotypes, roles and functions. As stated before these elements are the basis to classify and identify DC (reviewed by Vu Manh *et al.* (2015)).

The advent of multicolour flow cytometry enabled a deeper characterization of DC. While phenotypic and functional characterization of DC has been extensively done in mice and human, for domestic animals like pigs, the scarcity of reagents and the lack of unique markers for different subsets of DC are still serious bottlenecks for the study. For example, it is still

difficult to distinguish MoDC from macrophages (Merad *et al*., 2013). Moreover, DC are a rare cell population in the body, comprising fewer than 1% of leukocytes in the blood, and even less than 0.5% in lymphoid tissues (Autissier *et al.*, 2010). As a result, separation of DC from tissues usually yield a low number of cells, creating thus an additional difficulty for phenotyping or functional characterization of these cells. To solve this problem, most studies rely on the *in vitro* generation of MoDC and BMDC.

2.4 Dysregulation of DC is a strategy for viral survival

Viral pathogens have evolved mechanisms to escape the immune system by hijacking DC in the immune response. Table 3, summarizes some of these mechanisms.

Table 3. Some examples of evasion mechanisms of viruses based on the dysregulation or killing of DC.

Mechanism	Pathogen	Result
Inhibition of maturation	Influenza virus Murine cytomegalovirus	Impaired antigen presentation
Inhibition of migration	Hepatitis C virus Human immunodeficiency virus	Impaired antigen presentation
Cell death by apoptosis or by direct infection	Herpes simplex virus Human cytomegalovirus Epstein–Barr virus	Decreased capacity for efficient antigen presentation
Induction of IL-10	Several viruses	Down-regulation of cytotoxic responses
Induction of Tregs	Retrovirus	Lack of effective adaptive T-cell response

For example, some viruses may interfere with the maturation of DC (the case of influenza virus and murine cytomegalovirus) (Rinaldo and Piazza, 2004; van de Sandt *et al.*, 2012). Some other viruses may modulate trafficking of DC. In the infection of HCV, DC are trapped in the liver being inhibited to migrate to the draining lymph nodes (Nattermann *et al.*, 2006). Depletion of DC can happen as well. In HIV, the virus causes depletion of pDC by inducing

apoptosis upon their migration to the inflamed lymph nodes (Brown *et al.*, 2009). HIV may also take advantage of the migratory abilities of DC by firstly being sequestered by DC-SIGN in DC (Freer and Matteucci, 2009) and then being efficiently transferred to $CD4^+$ T cells in the lymph nodes.

Regulation of the cytokines patterns of DC is also a common mechanism for escaping the immune system. For instance, suppression of IL-12 and/or promotion of IL-10 may affect virus-specific Th1 cells and cytotoxic T lymphocytes (Steinman and Hemmi, 2006). In addition, some viruses promote apoptosis of infected DC (for example, herpes simplex virus) (Jones *et al.*, 2003) or of the effector T-cells through DC (for example, human cytomegalovirus and Measles virus) (Raftery *et al.*, 2001; Schneider-Schaulies *et al.*, 2003). And in some cases, antigen-loaded DC may induce the proliferation of regulatory T cells, which is an important mechanism for evasion of retroviruses (Balkow *et al.*, 2007).

2.5 Porcine DC generated *in vitro*

Several studies have identified in mice the essential cytokines and transcription factors required for differentiation of bona fide DC, MoDC, and LC. The well-known cytokines and growth factors include:

- Fms-related tyrosine kinase 3 (Flt3) ligand (Flt3L): This is an essential growth factor for the development of both cDC and pDC (Murphy *et al.*, 2016).
- Granulocyte-macrophage colony-stimulating factor (GM-CSF): Also known as colony stimulating factor 2 (CSF2). It is critical to develop MoDC and is used to generate DC from bone marrow as well (Carrasco *et al.*, 2001).
- Macrophage-CSF: This growth factor is for the generation of LC (Murphy *et al.*, 2016).

2.5.1 Monocyte-derived DC (MoDC)

In comparison to other species, porcine MoDC can be generated by stimulating blood monocytes with GM-CSF and IL-4 (Carrasco *et al*., 2001). Blood monocytes are initially isolated by adhering porcine peripheral blood mononuclear cells (PBMC) to plastic for 16 h and removing then the non-adherent cells or, alternatively, by separating the $CD14^+$ cells present in blood. Cells are fed every 3 days with 150 ng/ml rGM-CSF and 100U/ml rpIL-4. After 7 days, the non-adherent and loosely adherent cells are harvested. When examined by electron microscopy, the resulting cells show the ultrastructural characteristics of DC with large diameter, membranous protrusions, and contain abundant multivacuolar and multilamellar vesicles (Carrasco *et al*., 2001). With the same method, Chamorro *et al*. (2004) produced MoDC starting from $CD163^+$ and $CD163^-$ monocytes. Compared to $CD163^-$ MoDC, $CD163^+$ MoDC possess more advanced maturation characteristics.

It is worth to note here that, in spite that the standard method for obtaining MoDC uses IL-4, the role of this cytokine in pigs is controversial and most of the activity attributed to IL-4 in other species is thought to be done by IL-13 in pigs. Actually, a method using IL-13 has been reported (Bautista *et al.*, 2007). Other cytokines can also be included in the process of generating MoDC. Addition of IFN-α to the GM-CSF/IL-4 cocktail results in MoDC with enhanced ability to stimulate cell proliferation in a mixed leukocyte culture (Johansson *et al.*, 2003). Adding TGF-β1 resulted in the production of DC-like cells with moderate amounts of Birbeck granules (Paillot *et al.*, 2001) compatible with LCs.

2.5.2 Bone marrow-derived DC (BMDC)

Bone marrow-derived DC (BMDC) are generated by stimulating bone marrow hematopoietic cells (BMHC) with 25 ng/ml rpGM-CSF for 8 days (Carrasco *et al*., 2001). Addition of TNF-α results in further maturation. After 8 days, the culture consists of a mixture of adherent and

non-adherent cells. Most of the non-adherent and loosely adherent cells show DC-like morphology and are designated as BMDC. This is a highly heterogeneous population that may contain some immature precursors and granulocytic cells. The adherent population is mainly comprised by macrophage-like cells. Addition of TNF-α has no effect on morphological/phenotypic characteristics of BMDC, except for enhancing their T-cell stimulatory capacity.

In both cases, MoDc and BMDC, mainly represent inflammatory DC, although they are convenient for *in vitro* studies. MoDc and BMDC do not harbour CD135 (Flt3), a pan-marker of cDC1 and cDC2 (Guzylack-Piriou *et al.*, 2010). This is consistent with the transcriptional studies on human and mouse DC where Flt3 expression was observed to be restricted to blood cDC and pDC but not on GM-CSF-derived DC (Robbins *et al.*, 2008).

2.6 Modulation of DC by PRRSV infection

2.6.1 Modulation of MHC-II, CD80/86 and other immunologically relevant molecules

Both MoDC and BMDC can be infected by PRRSV (Chang *et al*., 2008; Flores-Mendoza *et al*., 2008; Gimeno *et al*., 2011; Park *et al*., 2008; Peng *et al*., 2009; Wang *et al*., 2007). Given the frequency of secondary infections in PRRSV-infected animals, the study of the effects of the viral infection on antigen presentation has been an obvious subject.

A correct antigen presentation requires three signals: a) presentation of the antigen in the histocompatibility complex, b) The expression of co-stimulatory molecules such as CD80/86 and, c) the production and release of the adequate cytokines to create a correct milieu. These three elements have been the focus of some studies.

The examination of the expression of MHC-II and CD80/86 in infected DC produced conflicting results. While some authors reported a down-regulation of MHC-II (Chang *et al*., 2008; Flores-Mendoza *et al*., 2008; Gimeno *et al*., 2011; Park *et al*., 2008; Peng *et al*., 2009; Wang *et al*., 2007), others did not find obvious changes (Silva-Campa *et al*., 2010). For the CD80/86 costimulatory molecules, both up- and down-regulation have been observed (Chang *et al*., 2008; Flores-Mendoza *et al*., 2008; Gimeno *et al*., 2011; Park *et al*., 2008; Peng *et al*., 2009). However, and at least for PRRSV1, the effect of the infection upon the expression of markers related to antigen presentation or maturation seem to be largely dependent on the isolates used (Gimeno *et al*., 2011; Weesendorp *et al*., 2013). Thus, using four PRRSV1 subtype 1 isolates to infect BMDC (Gimeno *et al*., 2011), three of them down-regulated MHC I while the expression of MHC-II, CD80/86, CD14 and CD163, varied depending on the strain. Again, with BMDC the infection with isolate Lena (PRRSV1 subtype 3) resulted in a decreased expression of CD14, CD172a and CD163. Also, MHC-II was downregulated in $CD163^+$ cells. No significant differences were detected for MHC II and CD80/86. Similarly, with MoDC different effects on MHC-II and CD80/86 were reported (Park *et al*., 2008; Silva-Campa *et al*., 2010).

Consistent with the conflicting results in the expression of MHC-II and CD80/86 on PRRSV-infected BMDC or MoDC, the ability of these cells to stimulate T cells are also under debate. Whereas Rodriguez-Gomez *et al*. (2015) did not find obvious inhibition by PRRSV1- or PRRSV2-infected MoDC on T-cell proliferation, Park *et al*. (2008) observed the mixed leucocytes were less stimulated when co-cultured with PRRSV2-infected BMDC. By contrast, Charerntantanakul *et al.* (2006) clarified the suppressive ability of PRRSV on T-cell responses was not associated with infected-DC or macrophages, but the infected monocytes. A recombinant PRRSV antigen targeting to porcine DC-SIGN (pDC-SIGN) has been proved to induce a higher number of antigen-specific $CD4^+$ T-cell immunity, indicating that pDC-SIGN

targeting might serve as a complementary strategy to existing vaccines (Subramaniam *et al.*, 2014).

2.6.2 Modulation of cytokine production

With respect to the impact of PRRSV infection on the cytokine patterns of DC, Gimeno *et al.* (2011) reported that different PRRSV1 isolates (n=39) induced different patterns regarding the secretion of IL-10 and TNF-α in BMDC, peripheral blood CD172a^{+} (SwC3^{+}) cells and alveolar macrophages. Actually, the four potential combinations resulting from the induction or not of TNF-α and IL-10 were observed in BMDC. Park *et al*. (2008) observed a significant increase in IL-10 expression when using MoDC infected with a PRRSV2 strain.

Regarding other cytokines, the results seem to be more uniform. Thus, Gimeno *et al*. (2011) showed that all PRRSV1 examined isolates induced high levels of IL-8 and IL-1 in PAM and also in DC (personal communication) and none of the examined isolates induced significant amounts of IFN-α in BMDC or other types of DC, set aside pDC (Baumann *et al*., 2013; Calzada-Nova *et al.*, 2010; Calzada-Nova *et al*., 2011). Lung DC (Loving *et al*., 2007) and pDC (Baumann *et al*., 2013; Calzada-Nova *et al*., 2011) are reported to be refractory to PRRSV infection.

2.6.3 Generation of Tregs

In the last 10 years, Tregs, either natural or antigen specific, have been in the centre of the debate for explaining the unusual adaptive immune response against PRRSV (Mateu and Diaz, 2008). Silva-Campa *et al*. (2010) and Wongyanin *et al*. (2010) demonstrated an *in vitro* induction of Tregs when PRRSV2-infected MoDC were seeded into peripheral blood mononuclear cells (PBMCs). In contrast, Charerntantanakul *et al*. (2006) showed that T-cell suppression was not associated with PRRSV2-infected MoDC. In other experiments with

PRRSV1 strains or with a high virulence PRRSV2 strain (VR2385) in MoDC, Tregs were not induced (Cecere *et al.*, 2012; Silva-Campa *et al*., 2010). Since Tregs have been shown to develop with PRRSV2 *in vivo* (Silva-Campa *et al*., 2012), the precise role of DC in this phenomenon remains to be fully clarified.

2.6.4 Inhibition of plasmacytoid DC (pDC)

pDC are anti-viral DC and the main source of natural IFN-α. In an experiment conducted by Calzada-Nova *et al*. (2010), pDC were refractory to PRRSV infection and PRRSV-related inhibition of IFN-α responses in these cells happened regardless of virus viability. According to Calzada-Nova *et al*. (Calzada-Nova *et al*., 2010; Calzada-Nova *et al*., 2011), the inhibitory effect was related with the unaltered or reduced intracellular interferon regulatory factor 7 (IRF-7), which is required for IFN-α gene transcription. This contrasted with the abundant IFN-a transcripts detected in PRRSV-infected MoDC, although no IFN-α in protein level occurred (Zhang *et al*., 2012).

In contrast, Baumann *et al*. (2013) showed that PRRSV1 and most PRRSV2 did not significantly suppress IFN-α responses in pDC. An exception was a highly virulent PRRSV2 isolate from China. Consistently with this notion, Garcia-Nicolas *et al.* (2016) showed that the highly virulent PRRSV1 subtype 3 isolate Lena did not induce IFN-α production in pDC while other isolates did. Interestingly, in the same paper, the authors showed the production of IFN-α by pDC increased when a tight contact between PRRSV-infected macrophages and pDC was permitted. These results indicated that pDC may sense PRRSV better than thought previously, but for some strains the inhibition of IFN-α may indeed exist. Overall, the induction of IFN-α in pDC by PRRSV is an extremely complex issue that is far from being fully understood.

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Hypotheses and objectives

Rationale and hypotheses

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically important pathogens for the swine industry. There are two recognized species: PRRSV1 and PRRSV2, with a high degree of genetic variation intra- and inter-species and diverse virulence.

PRRSV can establish long-term infections in pigs. This is thought to be the result, at least partially, of a delayed development of protective responses, namely neutralizing antibodies and cell-mediated immunity effectors. One possible cause for this delayed response is the interaction of the virus with the immune system. Macrophages and dendritic cells (DC) – professional antigen-presenting cells- are susceptible to PRRSV infection. However, while the infection of macrophages has been thoroughly studied, much less is known about the infection of DC.

In other studies, different PRRSV1 isolates were shown to have different immunobiological properties, including the effect of the replication in DC on the cytokine patterns or on the expression of cell surface molecules important for the functionality of the immune system. Nevertheless, the replication of the virus in DC has not been studied in depth.

Based on those previous observations, we hypothesize that different PRRSV1 isolates may have different behaviours when interacting with DC regarding replication kinetics or yield and the induction of apoptosis.

In addition, the maturation stage of the DC could play a role in the susceptibility to infection. As CD163 is considered as the essential receptor for PRRSV infection, and porcine sialoadhesin (PoSn) is involved in the interaction with the virus, the expression of these

receptors on the cell surface could be related to the susceptibility. Some reports indicated that in nasal explants and in polarized porcine monocytic cells, apparently $CD163^-$ cells were found to be infected when certain PRRSV isolates were used for the infection. Those observations suggested that other receptors might exist.

Objectives

1. To characterize the kinetics of replication of three PRRSV1 isolates in immature and mature bone marrow-derived DC (BMDC).

2. To examine the attachment of three PRRSV1 isolates on immature BMDC and mature BMDC.

3. To examine the attachment and replication of the three PRRSV1 isolates on immature BMDC based on the expression of PoSn and CD163.

4. To examine the effect of heparan sulphate removal from the cell surface of BMDC on the attachment of different PRRSV1 isolates.

5. To examine the susceptibility of $CD163^-$ BMDC to PRRSV1 infection.

6. To characterize the apoptosis induced by PRRSV1 infection in BMDC.

PART II: Studies

Study 1 Different isolates of *Porcine reproductive and respiratory syndrome virus 1* (PRRSV1) produce different patterns of infection in bone marrow-derived dendritic cells and apparently may infect PoSn/CD163 negative cells.

Study 2 Apoptosis induced by different PRRSV1 isolates occurs in both infected and bystander cells and depends on the isolate used and on IL-10 presence.

Study 1 Different isolates of *Porcine reproductive and respiratory syndrome virus* 1 (PRRSV1) produce different patterns of infection in bone marrow-derived dendritic cells and apparently may infect PoSn/CD163 negative cells.

