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Role of CD163 and other biomarkers of interest in the immunopathogenesis of PRRSV-1 at lung level

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AUTOR: *José María Sánchez Carvajal*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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Trabajo presentado por el Graduado en Veterinaria
D. José María Sánchez Carvajal
para optar al grado de Doctor por la Universidad de Córdoba

Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología
Córdoba, 2020

UNIVERSIDAD DE CÓRDOBA
FACULTAD DE VETERINARIA

DEPARTAMENTO DE ANATOMÍA Y ANATOMÍA PATOLÓGICA COMPARADAS Y TOXICOLOGÍA

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Facultad de Veterinaria
Universidad de Córdoba

Campus Universitario de Rabanales
Ctra. de Madrid – Cádiz Km 396
14014 - Córdoba (España)

Irene M. Rodríguez Gómez, profesora sustituta interina del Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología de la Universidad de Córdoba

Informa,

que el Graduado en Veterinaria D. **José María Sánchez Carvajal** ha realizado bajo mi tutela el trabajo titulado **"El papel del CD163 y otros biomarcadores de interés en la inmunopatogenia del PRRSV-1 a nivel pulmonar"**, el cual considero que reúne las condiciones y calidad científica necesarias para optar al Grado de Doctor por la Universidad de Córdoba.

Y para que conste y surta los efectos oportunos, firmo el presente informe en Córdoba a once de junio de 2020.

Fdo: Irene M. Rodríguez Gómez

Facultad de Veterinaria
Universidad de Córdoba

Campus Universitario de Rabanales
Ctra. de Madrid – Cádiz Km 396
14014 - Córdoba (España)

Jaime Gómez-Laguna, contratado "Ramón y Cajal" adscrito al Departamento de Anatomía y Anatomía Patológica Comparadas y Toxicología de la Universidad de Córdoba

Informa

Que el Graduado en Veterinaria D. **José María Sánchez Carvajal** ha realizado bajo mi dirección y asesoramiento el trabajo titulado **"El papel del CD163 y otros biomarcadores de interés en la inmunopatogenia del PRRSV-1 a nivel pulmonar"**, que considero que reúne las condiciones y calidad científica necesaria para optar al Grado de Doctor por la Universidad de Córdoba.

Y para que conste y surta los efectos oportunos, firmo el presente informe en Córdoba a once de junio de 2020.

Jaime Gómez Laguna

A mi madre,
por una camino que empezamos juntos

"However difficult life may seem, there is always something you can do, and succeed at. It matters that you don't just give up".

Stephen Hawking

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GLOSARIO DE TÉRMINOS - GLOSSARY OF TERMS

ABC	Avidin-Biotin-Complex; complejo avidina - biotina
ADE	Antibody-Dependent Enhancement
ADNr	Ácido Desoxirribonucleico Ribosómico
APCs	Antigen Presenting Cells; células presentadoras de antígenos
ARNr 16S	Ácido Ribonucleico
ARNm	Ácido Ribonucleico mensajero
BALF	BronchoAlveolar Lavage Fluids
BM-DCs	Bone Marrow-derived Dendritic Cells; células derivadas de médula ósea
cCasp	cleaved Caspase
Células NK	Natural Killer Cells; células asesinas naturales
CTLs	Cytotoxic T Lymphocytes; linfocitos T citotóxicos
dpi	days post-infection or -inoculation; días postinfección o inoculación
ELISA	Enzyme-Linked ImmunoSorbent Assay; ensayo por inmunoabsorción ligado a enzimas inmunoenzimática
FMDV	Foot-and-Mouth Disease Virus; Virus de la fiebre aftosa
GM-CSF	Granulocyte–Macrophage Colony-Stimulating Factor; Factor estimulante de colonias de granulocito-macrófago
HIV	Human Immunodeficiency Virus; Virus de la inmunodeficiencia humana
HP-PRRSV	Highly Pathogenic-PRRSV; PRRSV altamente patógeno
IFN-γ	InterFeróN γ
IFN-γ-SC	IFN-γ secreting cells; células secretoras de IFN-
IL	Interleukin; interLeuquina;
iNOS	inducible Nitric Oxide Synthase; enzima óxido nítrico sintetasa
LBP	Lipopolysaccharide Binding Protein; proteína de unión a lipopolisacáridos
LV	Lelystad Virus
MALDI–MSI	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging; espectrometría de masas con imagen por láser de desorción/ionización asistida por matriz
MHC-I	Major Histocompatibility Complex class I; complejo mayor de histocompatibilidad clase I

MHC-II	Major Histocompatibility Complex class II; complejo mayor de histocompatibilidad clase II
MLV	Modified Live Vaccine; vacuna viva atenuada
moDCs	monocyte-derived Dendritic Cells; células dendríticas derivadas de monocitos
NAbs	Neutralizing Antibodies; anticuerpos neutralizantes
Nsps	Non structural proteins; proteínas no estructurales
ORF	Open Reading Frame; fragmento de lectura abierta
PAMs	Pulmonary Alveolar Macrophages; macrófagos alveolares pulmonares
PAMPs	Pathogen-Associated Molecular Patterns; patrones moleculares asociados a patógenos
PBMCs	Peripheral Blood Mononuclear Cells; células mononucleares de sangre periférica
PIMs	Pulmonary Intravascular Macrophages; macrófagos intravasculares pulmonares
PRCV	Porcine Respiratory CoronaVirus; Coronavirus respiratorio porcino
PRDC	Porcine Respiratory Disease Complex; Complejo respiratorio porcino
PRRs	Pattern Recognition Receptors; receptores de reconocimiento de patrones
PRRS	Porcine Reproductive and Respiratory Syndrome; Sindróme reproductivo y respiratorio porcino
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus; Virus del sindróme reproductivo y respiratorio porcino
RCD	Regulated Cell Death; muerte celular regulada
RFS	Ribosomal Frameshift
RIG-I	Retinoic Acid Inducible Gene
ROIs	Regions-Of-Interest; áreas de interés
TNF-α	Tumor Necrosis Factor α; factor de necrosis tumoral α
SRCR	Scavenger Receptor Cysteina-Rich; receptor scavenger rico en cisteína
sCD163	soluble CD163; CD163 soluble
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2; Coronavirus 2 del síndrome respiratorio agudo grave

TGEV	Transmissible GastroEnteritis coronaVirus; Virus de la gastroenteritis transmisible
TLRs	Toll-Like Receptors; receptores tipo Toll
UTR	Untranslated Region; región no traducida

Introducción - Introduction

Imagen: Infobae

Como su propio nombre indica, el Síndrome Reproductivo y Respiratorio Porcino (PRRS, del inglés Porcine Reproductive and Respiratory Syndrome) integra dos patologías con características clínicas muy diferentes: (I) fallos reproductivos en hembras adultas caracterizados por abortos, fetos momificados y nacidos débiles; y (II) problemas respiratorios en cerdos en crecimiento y cebo, que tienen como consecuencia un menor índice de conversión y el desarrollo de infecciones bacterianas secundarias, dando lugar a la instauración del complejo respiratorio porcino (PRDC, del inglés Porcine Respiratory Disease Complex). En este último aspecto se centrará el desarrollo de la presente tesis doctoral.

Los primeros casos clínicos asociados al PRRS datan de finales de la década de los 80, casi simultáneamente en Europa y Estados Unidos, aislándose por primera vez el agente causal, el virus del PRRS (PRRSV), en 1991 en los Países Bajos. Actualmente, más de 30 años después de su primera aparición, el PRRS se ha convertido en una de las enfermedades víricas más importantes por su elevada contagiosidad y marcada variabilidad genética y antigénica. En este sentido, cabe destacar la emergencia de cepas de elevada virulencia, lo cual ha contribuido a dificultar la lucha frente a esta enfermedad, considerándose la mayor causa de pérdidas económicas para los sistemas de producción porcina actuales a nivel mundial. Estas pérdidas están asociadas de forma directa e indirecta a su clínica característica, suponiendo un aumento del índice de conversión y tratamientos asociados, tanto de tipo profiláctico como terapéutico.

El gran abanico de cepas del PRRSV complica el escenario de su prevención y erradicación, de forma que a pesar de existir un número considerado de vacunas comerciales disponibles, éstas sólo ofrecen protección homóloga, siendo incompleta parcial, en el mejor de los casos, la protección heteróloga. A pesar de ello, las vacunas tienen efectos positivos reduciendo tanto la transmisión del virus como su sintomatología clínica.

Tal y como se ha mencionado, la evolución del PRRSV ha dado lugar a la aparición de cepas de elevada virulencia caracterizadas por una elevada morbilidad y mortalidad, fiebre, lesiones de tipo hemorrágico y una marcada neumonía intersticial que evoluciona a bronconeumonía catarro-purulenta. Estos antecedentes ponen de manifiesto el interés en estudiar la inmunopatogenia del PRRSV, concretamente de su forma respiratoria, para intentar elucidar los mecanismos utilizados por el virus para

provocar una desregulación de la respuesta inmune a nivel respiratorio favoreciendo el desarrollo de infecciones bacterianas secundarias, así como evidenciar diferencias entre cepas de distinta virulencia. El empleo de nuevas herramientas de histología molecular representa una aproximación de interés en estos casos, ya que permite caracterizar mejor este tipo de procesos identificando marcadores implicados en el desarrollo de una lesión concreta o en estructuras específicas del tejido.

Así, el PRRS se ha consolidado como una enfermedad endémica en la mayoría de los países productores, ya que el virus es capaz de reintroducirse en regiones, granjas e incluso países tras costosos programas de erradicación. Esta frustrante enfermedad se ha convertido en uno de los mayores retos para la industria porcina y su comunidad científica.

Porcine Reproductive and Respiratory Syndrome (PRRS) includes two distinct clinical entities: (I) reproductive failure in sows or gilts characterised by abortions, foetal mummification and weak newborns; and (II) respiratory disorders in growing and fattening pigs which cause a drop in feed conversion rate as well as an increase of susceptibility to secondary bacterial infections, being considered as one of the main primary agents of porcine respiratory disease complex (PRDC). The present PhD dissertation will be focused on this last aspect of the disease.

The first clinical outbreaks associated with PRRS were almost simultaneously described in Europe and the United States at the end of the decade of 1980s. PRRS virus (PRRSV), the causative agent of PRRS, was then firstly isolated in The Netherlands in 1991. Nowadays, more than 30 years after its first emergence, PRRS has become one of the most important swine viral diseases due to its high contagiousness and marked genetic and antigenic variability. In this sense, it is noteworthy to mention that the emergence of virulent strains has made even more challenging to control PRRS, which is considered as one of the main causes of economic losses for the modern pig industry worldwide. Considering the full clinical range of this disease, the economic losses are associated with both direct and indirect costs, such as increased feed conversion ratio, cost of biosecurity measures or expenses associated with prophylactic and therapeutic strategies.

The wide range of PRRSV strains is struggling the prevention and/or eradication of PRRS, even though there are over 30 commercial vaccines available. These vaccines only provide homologous protection, being the heterologous protection partial or incomplete in the best-case scenario. Nonetheless, vaccines provide positive effects by reducing both the transmission of the virus and associated clinical symptoms.

As above mentioned, the rapid evolution of PRRSV has driven to the emergence of virulent strains characterised by high morbidity and mortality rates, fever, haemorrhagic lesions, acute respiratory clinical signs as well as severe interstitial pneumonia, which is frequently complicated with suppurative bronchopneumonia. These findings highlight the interest in studying the immunopathogenesis of PRRS, particularly its respiratory presentation, trying to elucidate the mechanisms involve in the dysregulation of the immune response at lung level, which favour the development of secondary bacterial infections, but also identifying differences between strains of

different virulence. In this context, in order to assess a better characterisation of PRRSV-associated lesions, the application of molecular histopathology would be an interesting approach to detect potential biomarkers involved in the development of each specific lesion or in specific tissue structures.

Therefore, PRRS has become an endemic disease in most of the swine producing countries, due to the frequent re-emergence of the virus in regions, farms and even countries after the application of costly control and eradication procedures. This frustrating disease has turned into one of the greatest challenges for the pig industry and its scientific community.

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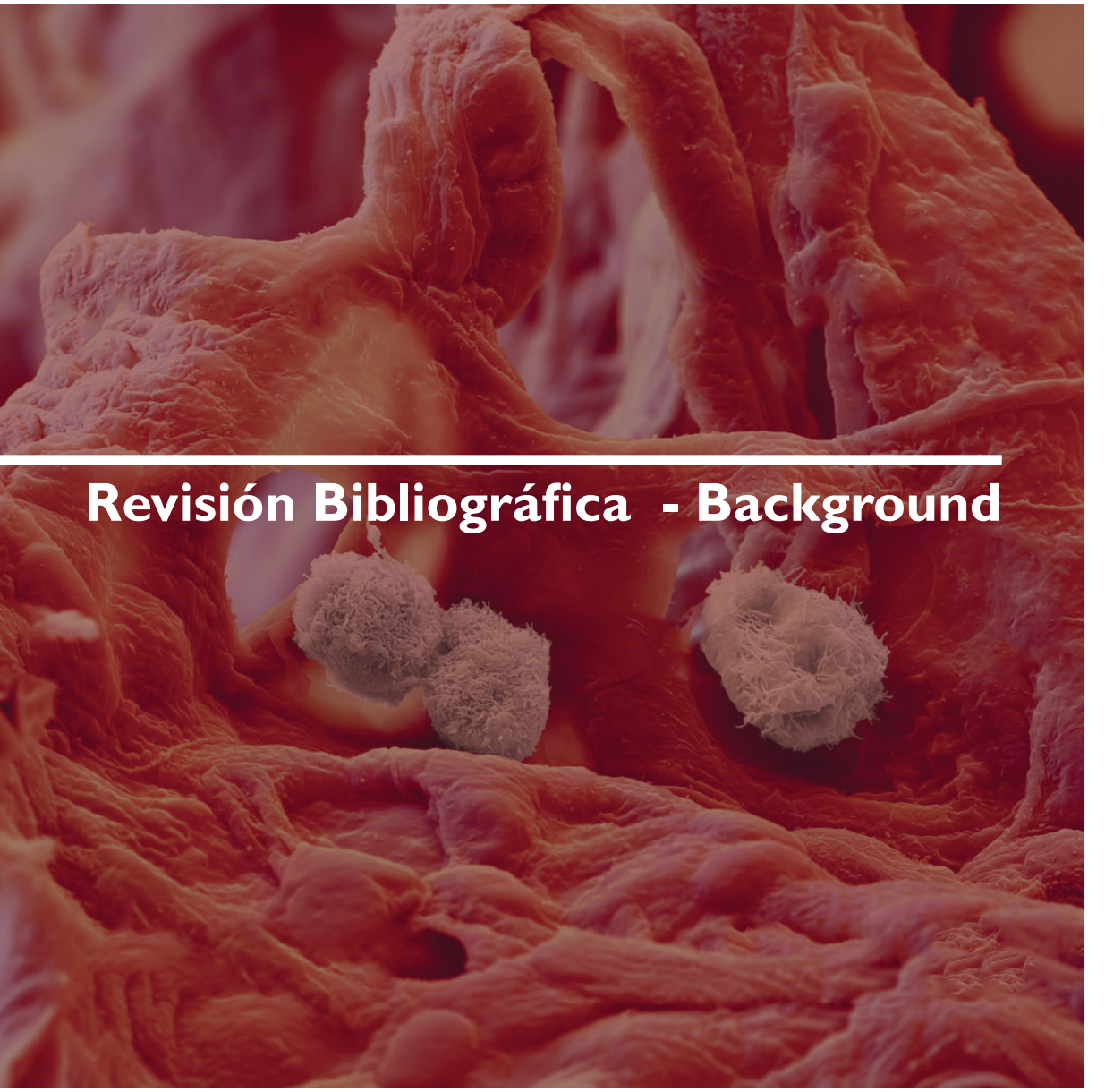

Revisión Bibliográfica - Background

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1.VIRUS DEL SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

1.1. Origen y evolución del PRRSV: causas y consecuencias de su diversidad genética

A finales de 1980 se describieron una serie de brotes en granjas porcinas localizadas en los estados de Carolina del Norte, Iowa y Minnesota, caracterizados por fallos reproductivos y neumonía en cerdos post destete (Keffaber, 1989; Loula, 1991). En Europa, brotes de características clínicas similares se describieron en Alemania, propagándose rápidamente por la mayoría de los países europeos (Conzelmann et al., 1993; Wensvoort et al., 1991; 1992; Plana et al., 1992; Yoon et al., 1992). En 1991, el agente etiológico del Síndrome Reproductivo y Respiratorio Porcino (PRRS del inglés Porcine Reproductive and Respiratory Syndrome) se aisló por primera vez en los Países Bajos, concretamente en los laboratorios de Lelystad (Wensvoort et al., 1991). Este primer aislamiento del virus del PRRS (PRRSV del inglés Porcine Reproductive and Respiratory Syndrome Virus) se denominó virus Lelystad (LV, del inglés Lelystad Virus) y es considerado el prototipo del PRRSV-1. Posteriormente, Collins et al. (1992) realizaron el primer aislamiento del virus en Estados Unidos, la cepa aislada se denominó VR-2332, prototipo del PRRSV-2. A pesar de que el aislamiento de ambos virus, LV y VR-2332, se hizo simultáneamente en Europa y Estados Unidos (Keffaber, 1989; Loula, 1991; Wensvoort et al., 1991) y de contar con características clínicas, epidemiológicas y ecológicas similares, se encontraron importantes diferencias genéticas entre ellos, clasificándose inicialmente en dos genotipos, el tipo 1 (o también conocido como europeo) y el tipo 2 (o americano).