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Keywords: *Porcine reproductive and respiratory syndrome virus*, bone marrow-derived dendritic cells, CD163, PoSn

Abstract

The present study was aimed to examine the replication and attachment of different PRRSV1 isolates in bone marrow-derived dendritic cells (BMDC). For this purpose, immature (i) and mature (m) BMDC were infected with three PRRSV1 isolates: 3249, 3262 and 3267. The kinetics of replication were assessed by titration of cell culture supernatants in macrophages. The attachment of PRRSV to different cell subsets was assessed by confocal microscopy. In the replication experiment, two isolates (3249 and 3262), reached peak titres earlier in iBMDC (24 hpi) compared to the titres in mBMDC (48 hpi). For the other isolate (3267) peak was reached at 24 hpi for both cell types. Moreover, the titres of all three isolates at 12 and 24 hpi were significantly ($p<0.05$) higher in iBMDC than in mBMDC. In both cell types, isolate 3262 showed the lowest titres at 12 and 24 hpi. These results indicated that iBMDC were more efficient in supporting PRRSV1 replication and this feature was not related to the proportion of $CD163^+$ cells or to the levels of IFN-α in the cultures. In the attachment experiments, isolates 3249 and 3267 were seen to attach on iBMDC regardless of the expression of CD163 or porcine sialoadhesin (PoSn). The attachment was not fully avoided after treatment with heparinase. Isolate 3262 showed the lowest attachment signal. When replication of the different isolates was examined by confocal microscopy, PRRSV1 isolates 3249 and 3267 apparently replicated in $CD163^-$ iBMDC. Additional sorting and flow cytometry examination showed that infected $CD163^-$ cells were only seen when $CD163^{hi}$ or $CD163^{lo}$ cells were included in the culture. Thus, the susceptible $CD163^-$ might arise because of the milieu created by the $CD163^+$ infected BMDC. Alternatively, these permissive $CD163^-$ subset could account for cells with a very low expression of CD163, beyond the technical sensitivity of the means used but enough to allow the infection.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the costliest diseases of pigs and many efforts are being directed to the control of the infection. Its causative agents are PRRS viruses (PRRSV1 and 2) enveloped, positive-strand RNA viruses belonging to the Genus Porateriviridae1, Family Arteriviridae, within the Order Nidovirales [1]. The PRRS virion consists of a nucleocapsid (protein N), with several membrane proteins embedded including major envelope proteins GP5/M, minor proteins GP2a, E, GP3 and GP4 and a recently discovered ORF5a protein.

PRRSV has a narrow tropism for cells of the monocyte/macrophage lineage, preferentially highly differentiated macrophages located in lungs, lymphoid tissues and placenta *in vivo* [4,5]. This restricted cell tropism is the result of the expression of receptors adequate for viral entry. At present CD163 is thought to be essential for the infection of macrophages [6,7,8]. Besides this, other receptors such as porcine sialoadhesin (PoSn) or CD151 have been identified [9,10,11].

The process of entry involves the interaction of PoSn with the M/GP5 heterodimers on the virus that triggers endocytosis. Upon internalisation, in early endosomes, CD163 interacts with the trimer GP2-GP3-GP4 resulting in genome release into cytoplasm initiating replication [9,10,12,13,14,15]. Heparan sulphate (HS) also plays a role in the attachment of the virus [16,17,18].

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

In several *in vitro* experiments, PRRSV could also productively infect bone marrow-derived (BMDC) and monocyte-derived DCs (MoDC), and 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 immune response [19-24].

Given the heterogeneity of DC [25] and the genetic diversity of PRRSV isolates, it was hypothesized that different PRRSV isolates and cells of 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 high health pigs 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 [22-24].

Bone marrow hematopoietic cells (BMHC) were aseptically isolated from the femora and humeri of 4-week-old pigs. Briefly, bones were cut into 1cm2 pieces and agitated in PBS at room temperature for 60 min. Then, the cell suspension was filtered through a 40 μm strainer after being depleted of erythrocytes by 0.15 M NH4Cl. Cells were washed and frozen in liquid nitrogen until use. 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*. [29] 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 [24,30] and 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 could have an impact on virus infectivity, PEG was removed by adding solid KCl and spinning at 12,000 g. Viral concentrates were titrated again and no differences in titres were seen, accordingly the centrifugation step was omitted afterwards. The PEG-concentrated virus was used in the attachment experiment to reach a higher MOI, while the original viral stock was used for the infection.

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

Plates (48-well, 2.5×105 cells/well) were seeded with PAM, iBMDCs or mBMDCs 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% fetal 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 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). Experiments were run in triplicates.

The replication in iBMDC at earlier times, 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) as the secondary antibody (ThermoFisher, Madrid, Spain). Cells were analysed on a FACSCalibur cytometer (BD Biosciences).

To test whether potential differences in replication between iBMDC and mBMDC were caused by cytokines, IFNα and TNF-α were tested with cell culture supernatants by capture ELISA. For IFN-α, K9 (R&D Systems) and biotinylated F17 (R&D Systems; Thermofisher) mAb were used 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). For TNF-α, a commercial TNF-α Swine ELISA Kit (Life Technologies, Madrid, Spain) was used according to the manufacturer's directions. In both cases, optical density (OD) of the mock-inoculated cultures were used to assess the background. Samples were examined in triplicate.

For the comparison of the CD163 expression in iBMDC, mBMDC and PAM, a flow cytometry assay was performed. Cells were fixed as above stained with mAb anti-pig CD163 (clone 2A10/11, BioRad, Oxford, UK) followed by anti-mouse IgG1 conjugated with Alexa Fluor

488 (ThermoFisher) and 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 the density of 2 × 105 cells/well. Cells were cooled down for 10 min on ice and then isolates 3249, 3262 and 3267 were added at a 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 room temperature (RT) for 10 min. For the double staining (PRRSV/CD163 or PRRSV/PoSn) the antibodies used were: mAb 1C5H anti-PRRSV N protein, mAb 3B11/11 anti-PoSn and 2A10/11 anti-CD163 (both from Bio-Rad). Antibodies conjugated with Alexa Fluor 610-RPE or Alexa Fluor 488 were used as secondary antibodies. Non-specific binding of secondary antibodies was reduced with a horse serum (10% in PBS) blocking. Samples without virus and samples omitted primary antibodies were used as negative controls. In the final step staining were mounted with ProlongGold Antifade mounting with DAPI (ThermoFisher). Images were captured with a Leica TCS SP5 confocal microscopy (Wetzlar, Germany). Channel merging and image processing was performed with ImageJ [31].

Since attachment of PRRSV1 was observed on either CD163 or PoSn negative cells, a three-color immunofluorescence labelling (plus DAPI nuclear staining) with a MOI 3 was produced to further assess whether CD163/PoSn double negative cells would permit viral attachment. In this case, only iBMDC were used and mAb anti-CD163:RPE (clone 2A10/11, Bio-Rad) in

combination with mAbs for PRRSV (Ingenasa) and PoSn (Bio-Rad) were used. Secondary antibodies conjugated (ThermoFisher) with Alexa Fluor 633 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 of the cells. Then, cells were subjected to the CD163/PoSn/PRRSV staining as stated above.

2.6. Visualisation of PRRSV1 replication 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 Cell sorting.

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 (clone 2A10/11, 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 antibody. Unstained cells, background from Alexa Fluor 647 alone and irrelevant isotype-matched mAb labelled with Alexa 647 were used as gating reference. Since the autofluorescence of BMDC was high, the fluorescence channel adjacent to that of Alexa 647 was examined at the same time to further discriminate between signals coming from labelling and autofluorescence. The staining divided BMDC into three populations based on the expression of CD163 that were defined as $CD163^{-}$, $CD163^{lo}$ 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. Because of the autofluorescence of live BMDC and the strict gating criterion, some contamination by $CD163^{-}$ cells was seen in the $CD163^{+}$ cells. Thus, the purity of sorted $CD163^{-}$ cells was 99.0% and 96.5% for the $CD163^{+}$. The second sorting assay, grouped together $CD163^{-}$ and $CD163^{lo}$ cells, and $CD163^{hi}$ cells were left in a separate population. The purities after sorting were 96.1% and 96.2%, respectively. The population of $CD163^{-}$ plus $CD163^{lo}$ were contaminated by only 0.2% of $CD163^{hi}$ cells.

The sorted BMDC were seeded in 24-well plates (5×10^{5} cells/well) and 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.

2.8 Analysis of PRRSV1 replication and CD163 expression in BMDC by flow cytometry.

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 ℃. 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 titrated for optimal staining performance beforehand. Cells were washed x3 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. The single-stained cells were used as fluorescence minus one (FMO) and compensation controls. The flow cytometry readings were analysed using FCS Express 6 (De Novo Software).

2.9. Statistical analysis.

Comparison of means in CPE assay, cytokine measurement and flow cytometry was 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.

Figure 1 depicts the kinetics of replication of the three PRRSV1 isolates examined in iBMDC and mBMDC. In iBMDC, all three isolates reached peak titres in the cell culture supernatants at 24 hpi. Also, for all three isolates, titres at 12 hpi were significantly ($p<0.05$) higher in iBMDC than in mBMDC. This difference tended to narrow at 24 hpi, but was still statistically significant. In contrast, in mBMDC 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 (Supplementary figure S1).

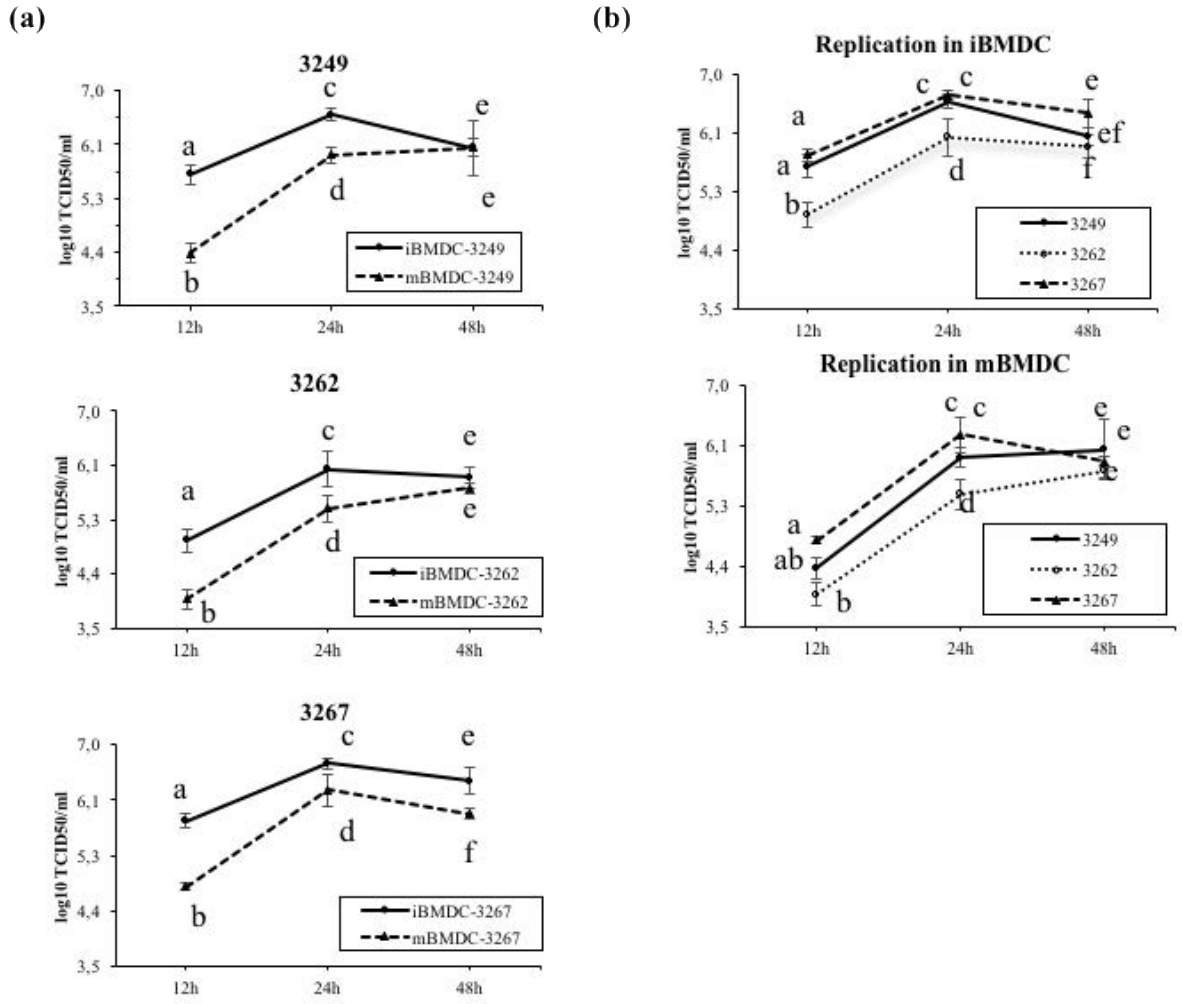


Figure 1. Titres of different PRRSV1 isolates in cell culture supernatants of immature (i) and mature (m) bone marrow-derived dendritic cells (BMDC). The figure shows the titres attained in cell culture supernatants of iBMDC and mBMDC when infected with different PRRSV1 isolates as determined by titration in PAM. (a) Replication of each isolate (3249, 3262 or 3267) in iBMDC and mBMDC was compared. (b) Replication of the three isolates in iBMDC or mBMDC was compared. Error bars account for standard deviations of three replicas. Different letters indicate significant differences ($p<0.05$).

Regarding the induction of cytokine expression, PRRSV was not able to induce detectable levels of IFN-α in any of the cultures. As expected from previous experiments, isolates 3249 and 3262 induced the release of TNF-α in iBMDC (up to 62 pg/mL) while isolate 3267 did not. TNF-α was not detected in mBMDC for all three isolates (data not shown).

Next, we examined the expression of CD163 in different cell types by flow cytometry. Before infection 66% of the mBMDC showed positive staining for CD163 and 57% of the iBMDC were $CD163^{+}$ (Supplementary figure S2).

When the kinetics of the infection in BMDC were examined by flow cytometry (Figure 2), significant differences ($p<0.05$) were seen between isolates. Thus, the proportion of infected cells for isolate 3262 was always lower, leading to think that an early event in the replication cycle, such as the attachment of the virus could be involved. Anyway, at 24h of incubation more than 30% of the iBMDC were infected (Supplementary figure S3 shows the results obtained in flow cytometry).

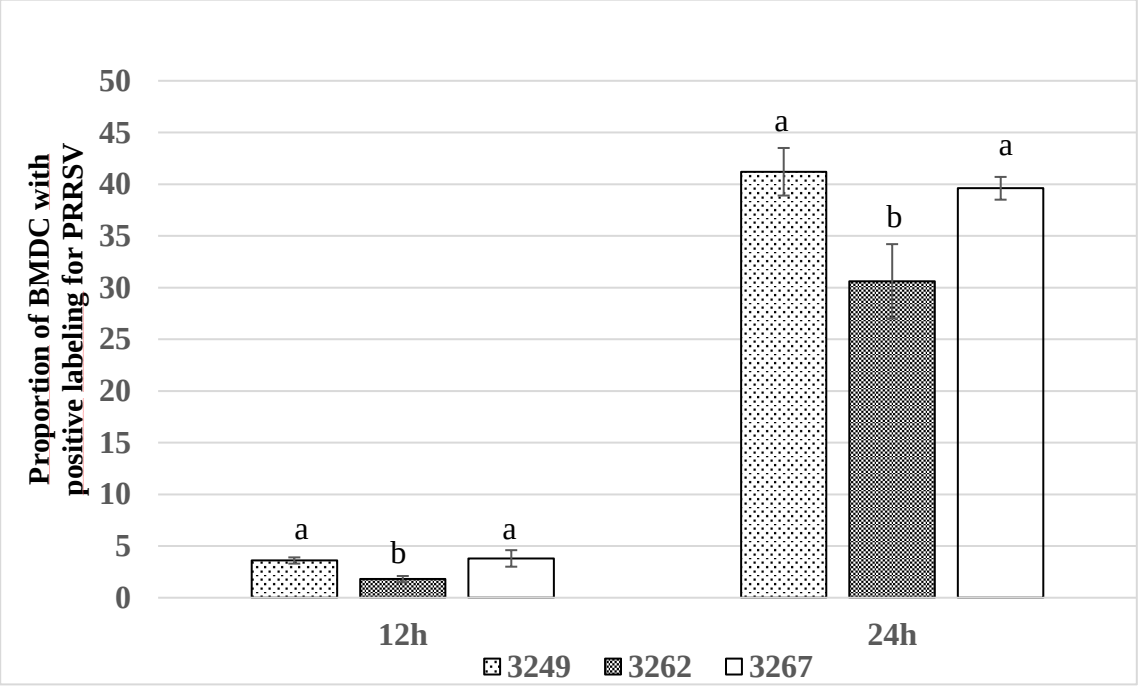


Figure 2. Proportion of virus-infected immature bone-marrow-derived dendritic cells (iBMDC) at different times. The graph shows the average proportion of infected cells with three PRRSV1 isolates after 12 or 24 h of culture (triplicates) as determined by flow cytometry. Bars depict the standard deviation. Different letters indicate statistically significant differences ($p<0.05$).

3.2. Intensity of the attachment to BMDC depends on the PRRSV1 isolate examined.

The examination of the attachment of different PRRSV1 isolates to iBMDC (Figures 3-5) 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 (supplementary figure S4 shows the comparison of isolate 3262 attaching to iBMDC and mBMDC). In PAM, the attachment of isolate 3262 was also lower than the other two isolates, but not so low as in iBMDC (Supplementary figure S5).

3.3. Colocalization of PRRSV1 attachment and PoSn/CD163 expression.

In the two-colour immunofluorescence labelling, PRRSV1 attachment to the $PoSn^-$ or $CD163^-$ subsets could be observed for both iBMDC and mBMDC. A further three-color labelling on iBMDC showed that besides double positive PoSn/CD163 cells, isolates 3249 and 3267 were also seen attached to single positive subsets ($PoSn^+/CD163^-$ and $PoSn^-/CD163^+$) and, more interestingly to the double negative subset $PoSn^-/CD163^-$ (Figures 3 and 5).

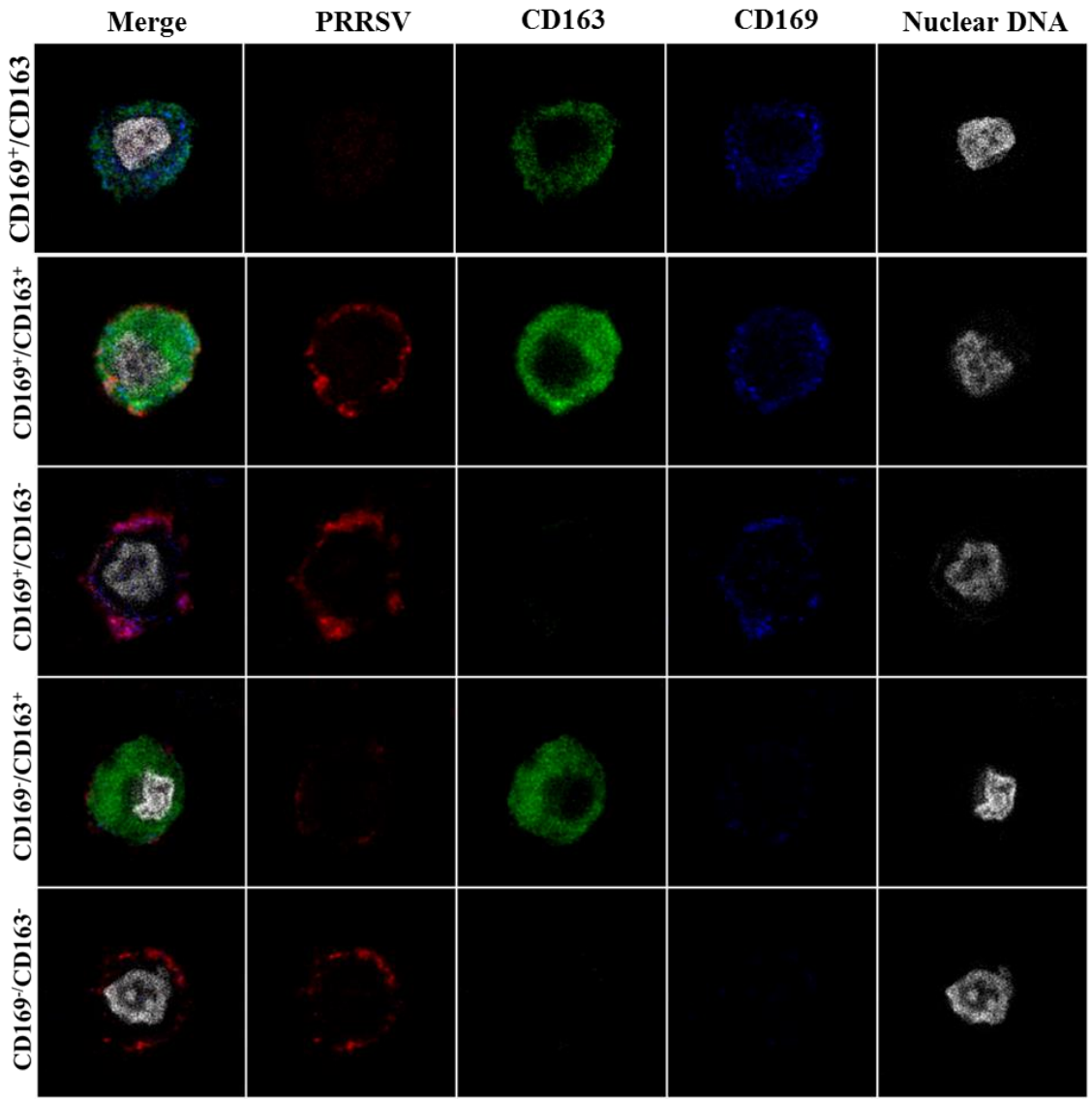


Figure 3. Attachment of isolate 3249 to different subsets of immature bone marrow-derived dendritic cells (iBMDC). The picture shows the attachment of isolate 3249 to the subsets defined by PoSn and CD163 as determined in confocal microscopy. The upper row shows uninfected cells.

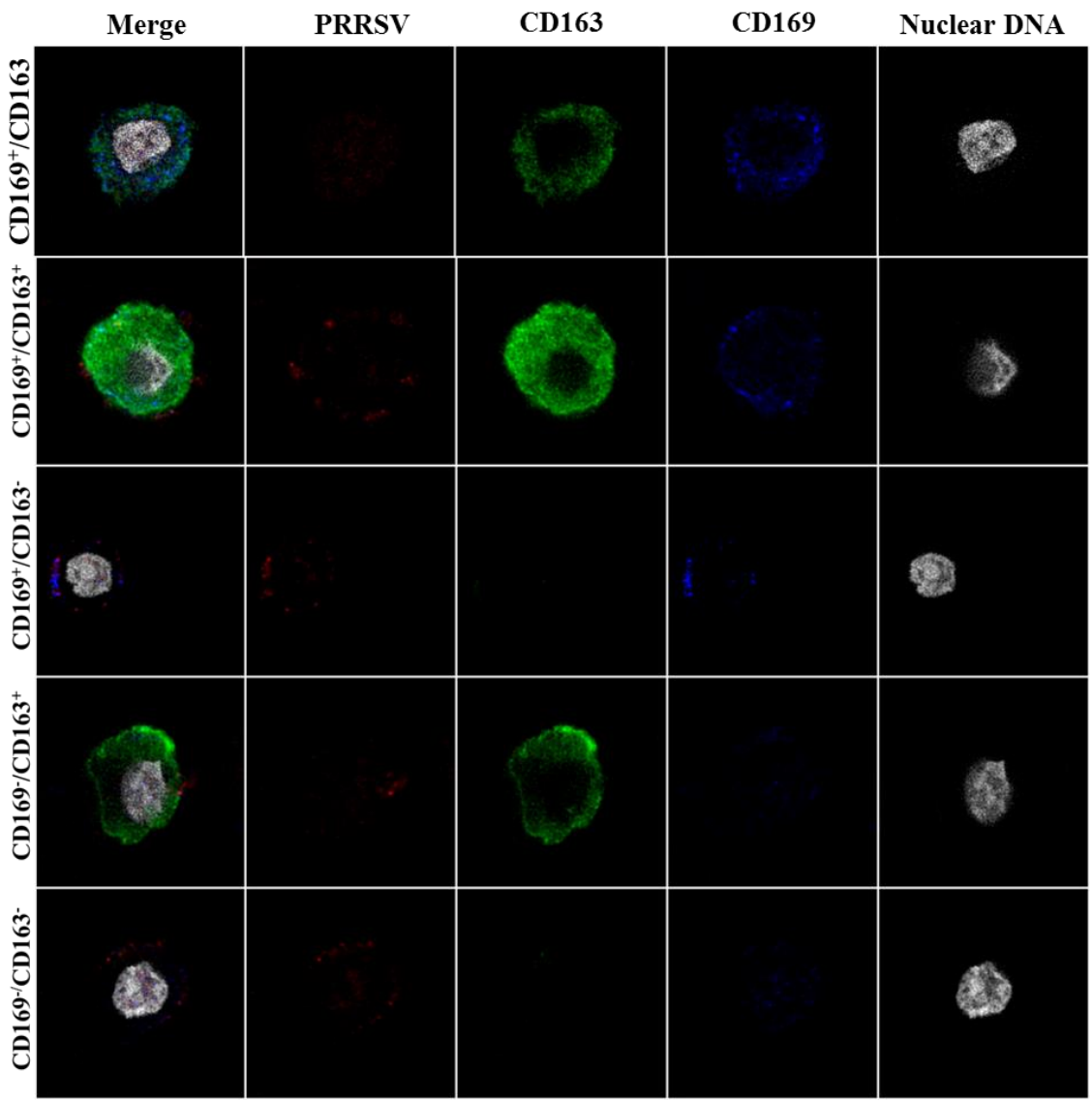


Figure 4. Attachment of isolate 3262 to different subsets of immature bone marrow-derived dendritic cells (iBMDC). The picture shows the attachment of isolate 3262 to the subsets defined by PoSn and CD163 as determined in confocal microscopy. The upper row shows uninfected cells.

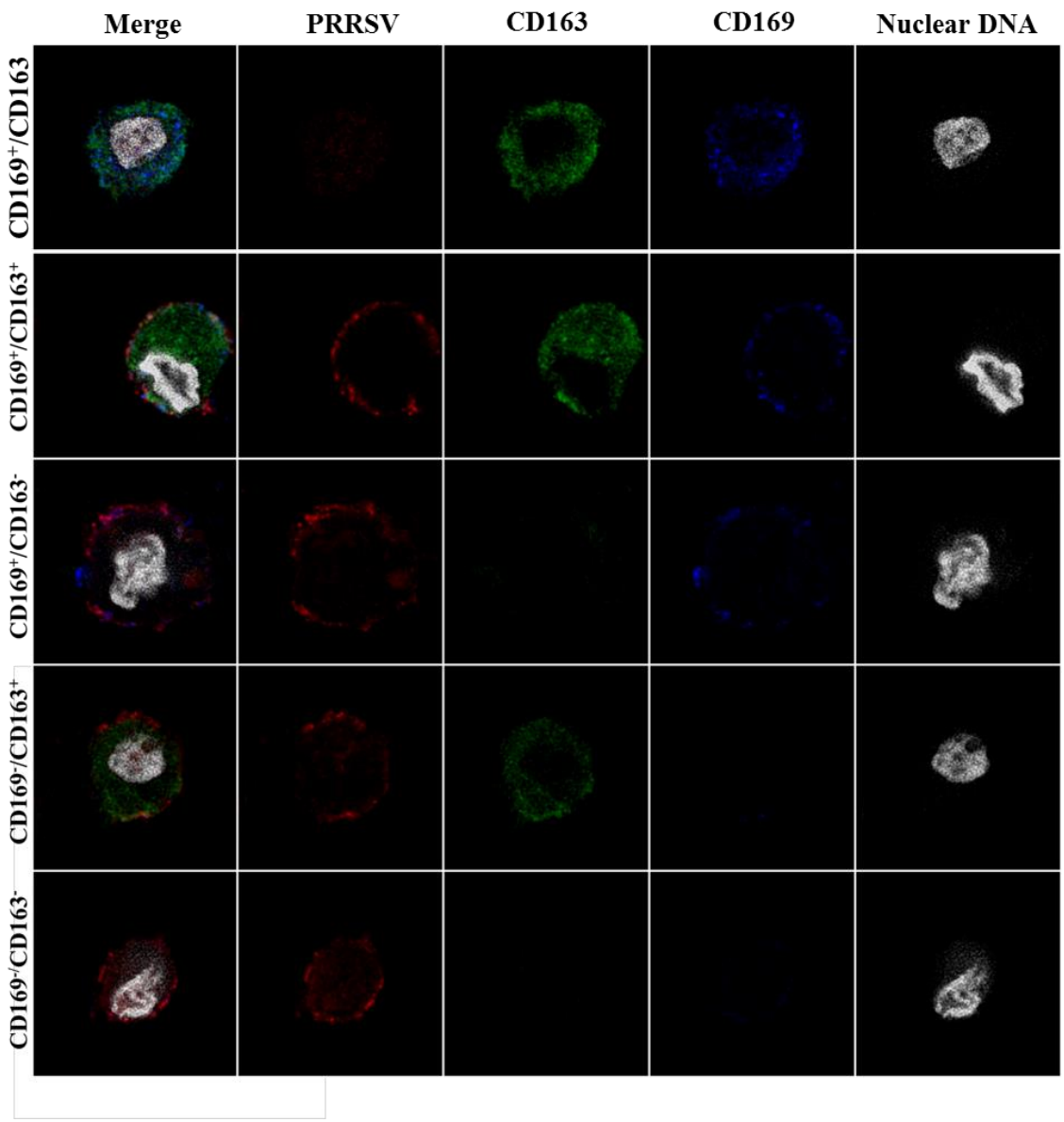


Figure 5. Attachment of isolate 3267 to different subsets of immature bone marrow-derived dendritic cells (iBMDC). The picture shows the attachment of isolate 3267 to the subsets defined by PoSn and CD163 as determined in confocal microscopy. The upper row shows uninfected cells.

3.4. Effect of the removal of heparan sulphate on the attachment of different PRSRV1 isolates.

By confocal microscopy, treatment of iBMDC with heparinase I resulted in some apparent reduction of the attachment by isolate 3267, but the attachment was not abolished (Figure 6). This reduction was not so evident for isolate 3249. After heparan sulphate removal, the attachment of isolates 3249 and 3267 to the different subsets defined by PoSn and CD163, could still be observed.

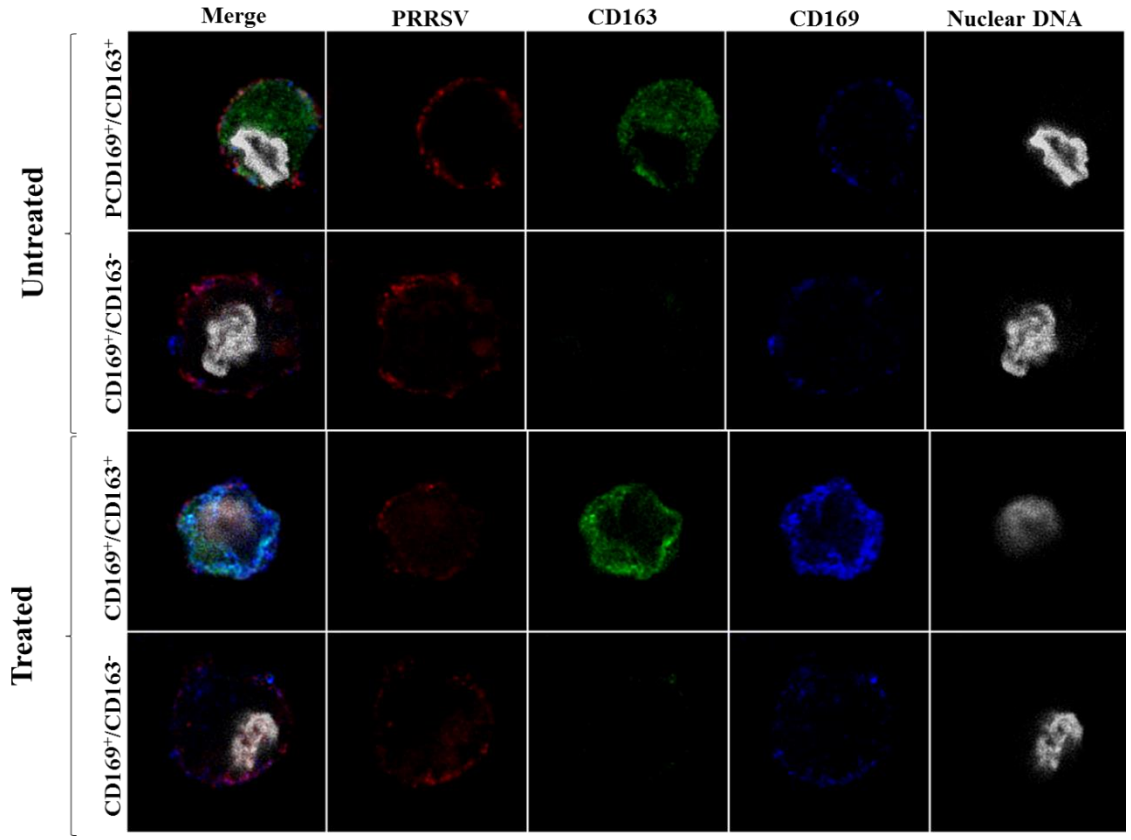


Figure 6. Effect of heparinase I treatment on the attachment of PRRSV1 isolate 3267. The figure shows the effect of removal of heparan sulphate by heparinise I on the attachment of isolate 3267 as determined by confocal microscopy.

3.5. PRRSV1 replication and PoSn/CD163 expression examined by confocal microscopy.

Cultures of iBMDC and mBMDC were examined for the replication of PRRSV1 isolates at 12h, 24h and 48h after inoculation. It was shown that at least isolates 3249 and 3267 replicated in iBMDC that apparently lacked CD163 expression. Figure 7 shows a confocal microscopy picture of infection with isolate 3249 as an example.