Se utilizaron diferentes nombres como "Enfermedad misteriosa porcina" o "Enfermedad de la oreja azul" para denominar a este síndrome, sin embargo, durante el primer congreso internacional de la enfermedad celebrado en St. Paul (1992, Minnesota, EE. UU) se adoptó su denominación actual.

Recientemente, PRRSV-1 y PRRSV-2 han sido considerados como dos especies víricas diferentes, Betaartevirus suid 1 y Betaarterivirus suid 2, respectivamente (Cavanagh, 1997; Gorbalenya et al., 2018). De este modo, ambos virus están incluidos dentro del nuevo género Betaarterivirus, perteneciente a la familia Arteriviridae, orden Nidovirales (Kuhn et al., 2016; Gorbalenya et al., 2018). PRRSV-1 y PRRSV-2

cuentan con una homología genética de tan sólo un 55-70%, presentando ambos virus una amplia diversidad genética (Stadejek et al., 2013) (Figura 1A).

Esta elevada variabilidad genética descrita dentro de cada virus, PRRSV-1 y PRRSV-2, queda patente por la gran cantidad de subtipos o linajes identificados para cada uno de ellos y que cuentan con una similitud intraespecie muy reducida (70% dentro del PRRSV-1; 78% dentro del PRRSV-2) (Balka et al., 2018; Snijder et al., 2013; Stadejek et al., 2013). Cabe destacar que la mayoría de los virus ARN tienden a la mutación y recombinación dentro de su ciclo de replicación (Domingo & Holland, 1997). En este sentido, el PRRSV presenta una de las tasas de mutación (ratio que mide el número de errores cometidos durante la replicación del virus) más elevadas de la naturaleza (entre 20 y 40 veces superior al virus de la inmunodeficiencia humana o al virus de la influenza aviar), junto con una elevada tasa de sustitución (ratio que mide el número de mutaciones que llegan a fijarse). Estos hechos se encuentran favorecidos porque el virus presenta una ARN polimerasa que no cuenta con la capacidad de corrección de errores introduciendo en el genoma mutaciones aleatorias a una gran velocidad (Hanada et al., 2005; Kappes & Faaberg, 2015; Murtaugh et al., 2001). Además, el PRRSV presenta una gran capacidad para establecer recombinaciones tanto con cepas de campo como con cepas vacunales atenuadas o MLV (del inglés Modified Live Vaccines) (Huang et al., 2009; Kappes & Faaberg, 2015; Li et al., 2009; Liu et al., 2011; Martin-Valls et al., 2014; Shi et al., 2013; Wang et al., 2019).

Actualmente, en base a análisis filogenéticos se describen 4 subtipos diferentes dentro del PRRSV-1, especie más prevalente en Europa, con una acentuada demarcación geográfica: el subtipo 1 o pan-europeo que circula principalmente en países del centro y oeste de Europa; los subtipos 2 y 3, en el que se incluyen cepas virulentas como Lena o SU1-bel y que se han aislado únicamente en países de Europa del Este; y el subtipo 4, con cepas procedentes de Letonia y Bielorrusia. Los subtipos de Europa del Este presentan una variabilidad filogenética más elevada que el subtipo 1 (Darwich et al., 2011; Stadejek et al., 2006; Stadejek et al., 2008, 2013); Balka et al. (2018) sugieren que los movimientos transfronterizos de animales infectados y de semen podrían haber facilitado esta mayor diversidad del PRRSV-1, aunque probablemente haya otros factores implicados (Figura 1B). Aunque el PRRSV-1 se aísle preferentemente en Europa, cabe destacar que brotes aislados asociados a cepas del PRRSV-1 también han sido descritos en países como Estados Unidos (Fang et al., 2004; Fang et al., 2007;

Ropp et al., 2004; Wasilk et al., 2004), Canadá (Dewey et al., 2000), Corea del Sur (Nam et al., 2009; Nguyen et al., 2014), China (Chen et al., 2011; Chen et al., 2017; Liu et al., 2017; Zhou et al., 2015) y Tailandia (Amonsin et al., 2009; Jantafong et al., 2015).

Por otro lado, en relación con el PRRSV-2, a pesar de no contar con subtipos claramente definidos, se describe la existencia de nueve linajes diferentes en base a análisis filogenéticos de la secuencia del fragmento de lectura abierta 5 (ORF5, del inglés Open Reading Frame). Es importante destacar que esta construcción filogenética no representa la diversidad completa del PRRSV-2, debido a la falta de información con relación a la secuencia de las cepas aisladas en Canadá (Brar et al., 2011; Shi et al., 2010a; 2010b).

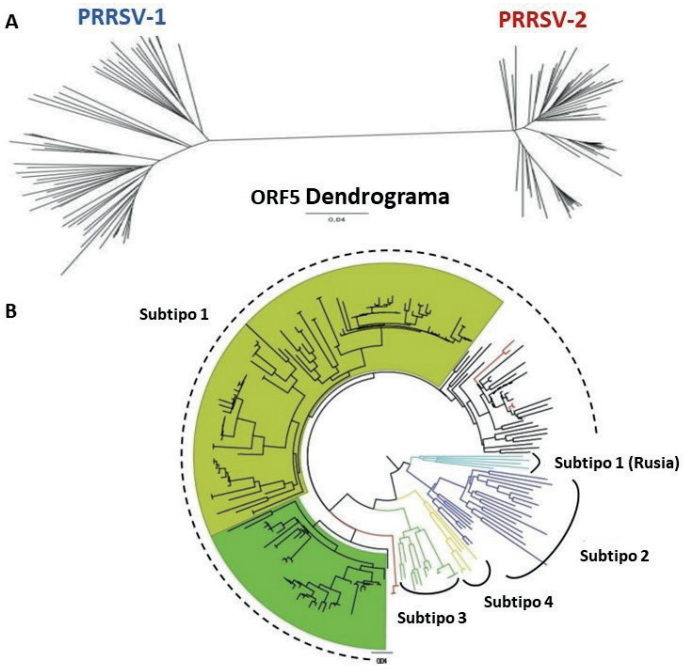

Figura 1. (A) *Modelo filogenético del PRRSV basado en la secuencia de nucleótidos de la ORF5 (modificado de Murtaugh et al., 2015).* ***(B)*** *Árbol filogenético del PRRSV-1 basado en la secuencia ORF5 (modificado de Franzo et al., 2015).*

La inestabilidad genómica del PRRSV junto con la fuerte selección inmunológica consecuencia del empleo sistemático de vacunas como estrategia de elección para controlar la enfermedad, han favorecido su rápida evolución genética, que se ha asociado a diferentes patrones de virulencia (Murtaugh et al., 2010). La gran diversidad genética y antigénica del PRRSV no sólo complica y limita su diagnóstico y control (Butler et al., 2014; Darwich et al., 2011; Geldhof et al., 2012; Murtaugh et al., 2010), sino que además se refleja en la aparición en la última década de cepas virulentas del PRRSV-1 (Karniychuk et al., 2010; Morgan et al., 2013; Stadejek et al., 2013) y del PRRSV-2 (Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008), aumentando la preocupación por la posible diseminación de éstas a nivel mundial, así como por los efectos que tendrían sobre el sector porcino.

Desde la aparición del PRRSV, algunas cepas desarrollaron una mayor virulencia dando lugar a brotes sobreagudos de enfermedad en diferentes países. A finales de los 90, se registraron los denominados inicialmente como brotes atípicos del PRRS en los estados de Iowa y Carolina del Norte (Estados Unidos), caracterizados por una elevada mortalidad y fallos reproductivos graves en explotaciones endémicas para el PRRSV (Halbur & Brush, 1997; Mengeling et al., 1999). Estos brotes se describieron como "formas agudas del PRRS" y se asociaron con aislados del virus que presentaban características genéticas similares, hasta que finalmente se describió la cepa virulenta MN184 del PRRSV-2 (Han et al., 2006). En el año 2006, se produjo un evento clave que cambió el concepto previo sobre la patogenia del PRRSV. En China, comenzaron a aparecer brotes asociados a cepas PRRSV-2 de elevada virulencia caracterizados por fiebre muy elevada (41-42 °C) acompañada de una intensa sintomatología respiratoria, elevada morbilidad y mortalidad que se denominaron como "enfermedad de la fiebre alta" (del inglés High Fever Disease), y actualmente se conoce como PRRSV altamente patógeno (HP-PRRSV, del inglés Highly Pathogenic-PRRSV). Esta nueva presentación clínica del PRRSV causó la muerte de millones de cerdos de todas las edades generando enormes pérdidas económicas en el sector porcino (Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008). Tras alcanzar Vietnam en el año 2007, estas cepas HP-PRRSV-2, procedentes de China, se propagaron por otros países del Sureste Asiático, como Laos, Filipinas, Camboya, Bután, Birmania, Malasia, y Singapur, alcanzando también Rusia y la India (An et al., 2011; Han et al., 2015; Rajkhowa et al., 2015; Tian et al., 2007; Tong et al., 2007; Zhou et al., 2008). Cabe destacar que, además, otras cepas virulentas del PRRSV-2 como las NADC30 y similares se han aislado

tanto en China como en Estados Unidos (Brockmeier et al., 2012; Li et al., 2016; Zhou et al., 2015). Aunque todavía es objeto de estudio, las cepas HP-PRRSV-2 parecen caracterizarse por una deleción de 30 aminoácidos en la proteína no estructural 2 (Nsp2) del virus, marcador molecular que no está relacionado con la virulencia de estas cepas y que podría permitir diferenciarlas.

En los últimos años también han aparecido en el contiene europeo un conjunto de cepas PRRSV-1, pertenecientes a los subtipos 1, 2 y 3 principalmente, que presentan una virulencia mayor que las cepas tradicionales del PRRSV-1. Las primeras cepas virulentas del PRRSV-1 se aislaron en Bielorrusia (2006-2010) pertenecientes al subtipo 3 de este virus (cepas LENA y SU1-bel) (Karniychuk et al., 2010; Morgan et al., 2013), lo que hizo que inicialmente se pensara que las cepas virulentas del PRRSV-1 podrían estar restringidas a este subtipo. Sin embargo, poco después se identificaron nuevas cepas virulentas pertenecientes tanto al subtipo 1 en países como Bélgica (13V091), Austria (AUT15-33) e Italia (PR40) (Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017), como al 2 del PRRSV-1 (BOR49) (Stadejek et al., 2017), abriendo el abanico de posibilidades y la complejidad dentro del PRRSV-1. Esta situación ha aumentado la preocupación por la diseminación de estas cepas del virus por el contiene europeo.

1.2. PRRSV, el virus

El PRRSV es un virus ARN, envuelto, con un tamaño aproximado de entre 50-65 nanómetros, monocatenario y de sentido positivo perteneciente a la familia Arteriviridae (Cavanagh, 1997; Meulenberg, 2000). Los arterivirus comparten una serie de características genéticas y biológicas únicas, como (I) una marcada variabilidad genética, (II) la organización del genoma y morfología del virión, (III) una especificidad celular elevada, ya que se replican principalmente en macrófagos alveolares, y (IV) una gran capacidad para causar infecciones persistentes y en algunos casos asintomáticas (Snijder et al., 2013).

El genoma del PRRSV tiene un tamaño aproximado de 15 kb (Meulenberg et al., 1993), está formado por una región no traducida 5'UTR (5'UTR del inglés, Untranslated Region), al menos 11 ORFs (ORF1a, ORF1a', ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5a, and ORF5–ORF7); una región no traducida 3'UTR y cuenta con

dos mecanismos de transcripción diferentes (RFS, del inglés Ribosomal Frameshift) (Johnson et al., 2011; Snijder et al., 2013; Kappes & Faaberg, 2015). A continuación del extremo 5'UTR (downstream) se localizan tres grandes ORFs superpuestos, ORF1a, ORF1a' y ORF1b, los cuales representan el 80% del genoma vírico y codifican al menos 14 proteínas no estructurales (Nsp's, del inglés Non structural proteins) relacionadas con la replicación vírica y la transcripción. Estas proteínas se expresan únicamente durante la fase de replicación y no forman parte de la estructura del virión. Los ORF2a, ORF2b, ORF3, ORF4, ORF5, ORF5a, ORF6 y ORF7 localizados en el extremo 3'UTR codifican hasta 8 proteínas víricas estructurales, las cuales se pueden dividir en proteínas estructurales menores y mayores (Firth et al., 2011; Johnson et al., 2011; Snijder et al., 2013).

- **Proteínas estructurales menores:** las glicoproteínas GP2a, GP3 y GP4 forman un complejo trimérico cuya interacción con la proteína GP5 es esencial para la entrada del virus en la célula. Este complejo trimérico también interacciona con la proteína E, siendo importante para la replicación vírica y para el tropismo celular del virus (Music & Gagnon, 2010; Snijder et al., 2013; Tian et al., 2012).

- **Proteínas estructurales mayores:** la GP5 es considerada la principal glicoproteína de la envuelta del virus, y junto con la proteína M, que deriva de la ORF6, forman heterodímeros que son esenciales para la formación del virión y la infectividad del virus (Snijder et al., 2013). La proteína ORF5a parece ser esencial para el proceso de replicación y la viabilidad del virión (Firth et al., 2011; Music & Gagnon, 2010; Tian et al., 2012). Por su parte, la proteína N (ORF7) de la nucleocápside, que se dispone de forma asimétrica tapizando el virus, interactúa con el ARN viral durante el ensamblaje de las partículas virales con capacidad infecciosa. Debido a su elevada expresión e inmunogenicidad, la proteína N es utilizada principalmente como antígeno para la detección de anticuerpos frente al PRRSV (Seuberlich et al., 2002; Sørensen et al., 1998).

Los viriones del PRRSV (Figura 2) son partículas esféricas con un diámetro aproximado de entre 45 y 80 nm. Estos viriones tienen una morfología asimétrica con una nucleocápside formada por la proteína N y el ARN viral, y rodeada por una envuelta de contenido lipídico (Spilman et al., 2009). La gran variabilidad genética y proteica que presenta el virus pone de manifiesto la complejidad y la plasticidad tanto del genoma del PRRSV como de la estructura de su virión.