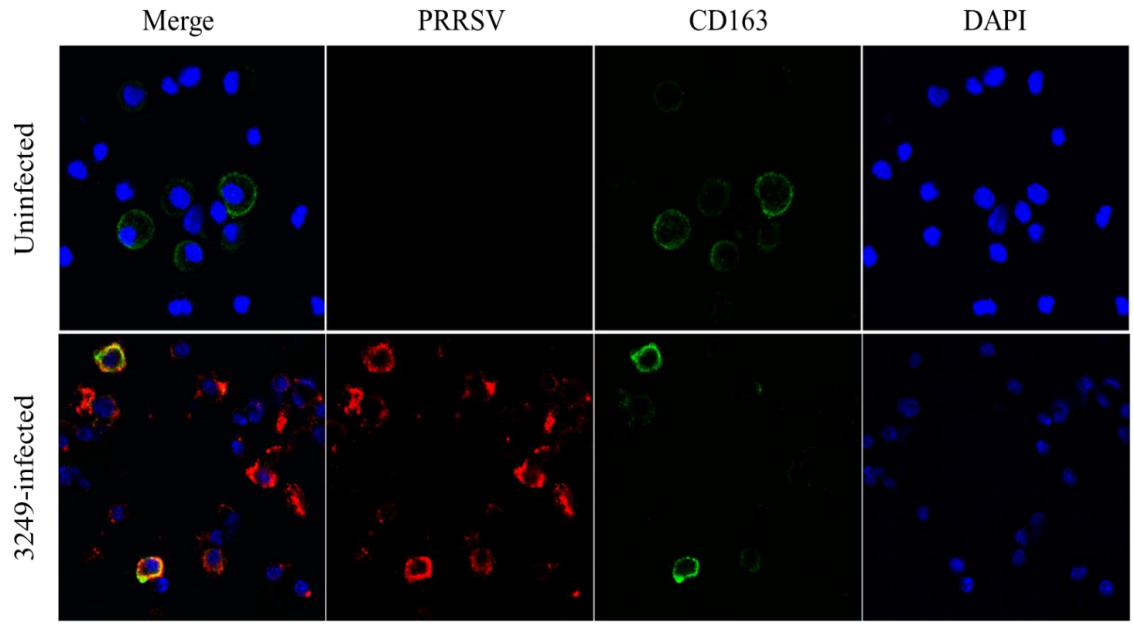


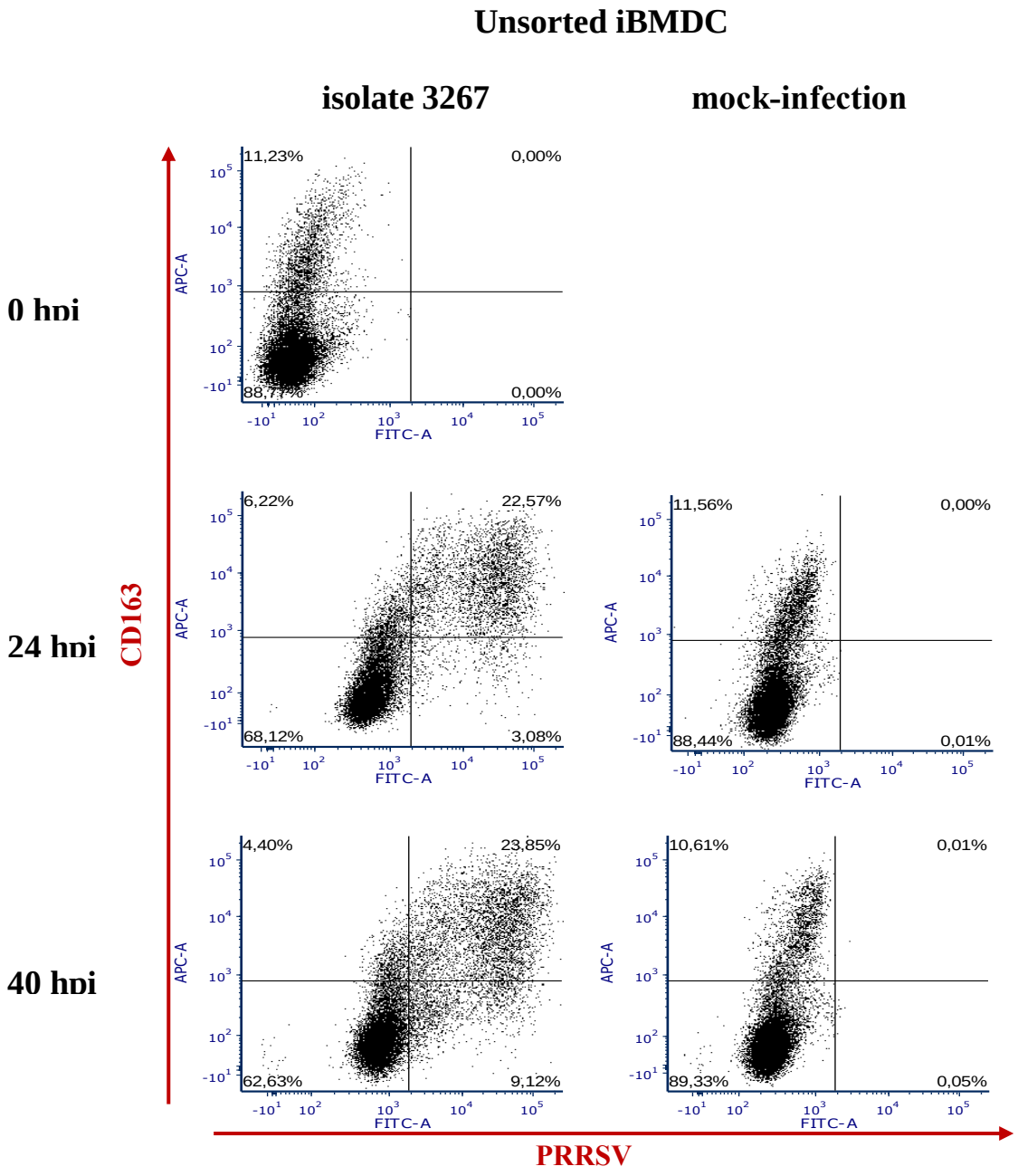
Figure 7. Replication of isolate 3249 in CD163 negative immature bone marrow-derived dendritic cells (iBMDC). The picture shows replication of isolate 3249 in CD163 negative iBMDC as determined by confocal microscopy. Upper row, uninfected cells; lower row, infected cells.

3.6. Flow cytometry analysis of PRRSV1 replication with regards to CD163 expression in iBMDC.

The replication of isolate 3267 in iBMDC was also analysed by a two-colour flow cytometry labelling. In unsorted iBMDC, 8.4% ± 0.5% cells of apparently $CD163^-$ were labelled as positive for PRRSV1 N protein by 40 hpi (3.3% ± 0.1% at 24 hpi) (Figure 8a). However, when pure $CD163^-$ cells were sorted, no infection could be detected in this population by 40 hpi

(Supplementary Figure S6). When CD163^{-} were sorted together with CD163^{lo} cells, 0.6% ± 0.07% of CD163^{-} cells were labelled by PRRSV1 N protein at 40 hpi (Figure 8b), and when the incubation was extended to 60 hpi, the percentage of infected CD163^{-} cells increased to 1.6% ± 0.08% (Figure 8b).

(a)



(b)

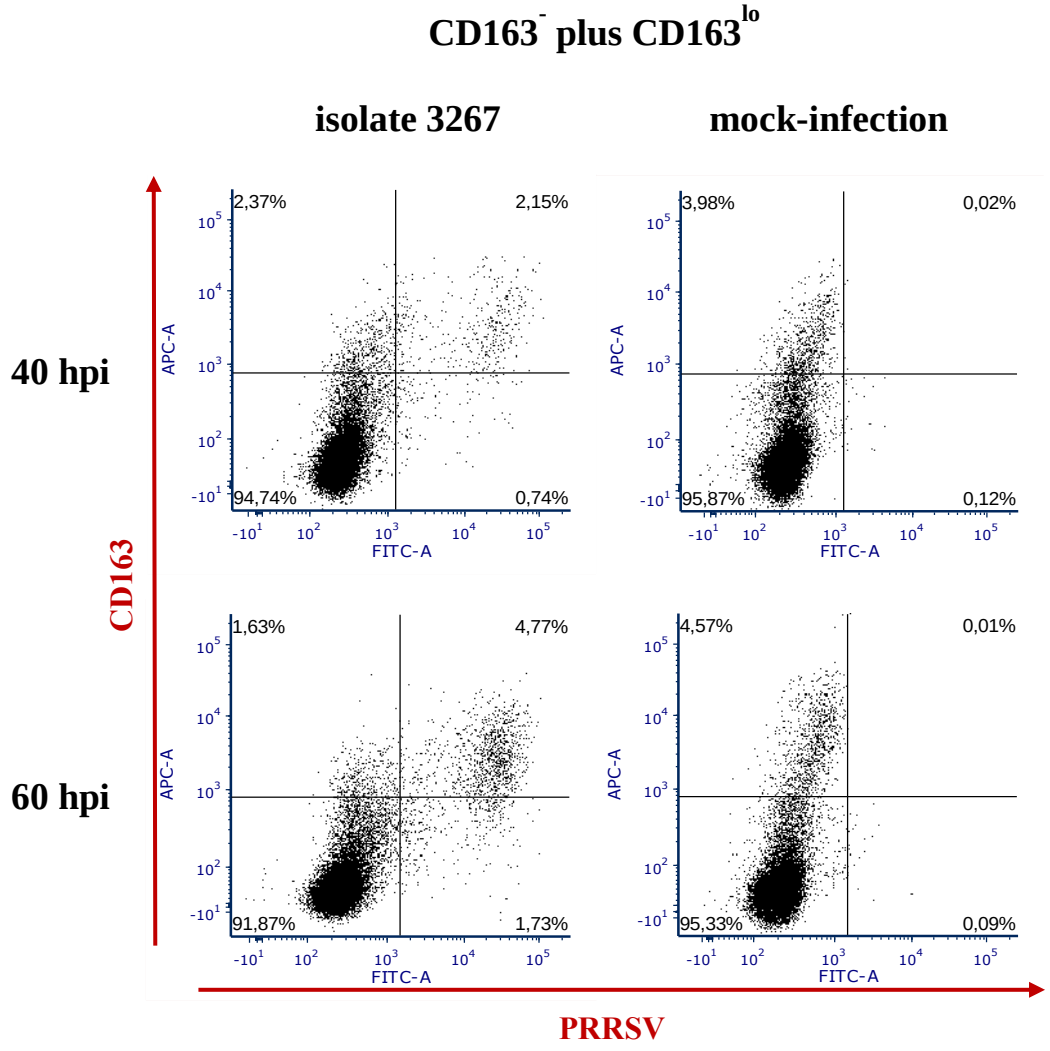


Figure 8. Replication of isolate 3267 in immature bone marrow-derived dendritic cells (iBMDC) with regards to the expression of CD163. (a) The picture shows the replication of isolate 3267 in unsorted iBMDC by 0, 24 and 40 hpi, with regards to the expression of CD163 as determined by flow cytometry. (b) The picture shows the replication of isolate 3267 in sorted iBMDC $CD163^{-}$ plus $CD163^{lo}$, by 40 and 60 hpi, with regards to the expression of CD163 assessed by flow cytometry.

4. Discussion

Classically, PAM have been considered the main target for either PRRSV 1 or 2 [4]. Nevertheless, previous works [19-24] indicated that DC can also be infected by PRRSV. The results of the present work suggest that iBMDC are not only infected but that the infection of

these cells is at least as productive, if not more, than the infection of PAM, at least for the PRRSV1 isolates examined. According to the titres attained in the culture supernatants, iBMDC appeared to be more efficient in supporting PRRSV1 replication than mBMDC. Since the proportion of CD163 positive cells in iBMDC was lower than in mBMDC, the productivity of the infection seemed not to be related to proportion of susceptible cells harbouring CD163. Moreover, the anti-viral cytokine IFN-α was not involved because there was no detectable IFN-α in cell culture supernatants of both iBMDC and mBMDC. Therefore, the higher or lesser productivity should reside in the intrinsic features of each cell type. The most plausible reason is that maturation resulted in the impaired antigen uptake ability of cells [38,39], and/or in some post-entry blocking during replication, as occurs for example in the case of HIV [40]. The induction of TNF-α in iBMDC can be an indication of the higher uptake ability of the cells.

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 consistent with its low virulence in pigs [41] and could be related to a lower attachment capability as later confirmed by confocal microscopy.

When iBMDC were treated with heparinase an apparent reduction of the attachment was seen by confocal microscopy but the attachment was not fully prevented in none of the four subsets defined by PoSn and CD163, indicating that in BMDC attachment may happen without the participation of these molecules. Other authors, working with transfected BHK-21 cells suggested that DC-SIGN (CD209), a C- type lectin that is expressed in the surface of DC, could act as a potential receptor [35]. Similarly, CD151 has been suggested as a potential PRRSV receptor [11] and can also be expressed in some types of DC of humans [36].

The fact that one of the isolates examined (3262) had a remarkably lower attachment to iBMDC but not to PAM (see figure S4) could be compatible with the notion that different

receptors exist in different cell types and that some isolates could preferentially use one or the other. Li *et al*. [37] showed that PRRSV2 GP5 harbour different sugars such as N-acetylglucosamine and N-acetyllactosamine that could bind receptors present in PAM. It is tempting to speculate that differences in the presence of sugar residues in the viral surface proteins may play a role in targeting the attachment.

Replication of PRRSV1 was found in cells apparently negative for CD163 expression examined by both confocal microscopy and flow cytometry. The results of the examination of the unsorted BMDC and of the sorting experiments do not permit to conclude beyond any doubt that $CD163^-$ BMDC are susceptible to PRRSV. This PRRSV-infected $CD163^-$ population appeared only when $CD163^{lo}$ or $CD163^{hi}$ were present in the culture. Since in the sorting experiments the number of infected cells at 60 hpi were higher than the original number of $CD163^+$ cells, the explanation is not simple. In our opinion, these observations indicate the subset of $CD163^-$ susceptible cells arose during infection, probably as a result of the milieu created by the infection. But it is also 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. It is very unlikely that the infected $CD163^-$ were $CD163^+$ cells that lost this molecule since this was not seen in the sorted $CD163^{hi}$ or $CD163^{lo+hi}$ that did not suffer any evident decrease in the percentage of $CD163^+$ cells after infection. Actually, $CD163^+$ cells increased by 10% during the course of infection in unsorted BMDC.

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. Previously, Frydas *et al*. [32] found that in nasal mucosa explants some PRRSV1 isolates may replicate in PoSn/CD163 double negative cells. Apparently, in the nasal mucosa dendritic-like cells were also found to be infected.

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*., 2016; Burkard *et al*., 2017; Wells *et al*., 2017). And 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 [33] and this could be different of what would happen in a very heterogeneous population such as BMDC. Secondly, the fact that PAM were affected by substitutions in CD163 does not preclude that in any other cell type a different mechanism or receptor for entry could exist.

In summary, we showed that iBMDC are relevant targets for PRRSV, at least *in vitro*, and that infection of those cells can happen without the apparent participation of CD163 or PoSn. Moreover, different PRRSV1 isolates seem to interact with the cell membrane receptors on BMDC in a different way. Our results emphasize the need for a more in-depth examination of the role of DC infection in PRRSV immunopathogenesis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All three authors contributed to the design of the experiments, writing of the paper and the scientific discussion of the results. YL performed the experimental work in the laboratory.

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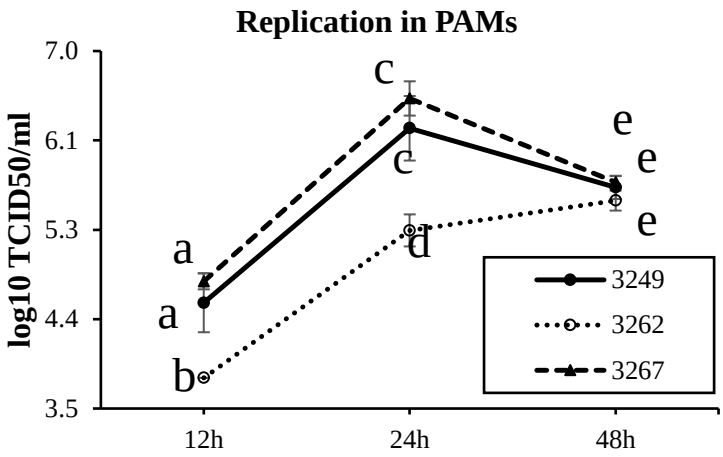
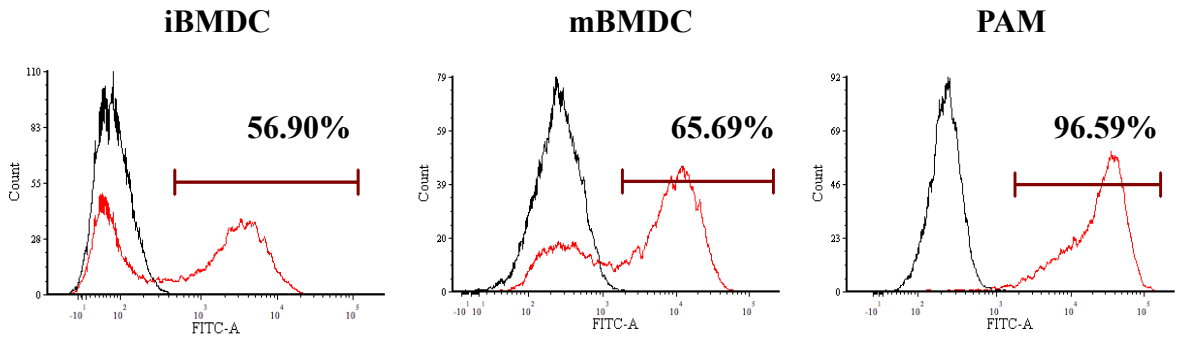
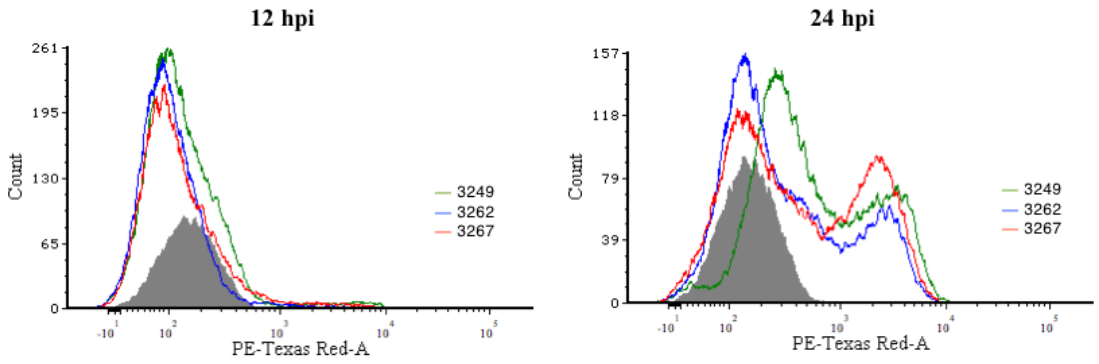


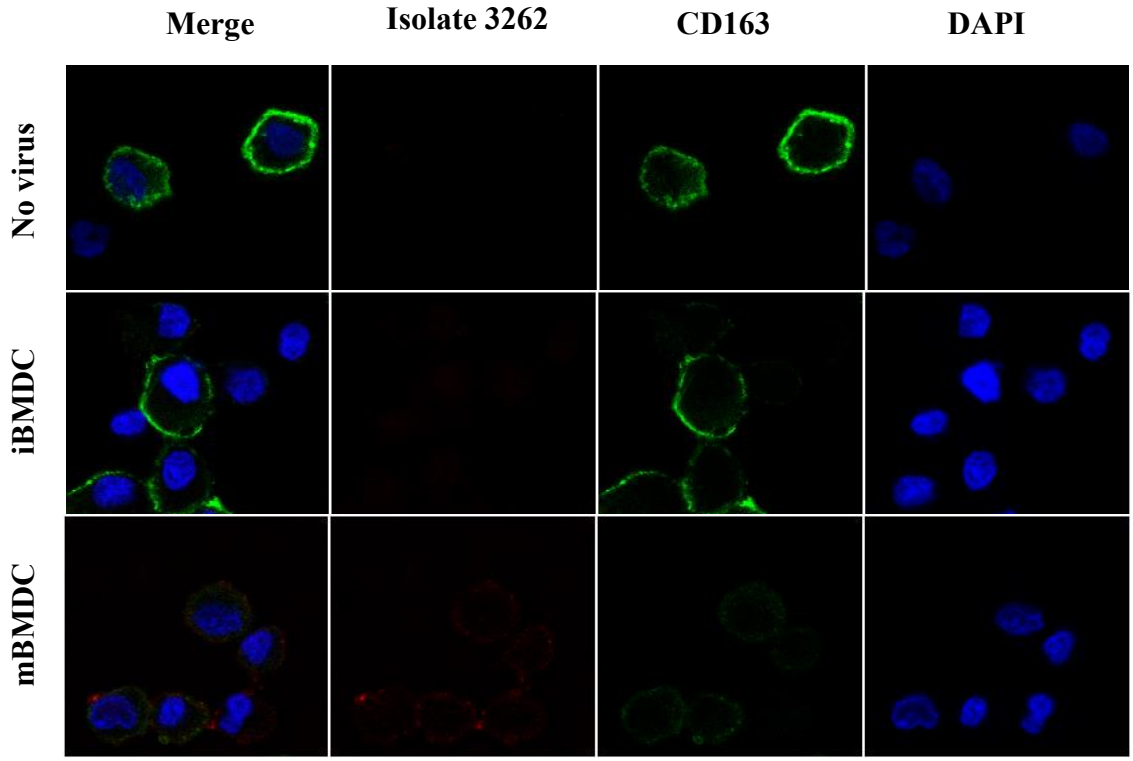
Supplementary Figure 1. Titres of different PRRSV1 isolates in cell culture supernatants of porcine alveolar macrophages (PAM). The figure shows the titres attained in cell culture supernatants of PAM when infected with PRRSV1 isolates (3249, 3262, 3267) as determined by titration in PAM. Error bars account for standard deviations of three replicas. Different letters indicate significant differences ($p<0.05$).



Supplementary Figure 2. Expression of CD163 as determined in flow cytometry for different types of cells. The graphs show the proportion of CD163 positive cells in immature (i) and mature (m) bone marrow-derived dendritic cells (BMDC) and porcine alveolar macrophages (PAM). Black line shows the results of the background (non-specific) staining.

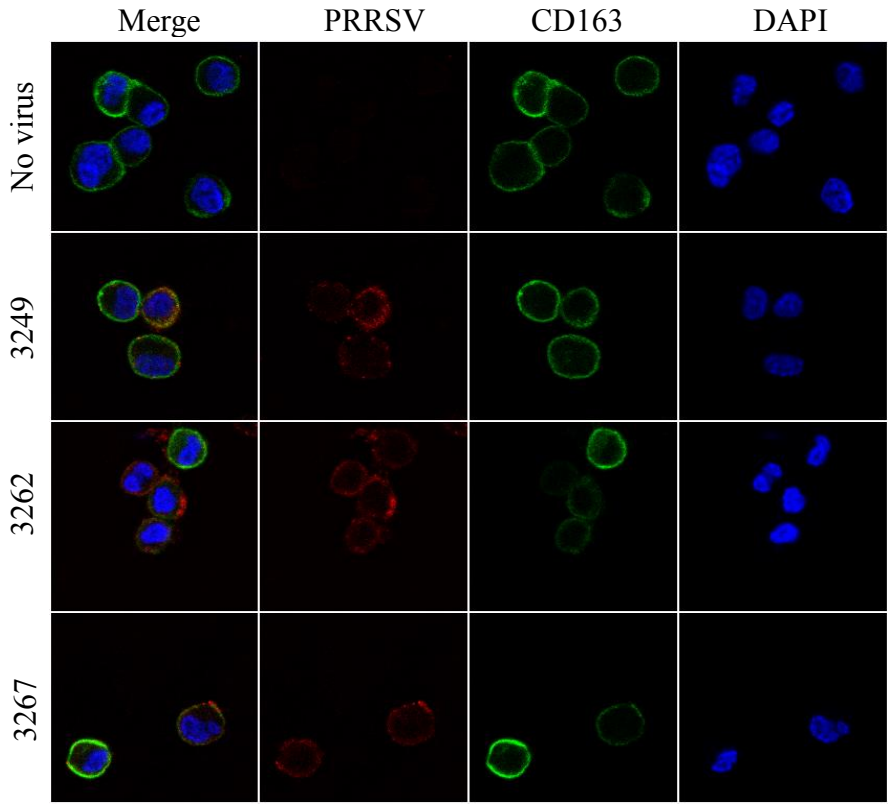


Supplementary figure 3. Overlaid histograms for immature bone marrow-derived dendritic cells (iBMDC) infected with different PRRSV isolates. The figure shows the flow cytometry results of the infection of iBMDC. The background created by the labeled secondary antibody (Alexa Fluor-610) was assessed in infected cells (shadowed histogram). Empty coloured histograms show the results of different isolates.

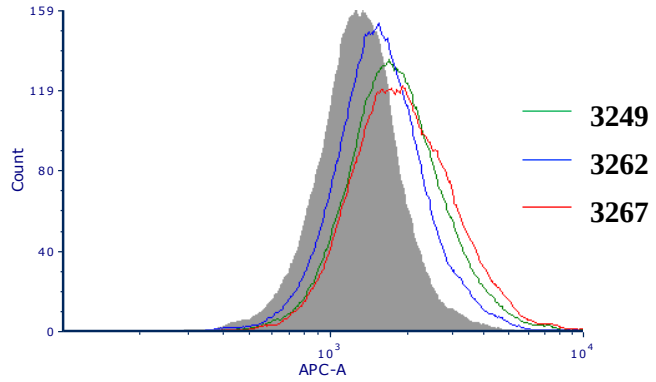


Supplementary Figure 4. Attachment of the PRRSV1 isolate 3262 to immature (i) and mature (m) bone marrow-derived dendritic cells (mBMDC). The figure shows confocal microscopy images for the attachment of isolate 3262 to iBMDC and mBMDC.

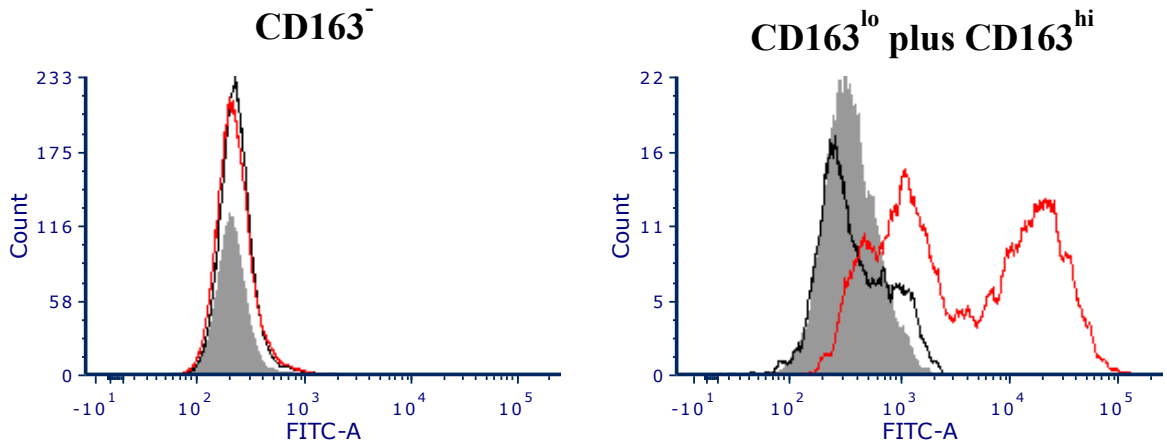
(a)



(b)



Supplementary Figure 5. Attachment of the different PRRSV1 isolates to porcine alveolar macrophages (PAM). (a) The picture shows the confocal microscopy images for the attachment of different PRRSV1 isolates to PAM. (b) The figure shows the overlaid histograms for the attachment of different PRRSV1 isolates to PAM assessed by flow cytometry. The shadowed histogram indicates the sample with no virus. The empty coloured histograms indicate cells attached by different isolates. By comparing median fluorescence intensity (MFI), 3249, 3262 and 3267 are respectively 36.4%, 19.4% and 44.7% higher than the sample without virus.



Supplementary Figure 6. Overlaid histograms for the infection of CD163$^-$ immature bone marrow-derived dendritic cells (iBMDC) with isolate 3267. The figure shows the flow cytometry results of the infection of CD163$^-$ iBMDC with isolate 3267 (red empty histogram). The isotype (shadowed histogram) and the mock-infected samples (black empty histogram) are used as negative controls. The infection of subset CD163^{lo} plus CD163^{hi} is used as the positive control.

Study 2 Apoptosis induced by different PRRSV1 isolates occurs in both infected and bystander cells and depends on the isolate used and on IL-10 presence.

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Keywords: *Porcine reproductive and respiratory syndrome virus*, bone marrow-derived dendritic cells, apoptosis, caspase-3

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) has been shown to induce apoptosis both *in vivo* and *in vitro*. In this study, we characterized the apoptosis induced by four PRRSV1 isolates with distinct cytokine release profiles 2988 (IL-10^{+}/TNF-α^{-}), 3249 (IL-10^{-}/TNF-α^{+}), 3262 (IL-10^{+}/TNF-α^{+}) and 3267 (IL-10^{-}/TNF-α^{-}) in porcine alveolar macrophages (PAM) and bone marrow-derived dendritic cells (BMDC). In PAM, apoptosis was observed in both infected and uninfected cells for all the isolates despite of their different replication efficiencies, indicative of the co-existence of the intrinsic and extrinsic pathways in this case. In BMDC, apoptosis was mainly found in uninfected cells suggesting the predominance of the extrinsic pathway. With moi 0.1, apoptosis in BMDC developed slower than in PAM. Further studies with BMDC showed that IL-10-induced isolates 2988 and 3262 produced very little apoptosis in BMDC while the two isolates (3249 and 3267) that had no IL-10 release in BMDC induced apoptosis and necrosis. Blocking of IL-10 with monoclonal antibodies in 3262-infected BMDC cultures resulted in apoptosis. In contrast, blocking of TNF-α in 3249-infected cultures did not abolish apoptosis. The balance of TNF-α and IL-10 production may influence the apoptosis induced by PRRSV1 but mechanisms other than the release of TNF-α should be involved since isolates 3267 -with neither IL-10 nor TNF-α induction- could also induce apoptosis.

1. Introduction

Porcine reproductive and respiratory syndrome viruses (PRRSV1 and 2), are enveloped, positive-strand RNA viruses classified in the genus *Porateriviridae* within the *Arteriviridae* family [1]. The main target for PRRS viruses are macrophages although certain types of dendritic cells are known to be susceptible [2-8].

The infection caused by these viruses has several unique features including an unusual immune response consisting of a delayed development of neutralizing antibodies and cell-mediated responses [9,10] and the persistence of the virus in the lymphoid tissues for weeks to months although it can be finally cleared [11,12].

For many years, researchers have been investigating the causes allowing the development of such long-term infection. One of the potential cause is virus-induced cell death. At present, virus-induced cell death is thought to happen by three mechanisms: necroptosis, pyroptosis or apoptosis. While necroptosis is a caspase-independent process, pyroptosis requires the intervention of caspase-1 and apoptosis involves caspase-3 activation. In any case, cell death mechanisms are a double-edged sword in viral infections. While they can contribute to the elimination of the infected cells, they can enhance viral release from infected cells as well. Danthi recently published an excellent and comprehensive review on this topic [13].

In the case of PRRSV, it was reported that cells could be resistant to apoptosis in early stages of infection [14]. Kim *et al*. [15] characterized PRRSV-induced apoptosis as a nontypical form that occurred in the late infection stage, resulting in a necrotic-like death. However, the precise mechanisms involved in PRRSV-induced apoptosis are yet undetermined and it is still controversial whether PRRSV triggers apoptosis mainly in infected cells or in bystanders. While in the infected animals most apoptotic cells in lymphoid tissues, lungs and testes were shown to be negative for PRRSV [17-21], there are also studies showing apoptosis in PRRSV-

infected cells [14, 16]. TNF-α and IL-10 were supposed to be potential effectors for apoptosis development in the bystander cells [22,23] although unregulated FasL could also be involved [24].

The aim of the present study was to examine the induction of apoptosis in bone marrow-derived dendritic cells (BMDC) using porcine alveolar macrophages (PAM) as a reference model. Considering the immunobiological diversity of PRRSV1, three different isolates were used. Also, the involvement of IL-10 and TNF-α was investigated in the BMDC model.

2. Materials and methods

2.1. Cells and viruses.

Four-week-old piglets were used as donors of porcine alveolar macrophages (PAM) and bone marrow hematopoietic cells (BMHC). PAM were obtained by bronchoalveolar lung lavage. BMHC, aseptically isolated from the femora and humeri, were stimulated with 100 ng/ml recombinant porcine granulocyte-monocyte colony stimulating factor (rpGM-CSF) (R&D Systems, Minneapolis, USA) *in vitro* for 8 days, to generate bone marrow-derived dendritic cells (BMDC) [25]. Cells were cultured at 37 ℃ in a humidified 5% CO_2 atmosphere.

Three PRRSV1 isolates designated as 3249, 3262 and 3267 were used (Genbank accession numbers JF276433, JF276431, JF276435). These isolates were selected based on their distinct cytokine-release profiles in BMDC: 3249 (IL-10$^-$/TNF-α$^+$), 3262 (IL-10$^+$/TNF-α$^+$) and 3267 (IL-10$^-$/TNF-α$^-$) that were characterized in previous studies [26,27]. For comparative purposes when evaluating the effect of IL-10, isolate 2988 was also used (with a cytokine-inducing profile IL-10$^+$/TNF-α$^-$). Viral stocks were a fifth passage in PAM.

2.2 Cleaved caspase-3 expression in PRRSV1-infected PAM and BMDC.

PAM were seeded in Lab-Tek Ⅱ slide chambers (ThermoFisher, Madrid, Spain) at a density of 100,000 cells/chamber and incubated overnight; then cultures were inoculated with isolates 3249, 3262 and 3267 at a multiplicity of infection (moi) of 1 or 0.1, or were mock-inoculated with an uninfected macrophage culture supernatant. The inoculum was removed after 1.5 h of incubation and was replaced by RPMI medium added with antibiotics and 10% fetal calf serum. Slides were collected at 0, 12, 24 and 48 hours post-inoculation (hpi). All cultures were done in duplicates.