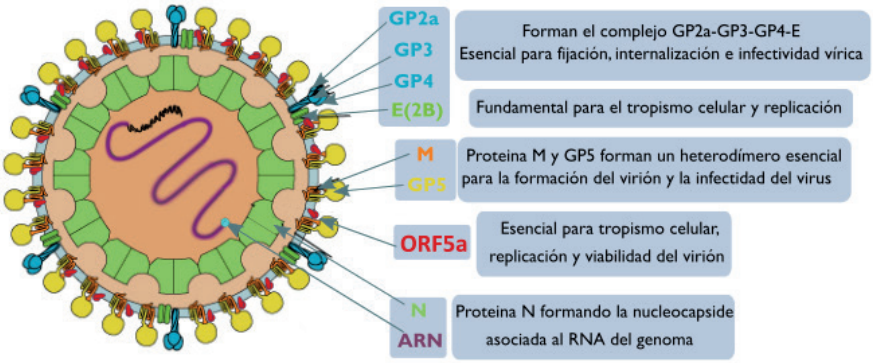

Figura 2. *Estructura del virión del PRRSV basado en Kappes & Faaberg, 2015; Snijder et al., 2013*

1.3. Replicación del PRRSV: CD163 y otros receptores de interés

El cerdo es el único hospedador natural del PRRSV conocido hasta la fecha. Como otros miembros de la familia Arteriviridae, el PRRSV ha desarrollado un tropismo celular restringido por células del linaje monocito/macrófago, siendo la subpoblación de macrófagos alveolares pulmonares (PAMs, del inglés Pulmonary Alveolar Macrophages) su principal célula diana (Duan et al., 1997). No obstante, estudios in vivo han identificado la presencia del virus en otras poblaciones de macrófagos residentes en el pulmón como los macrófagos intersticiales e intravasculares (PIMs, del inglés, Pulmonary Intravascular Macrophages), así como en las poblaciones de macrófagos presentes en órganos linfoides como el timo, bazo, tonsila y nódulos linfáticos, y en macrófagos intravasculares de la placenta (Duan et al., 1998; Gómez-Laguna et al., 2010; Halbur et al., 1996; Lawson et al., 1997; Rodríguez-Gómez et al., 2013; Snijder & Meulenberg, 1998; Thanawongnuwech et al., 2000). Además, en 2013, Hu et al., describieron cierto tropismo de cepas HP-PRRSV hacia células epiteliales, como sería el caso del aislado asiático JXwn06, tropismo que viene determinado por los ORFs del 2 al 4 (Tian et al., 2012). Estos datos sugieren que las cepas virulentas presentan un tropismo menos restringido, por lo que son capaces de replicarse en una variedad más amplia de tejidos como células endoteliales, pulmón y órganos linfoides primarios y secundarios. Además, este tipo de cepas cuentan con una tasa de replicación mucho más alta que las cepas clásicas del PRRSV tanto in vivo como in vitro (Hu et al., 2013; Karniychuk et al., 2010). Una de las posibles hipótesis es que

las cepas virulentas puedan utilizar receptores alternativos para entrar en la célula, sin embargo, son necesarios futuros estudios profundizando en estos mecanismos relacionados con un incremento en la virulencia de este tipo de cepas.

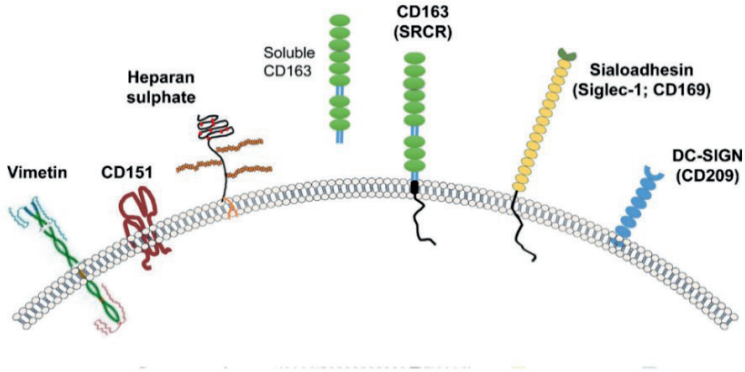

Figura 3. *Representación gráfica de las principales moléculas identificadas como potenciales receptores del PRRSV: vimentina, heparán sulfato, CD151, CD163, CD169 (Sn) y DC-SIGN (modificado de Zhang & Yoo, 2015).*

El proceso de entrada del PRRSV al interior de su célula diana así como el inicio del ciclo de replicación ha sido estudiado en profundidad (Van Breedam et al., 2010). La infección del virus se inicia mediante el reconocimiento de receptores específicos en la membrana celular (Zhang & Yoo, 2015). Actualmente, se han identificado al menos 6 moléculas diferentes como potenciales receptores del PRRSV, incluyendo el heparán sulfato (HpS) (Jusa et al., 1997), el CD151 (Shanmukhappa et al., 2007), la vimentina (Kim et al., 2006), el CD163 (receptor "scavenger" del complejo hemoglobina – haptoglobina) (Calvert et al., 2007), la sialoadhesina (Sn; Siglec – 1, también conocido como CD169) (Duan et al., 1997) y el DC-SIGN (conocido como CD209) (Huang et al., 2009) (Figura 3). De entre todos ellos, el CD163 y el CD169 se han estudiado en profundidad en los últimos años, ya que juegan un papel clave en la infección por el PRRSV, sin embargo, la expresión e implicación de los otros receptores puede hacer que la infección sea más eficaz (Van Breedam et al., 2010; Zhang & Yoo, 2015). En este sentido, se ha sugerido la existencia de receptores alternativos que favorecen una mayor capacidad de replicación de las cepas de elevada virulencia del PRRSV-1 (Frydas et al., 2013).

El CD163 es una proteína transmembrana tipo I compuesta de nueve receptores "scavenger" ricos en cisteína (SRCR del inglés scavenger receptor cysteine-rich) de tipo B consecutivos, los cuales forman un gran dominio extracelular. Este dominio extracelular esta unido a un único segmento transmembrana y a una cola citoplasmática corta (Law et al., 1993; Ritter et al., 1999). Su función principal es mediar en la endocitosis de los complejos de hemoglobina y haptoglobina protegiendo a los tejidos del daño oxidativo generado por moléculas libres de hemoglobina (Hb) (Schaer et al., 2006; Van Den Heuvel et al., 1999). La expresión del CD163 está restringida al linaje celular monocito/macrófago, siendo un marcador de diferenciación celular (Sánchez et al., 1999; Van Den Heuvel et al., 1999). Así mismo, puede jugar un papel importante modulando la respuesta inflamatoria, esto explicaría que su expresión esté regulada por señales pro- y anti-inflamatorias (Etzerodt & Moestrup, 2013; Fabriek et al., 2009; Kowal et al., 2011). Así, la interleuquina (IL)-6, IL-10, la Hb, los glucocorticoides y el estrés oxidativo actúan como potentes estimuladores de la expresión del CD163, mientras que IL-4, lipopolisacáridos (LPS), factor de necrosis tumoral α (TNF-α del inglés Tumor Necrosis Factor) e interferón γ (IFN-γ), ligando 4 de quimioquinas (CXCL4), así como el factor estimulante de colonias de granulocito-macrófago (GM-CSF del inglés Granulocyte–Macrophage Colony-Stimulating Factor) disminuyen su expresión (Boyle et al., 2009; Buechler et al., 2000; Gleissner et al., 2010; Kaempfer et al., 2011; Morganelli & Guyre, 1988; Sulahian et al., 2000; Zwadlo et al., 1987)(Tabla 1). Por otro lado, el CD163 tiene un papel como receptor frente a agentes patógenos, actuando como sensor del sistema inmune innato frente a bacterias gram positivas y gram negativas e inductor de una respuesta inflamatoria a nivel local por parte de los macrófagos residentes (Fabriek et al., 2009). Además es utilizado por algunos virus, como el PRRSV o el virus de la peste porcina africana, como puerta de entrada al interior celular (Van Gorp et al., 2008; Sánchez-Torres et al., 2003). En el caso del PRRSV, hay que tener en cuenta que, si bien la Sn puede potenciar la unión del virus a la célula diana, la falta de expresión de CD163 impide que haya infección efectiva por el PRRSV (Prather et al., 2013); mientras que en células que expresan CD163, pero no Sn sí se produce la internalización del virus y la progenie de este (Zhang et al., 2015). En este sentido, la supresión del dominio SRCR5 del CD163 en la población de macrófagos, mediante el uso de nuevas técnicas de edición genética (CrisprCas9), ha demostrado conferir resistencia in vivo frente a la infección por un panel de diferentes cepas del PRRSV-1 y el PRRSV-2 (Burkard et al., 2017; Wells et al., 2017; Whitworth et al., 2016).

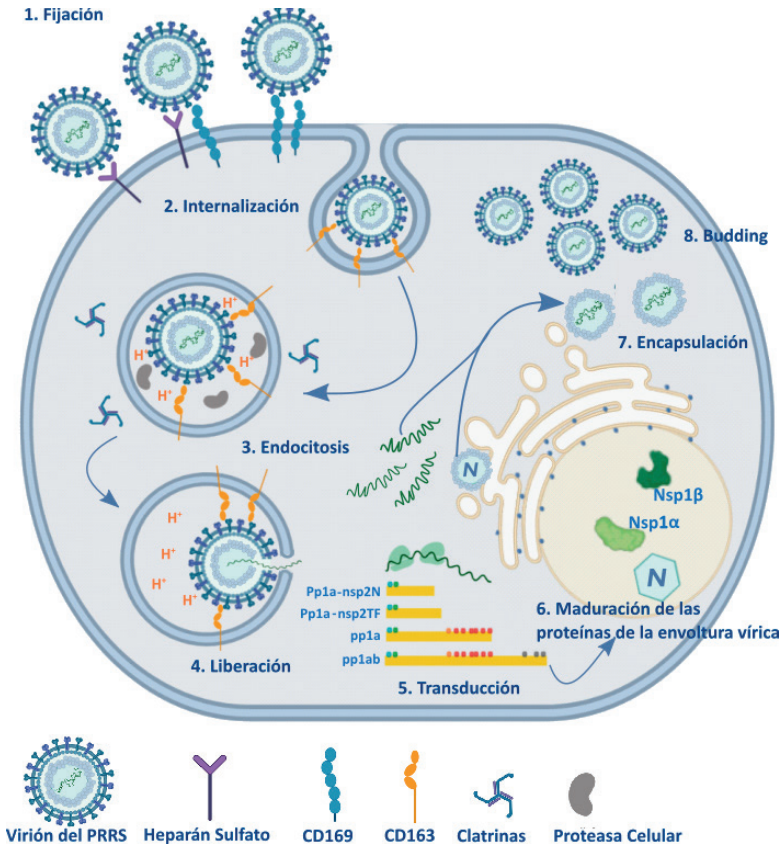

Figura 4. *Modelo de entrada del PRRSV al macrófago porcino propuesto por Van Breedam et al. (2010). Realizado con BioRender.com.*

El proceso de internalización del virus propuesto por Van Breedam et al., (2010) (Figura 4) se inicia con la unión entre las proteínas estructurales GP5 y M del virus y el HpS de la membrana celular de los macrófagos (Delputte et al., 2002; Jusa et al., 1997; Vanderheijden et al., 2001). El HpS incrementa la eficacia de la infección del PRRSV acercando los viriones del PRRSV a las proximidades de receptores específicos (Van Breedam et al., 2010). Posteriormente, el CD169 fija el virión a la superficie celular induciendo su internalización al interaccionar con los ácidos siálicos presentes en la envoltura proteica de éste (Delputte et al., 2005; Vanderheijden et al., 2003) mediante un proceso de endocitosis mediado por clatrinas (Nauwynck et al., 1999). Finalmente, el virus es liberado del endosoma temprano por acción del receptor CD163 y por la

acidificación del microambiente de dicho endosoma (Nauwynck et al., 1999; Van Gorp et al., 2008). Además, el CD163 es fundamental en la liberación del genoma vírico en el citoplasma celular por interacción con el complejo proteico GP2a-GP3-GP4 del virión (Figura 5) (Das et al., 2010; Van Breedam et al., 2010). Tras la internalización y liberación del genoma vírico se inician los procesos de transcripción y transducción necesarios para la formación de nuevos viriones del PRRSV.

Además, el CD163 cuenta con una forma extracelular soluble (sCD163 del inglés soluble CD163) la cual ha sido identificada en plasma en individuos sanos (Møller et al., 2002). La liberación del sCD163 por parte de los macrófagos y monocitos residentes en los tejidos es un proceso mediado por metaloproteinasas y en el cual está involucrada la enzima conversora de TNF-α (TACE/ADAM17) (Etze rodt et al., 2010, 2014; Hintz et al., 2002), sugiriendo que el sCD163 actuaría como mediador de la inflamación. En este sentido, se ha descrito un incremento en la concentración plasmática de sCD163 en diversos procesos inflamatorios agudos y crónicos con implicación de macrófagos (Buechler et al., 2013; Møller, 2012), por lo que el sCD163 se ha propuesto como un interesante biomarcador de la actividad macrofágica en enfermedades con inflamación sistémica (Buechler et al., 2013; Etzerodt & Moestrup, 2013). Sin embargo, aunque hasta la fecha el papel fisiológico del sCD163 no se conoce con exactitud, podría jugar un papel inhibiendo la proliferación de linfocitos T y en la opsonización de bacterias en situaciones de sepsis (Etzerodt & Moestrup, 2013; Högger & Sorg, 2001; Kneidl et al., 2012). Además recientemente, se ha descrito que sCD163 estaría formado por dos subcomponentes, un ectodominio soluble del CD163, que se incrementa en procesos inflamatorios sistémicos agudos como la fase inicial de una sepsis, mientras que EV-CD163 lo hace en fases más avanzadas de la respuesta inflamatoria (Etzerodt et al., 2017). En porcino se han hecho en los últimos años algunas aproximaciones experimentales midiendo los niveles de sCD163 en suero utilizando técnicas ELISA (ensayo por inmunoabsorción ligado a enzimas) como biomarcador para enfermedades como el PRRS o la enfermedad de Glasser (Costa-Hurtado et al., 2013; Pasternak et al., 2019; Pérez et al., 2008). En este sentido, las cepas virulentas de *Haemophilus parasuis* provocan un incremento de los niveles de sCD163 en sangre asociado a una disminución en la expresión del CD163 en los PAMs (Costa-Hurtado et al., 2013). En el caso del PRRSV, Pasternak et al., (2019), han descrito un incremento in vitro en el sobrenadante de PAMs estimulados tanto con LPS como infectados con PRRSV-2, sugiriendo estos resultados que el sCD163 podría funcionar como biomarcador para ambas enfermedades en situaciones de septicemia.

Tabla 1. Moléculas que regulan la expresión del CD163 en monocitos y macrófagos in vitro (Etzerodt & Moestrup, 2013

Molécula	Incremento o disminución de la expresión	Referencia
IL-6	Incremento	Buechler et al., 2000
IL-10		Buechler et al., 2000; Sulahian et al., 2000; Boyle et al., 2009
GM-CSF		Buechler et al., 2000
Hb		Kaempfer et al., 2011
Glucocorticoides		Sulahian et al., 2000
Estrés oxidativo		Zwadlo et al., 1987; Gleissner et al., 2010
IL-4	Disminución	Buechler et al., 2000
CXCL4		Gleissner et al., 2010
IFN-γ		Buechler et al., 2000; Morganelli & Guyre, 1988
TFN-α		Buechler et al., 2000
LPS		Buechler et al., 2000

En resumen, el receptor CD163 es esencial y necesario para la infección del PRRSV, jugando un papel fundamental tanto en la internalización como en la liberación del genoma del virus en el citoplasma celular.

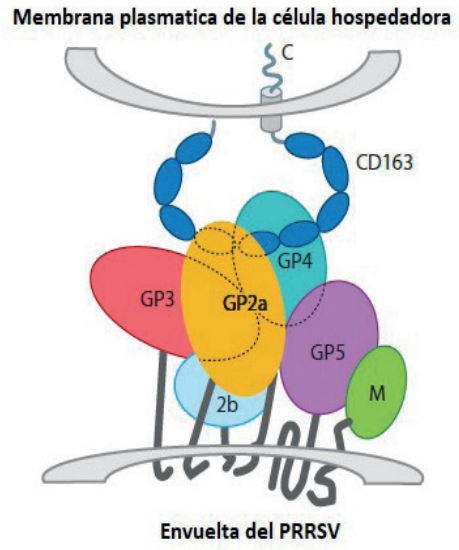

Figura 5. *Esquema de la interacción del complejo proteico del PRRSV con el receptor CD163 presente en la membrana plasmática de la célula hospedadora (basado en Das et al., 2010).*

2. INMUNOPATOGENIA DEL PRRSV

El PRRSV entra en el organismo del cerdo por vía oro-nasal principalmente, infectando mayoritariamente a los PAMs localizados en el pulmón, y en menor medida a los PIMs y macrófagos intersticiales (Gómez-Laguna et al., 2010a; Lawson et al., 1997; Rossow et al., 1995; Snijder et al., 2013; Snijder & Meulenberg, 1998). El virus puede detectarse en sangre (viremia) a partir de las 6 – 12 horas post infección, así como en otros órganos que cuenten con subpoblaciones de macrófagos maduros susceptibles al virus (Rossow et al., 1995), observándose un pico de replicación vírica entre los 7 y 14 días post infección (dpi). La duración de la viremia está relacionada con el tipo de cepa y su virulencia, con la edad de los animales y con el estatus inmunitario de los mismos. En este sentido, la viremia puede durar 3-4 semanas en los lechones, mientras que en cerdos adultos podría reducirse a 5–7 días (Cho et al., 2006; Díaz et al., 2012; van der Linden et al., 2003). Por otro lado, estudios in vivo con cepas de diferente virulencia del PRRSV-1 describen que las cepas de elevada virulencia del PRRSV-1 producen un pico de viremia mayor y más temprano (6 dpi aproximadamente), pero la eliminación del virus se produce antes en comparación con cepas PRRSV-1 de baja virulencia (Renson et al., 2017; Weesendorp et al., 2014). Este hecho puede deberse a dos escenarios: (I) la inducción de una respuesta inflamatoria más marcada por parte de las cepas virulentas, lo que favorece la eliminación temprana del virus del organismo; o (II) la pérdida importante de células susceptibles $CD163^{+}$ (Chand et al., 2012; Morgan et al., 2013; Weesendorp et al., 2013a; Weesendorp et al., 2014) limitándose así la capacidad de replicación y persistencia del virus.