For the fluorescent staining of cleaved caspase-3 and PRRSV, slides were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized with 100% methanol for 10 minutes at -20 ℃. PBS containing 0.3% Triton X-100 (PBST) (Sigma, Madrid, Spain) was used to wash the slides between each step. Blocking of unspecific binding sites was performed with PBS containing 10% horse serum. Afterwards, anti-cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Leiden, The Netherlands) was added at a 1:200 dilution and slides were incubated overnight at 4 ℃. Alexa Fluor 488-labeled goat anti-rabbit IgG (10 μg/ml, Invitrogen, Madrid, Spain) was used as the secondary antibody (2 hours at RT). For labelling of PRRSV, mAb anti-PRRSV1 nucleocapsid 1C5H (1:20, 10 hours, 4 ℃) (Ingenasa, Madrid, Spain) and Alexa 610-R-Phycoerithrin goat anti-mouse IgG (5 μg/ml, 60 min, RT) (Invitrogen) were combined. The staining was mounted with Prolong Gold Antifade mounting with DAPI (ThermoFisher), and analyzed by fluorescence microscopy (Nikon Eclipse 90i).

For BMDC, cells were cultivated in 48-well plates and inoculated as above but using only a moi of 0.1 since higher moi induced death of most cells at 12h for isolates 3249 and 3267. At 0, 12, 24 and 48 hpi, cells were collected and a 5μL volume containing approximately 50,000 cells were transferred onto a microscope slides (ThermoFisher). Slides were dried and then were fixed, permeabilised and stained as described above.

2.3. Assessment of PRRSV-induced apoptosis and necrosis in BMDC by flow cytometry.

Duplicate cultures of freshly derived BMDC ($1x10^6$ cells) were inoculated with three PRRSV1 isolates 3249, 3262 and 3267 at 0.01 moi as above. At 48 hpi, cells were recovered and subjected to Annexin V-FITC/propidium iodide (PI) double staining for flow cytometry. The Annexin V staining was performed using a commercial flow cytometry kit (Annexin V FITC kit, AbD Serotec, Spain). Annexin V-$FITC^+/PI^-$ was considered as early-stage apoptotic cells, Annexin V-$FITC^+/PI^+$ as late-stage apoptotic cells, and Annexin V-$FITC^-/PI^+$ as necrotic cells. This procedure was repeated twice in separate days including non-inoculated cells and cells inoculated with heat-treated virus (60°C, 60 min). Cells were analysed on a FACSCalibur cytometer (BD Biosciences).

2.4. Effect of the blocking of IL-10 and TNF-α on apoptosis and necrosis of BMDC.

BMDC were produced and inoculated with PRRSV1 isolates as above. In this case isolate 2988 was included for comparative purposes. In the first section of the experiment, BMDC were cultured in the presence of an anti-IL-10 neutralizing antibody (Thermofisher) in an excess amount with regards to the expected IL-10 production induced by PRRSV1 in BMDC (0.15 µg/ml). In a second section, cells inoculated with PRRSV1 isolate, were incubated in the presence of an anti-TNF-α neutralizing antibody (0.20 µg/ml, R&D Systems). Necrosis and apoptosis were evaluated as above with the Annexin V kit (AbD Serotec) by flow cytometry.

3. Results

3.1. In PAM apoptosis is observed in both infected and uninfected cells.

Figures 1-2 show the labelling of PRRSV and cleaved caspase 3 at different times of infection in PAM. With moi 1, at 12 hpi a notable proportion of PAM were infected with isolates 3249 and 3267 but when isolate 3262 was used the rate of infection was very low. At that time, caspase 3 labelling was observed in both, infected and bystander cells. At 24 hpi, in cultures inoculated with isolates 3249 and 3267, cell death was massive, while the cultures inoculated with 3262 were largely infected and started developing apoptosis. For cultures infected at moi 0.1, the difference between the three isolates were not so obvious although apoptosis was observed again in both infected and uninfected cells. For moi 0.1 the caspase 3 signal peaked at 24 hpi (Supplementary figure 1).

In BMDC, where m.o.i 0.1 was used, cleaved caspase 3 signal was rarely detected at 12 hpi and 24 hpi, but at 48 hpi the signal was evident (Figure 3). In this case the majority of labelled cells were uninfected.

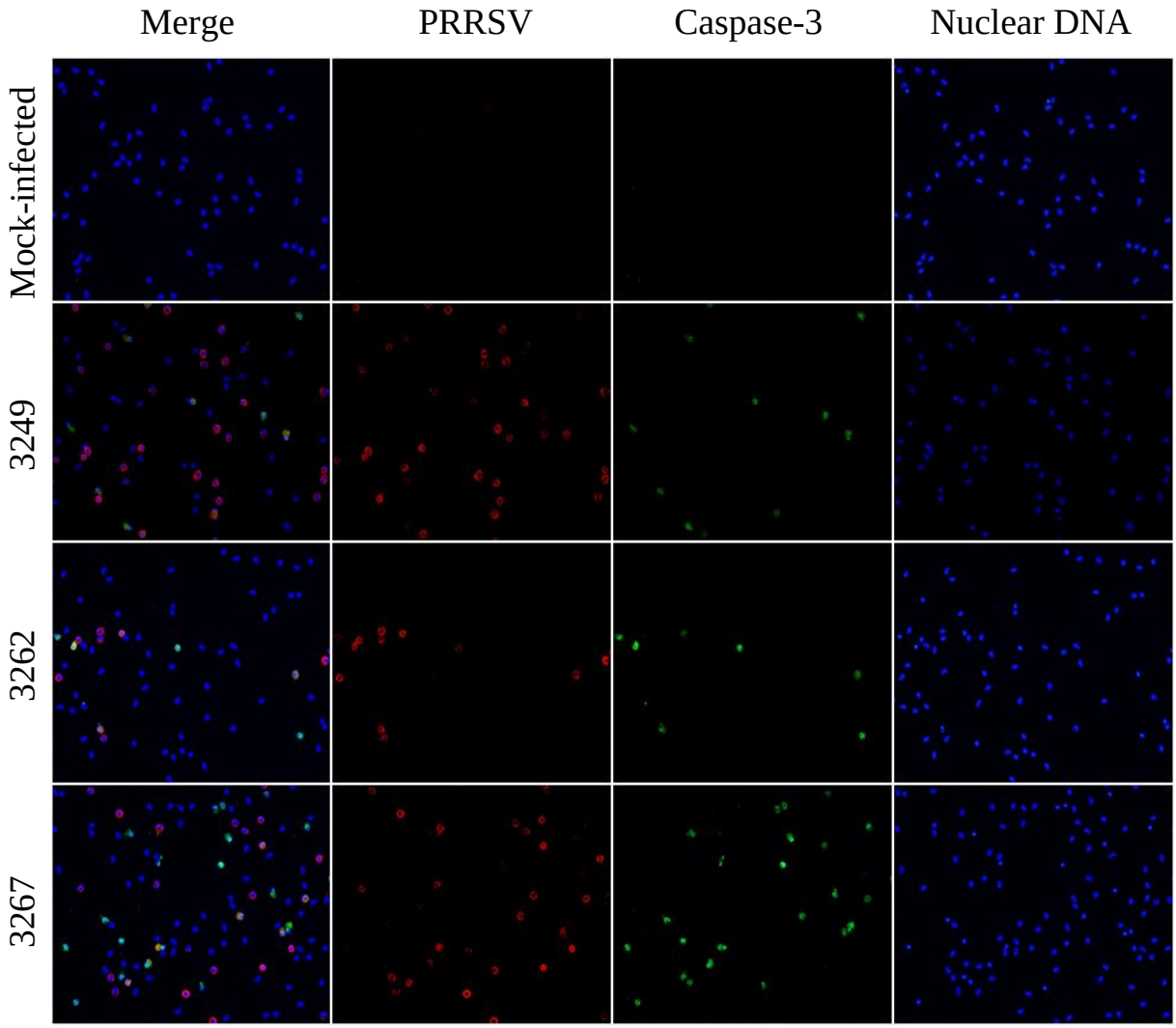

Figure 1. Colocalization of PRRSV (red), caspase-3 (green) and nuclear DNA (blue) in porcine alveolar macrophages (PAM) inoculated by three PRRSV1 isolates (3249, 3262 and 3267) for 12 hours at a MOI of 1.

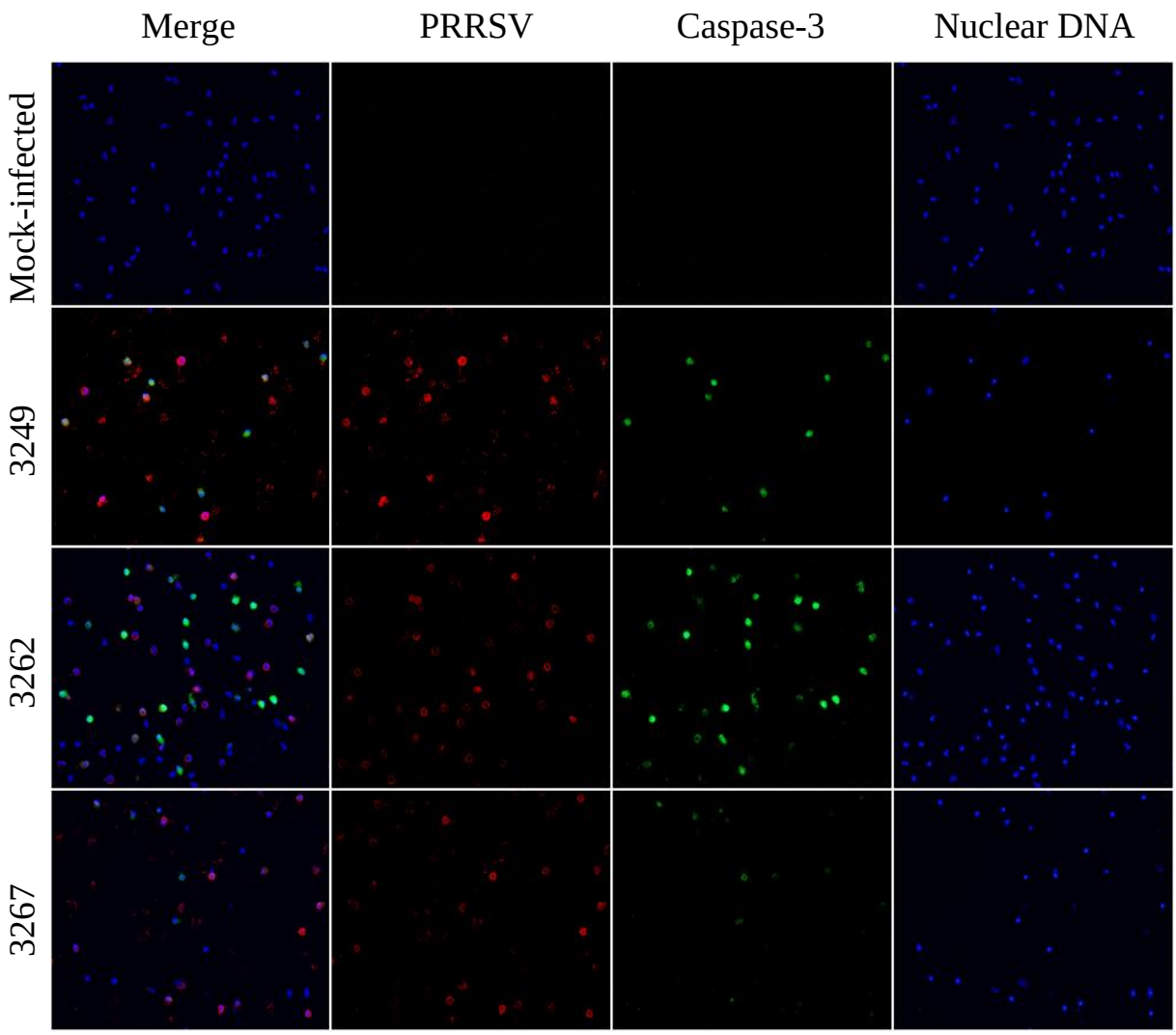

Figure 2. Colocalization of PRRSV (red), caspase-3 (green) and nuclear DNA (blue) in porcine alveolar macrophages (PAM) inoculated by three PRRSV1 isolates (3249, 3262 and 3267) for 24 hours at a MOI of 1.

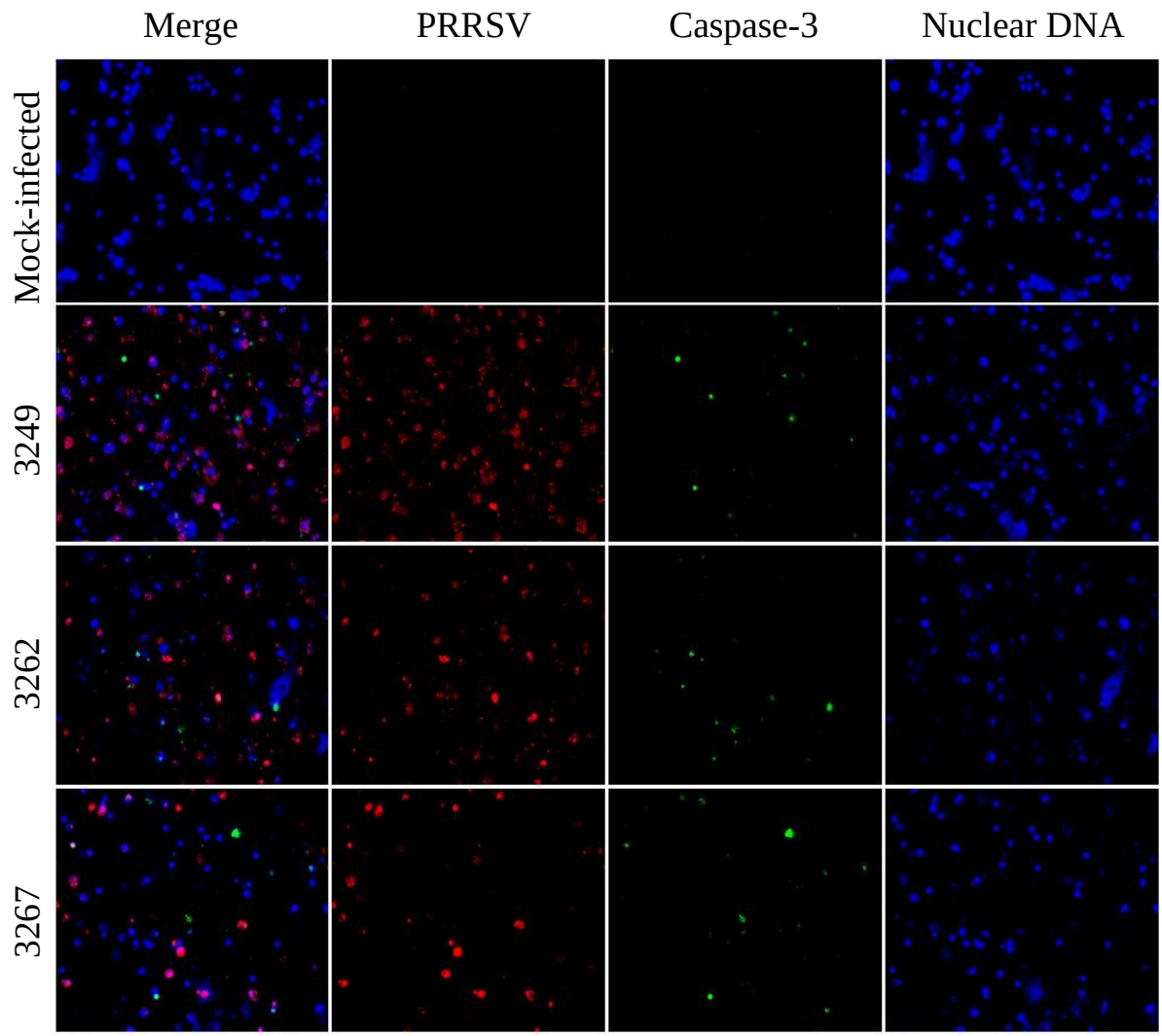

Figure 3. Colocalization of PRRSV (red), caspase-3 (green) and nuclear DNA (blue) in bone marrow-derived dendritic cells (BMDC) inoculated by three PRRSV1 isolates (3249, 3262 and 3267) for 48 hours at a MOI of 0.1.

3.2. PRRSV-induced necrosis and apoptosis is isolate-dependent.

Inoculation of BMDC with different isolates produced different morphological and biological alterations. Thus, at 48 hpi, BMDC infected with isolates 3249 or 3267 showed clear signs of cell death while cells inoculated with isolate 3262 had much less evident signs of cell death.