A continuación, por vía linfohemática el virus alcanza diferentes órganos linfoides como la tonsila, el timo y los nódulos linfáticos, donde se acantona durante semanas o meses hasta que finalmente es eliminado del animal (Allende et al., 2000; Lawson et al., 1997; Rossow et al., 1995; Zimmerman et al., 2019). En estos órganos linfoides se ha aislado virus con capacidad infectiva hasta 157 dpi, y antígeno vírico hasta 251 dpi, lo que pone de relieve la importancia del papel que juegan los órganos linfoides en la persistencia del virus en el organismo (Wills et al., 2000; 2003). Los mecanismos implicados en la persistencia del virus en los animales infectados no se han descrito todavía, sin embargo, diversos estudios sugieren que tanto la regulación negativa del sistema inmune del hospedador como la selección de una serie de subpoblaciones o quasiespecies víricas durante el proceso de infección podrían jugar un papel importante (Cortey et al., 2017; 2018; Lu et al., 2017; Mateu & Diaz, 2008).

La sintomatología clínica y las lesiones que se observan durante la infección por el PRRSV se deben a diferentes mecanismos entre los que destacan: (I) procesos de muerte celular, apoptosis y necrosis, que afectan tanto a los macrófagos infectados como a células vecinas no infectadas; (II) la inducción de una fuerte respuesta inflamatoria mediada por citoquinas; (III) la activación policlonal de linfocitos B; (IV) cambios en las subpoblaciones de linfocitos T; y (V) la disminución de la capacidad de presentación de antígeno tanto en macrófagos como en células dendríticas. Consecuencia de esta desregulación de la respuesta inmune, se favorece la replicación y persistencia del virus a la vez que aumenta la susceptibilidad de los animales a un amplio rango de patógenos secundarios.

2.1. Papel de los macrófagos en el PRRS

Los macrófagos se originan a partir de monocitos sanguíneos que dejan la circulación madurando en diferentes tejidos del organismo. Los macrófagos juegan un papel esencial en el sistema inmune del hospedador, siendo células especializadas en la detección, fagocitosis y destrucción de bacterias y otros patógenos. Además, están implicados en la presentación de antígenos a los linfocitos T y en el inicio de la respuesta inflamatoria mediada por la liberación de citoquinas proinflamatorias, activando así otras células del sistema inmune (Elhelu, 1983; Gordon & Plüddemann, 2017).

wEl sistema mononuclear fagocítico del pulmón está formado por los PAMs, los macrófagos intersticiales pulmonares y, en el caso del cerdo y otras especies, por los PIMs (Longworth, 1997). Mientras que los PAMs se encuentran libres en los alvéolos, donde fagocitan las partículas inhaladas, los PIMs se localizan dentro de los capilares pulmonares y adheridos a las células endoteliales, donde eliminan partículas extrañas en circulación siendo además capaces de migrar hacia zonas lesionadas del pulmón donde liberan mediadores proinflamatorios que regulan la homeostasis pulmonar (Carrasco et al., 2002; Chitko-McKown et al., 1991; Sierra et al., 1990)

La función principal de los PAMs es establecer una primera línea de defensa fagocítica frente a infecciones microbianas, mientras que los macrófagos septales (macrófagos intersticiales y PIMs) están más relacionados con la liberación de citoquinas proinflamatorias (De Baere et al., 2012; Gómez-Laguna et al., 2010). No

obstante, la replicación del PRRSV en los PAMs y en los macrófagos septales modifica las funciones básicas de éstos, como son la fagocitosis, consecuencia de la interacción del virus con el receptor CD163, la presentación de antígenos y la producción de citoquinas proinflamatorias (Darwich et al., 2010; De Baere et al., 2012; Gómez-Laguna et al., 2013a; Sang et al., 2011; Thanawongnuwech et al., 2001). Además, tras la infección se ha descrito una polarización M2 de los macrófagos pulmonares lo que estaría relacionado con la liberación de citoquinas antiinflamatorias que favorecen la resolución de la inflamación (García-Nicolás et al., 2014; Wang et al., 2017). También se produce la muerte celular temprana de PAMs infectados, así como necrosis y apoptosis de macrófagos y linfocitos en pulmón y en órganos linfoides (Amarilla et al., 2016; Gómez-Laguna et al., 2013b; Rodríguez-Gómez et al., 2014; Ruedas-Torres et al., 2020). Todas estas alteraciones en su conjunto provocan un desequilibrio de la respuesta inmune en los animales infectados incrementando su susceptibilidad a un amplio rango de patógenos respiratorios tanto de etiología vírica (virus influenza, circovirus porcino tipo 2 o coronavirus respiratorio porcino) como bacteriana (*Actinobacillus pleuropneumoniae, Glaesserella parasuis, Mycoplasma hyopneumoniae* o *Streptococcus suis*) (Brockmeier et al., 2001, 2017; Pallarés et al., 2002; Thacker et al., 1999; Thanawongnuwech et al., 2000; Van Reeth et al., 1996). Este tipo de infecciones mixtas, propias del PRDC dan lugar a un incremento de la intensidad de los signos clínicos y de las lesiones a nivel pulmonar (Gómez-Laguna et al., 2013b).

El papel del PAM en la patogenia del PRRS viene marcado por el repertorio de moléculas que expresa con capacidad para polarizar la respuesta inmune del hospedador en uno u otro sentido. Entre estas moléculas, además del CD163, cabe destacar el papel de la molécula CD107a (Bullido et al., 1997; Domenech et al., 2003), también conocida como proteína de membrana 1 asociada a lisosomas (LAMP-1). Es una glicoproteína expresada principalmente en la membrana de lisosomas y endosomas antes de la degranulación (Eskelinen 2006), aunque también se ha descrito su expresión en la membrana plasmática de macrófagos activados y en linfocitos T citotóxicos (CTLs, del inglés Cytotoxic T Lymphocytes). Estudios in vitro e in vivo sugieren una disminución en la actividad citotóxica de los CTLs hacia PAMs infectados con PRRSV-1 (Cao et al., 2013; Costers et al., 2009); sin embargo, mientras que no se han observado modificaciones en la expresión de CD107a en condiciones in vitro (Cao et al., 2013), no hay estudios in vivo sobre la expresión de CD107a en macrófagos tras la infección por PRRSV.

2.2. Características clínicas de la forma respiratoria del PRRS

El PRRS es una enfermedad multisistémica, cuyas características clínicas más relevantes son fallos reproductivos en hembras adultas y problemas respiratorios en cerdos en crecimiento. Se considera una enfermedad distribución mundial y su presentación es generalmente de forma endémica. La presentación epidémica de la enfermedad se asocia con la forma reproductiva (Dewey et al., 1999) y se produce normalmente cuando la enfermedad aparece por primera vez en un país libre de la enfermedad o cuando una nueva cepa, con una baja homología con las cepas circulantes en la zona, ingresa en la explotación. Por su parte, la forma endémica está relacionada con un fallo en la respuesta inmunológica y la presentación de la forma respiratoria (Drew, 2000). Asimismo, la clínica y las lesiones provocadas por la infección por el PRRSV pueden variar en función de la virulencia de la cepa, del estado inmunitario y edad de los animales, de la coexistencia de otros patógenos, así como de las condiciones de manejo dentro de la propia granja (Chand et al., 2012; Halbur et al., 1995; Karniychuk et al., 2010).

La fase aguda de la infección se caracteriza clínicamente por un periodo de letargia y anorexia como consecuencia de la viremia en animales de todas las edades. Los cerdos en crecimiento pueden presentar además hiperemia cutánea que suele ser transitoria, signos característicos de enfermedad respiratoria (disnea, taquipnea y dificultad respiratoria), pelaje hirsuto y una disminución variable de la ganancia media diaria. Sin embargo, en cerdos adultos, machos y hembras reproductoras, la infección por el PRRSV provoca una fase transitoria de fiebre y anorexia ocasional (Labarque et al., 2002; Rossow et al., 1995; Kristien Van Reeth et al., 1996; Zimmerman et al., 2019).

Las cepas de elevada virulencia PRRSV-1 y PRRSV-2 dan lugar a un cuadro clínico más grave caracterizado por fiebre muy elevada, que puede alcanzar los 42°C, una elevada capacidad de contagio, un marcado distrés respiratorio, así como unas tasas de mortalidad y morbilidad muy altas de hasta el 20-80% asociados tanto a la virulencia de la cepa como a una mayor incidencia de infecciones bacterianas secundarias. También se han observado signos neurológicos y diarrea (Canelli et al., 2017; Han et al., 2017; Morgan et al., 2013; Sinn et al., 2016a; 2016b; Stadejek et al., 2017; Weesendorp et al., 2013a; Zhou & Yang, 2010).

2.3. Dominantes patológicas a nivel pulmonar y su papel en el complejo respiratorio porcino

El pulmón es el órgano diana de este virus y donde se produce la replicación de este entre los 4 y 28 dpi. En este sentido, aunque la gravedad y la distribución de las lesiones en el aparato respiratorio son relativamente homogéneas entre grupos experimentales en infecciones controladas, a nivel de campo pueden variar considerablemente dando lugar a patrones mixtos de lesión consecuencia de infecciones secundarias con otros virus o bacterias. Además, tradicionalmente se ha descrito que las cepas del PRRSV-2 son más neumovirulentas que las PRRSV-1, dando lugar a lesiones respiratorias más intensas (Martínez-Lobo et al., 2011).

El PRRSV provoca una neumonía intersticial de leve a grave, con una distribución de multifocal a difusa. Esta neumonía suele ir acompañada de un aumento de tamaño en los nódulos linfáticos regionales (traqueobronquial y mediastínico) (Gómez-Laguna et al., 2010a; Halbur et al., 1995; 1996). Macroscópicamente podemos observar un pulmón en cúpula, no colapsado, con incremento en la consistencia y un aspecto moteado multifocal de color gris-marrón, siendo los lóbulos apical y medial del pulmón en sus porciones craneoventrales los más afectados. En los casos más graves, la lesión aparece de forma difusa dando lugar a pulmones de consistencia firme, húmedos y de color rojizo (Zimmerman et al., 2019).

A nivel microscópico, esta lesión se caracteriza por un engrosamiento de los septos alveolares, debido a un infiltrado inflamatorio mixto, compuesto principalmente por macrófagos y en menor medida por linfocitos, y por la hipertrofia e hiperplasia de los neumocitos tipo II (Balka et al., 2013; Halbur et al., 1995; 96). Es frecuente la presencia de resto celulares, macrófagos descamados y sincitios celulares característicos de la infección por el PRRSV. La instauración de esta neumonía se debe a la liberación de citoquinas proinflamatorias como IL-1α, IL-1β, IL-6 y TNF-α por los macrófagos septales como respuesta a la infección vírica, las cuales presentan principalmente un efecto paracrino ejerciendo su función a nivel del parénquima pulmonar, mostrando sólo cambios leves en la concentración sérica de estas citoquinas (Amarilla et al., 2015; Borghetti et al., 2011; Gimeno et al., 2011; Gómez-Laguna et al., 2010a; 2010b; 2013a).

Además de estas citoquinas proinflamatorias, en el parénquima pulmonar de cerdos infectados con diferentes cepas del PRRSV también se produce un incremento en la expresión de citoquinas antiinflamatorias como IL-10 y TGF-β con el objetivo de regular la proliferación de células inflamatorias (Charavaryamath et al., 2006; Gómez-Laguna et al., 2010b; 2012; 2013b; Johnsen et al., 2002; Spight et al., 2005; Weesendorp et al., 2014). En este sentido, se ha descrito una correlación entre la expresión de estas citoquinas y la proteína N del PRRSV a nivel pulmonar, sugiriendo una modulación por parte del virus de los macrófagos septales para la liberación de IL-10 y TGF-β regulando así la respuesta inflamatoria que tiene lugar a nivel pulmonar (Gómez-Laguna et al., 2010b; 2012; 2013b).

Las cepas de elevada virulencia, tanto del PRRSV-1 como del PRRSV-2, se caracterizan por provocar lesiones pulmonares más intensas y agudas, asociadas a una mayor eficiencia de replicación del virus, lo que da lugar a una disminución importante de la población de PAMs, así como a una exacerbada respuesta inflamatoria a nivel pulmonar desencadenando el desarrollo de una bronconeumonía catarro-purulenta (Amarilla et al., 2015; Frydas et al., 2013; Han et al., 2014; Han et al., 2017; Karniychuk et al., 2010; Morgan et al., 2016; Renson et al., 2017). De esta manera, acompañando a la neumonía intersticial grave característica de esta enfermedad se observan también lesiones de bronquitis, bronquiolitis y bronconeumonía caracterizadas por un marcado infiltrado inflamatorio formado por neutrófilos y macrófagos, así como abundantes restos celulares, que ocluyen los espacios alveolares y la luz de bronquios y bronquiolos (Figura 6) (Han et al., 2014; Karniychuk et al., 2010; Zhou & Yang, 2010). Estas lesiones se deben en parte a la fuerte respuesta inflamatoria media por IL-1α/IL-1β que se ha descrito en cepas de elevada virulencia (Amarilla et al., 2015; Liu et al., 2010; Renson et al., 2017; Weesendorp et al., 2014) En función de la intensidad del proceso se pueden observar otras dominantes patológicas como neumonía intersticial necrotizante, pleuritis fibrinosa, extensas áreas hemorrágicas, o diferentes grados de vasculitis caracterizadas por un marcado infiltrado mononuclear perivascular (Figura 6) (Canelli et al., 2017; Grau-Roma & Segalés, 2007; Han et al., 2017).

Teniendo en cuenta estudios *in vitro* donde se ha llevado a cabo la polarización de macrófagos derivados de monocitos (García-Nicolás et al., 2014; Wang et al., 2017), podemos hipotetizar que a lolargo de la infección por PRRSV los macrófagos pulmonares pasarían por diferentes estados de activación. Una fase inicial de activacion clásica como macrófagos M1 los cuales desarrolloan una potente actividad antimicrobiana (Gordon & Martinez, 2010; Mosser, 2003; Mosser & Edwards, 2008). Posteriormente, los macrófagos pasarían por una fase de activación alternativa como macrófagos M2, aunque más sensibles a la infección por PRRSV (García-Nicolás et al., 2014; Wang et al., 2017), cuenta con capacidad antinflamatoria favoreciendo la resolución del proceso inflamatorio (Gordon & Martinez, 2010; Mosser, 2003; Mosser & Edwards, 2008).

2.4 Regulación de la respuesta inflamatoria a nivel pulmonar

La aparición de una bronconeumonía catarro-purulenta asociada a la infección por el PRRSV está ligada a la complicación con infecciones bacterianas secundarias fruto del daño provocado por el virus en las diferentes poblaciones de macrófagos (PAMs y PIMs) (Brockmeier et al., 2017; Thanawongnuwech et al., 1997; 1998; 2000). Existe un efecto sinérgico entre las coinfecciones y la patogénesis del PRRSV, ya que la acción conjunta del PRRSV y agentes bacterianos desencadena una cascada de citoquinas proinflamatorias, intensificando el daño pulmonar (Van Gucht et al., 2004). Aunque los mecanismos implicados en el desarrollo de estas coinfecciones y en la potenciación del daño tisular a nivel pulmonar están siendo objeto de estudio en la actualidad, se han propuesto varios mecanismos que podrían estar implicados. El primero de los mecanismos consistiría en un incremento en la expresión de CD14, receptor principal del complejo LBP (del inglés Lipopolysaccharide binding protein)-LPS, en monocitos y macrófagos intersticiales poco diferenciados (CD14high) (Zanoni & Granucci, 2013). Se ha descrito que este receptor participa en el reconocimiento de moléculas localizadas en la pared de bacterias gram positivas y gram negativas, iniciando la respuesta inflamatoria frente a estos microorganismos. Por tanto, un incremento en la expresión del CD14 predispondría al tejido pulmonar hacia una mayor producción de citoquinas proinflamatorias tras la exposición bacteriana dando lugar a daño pulmonar y sintomatología respiratoria más intensa (Thanawongnuwech et al., 2004; Van Gucht et al., 2004, 2005). Otro mecanismo implicado sería la influencia de la microbiota respiratoria en la respuesta inmune frente al virus, ya que algunas de las bacterias que se aíslan en estos procesos suelen ser comensales de las vías respiratorias del cerdo (Mycoplasma hyopneumoniae, Streptococcus suis, Glaesserella parasuis y Actinobacillus suis) (Brockmeier et al., 2017; Karst, 2016). En este sentido, el daño ocasionado por el PRRSV en el pulmón favorecería el crecimiento y proliferación de estos microorganismos, dando lugar al desarrollo de procesos neumónicos más complejos. El tercer mecanismo que estaría jugando un papel en las lesiones observadas a nivel pulmonar sería el incremento en la síntesis y liberación de IL-1α/IL-1β, citoquina que actúa como mediador de la respuesta inflamatoria, inducida por macrófagos pulmonares activados y que se ha asociado a infecciones tanto con cepas de diferente virulencia del PRRSV, como se ha comentado previamente, (Gómez-Laguna et al., 2010b; Amarilla et al., 2015; Weesendorp et al., 2014), como por *Glaesserella parasuis y Mycoplasma hyopneumoniae* (Kavanová et al., 2015; Thanawongnuwech et al., 2004). De

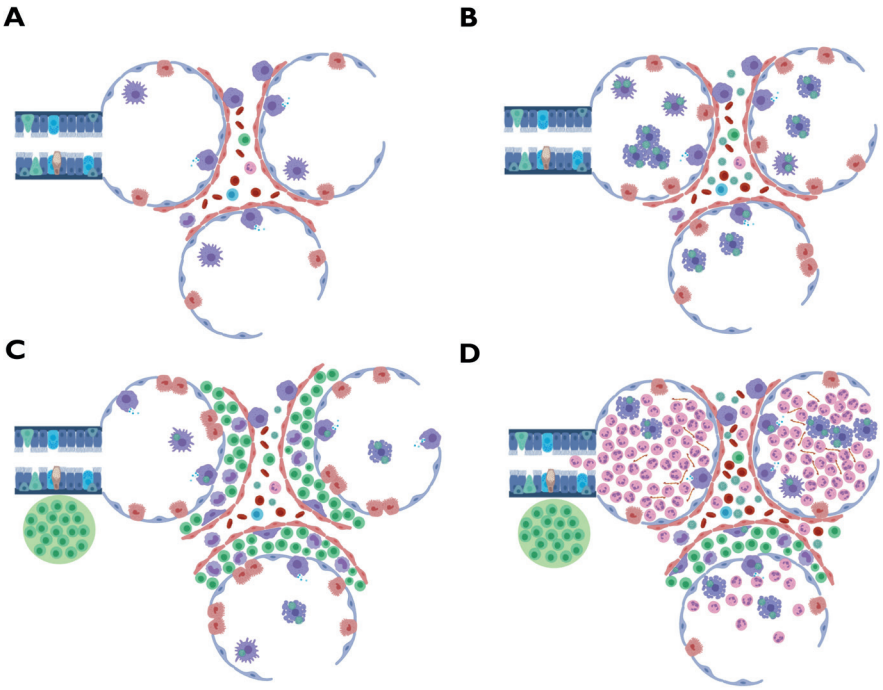

Figura 6. *Representación gráfica de las lesiones pulmonares provocadas por cepas de diferente virulencia del PRRSV-1. A. Pulmón sano. B. Fase inicial de la infección respiratoria en un animal caracterizada por la replicación del virus en macrófagos alveolares, algunos de ellos en apoptosis. C. Neumonía intersticial característica de la infección por el PRRSV. D. Patrón mixto de bronconeumonía consecuencia de la infección bacteriana secundaria y neumonía intersticial. Realizado con BioRender.com.*

este modo, la acción sinérgica de estas bacterias comensales del aparato respiratorio del cerdo y el virus del PRRSV provoca una mayor activación del inflamasoma dando lugar a una mayor liberación de citoquinas proinflamatorias (Qiao, Feng, et al., 2011; Zhang et al., 2013). No obstante, estos mecanismos representan sólo algunas de las rutas activadas durante la infección y el desarrollo del daño pulmonar en animales infectados por el PRRSV, siendo necesario profundizar en el papel de otras moléculas de interés implicadas tanto en la respuesta pro y antiinflamatoria como en el daño tisular para entender mejor la patogenia de esta enfermedad.

La enzima óxido nítrico sintetasa (iNOS del inglés inducible nitric oxide synthase) incrementa su expresión en células fagocíticas como respuesta a diferentes estímulos, ya sean citoquinas inflamatorias o procesos infecciosos, tanto víricos como bacterianos, dando lugar a la sobreproducción de óxido nítrico (NO) (Akaide and Maeda, 2000; Wink et al., 2011). En este sentido, la infección por PRRSV induce un incremento en la expresión de la iNOS y por tanto de NO provocando daño a nivel pulmonar consecuencia del estrés oxidativo, la inflamación y la apoptosis (Akaike & Maeda, 2000; Yan et al., 2017).

EL FoxP3 es el factor de transcripción que controla el desarrollo y función de los linfocitos T reguladores (Tregs), y, por tanto, se considerada como el principal marcador molecular de estas células (Pereira et al., 2017). En el cerdo, se han descrito dos subpoblaciones de Tregs caracterizadas como $CD4^+CD8^-CD25^+Foxp3^+$ y $CD4^+CD8^+CD25^+Foxp3^+$ (Käser et al., 2008). Los Tregs están especializados en la regulación negativa de respuestas inflamatorias exacerbadas consecuencia tanto de agentes infecciosos como no infecciosos, ayudando así al mantenimiento de la homeostasis y los procesos de tolerancia inmunológica (Pereira et al., 2017). En el caso del PRRS, el estudio de los Tregs se ha limitado a estudios in vitro o bien a la cinética de esta subpoblación en sangre periférica (explicados más adelante en esta Revisión Bibliográfica), no habiéndose estudiado el papel de esta subpoblación celular en el pulmón.

El CD200R1 podría tener un papel importante inhibiendo la producción de citoquinas proinflamatorias (Vaine & Soberman, 2014). En el cerdo, la expresión de este receptor se ha observado en células mieloides, como monocitos, macrófagos, granulocitos o células dendríticas, así como en linfocitos B (Poderoso et al., 2019). El

CD200R1 podría tener un papel dicotómico en el control de la respuesta inflamatoria. En modelo murino, la infección por el virus Influenza provoca un incremento en la expresión del CD200R1, disminuyendo la capacidad de respuesta de los macrófagos e incrementando la sensibilidad a infecciones bacterianas, induciendo así mayor daño pulmonar (Vaine & Soberman, 2014). Sin embargo, en infecciones por coronavirus la activación de las vías de señalización CD200/CD200R1 limitan la producción de IFN tipo I, protegiendo así al hospedador de una tormenta de citoquinas (Vaine & Soberman, 2014). En cerdos infectados con PRRSV, el eje CD200/CD200R1 se ha estudiado únicamente a nivel del sistema nervioso central como modelo experimental para evaluar el efecto de una infección vírica respiratoria sobre la activación de la microglía (Elmore et al., 2014). En este sentido, a nivel de la microglía el PRRSV causó una disminución en la expresión del CD200 asociada a un incremento del CD200R1 (Elmore et al., 2014), el cual ejerce un papel inhibidor sobre la respuesta inflamatoria regulando la liberación de moléculas proinflamatorias como iNOS o TNF-α (Vaine & Soberman, 2014).

2.5. Lesiones en órganos linfoides: el papel del timo como órgano linfoide primario

Además de en el parénquima pulmonar, el PRRSV también se replica en los órganos linfoides de los animales infectados, tanto en órganos linfoides primarios (timo y médula ósea) como en órganos linfoides secundarios (nódulo linfático, tonsila, placas de Peyer y bazo) dando lugar a fenómenos de muerte celular.

El timo, como órgano linfoide primario, es fundamental para la maduración y diferenciación de los linfocitos T, así como para el desarrollo del sistema inmune del animal adulto. Macroscópicamente, se ha descrito una disminución en el tamaño del timo en lechones infectados tanto con cepas de baja virulencia como con cepas virulentas del PRRSV-2 (Feng et al., 2002; Wang et al., 2014). Aunque a nivel microscópico, las cepas de baja virulencia del PRRSV provocan una depleción linfoide de leve a moderada en la corteza del timo; las cepas virulentas de PRRSV-1 y PRRSV-2 provocan una marcada atrofia tímica, caracterizada por una pérdida total de la diferenciación del límite cortico-medular y una intensa disminución de la ratio corteza:médula acompañada de un incremento del estroma (Figura 7). En la corteza del timo se observa la presencia de un número elevado de macrófagos de cuerpo

tingible y cuerpos apoptóticos. Esto da lugar a una marcada reducción del número de timocitos y de linfocitos T $CD3^+$ maduros (precursores de los linfocitos T $CD8^+$ o citotóxicos y de los linfocitos T $CD4^+$ o colaboradores) (Amarilla et al., 2016; Guo et al., 2013; He et al., 2012; Li et al., 2012; Ruedas-Torres et al., 2020).

Como consecuencia de la reducción en el número de linfocitos T maduros, se produce un deterioro en la respuesta inmune celular mediada por linfocitos T (Rodríguez-Gómez et al., 2013), aumentando la susceptibilidad del hospedador a infecciones bacterianas secundarias y dando lugar a una respuesta celular débil y tardía, como analizaremos posteriormente. Estos fenómenos de apoptosis observados tanto en el timo como en órganos linfoides secundarios han sido relacionados con el efecto directo del virus en las células infectadas así como con otros factores indirectos como la liberación de citoquinas proapoptóticas por parte de macrófagos y de otros mediadores proapoptóticos, que ejercen su acción en células próximas (Barranco et al., 2011; Costers et al., 2008; Gómez-Laguna et al., 2013b; Rodríguez-Gómez et al., 2014; Sirinarumitr et al., 1998; Sur et al., 1998). Está inducción de los fenómenos de apoptosis parece ser mucho más intensa en las cepas virulentas PRRSV-1 y HP-PRRSV-2 que en las cepas tradicionales del virus (Han et al., 2017; He et al., 2012; Li et al., 2014; Ruedas-Torres et al., 2020).

3. Respuesta inmune frente al PRRSV: una aproximación a la respuesta inmune innata y a la respuesta inmune adaptativa

Los virus son microorganismos intracelulares obligados, que permanecen en el interior de las células del hospedador utilizando su maquinaria celular para reproducirse e infectar a un nuevo huésped. Para ello, algunos virus provocan una desregulación del sistema inmune como el virus de la inmunodeficiencia humana (HIV del ínglés Human Immunodeficiency Virus) o el virus de la fiebre aftosa (FMDV del inglés Foot-and-Mouth Disease Virus), mientras que otros revierten a un estado de baja virulencia sobreviviendo en el organismo del hospedador como los herpesvirus (Butler et al., 2014; Rinaldo, 1994). En el caso de la reciente pandemia por el coronavirus 2 del síndrome respiratorio agudo grave (SARS-CoV-2 del inglés, Severe Acute Respiratory Syndrome Coronavirus 2), se ha descrito una activación de la respuesta innata y adaptativa, provocando en algunos casos una respuesta inflamatoria innata descontrolada junto con una desregulación de la respuesta adaptativa que conduce a un intenso daño tisular tanto a nivel local como sistémico (Cao, 2020; di Mauro et al., 2020)

El PRRSV se caracteriza por su capacidad para modular, e incluso evadir, la respuesta inmune del hospedador, favoreciendo su replicación, distribución y latencia en el organismo. Así, el virus es capaz de alterar varios de los mecanismos que intervienen en la homeostasis del sistema inmune, como la producción y señalización de interferones (IFNs), la modulación de la expresión de diferentes citoquinas, la inducción de fenómenos de muerte celular en células inmunitarias y la regulación de la inmunidad adaptativa (Butler et al., 2014; Han et al., 2017; Huang et al., 2015; Lunney et al., 2016; Mateu and Diaz, 2008; Mair et al., 2014). Aunque, cada vez hay un mayor consenso dentro de la comunidad científica, algunos puntos importantes relacionados con la evasión de la respuesta inmune no se conocen con exactitud, ya que varían entre diferentes cepas del PRRSV. En este sentido, la aparición de aislados de mayor virulencia ha incentivado aún más el interés por descifrar qué mecanismos utilizan estas cepas para burlar el sistema inmune y que actualmente se ignoran.

Para comprender cómo se desarrolla la respuesta inmune frente al PRRSV y facilitar su comprensión, desglosaremos esta respuesta en inmunidad innata y adaptativa, aunque en la realidad se trata de un proceso continuo en el que intervienen un gran número de mecanismos e interacciones moleculares.

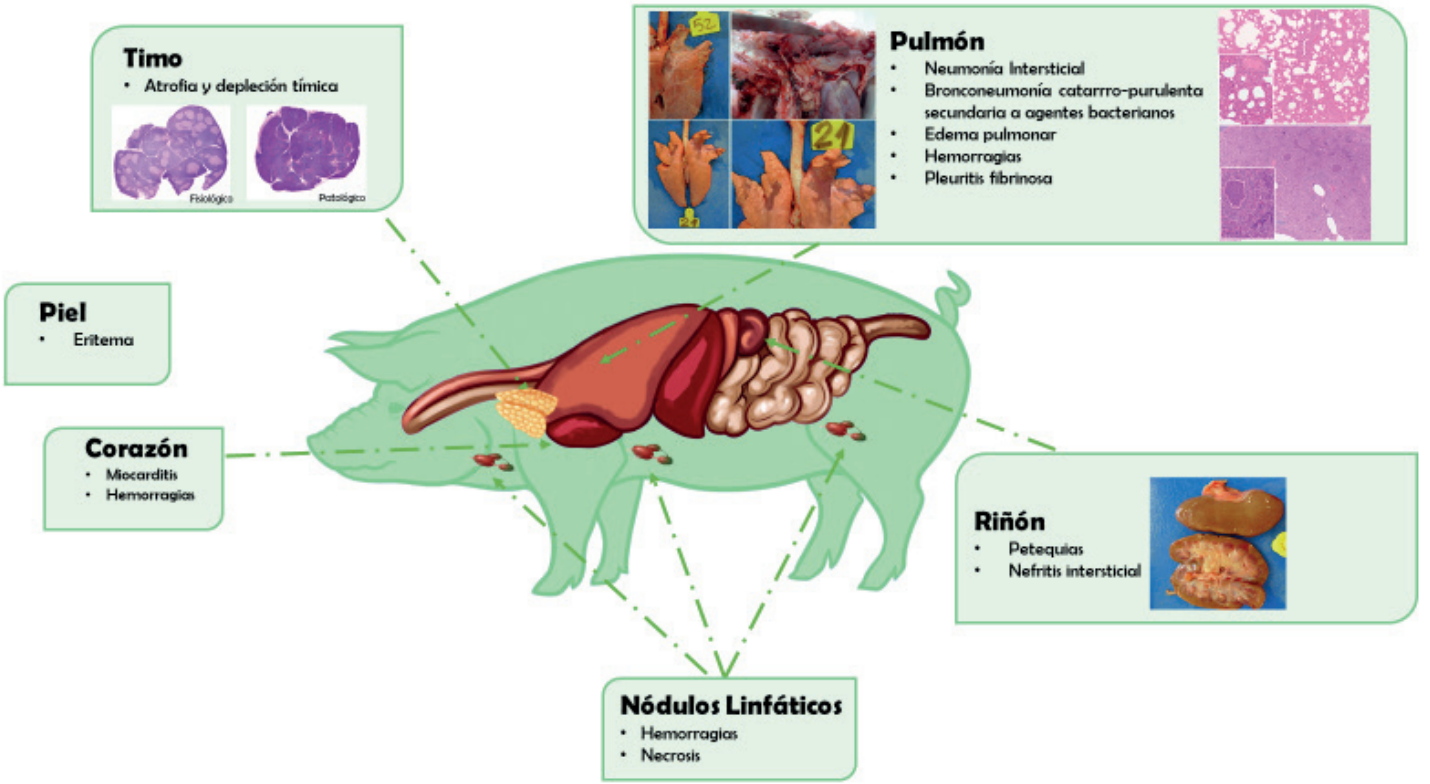

Figura 7. Principales lesiones observadas en cerdos infectados con la cepa Lena, prototipo de cepa virulenta PRRSV-1.