Examination of inoculated BMDC cultures for necrosis/apoptosis by flow cytometry (Figure 4) showed significant differences between isolates, and the development of necrosis or apoptosis was related to the viability of the virus. Specifically, BMDC inoculated with the heat-treated virus showed a very low proportion (always <10%) of propidium iodide (PI)-positive cells and almost no annexin V labelling after 48 h of culture. In contrast, at 48 hpi, for isolate 3267, 58.4% cells were labelled as apoptotic (including 27.7% of early-stage and 30.7% of late-stage apoptotic cells) with additional 9.6% necrotic cells. For isolate 3249, there were 27.5% apoptotic cells (including 13.9% of early-stage and 13.6% of late-stage apoptotic cells) and 15.1% necrotic cells.

However, for isolates 2988 and 3262 (IL-10^{+}/TNF-α^{+}), a small proportion of the inoculated BMDC were labelled by PI (5% and 12%, respectively) but only a very low proportion (less than 2%) of them were labelled with the Annexin V antibody (Figure 4).

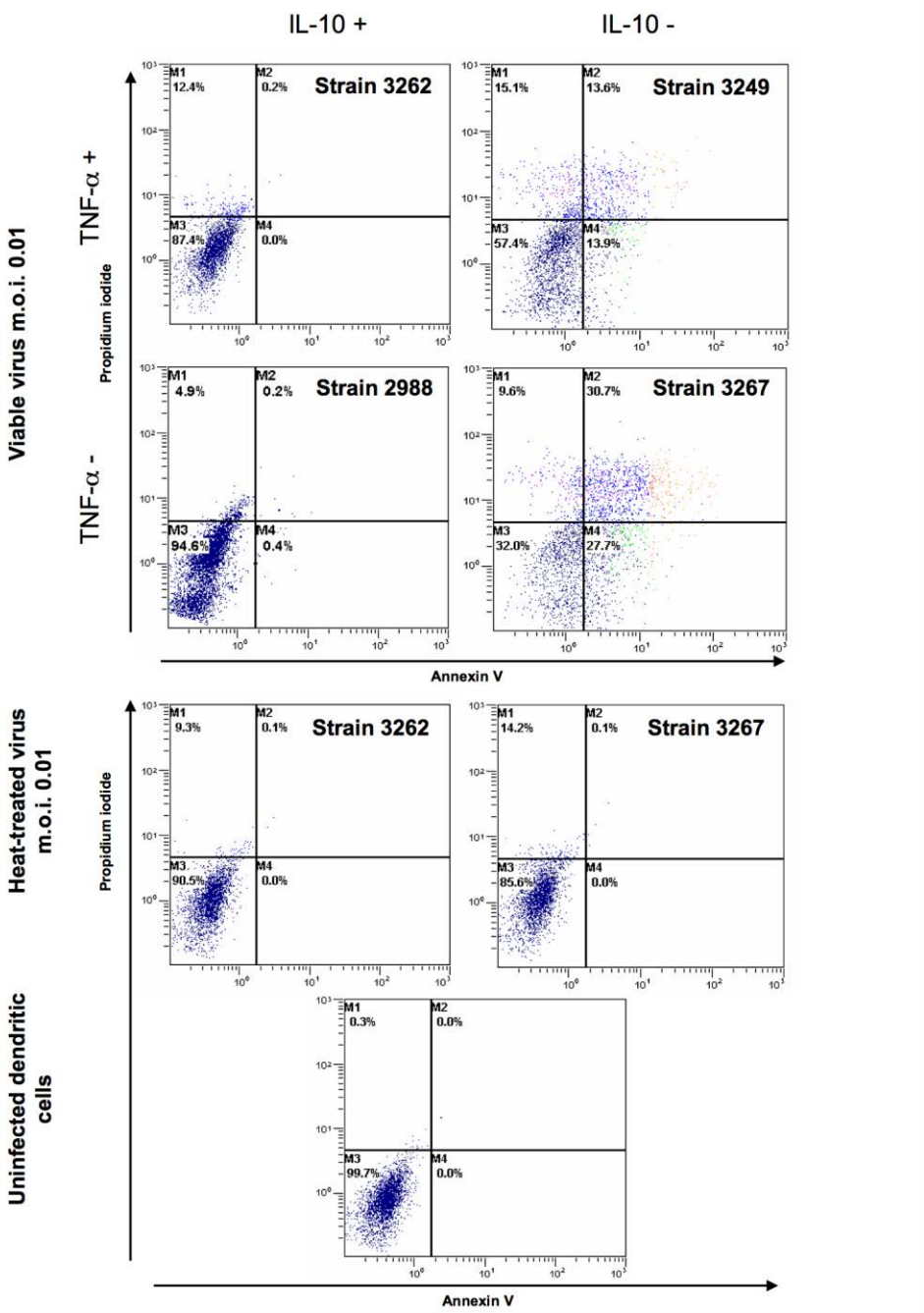

Figure 4. Flow cytometry analysis for necrosis (propidium iodide staining) and apoptosis (Annexin V labeling) of bone marrow-derived dendritic cells (BMDC) incubated for 48 h at a 0.01 multiplicity of infection (viable virus –upper panel- or heat-inactivated virus –lower panel) of four PRRSV strains representative of different cytokine-inducing profiles. IL-10 +/- and TNF- α +/- indicate the ability of a given strain to induce the release of a given cytokine in the BMDC model.

Next, two isolates, 2988 (IL-10^{+}/TNF-α^{-}) and 3262 (IL-10^{+}/TNF-α^{+}) were chosen to study the effect of IL-10 blocking. As shown in Figure 5, both isolates did not induce significant apoptosis in BMDC after 48 h of incubation. When IL-10 was blocked by using a neutralizing monoclonal antibody, 7.8% of BMDC inoculated with 3262 were positive for Annexin V at 48 hpi while cells inoculated with isolate 2988 remained mostly (>99%) negative for Annexin V, indicating that IL-10 played different roles in each case. Effect of the blocking of TNF-α was then examined similarly for isolate 3249. No substantial effect on the reduction of apoptosis in BMDC was seen (data not shown).

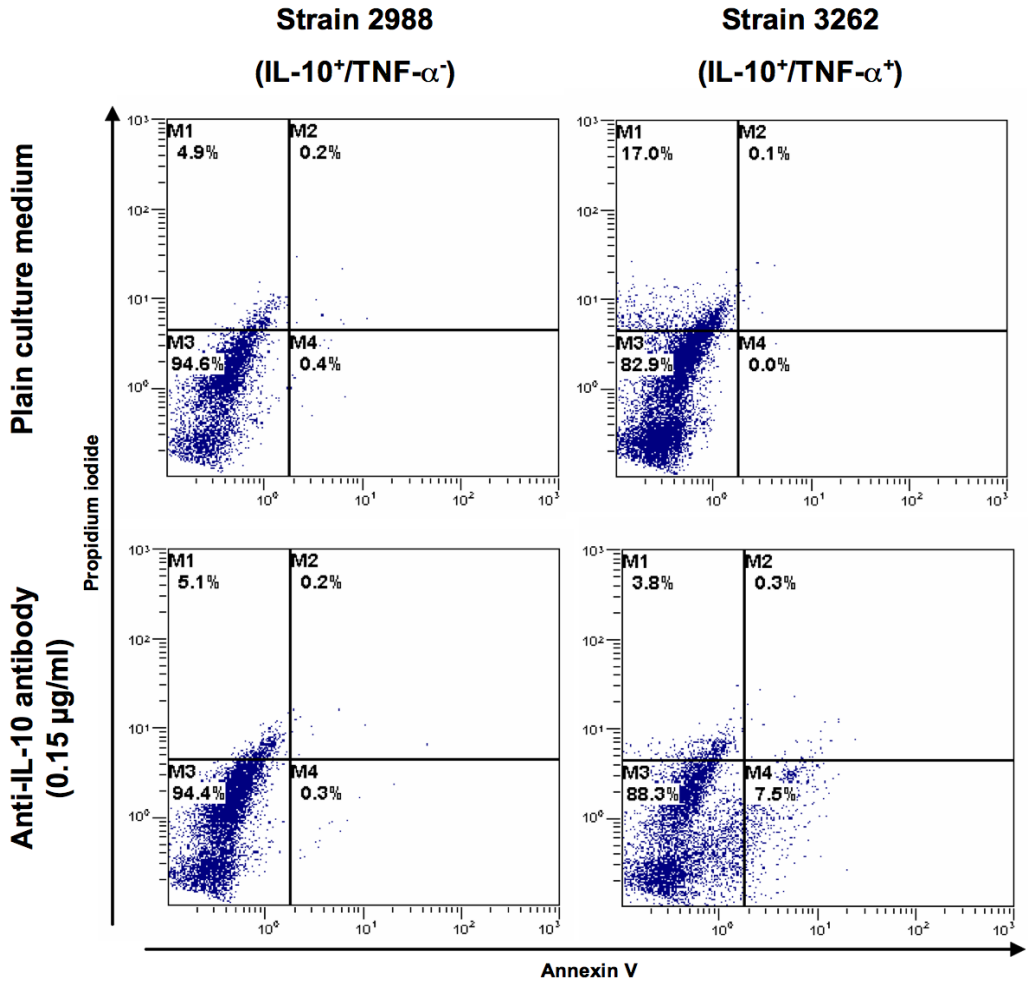

Figure 5. Annexin V and propidium iodide staining of BDMC incubated 48 h with two different IL-10 inducing strains in presence of an anti-IL-10 neutralizing antibody. Upper panel BDMC culture with plain culture medium; lower panel cultures where IL-10 was blocked.

4. Discussion

The studies dealing with the development of PRRSV-induced apoptosis have produced results that are somewhat contradictory when they are taken together. Thus, Sirinarumitr *et al*. [19] showed that in infected animals a large number of PAM in the lungs died because of apoptosis in spite of being not infected by the virus. Labarque *et al*. [23] also detected apoptotic cells in the bronchoalveolar lavages of infected pigs. In contrast, several *in vitro* studies [14, 16] showed that apoptosis happened in infected PAM. Kim *et al*. [15] further characterised the apoptotic infected cells as detached cells in the late infection stage *in vitro*.

In the present study that took an *in vitro* approach, apoptosis was observed in both infected and uninfected PAM which were still attached. This happened with all three used isolates despite of their distinct replication efficiencies and is an evidence supporting the idea that PRRSV-induced apoptosis in PAM happens by both the intrinsic and extrinsic pathways. At 12 hpi, the percentage of apoptotic cells in PAM was clearly inferior to the number of cells that were infected. This would be consistent with the previously reported anti-apoptotic state of macrophages in early PRRSV infection [14]. Non-structural viral proteins are supposed to be involved in this process and also the upregulation of several host genes [14, 28, 29].

In contrast, for BMDC, that were productively infected by the virus, apoptosis was mainly detected in uninfected cells, indicating that extrinsically triggered apoptosis was predominant. TNF-α release could be thought as a candidate for such induction of apoptosis. However, the fact that isolate 3267 showed high levels of apoptosis in bystander cells while did not induce TNF-α release pointing towards another mechanism for triggering the cell death cascade. In addition, at a moi 0.1 the caspase 3 signal in BMDC peaked at 48 hpi, later than in PAM (24 hpi). This slower development of apoptosis may allow more cycles of viral replication, resulting in higher viral yields in BMDC.

Moreover, when apoptosis was examined in BMDC after 48h of incubation with the virus, the percentage of cells with annexin V labeling (early and late apoptosis) was higher than those with single PI labelling (necrosis), suggesting that apoptosis is a major contributor to PRRSV-induced cell death. Costers *et al*. [14] and Kim *et al*. [15] reached a similar conclusion when studied the infection in MARC-145 cells and macrophages, namely, that PRRSV-infected cells died by caspase-dependent apoptosis which culminate in apoptotic necrosis. In any case, apoptosis was only seen using viable replicative virus and therefore, if GP5 participates in the induction of apoptosis as others noted [30], this would not probably occur by simple interaction of GP5 with the viral receptor in the cell membrane.

Rodríguez-Gómez *et al*. described a positive correlation between the expression of markers of apoptosis and the expression of certain cytokines with the ability to regulate apoptosis (IL- 10, IL-6 and TGF-β) [31]. In our case, the neutralization of IL-10 resulted in the development of apoptosis for one IL-10 inducing isolate (3262) while did not have any effect for the other (2988). Isolate 3262 induced TNF-α in BMDC and therefore, it can be thought that blocking of IL-10 eliminated the anti-apoptotic effect of this cytokine and thus no counteracting of the TNF-α action existed. Since 2988 did not induce TNF-α release, blocking of IL-10 should have no effect on apoptosis, as was the case.

Nevertheless, in the next experiment, the blocking of TNF-α did not result in a significant change in the proportion of apoptotic cells for 3262 or 3249. The role of TNF-α in the development of apoptosis in PRRSV-infected BMDC remains unclear.

In summary, the present paper shows that apoptosis occurs in a somewhat different manner in PAM and BMDC and that the development of apoptosis seems to be related, at least to some extent, with the infecting isolate. In BMDC, virus-induced IL-10 release seems to play an anti-

apoptotic role. The role for TNF-α is unclear yet. In any case, several mechanisms of apoptosis are probably involved.

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Competing interests

No competing interests.

Authors contributions

All authors contributed to the design of the experiments, to the scientific discussion and to the writing of the paper. YL, MG and IRG performed the laboratory work.

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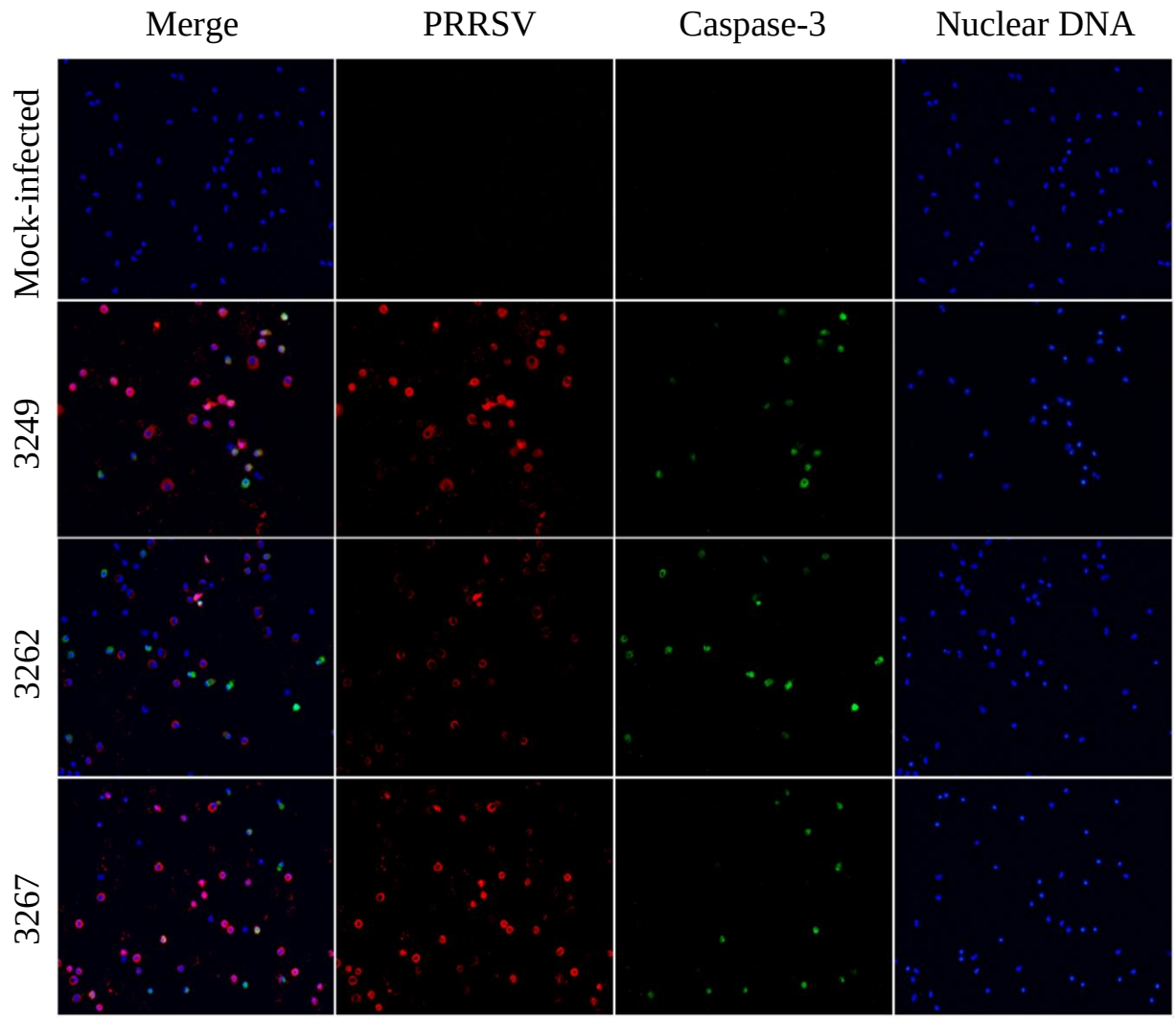

Supplementary figure 1. Colocalization of PRRSV (red), caspase-3 (green) and nuclear DNA (blue) in PAM cultured with three PRRSV1 isolates for 24 hours at a MOI of 0.1.