3.1. Evasión de la respuesta inmune innata

La respuesta inmune innata, primera línea de defensa frente a infecciones víricas, inducida por el PRRSV es débil y da lugar a una eliminación incompleta o parcial del virus en la mayoria de los animales en función de la edad y el estatus sanitario, obstaculizando la construcción de una respuesta inmune adaptativa eficiente (Butler et al., 2014; Han et al., 2017; Lunney et al., 2016; Mair et al., 2014). Los receptores de reconocimiento de patrones (PRRs, del inglés Pattern Recognition Receptors), están presentes en células que participan en la respuesta innata y son capaces de reconocer patrones moleculares asociados a patógenos (PAMPs, del inglés Pathogen-Associated Molecular Patterns), cuya activación desencadena la liberación de citoquinas y quimioquinas inflamatorias (Takeuchi & Akira, 2010). Los receptores tipo Toll (TLRs, del inglés Toll-Like Receptors), concretamente el TLR3, TLR7/8 y TLR9, así como los receptores tipo RIG-I (RIG-I del inglés, Retinoic Acid Inducible Gene) son PRRs involucrados en el reconocimiento de virus ARN (Bowie & Unterholzner, 2008; Thompson et al., 2011). Mientras que no está claro si los TLR7 y TLR9 se activan en PAMs tras la infección con PRRSV-1 (Kuzmetseva et al., 2104), sí que se ha demostrado que la regulación de las cascadas de señalización activadas por TLR3 y RIG-I en macrófagos y células dendríticas podría depender del tipo de cepa involucrada, dando lugar a una mayor o menor producción de citoquinas inflamatorias según proceda (Chaung et al., 2010; Huang et al., 2014; Kuzmetseva et al., 2104; Liu et al., 2009; Sang et al., 2008; Sun et al., 2016).

a. Supresión de las vías de señalización e inducción de los IFNs tipo I

Los IFNs tipo I son uno de los mecanismos más efectivos de la respuesta innata frente a las infecciones víricas, limitando la replicación y la diseminación por el organismo (Theofilopoulos et al., 2005). Estudios previos han demostrado que el PRRSV es sensible al IFN-α tanto en experimentos in vitro como in vivo (Albina et al., 1998; Brockmeier et al., 2001, 2012; Overend et al., 2007). Sin embargo, a diferencia de otros virus, como el virus de la gastroenteritis transmisible (TGEV, del inglés Transmissible GastroEnteritis coronaVirus) y el coronavirus respiratorio porcino (PRCV, del inglés Porcine Respiratory CoronaVirus), que inducen elevados niveles de IFN-α, PRRSV-1 y PRRSV-2, incluyendo cepas de elevada virulencia, son capaces de inhibir la producción de IFN-α tanto en macrófagos como en células dendríticas

plasmacitoides, una de las principales fuentes de IFN tipo I en el cerdo (Albina et al., 1998; Baumann et al., 2013; Brockmeier et al., 2012; Buddaert et al., 1998; Calzada-Nova et al., 2010; 2011). En concreto, las Nsp 1α, 1β, 2, 4, 5 y 11, así como la proteína N parecen estar involucradas en este proceso actuando como antagonistas del IFN-α/β (Chen et al., 2010; Huang et al., 2014; Yoo et al., 2010). Esto da lugar a un ambiente muy favorable para la replicación del virus, explicando así los bajos niveles de IFN detectados en suero y pulmón en animales infectados con diferentes cepas del PRRSV.

b. Células "asesinas naturales" (NK)

Los linfocitos citotóxicos naturales (NK, del inglés Natural Killer cells) son una subpoblación de linfocitos T innatos que tienen la capacidad de reconocer y eliminar células infectadas. Además, las células NK se consideran como la principal fuente de liberación de IFN-γ durante la respuesta innata (Lodoen & Lanier, 2006). Durante la infección con diferentes cepas del PRRSV se produce una supresión importante de la actividad citotóxica de las células NK, disminuyendo además la secreción de IFN-α (Albina et al., 1998; Cao et al., 2013; Dwivedi et al., 2011a; Renukaradhya et al., 2010), hechos que se han observado en infecciones con cepas de campo, virus inactivado y en vacunas MLV administradas tanto por vía intramuscular como intranasal (Dwivedi et al., 2011a; 2011b; 2012; Renukaradhya et al., 2010). Esta reducción en la actividad citotóxica inducida por el PRRSV es independiente de la frecuencia de células NK, la cual se recupera semanas después de la infección (Dwivedi et al., 2011b; Renukaradhya et al., 2010). Cao et al., (2013) investigaron in vitro la interacción entre células NK y PAMs infectados con PRRSV-1, observando una marcada reducción en la susceptibilidad de los PAMs hacia la reacción citotóxica mediada por las células NK. Estos datos sugieren que el PRRSV es capaz de modular la actividad de las células NK como una de las posibles estrategias para evadir la respuesta inmune del hospedador (Cao et al., 2013; Lunney et al., 2016).

Los linfocitos Tγδ, al igual que las células NK, cuentan con actividad citotóxica, una rápida respuesta frente a infecciones y la capacidad para producir citoquinas. Se ha descrito que esta población de linfocitos puede generar y retener memoria inmunológica, siendo un puente de unión entre la respuesta inmune innata y la adaptativa (Gerner et al., 2009; Mair et al., 2012, 2016; Takamatsu et al., 2006). Durante la infección por el PRRSV se produce una modulación en la población de linfocitos Tγδ, los cuales son una fuente

importe de IFN-γ juntos con las células NK, sin embargo, debido al número limitado de estudios no se ha podido establecer ninguna hipótesis sobre su papel en la respuesta inmune frente al PRRSV (Lamontagne et al., 2003; Olin et al., 2005; Sinkora et al., 2014).

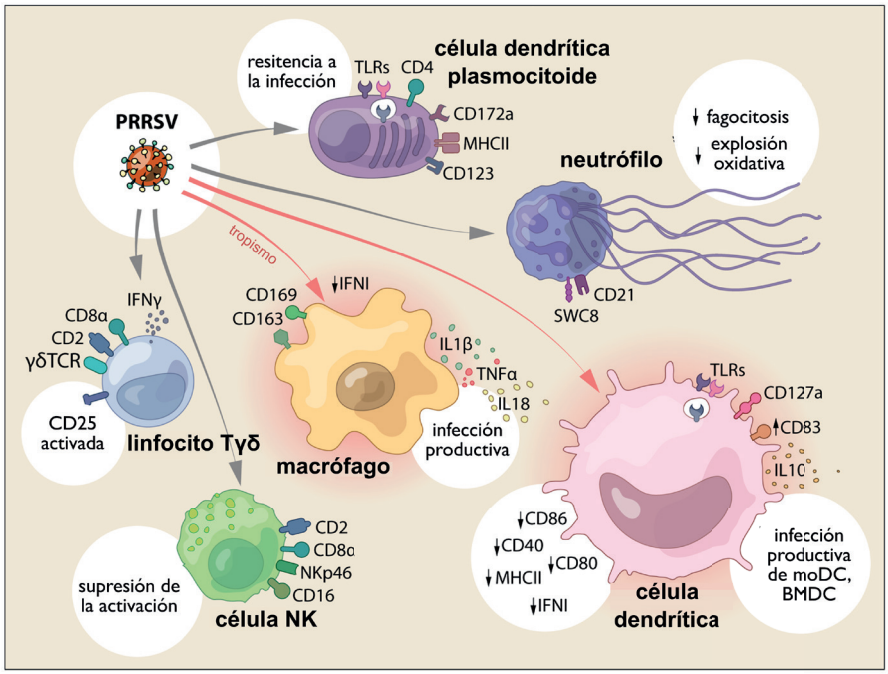

Figure 8. *Interacción del PRRSV con las células inmunes que forman parte de la respuesta inmune innata (modificado de Crisci et al., 2019).*

En cuanto a las cepas virulentas del PRRSV-1, SU1-bel y Lena producen un incremento progresivo en la frecuencia de células NK en el torrente sanguíneo de forma similar a las cepas de baja virulencia LV y Belgium A (Morgan et al., 2013; Weesendorp, et al., 2013a). Sin embargo, para la cepa virulenta PR40 se describe un incremento temprano de las células NK coincidiendo con el pico de viremia (Canelli et al., 2017, Ferrari et al., 2018), por lo que podría estar relacionado con la eliminación del virus del torrente sanguíneo. Ferrari et al. (2018) también evaluaron la frecuencia de linfocitos Tγδ observando una disminución durante la fase temprana de infección tanto en PR40 como en la cepa de baja virulencia PR11. En infecciones con cepas virulentas PRRSV-2 han asociado esta disminución en los linfocitos Tγδ con procesos de muerte celular en las subpoblaciones de linfocitos en órganos linfoides secundarios (Wang et al., 2014).

c. Modulación de la producción de citoquinas pro- y anti-inflamatorias

La respuesta inflamatoria aguda es una pieza clave para la instauración de la respuesta innata del hospedador. A diferencia de otras enfermedades víricas del cerdo que inducen una fuerte respuesta inflamatoria con una marcada liberación de citoquinas proinflamatorias al torrente circulatorio, los niveles séricos de estas citoquinas durante la infección por PRRSV es limitada (Carrasco et al., 2002; Gómez-Laguna et al., 2010b; Khatri et al., 2010; Van Reeth et al., 1999), favoreciendo una activación débil y tardía de la respuesta adaptativa. Esta ausencia de cambios marcados en la concentración sérica de citoquinas proinflamatorias, como IL-1, IL-6 y TNF-α, se ha descrito tanto para PRRSV-1 como para PRRSV-2 asociándose a la aparición de una sintomatología clínica discreta a nivel respiratorio y sistémico propia del PRRS (García-Nicolás et al., 2015; Gómez-Laguna 2010b; Lunney et al., 2010; Renukaradhya et al., 2010; Thanawongnuwech et al., 2001; Van Reeth et al., 1999). La secrección de algunas de estas citoquinas inflamatorias (IL-8, IL-1β, IFNγ) se correlaciona con la carga vírica (Lunney et al., 2010), y puede variar en función de la cepa (Darwich et al., 2011; Gimeno et al., 2011). Estudios in vitro han relacionado las Nsp 1α y 1β del virus con la baja producción de TNF-α (Chen et al., 2010).

En contraste, a nivel pulmonar se produce un incremento in situ de la expresión de IL-1, IL-6 y TNF-α en cerdos infectados con PRRSV-1, observándose una correlación con el desarrollo de neumonía intersticial (Gómez-Laguna et al., 2010a; Labarque et al., 2000; Van Gucht et al., 2003). La ausencia del reflejo de la respuesta inflamatoria en el pulmón a nivel sanguíneo podría ser una de las estrategías del virus para escapar de la respuesta inmune del hospedador (Gómez-Laguna et al., 2013a; Lunney et al., 2016).

Sin embargo, las cepas de elevada virulencia PRRSV-1 y PRRSV-2, provocan una intensa respuesta inflamatoria a nivel sistémico asociada a niveles elevados de IL-1α/β, IL-6 y TNF-α en el torrente sanguíneo, que se traduce en la aparición de un cuadro febril marcado (40.5 - 42oC) a partir de los 2 – 3 dpi, indicando el desarrollo de una respuesta inflamatoria intensa, característica de estas cepas más virulentas (Liu et al., 2010; Guo et al., 2013; Morgan et al., 2013; Han et al., 2014, 2015; Sinn et al., 2016; Renson et al., 2017). A nivel pulmonar, como ya se ha comentado con anterioridad, se ha descrito que las cepas virulentas del PRRSV-1, Lena y SU1-bel, inducen una mayor expresión IL-1α/β que cepas de baja virulencia como LV o Belgium A, dando lugar a

una neumonia intersticial más intensa (Amarilla et al., 2015; Weesendorp et al., 2014). En el caso de las cepas virulentas del PRRSV-2, también se ha descrito una mayor expresión de IL-1 por parte de los PAMs (Qiao et al., 2011a; Zhang et al., 2013a). Aunque estos niveles elevados de citoquinas inflamatorias podrían favorecer una rápida eliminación del virus (Morgan et al., 2016; Weesendorp et al., 2014), no hay que olvidar que también tendrían efectos adversos provocando daño tisular mas intenso, e incluso un fallo multiorgánico asociado a una "tormenta de citoquinas" si estos niveles se mantienen elevados durante varios días (Han et al., 2017), como ocurre con SARS-CoV-2 (Cao, 2020; di Mauro et al., 2020)Este hecho, podría ser uno de los factores asociados a la mayor morbilidad y mortalidad observada en los animales infectados con cepas virulentas del PRRSV.

La respuesta inflamatoria esta regulada por el equilibrio entre moléculas pro- y anti-inflamatorias. De este modo, la infección con algunas cepas del PRRSV, incluyendo las de mayor virulencia, inducen la liberación de IL-10. Las proteínas N y GP5 del PRRSV regulan la producción de IL-10 por parte de los macrófagos (Song et al., 2013; Wongyanin et al., 2012). Esta potente citoquina anti-inflamatoria es capaz de controlar el daño tisular provocado por la respuesta inflamatoria pero a su vez debilita la respuesta inmune Th1 y, por tanto, la respuesta inmune adaptativa en animales infectados por el PRRSV (Díaz et al., 2005b; Guo et al., 2013; Han et al., 2015; Moore et al., 2001; Sabat et al., 2010). Esta inducción de IL-10 es capaz de contrarrestar el efecto del IFN-γ además de potencialmente estimular la proliferación de Tregs (Díaz et al., 2006; Dwivedi et al., 2012). Algunos estudios indican que la infección por PRRSV induce un incremento de los niveles de IL-10 (Díaz et al., 2005; 2006; Flores-Mendoza et al., 2008; Gómez-Laguna et al., 2010b; Suradhat et al., 2003), sin embargo, otros no han sido capaces de detectar estos cambios en la expresión de esta citoquina (Klinge et al., 2009; Subramaniam et al., 2011). Esta falta de consenso entre estudios se puede explicar teniendo en cuenta que no todas las cepas del PRRSV son capaces de estimular la liberación de IL-10, habiendo gran variabilidad entre cepas que estaría determinada por la virulencia de la cepa y por la capacidad para interaccionar con las células dendríticas (Darwich et al., 2011; Gimeno et al., 2011; Klinge et al., 2009; Silva-Campa et al., 2010; Zhang et al., 2013b).

En este sentido, la respuesta inflamatoria puede ser un arma de doble filo, ya que cuando es insuficiente, como sería el caso de las cepas de baja virulencia, el

hospedador es incapaz de montar una respuesta inmune eficaz frente al virus; pero si fuera demasiado potente, como en cepas virulentas, provocaría una mayor intensidad de la clínica y de las lesiones a nivel del parénquima pulmonar, lo que aumentaría la predisposición a infecciones bacterianas secundarias.

d. Modulación de la apoptosis

Los fenómenos de muerte celular regulada (RCD del inglés Regulated Cell Death) tienen como función principal el mantenimiento de la homeostasis, sin embargo, también se consideran como un mecanismo innato de defensa del hospedador frente a agentes patógenos (Galluzzi et al., 2018; Jorgensen et al., 2017), siendo la apoptosis una de los más estudiados (Kerr et al., 1972; Singh et al., 2019). Sin embargo, cuando la muerte celular se produce en condiciones fisiológicas, entonces se conoce como muerte celular programada (Galluzzi et al., 2018). En este sentido, la apoptosis es un mecanismo evolutivo conservado de RCD que se inicia como consecuencia de estímulos externos o internos, y que requiere de la activación de un sistema de enzimas conocidas como caspasas provocando así la degradación de los componentes celulares (D'Arcy, 2019; Kerr et al., 1972; Lockshin & Zakeri, 2004; Logue & Martin, 2008). En la cascada de señalización que daría lugar a la apoptosis, la caspasa 8 y la caspasa 9 pertenecen a la vía extrínseca e intrínseca de la apoptosis respectivamente, aunque la caspasa 8 podría jugar un papel importante como "interruptor celular" entre ambos mecanismos (Fritsch et al., 2019; Tait & Green, 2013). Sin embargo, en los últimos años han surgido otros tipos de RCD como la necroptosis, piroptosis o netosis (Galluzzi et al., 2018), los cuales son fundamentales en el desarrollo de una respuesta antiviral (Man et al., 2017; Nailwal & Chan, 2019; Orzalli and Kagan, 2017).

En el caso de las infecciones víricas, los diferentes fenómenos de RCD interrumpen la replicación del virus eliminando las células infectadas y disminuyendo el reservorio celular para estos patógenos intracelulares, siendo además esencial para montar una respuesta inflamatoria frente al virus en cuestión (Davidovich et al., 2014; Jorgensen et al., 2017; Orzalli & Kagan, 2017). Algunos virus han desarrollado mecanismos para inhibir o retrasar estos fenómenos de RCD, incrementando así el tiempo de supervivencia celular hasta que se produce la replicación y liberación de los nuevos viriones (Banadyga et al., 2009; Cai et al., 2011; Shen et al., 2013). Por el contrario, también hay virus que inducen RCD en las células infectadas con el

objetivo de favorecer su diseminación y evitar la respuesta inmune del hospedador (Jan et al., 2000; Medigeshi et al., 2007; Metz et al., 2014). En este sentido, existen numerosas señales intracelulares entrelazadas entre los diferentes mecanismos de RCD, de forma que la señalización celular estaría bien protegida frente al ataque del virus (Fritsch et al., 2019; Galluzzi et al., 2018; Jorgensen et al., 2017).

En el caso del PRRSV, la modulación de la apoptosis juega un papel fundamental en la patogénesis de la infección (Y. He et al., 2012; Karniychuk et al., 2013; Labarque et al., 2003; Sur et al., 1997). En estudios in vitro se ha descrito que el virus es capaz de modular los fenómenos de apoptosis en PAMs a lo largo de la infección, de forma que durante las fases tempranas de la infección el virus podría inhibir la apoptosis mediante un proceso mediado por la proteína GP2a, favoreciendo así la replicación del virus; mientas que induciría la apoptosis en fases posteriores, ayudando así a la liberación y dispersión de las partículas víricas (Costers et al., 2008). La GP5 y la Nsp4 del PRRSV se han asociado con la inducción directa de fenómenos de apoptosis por la acción del virus mediada por la vía de señalización de la quinasa c-Jun N-terminal (Costers et al., 2008; Huo et al., 2013; Pujhari et al., 2014; Suárez et al., 1996; Yin et al., 2012). En estudios in vivo, el PRRSV induce apoptosis y necrosis por acción directa del virus, asi como de manera indirecta en órganos linfoides primarios y secundarios mediante la liberación de diferentes mediadores/moléculas proapoptóticas (Gómez-Laguna et al., 2013b; Rodríguez-Gómez et al., 2014; Sirinarumitr et al., 1998; Sur et al., 1998). A nivel pulmonar, estos fenómenos de apoptosis y necrosis se han observado principalmente en células no infectadas por el virus localizadas en el parénquima pulmonar así como en macrófagos intersticiales y monocitos, sugiriendo que citoquinas inflamatorias como la IL-1 o la IL-10 podrían jugar un papel importante (Labarque et al., 2003).

Por otro lado, la capacidad del virus para inducir apoptosis y necrosis podría depender de la virulencia de la cepa, como se ha demostrado para cepas virulentas del PRRSV-1 y PRRSV-2 capaces de inducir necrosis y apoptosis en órganos linfoides primarios y secundarios con mayor intensidad que las cepas de baja virulencia, caracterizando además los mecanismos celulares implicados (Amarilla et al., 2016; He et al., 2012; Li et al., 2014; Morgan et al., 2016; Ruedas-Torres et al., 2020; Wang et al., 2014).

Aunque los mecanismos de RCD han sido evaluados en otras enfermedas víricas que afectan al ganado porcino como la peste porcina africana o la gripe pocina (Atkin-Smith et al., 2018; Ma et al., 2019), estos mecanismos no han sido estudios en PRRS. En este sentido, al igual que el papel de la apoptosis en la inmunopatogenia del PRRSV parece evidente, se puede hipotetizar que otros mecanismos de RCD podrían estar implicados contribuyendo a la desregulación de la respuesta inmune innata y adaptativa a nivel de órganos linfoides y del pulmón, disminuyendo las poblaciones de macrófagos y linfocitos T y, por tanto, incrementado la susceptibilidad a infecciones bacterianas secundarias (Gómez-Laguna et al., 2013a; Labarque et al., 2003).

En resumen, el PRRSV tiene un efecto negativo sobre algunos de los principales mecanismos implicados en el desarrollo de la respuesta inmune innata, dando lugar a una respuesta adaptativa celular y humoral frágil e incompleta.

3.2. Evasión de la respuesta inmune adaptativa

La respuesta inmune adaptativa, fundamental para el desarrollo de una inmunidad protectora, se caracteriza por la producción de anticuerpos y linfocitos T y B, algunos de los cuales permanecerá como células de memoria que se activarán rapidamente en encuentros futuros con el patógeno en cuestión.

a. Respuesta humoral

El PRRSV da lugar a la aparición temprana de anticuerpos específicos (7 – 9 dpi), que alcanzan su máxino nivel a los 14 - 21dpi (Figura 8)(Loemba et al., 1996; Lopez & Osorio, 2004; Loving et al., 2015; Vézina et al., 1996; Yoon et al., 1992). Estos anticuerpos carecen de capacidad neutralizante, es decir, no están correlacionados con la protección, y están dirigidos principalmente frente a las proteínas N, GP5, Nsp1 y Nsp2 (de Lima et al., 2006; Lopez et al., 2007; Lopez & Osorio, 2004; Oleksiewicz et al., 2001). Los anticuerpos neutralizantes (NAbs, del inglés Neutralizing Antibodies) aparecen de manera inconsistente y normalmente a partir de las 4 semanas post-infección, con títulos muy bajos, que van aumentando lentamente hasta alcanzar su máximo nivel varias semanas o meses después, cuando la viremia ya ha disminuido o se ha eliminado el virus de la circulación sanguínea (Figura 9) (Díaz et al., 2005; Nelson et al., 1994; Vézina et al., 1996; Yoo et al., 2010). Los NAbs transfieren protección pasiva

frente al virus, previniendo de una infección transplacentaria y confiriendo inmunidad esterilizante frente al PRRSV, aunque es necesario una elevada concentración de los mismos (Lopez et al., 2007; Osorio et al., 2002). Es importante destacar que aunque los NAbs protegen frente a la infección, la viremia ocasionada por el PRRSV se resuelve incluso en ausencia de niveles detectables de estos (Figura 9) (Mateu & Diaz, 2008; Nelson et al., 1994; Vézina et al., 1996). Inicialmente se pensaba que el principal epítopo relacionado con la inducción de NAbs era la GP5 (Plagemann et al., 2002; Wissink et al., 2003; 2005), sin embargo, actualmente se hipotetiza que los NAbs se dirigen también hacia las GP2, GP3 y GP4, heterodímero que determina el tropismo del virus e interactúa con el receptor del virus, la molécula CD163 (Das et al., 2010; Tian et al., 2012).

En cuanto a las cepas virulentas del PRRSV, la mayoria de los estudios con NAbs se centran en evaluar estas cepas como posibles candidatos vacunales. Pero, al igual que las cepas de baja virulencia, éstas inducen un incremento tardío de NAbs (Galliher-Beckley et al., 2015; Tian et al., 2009).

Además de los NAbs, existe otro mecanismo basado en anticuerpos antivirales no-neutralizantes conocido como estimulación dependiente de anticuerpos (ADE, del inglés Antibody-Dependent Enhancement), sin embargo, este mecanismo actúa como un caballo de Troya para el virus, facilitando su internalización mediante el receptor Fc que media la endocitosis en PAMs, favoreciendo así la replicación vírica (Bao et al., 2013; Mateu & Diaz, 2008; Qiao et al., 2011)

Uno de los grandes retos en la lucha frente al PRRSV es el desarrollo de una respuesta humoral fuerte con capacidad de neutralización cruzada frente a diferentes cepas. Actualmente, las vacunas ofrecen una protección limitada que requiere de múltiples dosis vacunales para obtener unos niveles óptimos de NAbs, confiriendo una protección parcial, específica frente a la cepa vacunal (homóloga) y con escasa o nula capacidad de neutrilización cruzada (heteróloga) (Díaz et al., 2012; Robinson et al., 2015).

b. Respuesta celular

En la mayoría de las infecciones víricas el desarrollo de una respuesta celular es crucial para la eliminación de las células infectadas, y por tanto del virus. En el caso del PRRSV, la respuesta celular se considera débil, tardía y variable, apareciendo entre las 2 y 8 semanas post infección (Bautista & Molitor, 1997; 1999; Xiao et al., 2004).

b.1. El papel del complejo mayor de histocompatibilidad y de los linfocitos T CD4 y linfocitos T CD8 en el PRRSV

Uno de los mecanismos que parece utilizar el PRRSV para evadir la respuesta inmune del hospedador consiste en reducir la expresión del complejo mayor de histocompatibilidad clase I (MHC-I, del inglés Major Histocompatibility Complex class I) y clase II (MHC-II) por parte de las células presentadoras de antígeno (APCs, del inglés Antigen Presenting Cells), así como de las moléculas coestimuladoras CD80/CD86. Este mecanismo se ha descrito in vitro en células dendríticas derivadas de monocitos (moDCs, del inglés monocyte-derived Dendritic Cells) (Wang et al., 2007) y en células dendríticas derivadas de medula ósea (BM-DCs, del inglés Bone Marrow-derived Dendritic Cells) (Chang et al., 2008; Peng et al., 2009; Weesendorp, et al., 2013b), así como in vivo en órganos linfoides de cerdos infectados con cepas del PRRSV-1 (Rodríguez-Gómez et al., 2013a). Sin embargo, los resultados obtenidos en distintos estudios ponen en evidencia una amplia variabilidad en este mecanismo de evasión en función de la cepa del PRRSV (Rodríguez-Gómez et al., 2013b). Por otro lado, además de modificar la expresión de estas moléculas en las APCs, el PRRSV es capaz también de inducir apoptosis y necrosis de las mismas (Rodríguez-Gómez et al., 2013b). Como consecuencia de estos mecanismos, las poblaciones de células T $CD4^+$ permanecen en niveles bajos y constantes después de la infección (Bautista & Molitor, 1997;1999; Feng et al., 2002; López-Fuertes et al., 1999; 2000; Xiao et al., 2004), dando lugar a una respuesta celular tardía y débil tras la infección por el PRRSV. Por su parte, los linfocitos T $CD8^+$ o CTLs inducen la muerte de células infectadas por cualquier virus frenando así la producción de nuevas partículas víricas (Murphy et al., 2016). La producción de CTLs durante el transcurso de la infección por PRRSV es débil y lenta. Aunque estos CTLs pueden incrementar su frecuencia de forma pasajera tras la primera semana post-infección (Costers et al., 2009; Gómez-Laguna et al., 2009; Lamontagne et al., 2003), no desarrollan actividad citotóxica frente al PRRSV (Costers et al., 2009).

Las cepas virulentas PRRSV-2 inducen una disminución en la expresión de la mólecula MHC-I en APCs (Du et al., 2015; Cao et al., 2016; Qi et al., 2017). Du et al. 2015 describe que esta disminución en la expresión de la molecula MHC-1 en PAMs estaría relacionada con el sistema ubiquitina-proteosoma (Du et al., 2015) y con la Nsp4 del virus (Qi et al., 2017). En este sentido la cepa virulenta Lena (PRRSV-1) induce una fuerte disminución de la expresión de las moleculas MHC-I, MHC-II y CD80/86 en APCs in vitro, mientras que in vivo esta disminución se observó solo para MHC-II. En ambos casos las cepas de baja virulencia Belgium A y LV, apenas tuvieron efectos sobre la expresión de estas móleculas (Weesendorp et al., 2013b), lo cual sugiere un fallo en la activación de las mismas y, por tanto, de la respuesta celular.

En cuanto a los niveles de linfocitos $CD4^+CD8^+$ (CTLs) y linfocitos T CD4-$CD8\alpha\beta^+$ (linfocitos de memoria), la cepa PR40 provoca un incremento de ambas poblaciones de linfocitos a partir de los 21 dpi comparado con las cepa de baja virulencia y el grupo control (Ferrari et al., 2018). Asimismo, para las cepas virulentas SU1-bel y Lena, se ha observado un incremento similar en las poblaciones de linfocitos $CD4^+CD8^+$ (CTLs), lo que pudo contribuir a una disminución más rápida de la viremia en los animales infectados (Morgan et al., 2013; Weesendorp et al., 2013b).

b.2. Células secretoras de IFN-γ

La determinación del número de células secretoras de IFN-γ (IFN-γ-SC, del inglés IFN-γ secreting cells) específicas frente a PRRSV mediante ELISPOT ha sido ampliamente utilizado para evaluar la respuesta celular frente a este virus (Díaz et al., 2005; 2006; Dwivedi et al., 2012; Meier et al., 2003). Estas IFN-γ-SC están compuestas principalmente por linfocitos $CD4^+CD8^+$ y en un pequeño porcentaje por linfocitos T CD4-$CD8\alpha\beta^+$ (Schroder et al., 2004). En la infección por PRRSV, niveles elevados de IFN-γ-SC se correlacionan con protección frente al virus, sin embargo el PRRSV es un inductor débil de IFN-γ-SC (Batista et al., 2004; Díaz et al., 2005;2006). En este sentido, la frecuencia de IFN-γ-SC específicas frente al PRRSV tras vacunación o infección son entre 3 o 4 veces menores comparadas con la generada por la vacunación frente a la enfermedad de Aujeszky (Meier et al., 2003). En órganos linfoides secundarios, como nódulo linfático y tonsila, donde el virus es capaz de persistir de forma crónica, los niveles IFN-γ-SC son similares durante la fase virémica y postvirémica, disminuyendo la carga viral en estos órganos independientemente de los niveles de IFN-γ-SC.

En la infección con cepas virulentas del PRRSV-1, los niveles de IFN-γ-SC incrementan partir de los 21 dpi en animales infectados con la cepa PR40 y la cepa SU1-bel comparado con las cepas de baja virulencia y con los animales control, que no experimentaron dicho incremento (Ferrari et al., 2018; Morgan et al., 2013). Sin embargo, en animales infectados con la cepa Lena los niveles de IFN-γ-SC permanecieron bajos a lo largo de la infección (Weesendorp et al., 2013b). Aunque este incremento de las IFN-γ-SC se ha asociado en la cepa SUI-bel a una disminución temprana de la viremia, está asociación no se ha podido demonstrar en el caso de la cepa PR40 (Canelli et al., 2017; Morgan et al., 2013).

b.3. Linfocitos T reguladores (Tregs)

Dentro de las diferentes poblaciones de linfocitos T, los Tregs son fundamentales en la regulación de la intensidad de la respuesta inmune frente a infecciones, así como en la capacidad de mitigar una inflamación excesiva que diera lugar a una mayor intensidad del daño tisular (Belkaid, 2007). En el cerdo, las poblaciones de Tregs se caracterizan por la expresión del factor de transcripción FoxP3 (Käser et al., 2008). En el PRRSV se ha hipotetizado sobre la inducción por el virus de una fuerta respuesta inmunomoduladora que restrasaría el inicio de la respuesta inmune Th1, y que estaría asociada principalmente a dos eventos, el incremento coordinado de la frecuencia de Tregs FoxP3$^+$ y la producción de IL-10 y TFG-β (Darwich et al., 2010; Dwivedi et al., 2011b; Gómez-Laguna et al., 2012; Renukaradhya et al., 2010; Suradhat et al., 2003; Wongyanin et al., 2012). Cabe destacar que en la infección por otros virus como HIV o el virus de la hepatitis C está ampliamente aceptada la instauración de una respuesta inmunosupresora como mecanismo de evasión del sistema inmune del hospedador favoreciendo así su persitencia en el mismo (Kinter et al., 2004; Kui et al., 2005; Smyk-Pearson et al., 2008; Vahlenkamp et al., 2005).

Si bien los Tregs FoxP3$^+$ inducidos por el PRRSV podrían bloquear la respuesta antiviral y favorecer su persistencia en el organismo, su papel en esta enfermedad es controvertido ya que los datos son incosistentes debido probablemente a la diversidad de cepas del virus. En este sentido, mientras que algunos estudios señalan que el PRRSV-2 podría activar a los Tregs FoxP3$^+$ tanto in vitro como in vivo (Cecere et al., 2012; LeRoith et al., 2011; Manickam et al., 2013; Silva-Campa et al., 2009, 2012; Wongyanin et al., 2010), no está claro que las cepas pertenecientes al PRRSV-1 sean

capaces de inducir células Treg aunque sí puedan dar lugar a un incremento en la producción de IL-10 (Silva-Campa et al., 2010) . En este sentido, Rodríguez-Gómez et al. (2015) describieron la ausencia de cambios tanto en la frecuencia como en la proliferación de linfocitos Treg dentro de la poblacion de células T $CD4^+$ co-cultivados con moDCs infectadas con cepas PRRSV-1 y PRRSV2.