PART III: General discussion and conclusions

General discussion

Conclusions

General discussion

Attachment and internalization are the first steps in virus infection. In the case of PRRSV, our understanding about the process of viral entry has changed with time. At present, attachment is thought to occur by means of unspecific and specific mechanisms. Heparan sulphate -a linear polysaccharide that binds to a variety of protein ligands- is known to accumulate PRRSV particles on the surface of macrophages (Delputte *et al.*, 2002; Jusa *et al.*, 1997; Vanderheijden *et al.*, 2001).. By contrast, porcine sialoadhesin (PoSn, also denominated CD169 or Siglec-1) binds specifically to sialic acid on viral structural proteins, triggering endocytosis (Delputte and Nauwynck, 2004; Van Breedam *et al.*, 2010; Van Gorp *et al.*, 2009; Vanderheijden *et al.*, 2003). Recently, another siglec, siglec-10, has been supposed to have a function comparable to PoSn (Xie *et al.*, 2017). Whereas, both, heparan sulphate and PoSn, are thought to be non-essential for PRRSV infection, CD163 is considered essential as shown *in vitro* and *in vivo* experiments (Burkard *et al.*, 2017; Calvert *et al.*, 2007; Van Gorp *et al.*, 2008; Wells *et al.*, 2017; Whitworth *et al.*, 2016). CD163 participates in the viral genome release by interacting with GP2a and GP4 glycoproteins on PRRS virions (Das *et al.*, 2010; Tian *et al.*, 2012; Van Gorp *et al.*, 2010).

Based on the expression of CD163, some macrophages, monocyte-derived DC and subsets of bone marrow-derived DC (either derived with GM-CSF or Flt3L) would be susceptible to PRRSV (Flores-Mendoza *et al.*, 2008; Gimeno *et al.*, 2011; Loving *et al.*, 2007; Park *et al.*, 2008; Peng *et al.*, 2009; Rodriguez-Gomez *et al.*, 2015; Weesendorp *et al.*, 2013). However, in studies aimed to evaluate the replication of different PRRSV1 isolates in nasal mucosa, it was shown that some isolates infected dendritic-like cells that were apparently $CD163^-$ (Frydas *et al.*, 2013).

In the present study, three PRRSV1 isolates (3249, 3262 and 3267) that had been extensively characterized before and were shown to have different immunobiological properties (Darwich *et al.*, 2011; Diaz *et al.*, 2012; Gimeno *et al.*, 2011), were evaluated for their attachment and replication in GM-CSF-derived BMDC. For two isolates (3249 and 3262), replication peaked earlier in iBMDC (24 h) compared to mBMDC (48 h) while for the other (3267) peak was reached at 24 h in both cases. Moreover, the productivity of virus at 12 and 24 hpi were significantly higher in iBMDC than in mBMDC. These observations indicate that iBMDC are more efficient than mBMDC in uptaking/replicating the virus although the proportion of $CD163^{+}$ cells in iBMDC is lower than in their mature counterparts. Another fact that could explain these differences is that mBMDC have been pre-conditioned by anti-viral cytokines released during maturation with LPS but not during PRRSV infection since no detectable IFN-α was found in cell cultures of both iBMDC and mBMDC. In our opinion, the most plausible reason is that maturation resulted in the impaired antigen uptake ability of cells (Lambotin *et al.*, 2010; Platt *et al.*, 2010), and/or a post-entry blocking during replication happened, as the case of HIV (Canque *et al.*, 1999).

In addition, the replication efficiency was strain-dependent. Isolate 3262 showed the lowest titres in both cell types at 12 and 24 hpi. This is consistent with its low virulence in pigs (Diaz *et al.*, 2012). When replication kinetics were examined by flow cytometry, it became more evident that the proportion of cells infected by 3262 at earlier times was significantly lower than with other isolates. This pointed towards a decreased attachment and internalization of this isolate.

Then, the attachment and replication of the three isolates was studied with regards to the expression of PoSn, CD163 and heparan sulphate. Using a two-colour confocal microscopy staining, attachment and replication was observed in $PoSn^{-}$ or $CD163^{-}$ cells of both iBMDC and mBMDC. A further three-color staining (PoSn, CD163 and PRRSV) on iBMDC showed

that besides double positive $PoSn^{+}CD163^{+}$ cells, isolates 3249 and 3267 were also seen attached on the single positive subsets $PoSn^{+}$ $CD163^{-}$ and $PoSn^{-}$ $CD163^{+}$ and, more interestingly on the double negative subset $PoSn^{-}$ $CD163^{-}$. After removal of heparan sulphate by treatment with heparinase, attachment of isolates 3249 and 3267 could still be observed on the four subsets defined by PoSn and CD163. These results indicated either the attachment on BMDC occurred without the intervention of HS, PoSn and CD163, or the attachment was on cells expressing such low amounts of those molecules that were below the detection limit of confocal microscopy. Compared with isolates 3249 and 3267, isolate 3262 had lower binding capability on both iBMDC and mBMDC, which to some extent explained the lower titres detected in the cell cultures inoculated by this isolate.

With regards to replication, a two-colour immunofluorescence labelling (CD163 and PRRSV) showed that replication of the isolates 3249 and 3267 happened in iBMDC that were apparently $CD163^{-}$. When this was further examined by flow cytometry with isolate 3267, 8.4% ± 0.5% of apparently $CD163^{-}$ cells were labelled as positive for PRRSV N protein. At this point, the essential role of CD163 in PRRSV infection was questioned. However, in the heterogeneous cell population iBMDC, at least three subsets can be recognized: $CD163^{-}$, $CD163^{lo}$ and $CD163^{hi}$ with no clear cut-off points between each one. Thus, the possibility that cells apparently $CD163^{-}$ were indeed $CD163^{very\ low}$ could not be excluded despite with controls (uninfected cells, irrelevant antibodies, etc.).

The next experiment to solve this issue was to sort out different subsets defined by CD163. However, soon it became evident that some problems existed. One was the high levels of autofluorescence presented by live iBMDC that could not be solved with conventional methods (phenol red free medium, etc.). As a result, if a stringent criterion to avoid inclusion of $CD163^{+}$ cells within the $CD163^{-}$ compartment had to be used, the sensitivity would be decreased, namely, $CD163^{-}$ cells with higher autofluorescence would be excluded. On the contrary, being

stringent for $CD163^{+}$ cells would result in classifying $CD163^{lo/very\ low}$ as $CD163^{-}$. The other problem is that the cell separation will conceal the effect of potential cell-cell contact. During the infection of iBMDC, cytokines or other factors released by infected cells may induce some $CD163^{-}$ cells to express CD163 in very low amounts, however enough to permit infection, or to express some other molecules -yet unknown- that may act as PRRSV receptors. If so, these permissive cells arising from the $CD163^{-}$ subset would only appear during the infection of the original heterogeneous population of iBMDC. It is worth mentioning that in the unsorted iBMDC, the infected cells that apparently were $CD163^{-}$ were not evident by 24 hpi, but noticeably arose after another 24 hours' incubation, being in favour of the abovementioned idea.

Two sorting experiments were designed. Since the autofluorescence of iBMDC was higher in channels of green and yellow-to-red, a far-red fluorophore was chosen and the adjacent channel was examined at the same time to further discriminate signals between labelling and autofluorescence. The first experiment focused on sorting "beyond doubt" $CD163^{-}$ cells. For this, $CD163^{-}$ cells without $CD163^{lo}$ contamination were grouped to detect whether cells without CD163 expression were susceptible to PRRSV. In the second experiment, sorting was carried out grouping truly $CD163^{-}$ cells together with $CD163^{lo}$ to see whether infected $CD163^{lo}$ cells would have effect on the susceptibility of $CD163^{-}$ subset.

Again in the unsorted iBMDC, 5.7 ± 0.2% of cells apparently $CD163^{-}$ were detected as PRRSV-positive. But in the first sorting experiment, no infection could be observed by 40 hpi suggesting that the "beyond doubt" $CD163^{-}$ cells were not permissive to PRRSV1 infection. However, when $CD163^{-}$ cells were sorted together with $CD163^{lo}$, the infected $CD163^{-}$ population (0.6 ± 0.07%) appeared again by 40 hpi and when the incubation was extended to 60 hpi, the percentage of infected $CD163^{-}$ cells increased more (1.6% ± 0.08%).

These results could be interpreted in different ways. Firstly, the subset of $CD163^-$ susceptible cells that arose during the infection, could be the result, at least partially, of the milieu created by the infection. Secondly, it is still possible that the infected $CD163^-$ cells were those with an extremely low expression of CD163 (beyond the sensitivity of the flow cytometry technique) but enough to permit the infection. Thirdly, some $CD163^+$ could had undergone a phenotypic change to $CD163^-$. This is very unlikely since the sorted cells $CD163^{hi}$ or $CD163^{lo+hi}$ did not have obvious changes in the percentage of $CD163^+$ after infection. Furthermore, it cannot even be excluded that the infected $CD163^-$ cells represented a subset with particular abilities to uptake the virus by macropynocitosis or other mechanisms. But if so, more cells would have been observed to be infected.

To some extent, our results 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 (Burkard *et al*., 2017; Wells *et al*., 2017; Whitworth *et al*., 2016). And substitution of SRCR5 domain with a homolog humane counterpart could impair pigs' susceptibility (Wells *et al*., 2017). From another point, our results and those of others are not necessarily contradictory. Firstly, it is worth mentioning that in our experiment only a small proportion of susceptible $CD163^-$ BMDC were observed and those cells arose in the course of infection with other CD163-positive cells. Whereas in CD163 edited animals, the lack of infection of macrophages -a much larger population than DC- would result in no viremia and no detectable virus in spite that a small subset of DC could be infected in lymphoid tissues. Secondly, from our previous results it seems clear that infection of DC, to some extent, depends on the isolate. So, differences between PRRSV1 and PRRSV2 may exist and even between isolates of the same species. Thirdly, there are still some evidences about the possibility of the existence of other receptors. For example, in nasal explants Lena showed a tropism towards $CD163^-$ cells (Frydas *et al*., 2013). Sang *et al.* (2014) described a PRRSV infection in $CD163^-$

population among polarized porcine monocytic cells. And Doeschl-Wilson *et al.* (2016) demonstrated an increasing proportion of PRRSV-infected $CD163^-$ PAM at the later incubation stages.

In conclusion, the present results reinforce the idea that CD163 negative cells may support the replication of PRRSV and other receptors or mechanisms of entry might exist. The fact that the proportion of infected $CD163^-$ cells was low, does not preclude anything on the biological significance of this population before any further characterization.

The second part of the present thesis was devoted to characterising the apoptosis induced by three PRRSV1 isolates (3249, 3262 and 3267). In PAM, positive signals for caspase-3 was observed in both infected and bystander cells for all three examined isolates despite of their different replication kinetics. The most likely explanation for this observation is that PRRSV1 may trigger apoptosis in PAM through both intrinsic and extrinsic pathways and, probably, this is mediated by cytokines. Differently, in BMDC, apoptosis was mainly found in bystander cells suggesting the predominance of the extrinsic pathway. Moreover, at a low MOI (0.1) the caspase 3 signal in BMDC peaked at 48 hpi, later than in PAM (24 hpi). This slower development of apoptosis may allow more cycles of viral replication, resulting in higher viral yields in BMDC. For PRRSV as well as for other viruses it has been reported that preventing or delaying apoptosis is a commonly used strategy to evade the host response (Banadyga *et al.*, 2009; Lu *et al.*, 2011; Thomson, 2001). The mechanisms involved in this particular case is not known.

Further examination of inoculated BMDC cultures for apoptosis/necrosis showed significant differences between isolates. Whereas 3249 and 3267 apparently induced apoptosis and necrosis at 48 hpi, cells infected by 3262 only had minor changes, corresponding with its lower replication rate. Thus, for isolate 3267, 58.4% cells were labeled as apoptotic (including early-

and late-stage apoptotic cells) with additional 9.6% necrotic cells. For isolate 3249, there were 27.5% apoptotic cells and 15.1% necrotic cells. These results indicate that apoptosis is a major contributor of PRRSV-induced cell death. Kim *et al.* (2002) and Costers *et al.* (2008) made a similar conclusion when studied the infection in MARC-145 cells and macrophages, that PRRSV-infected cells died by caspase-dependent apoptosis which culminate in apoptotic necrosis. In contrast, isolate 3262 induced very little apoptosis, a fact that is difficult to explain. However, it is worth noting that this isolate induced IL-10 in BMDC while 3249 and 3267 did not. Neutralization of IL-10 resulted in the emergence of apoptosis but this did not happen with a second IL-10 inducing isolate (designated as 2988). In contrast, blocking of TNF-α in 3249-infected cultures (induce TNF-α+ but not IL-10) did not abolish apoptosis. Therefore, the balance of TNF-α and IL-10 production may influence the apoptosis induced by PRRSV1, but mechanisms other than the release of TNF-α probably are involved because isolates 3267 (with neither IL-10 nor TNF-α induction) could also induce apoptosis.

In fact, molecular mechanisms mediating pathogen-induced cell death are more complicated than have always been appreciated. In single dying cells, multiple death pathways can be activated and cross-talk between these pathways may exist and lead to the ultimate outcome. Therefore, it is not strange that inhibiting/activating one route or one factor may not result in survival/death but the opposite.

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Conclusions

1. The PRRSV1 titres attained in immature (i) bone marrow-derived DC (BMDC) cultures were always higher than those obtained in mature (m) BMDC. Moreover, the replication peaked earlier, at least for two isolates (3249 and 3262), in iBMDC (24 hpi) than in mBMDC (48hpi). Since the proportion of $CD163^+$ cells in mBMDC was higher than in iBMDC and none of the isolates induced IFN-α, the differences are probably attributable to the intrinsic ability of iBMDC for supporting PRRSV1 replication.
2. Significant differences in the viral titres were observed between the examined isolates. This result indicates that viral diversity may also affect the ability to infect BMDC.
3. Attachment of isolates 3249 and 3267 to iBMDC and mBMDC was seen similar in confocal microscopy; in contrast, isolate 3262 attached much less. In addition, the proportion of 3262-infected cells at 12h post-inoculation was significantly lower as determined by flow cytometry. These observations reinforce the notion that not all PRRSV1 are equally efficient to infect BMDC.
4. Attachment of PRRSV1 isolates 3249 and 3267 on iBMDC was observed in the four subsets defined by the expression of CD163 and porcine sialoadhesin. The removal of heparan sulphate did not abolish the attachment. These results are compatible with the existence of additional specific or unspecific receptors for the virus. It would be also possible that the virus attached to cells expressing tiny amounts of the examined receptors below the sensitivity of confocal microscopy.
5. The apparently permissive $CD163^-$ cells observed in flow cytometry experiments were only observed when $CD163^+$ cells were included in the culture. The results were compatible with two scenarios. First, true $CD163^-$ permissive cells arose because of the effect of the milieu created by the presence of $CD163^+$ infected BMDC. Second, there

is a continuous generation of cells expressing very low levels of CD163 during the incubation, enough to allow infection but beyond the detection capability of flow cytometry.

6. PRRSV1 infection induced apoptosis in both infected and bystander porcine alveolar macrophages while in BMDC, apoptosis was mainly seen in bystander cells and developed slower than in macrophages. These observations confirm that PRRSV1-induced apoptosis may happen by the intrinsic and the extrinsic pathways. BMDC could be somehow protected from apoptosis at earlier stage of infection.
7. Different PRRSV1 isolates induced different levels of apoptosis and necrosis. This observation agrees with the knowledge of different immunobiological properties of different PRRSV1 strains.

ANNEX

Presentations in International congresses about PRRSV

Y.L. Li, L. Darwich, E. Mateu. Resolution of genotype 1 PRRSV attachment on bone marrow-derived dendritic cells. 4th North American PRRS Symposium, December 3-4, 2016, Chicago, USA. (Oral communication and poster)

Y.L. Li, L. Darwich, E. Mateu. Attachment and replication of genotype 1 porcine reproductive and respiratory syndrome virus (PRRSV) on bone marrow-derived dendritic cells. 3rd North American PRRS Symposium, December 5-6, 2015, Chicago, USA. (Oral communication and poster)

Y.L. Li, M. Gimeno, L. Darwich, E. Mateu. Apoptosis induced by replication of different genotype 1 porcine reproductive and respiratory syndrome virus. 3rd North American PRRS Symposium, December 5-6, 2015, Chicago, USA. (Poster)

E. Pileri, E. Gibert, **Y. Li**, L.V. Alarcón, G. Martín-Valls, I. Díaz, J. Casal, M. Martín, L. Darwich, E. Mateu. Vaccination of piglets with a genotype 1 modified live PRRS virus vaccine delays transmission between pigs and decreases viral load in a one-to-one transmission experiment. 7th European Symposium of Porcine Health Management, April 22-24, 2015, Nantes, France. (Poster)

Publications about PRRSV

Ait-Ali, T., Díaz, I., Soldevila, F., Cano, E., **Li, Y.**, Wilson, A.D., Giotti, B., Archibald, A.L., Mateu, E., Darwich, L., 2016. Distinct functional enrichment of transcriptional signatures in pigs with High and Low IFN-gamma Responses after Vaccination with a Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). *Vet Res*, 47(1):104.