En cuanto a las cepas virulentas, nuevamente nos encontramos con datos contradictorios sobre la inducción de Tregs $FoxP3^+$, todo ello debido en gran medida a la utilización de aproximaciones experimentales y cepas diferentes. Fan et al. (2015) describen in vitro una inducción de linfocitos Treg $FoxP3^+$ en células mononucleares de sangre periférica (PBMCs, del inglés Peripheral Blood Mononuclear Cells) en co-cultivo con moDC infectadas con la cepa virulenta BB (PRRSV-2). En estudios in vivo con cepas virulentas del PRRSV-1, no se observaron cambios en las poblaciones de Tregs $FoxP3^+$ en animales infectados con la cepa virulenta SU1-bel (Morgan et al., 2013), mientras que la cepa virulenta PR-40 causó una disminución de esta subpoblación a partir de los 7 dpi en comparación tanto con el grupo control como con una cepa de menor virulencia (Ferrari et al., 2018). Estos resultados parecen indicar que la generación de células Treg $FoxP3^+$ no representa una estrategia explotada por el PRRSV-1 para evadir la respuesta inmune del hospedador a nivel sistémico. Sin embargo, sería de interés llevar a cabo estudios focalizados en el papel de esta subpoblación en órganos diana para determinar su papel tanto en el control de la respuesta inflamatoria como de la respuesta inmune a nivel local tras la infección con cepas de distinta virulencia.

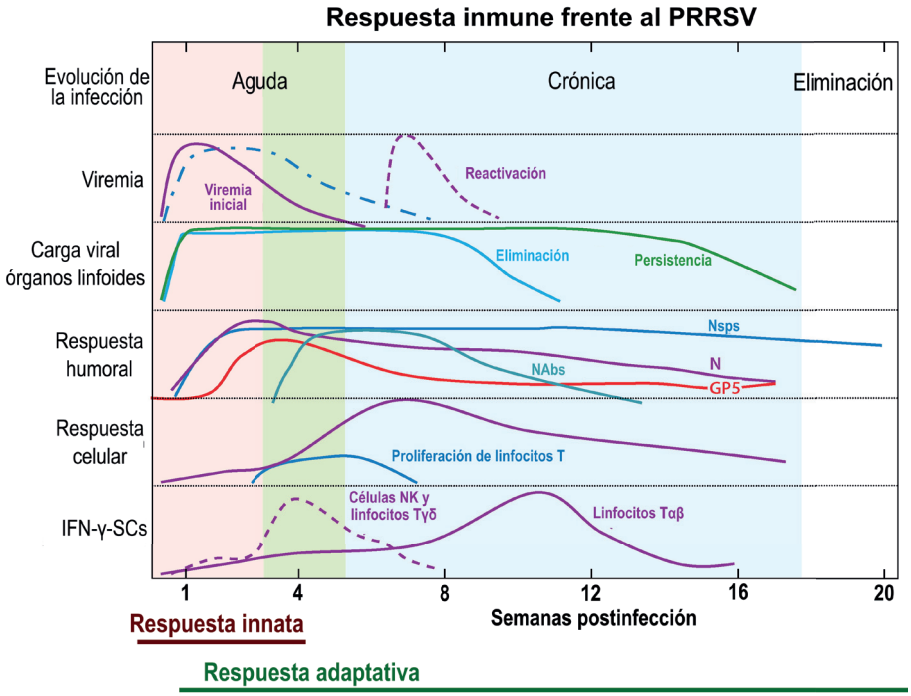

Figura 9. *Respuesta inmune frente a la infección por PRRSV (modificado Lunney et al., 2016). La carga viral en suero está representada por 3 líneas diferentes (eliminación rápida del virus, línea de color púrpura; eliminación lenta, línea de color azul; reactivación del virus, línea púrpura discontinua). La evolución temporal de la respuesta humoral está dividida en NAbs y anticuerpos no neutralizantes frente a las proteínas N, GP5 y Nsps. La respuesta inmune celular que es inicialmente débil alcanza su pico a las 8 – 12 semanas post infección, con la secreción de IFNγ por parte de las IFN-γ-SCs, inicialmente NK y linfocitos Tγδ, y posteriormente por linfocitos Tαβ. (modificado Lunney et al., 2016).*

4. MALDI–MSI: una aproximación a la histología molecular

Los tejidos están constituidos por un micro universo complejo formado por diferentes tipos de células, cada una de ellas especializada en una función específica y conectadas entre sí por una compleja red de interacciones (Kreeger et al., 2010).

Las lesiones producidas en los tejidos son la principal manifestación de un amplio espectro de enfermedades, conteniendo toda la información relativa a cambios morfológicos, genéticos y proteómicos, siendo la base para un estudio óptimo de la inmunopatogenia de las mismas (Schöne et al., 2013). En este sentido, conocer qué moléculas están implicadas en el desarrollo de cada enfermedad nos facilita una mayor comprensión sobre su fisiopatología, así como su interpretación histopatológica. Sin embargo, algunas de las técnicas disponibles actualmente, como la evaluación histopatológica, inmunohistoquímica o el análisis proteómico tras micro-disección láser presentan en menor o mayor medida algunas limitaciones técnicas (Banks et al., 2000; Craven & Banks, 2001; Gutstein & Morris, 2007; Kreeger et al., 2010). La histopatología tradicional, aunque es considerada como el "gold-standard" para la valoración y clasificación de algunas lesiones, como serían determinados tipos de tumores, necesita de un patólogo muy experimentado para evitar llevara a cabo una valoración subjetiva de la lesión (Aichler & Walch, 2015; He et al., 2012; Lazova et al., 2016). Por otro lado, esta técnica no nos permite ir más allá e identificar que moléculas o marcadores de interés pueden estar implicados en el desarrollo de cada proceso y pueden resultar de interés de cara al diagnóstico o monitorización de una patología. Ese sería el caso de la inmunohistoquímica, que nos ofrece una visión in situ de la distribución espacial de un determinado marcador, aunque normalmente sólo nos permite visualizar un número limitado de moléculas, de una a cuatro, en el mismo tejido, dependiendo de la calidad de los anticuerpos y reactivos. Además, esta técnica es laboriosa y exige mucho tiempo, siendo en algunas ocasiones imprecisa (Aichler & Walch, 2015; Gustafsson et al., 2011). Por último, el análisis proteómico tras disección láser es una técnica que aísla distintas áreas del tejido para posteriormente ser analizadas, lo que resulta de gran interés para poder analizar en profundidad un área, estructura o grupo de células en concreto de un tejido, incluso podría permitir compararlo con otro grupo de células de interés, pero se perdería información con respecto al resto del tejido y su perfil de proteómica (Banks et al., 2000; Craven & Banks, 2001; Gutstein & Morris, 2007; Kreeger et al., 2010).

Actualmente, entre las herramientas disponibles para la investigación de procesos patológicos utilizando tejidos, la espectrometría de masas con imagen por láser de desorción/ionización asistida por matriz (MALDI–MSI, del inglés Matrix-Assisted Laser Desorption/Ionization Imaging Mass Spectrometry) o también conocida como histología molecular presenta numerosas ventajas frente a las técnicas mencionadas anteriormente, y está logrando cada día una mayor relevancia en el campo de la investigación. Se trata de una técnica de análisis masivo de proteínas, que combina la espectrometría de masas de alta resolución con la histología (Neagu, 2019; Norris & Caprioli, 2013) (Figura 10).

Una de estas ventajas es el análisis in situ, en el propio tejido, de un amplio rango de moléculas como proteínas, péptidos, lípidos, y pequeñas moléculas exógenas o endógenas, a las cuales no tendríamos acceso utilizando técnicas histológicas tradicionales, así como la flexibilidad para analizar todas estas moléculas en una misma matriz (Aichler & Walch, 2015; Neagu, 2019; Schöne et al., 2013). La evolución en los últimos años de la tecnología MALDI con respecto a una mayor precisión y resolución espacial de los resultados permite la identificación de estas moléculas mediante el análisis bioinformático de las mediciones obtenidas con el espectrómetro (Maier et al., 2013; Sun et al., 2014).

A diferencia de las técnicas que implican micro-disección del tejido, MALDI-IMS lleva a cabo una micro-disección virtual para definir una serie de áreas de interés (ROIs, del inglés regions-of-interest), y posteriormente extraer los perfiles espectrométricos mediante un software. Presenta la gran ventaja de que podemos analizar todas las partes del tejido de forma separada, pero en un mismo análisis aportando una visión global de la lesión (Aichler & Walch, 2015; Neagu, 2019). Otra de las grandes ventajas de MALDI-MSI es la posibilidad de correlacionar toda la información molecular con la histopatología o inmunohistoquímica convencional, manteniendo la localización espacial de cientos de miles de moléculas contenidas en una misma sección de tejido de forma simultánea. Esta información, a su vez, podría integrarse en herramientas para el análisis de rutas de señalización, permitiendo así la identificación de vías de señalización directamente desde tejidos y células individuales implicadas en los diferentes procesos patológicos (Aichler & Walch, 2015; Sun et al., 2014).

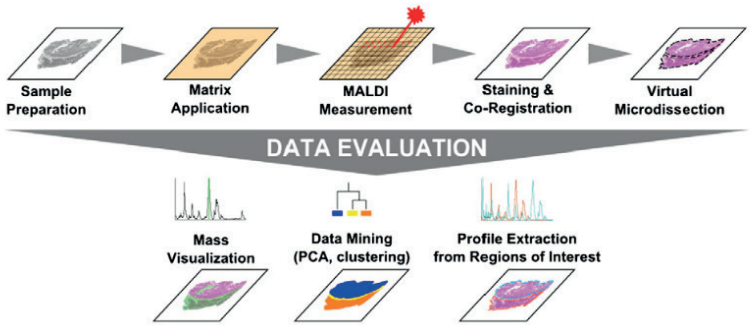

Figura 10. Para la espectrometría de masas con imagen por láser de desorción/ ionización asistida por matriz (MALDI–MSI), se coloca un corte del tejido sobre en un portaobjetos y se cubre con una matriz, que posteriormente se mide en una cuadrícula predefinida, registrándose un espectro de masas específico para cada punto del tejido. Posteriormente, se elimina la matriz, el corte de tejido se tiñe con H&E y se registra digitalmente junto con los datos espectrométricos obtenidos, lo que permite la evaluación histológica de cada una de las medidas mediante microdisección virtual. Los datos resultantes tras el análisis de MALDI-MSI pueden utilizarse para visualizar la distribución de las diferentes masas dentro de la muestra de tejido. Por último, mediante análisis bioestadístico del conjunto de datos se extraen los perfiles moleculares específicos de cada región del tejido en función de las características histopatológicas (modificado Schöne et al., 2013).

La combinación de la capacidad analítica de la espectrometría de masas con información histológica nos ayudaría a comprender los procesos moleculares y patológicos que están teniendo lugar en células específicas dentro de un tejido, así como potenciales biomarcadores y moléculas implicadas, complementando así la evaluación histopatológica e inmunohistoquímica (Aichler & Walch, 2015).

En los últimos años, MALDI–MSI se está utilizando para el análisis de diferentes tejidos y enfermedades, principalmente en investigación del cáncer, permitiendo identificar su perfil proteico así como su caracterización. Además se utiliza para evaluar el microambiente tumoral o la heterogeneidad intratumoral e intertumoral (Neagu, 2019; Aichler & Walch, 2015). En el campo de las enfermedades infecciosas

MALDI-MSI se ha utilizado para la evaluación y seguimiento del tratamiento aplicado frente al patógeno diana identificando biomarcadores relacionados con la respuesta terapéutica y la distribución del fármaco en áreas concretas de lesión (Flinders, 2013; Neagu, 2019; Prideaux et al., 2011; Schöne et al., 2013). En este sentido, Prideaux et al. (2011) utilizaron la tecnología MALDI-MSI para evaluar la distribución del moxifloxacino (quinolona de cuarta generación utilizada para el tratamiento de infecciones repiratorias) en conejos infectados con Mycobacterium tuberculosis que presentaba lesiones granulomatosas a nivel pulmonar. Por otro lado, Gonnet et al. (2020) identificaron biomarcadores de respuesta innata a nivel cutáneo relacionados con la eficacia vacunal tras la adminstración de una vacuna trivalente de la gripe utilizando explantes cutáneos humanos. No obstante, su utilización para la evaluación de enfermedades infecciosas es limitada y no está tan extendido como su uso en oncología.

En resumen, podemos considerar que MALDI-MSI es un microscopio molecular multicolor que investiga los cambios patológicos y fisiológicos que tienen lugar dentro del tejido reteniendo el contexto histológico (Li et al., 2017). Por tanto, resulta evidente que cada técnica cuenta con un grado de imprecisión, y es importante recordar que la histología molecular se considera un campo de la patología que necesita de la habilidad del patólogo para identificar el tipo de lesión, así como para hacer una evaluación y análisis de la información molecular (Aichler & Walch, 2015), lo que representa una de las principales desventajas cuando se pretende llevar a cabo por personal sin la formación adecuada.

En el contexto de la presente tesis doctoral, se plantea el uso del MALDI – MSI como una aproximación novedosa a la histología molecular que permitirá diseccionar la inmunopatogenia de los diferentes patrones de lesión observados en la infección con cepas de diferente virulencia del PRRSV, ayudando así a la identificación de potenciales biomarcadores relacionados con la activación de la respuesta inflamatoria a nivel pulmonar. Esta respuesta, como se ha descrito anteriormente, es mucho más intensa tras la infección con cepas de elevada virulencia del PRRSV, dando lugar a una mayor extensión y gravedad de las lesiones, lo que allanaría el camino a infecciones bacterianas secundarias.

02

Objetivos - Objectives

Imagen: Julia Yellow

OBJETIVOS

El **principal objetivo** de esta tesis doctoral ha sido evaluar la inmunopatogenia de la infección por PRRSV-1 con cepas de diferente virulencia en el pulmón, principal órgano diana del virus. Para alcanzar este objetivo, se han llevado a cabo tres estudios con los siguientes objetivos específicos:

Objetivo 1: Comparar la expresión, distribución y cinética del antígeno del PRRSV, del receptor CD163 y otras moléculas de interés en órganos diana a lo largo de una infección experimental con cepas de moderada y alta virulencia del PRRSV-1.

Estudio 1: "Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence". Este estudio ha sido publicado en la revista *Veterinary Research Communications*, 43(3), 187-195, https://doi.org/10.1007/s11259-019-09755-x y se presenta como indicio de calidad para la lectura y defensa de la tesis doctoral.

Objetivo 2: Evaluar la cinética de expresión de la molécula CD163 en células obtenidas a partir de lavado broncoalveolar de cerdos infectados con cepas de distinta virulencia del PRRSV-1 y determinar su asociación con la replicación del virus y los fenómenos de muerte celular.

Estudio 2: "Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells". Este estudio ha sido publicado en la revista *Veterinary Microbiology,* 235, 101-109, https://doi.org/10.1016/j.vetmic.2019.06.011 y se presenta como indicio de calidad para la lectura y defensa de la tesis doctoral.

Estudio 3: "Activation of regulated cell death in the lung of piglets infected with virulent PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8". Este estudio ha sido enviado a *Veterinary Research* y se encuentra actualmente bajo revisión.

Objetivo 3: Evaluar la expresión de biomarcadores de interés, como CD163s, proteínas de fase aguda o citoquinas, relacionados con la respuesta inflamatoria y respuesta inmune a nivel sistémico y/o local como indicadores de la infección y evolución por cepas de distinta virulencia del virus del PRRS.

Estudio 4: "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena" Este estudio ha sido publicado en la revista *Veterinary Microbiology*, 246, 108-115, https://doi.org/10.1016/j.vetmic.2020.108744.

Estudio 5: "Characterisation of PRRSV-1-induced lung lesion using MALDI mass spectrometry imaging".

OBJECTIVES

The **main objective** of this doctoral dissertation was to dissect the immunopathogenesis of PRRSV-1 infection with strains of different virulence in the lung, the target organ of PRRSV. To achieve this objective, four studies have been carried out with the following specific objectives:

Objective 1: Evaluation of the expression, distribution, and kinetics of PRRSV antigen, the scavenger receptor CD163 and other potential molecule in target tissues along an experimental study with PRRSV-1 strains of different virulence.

Study 1: "Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence". This study has been published in the journal *Veterinary Research Communications,* 43(3), 187-195, https://doi.org/10.1007/s11259-019-09755-x and it is presented as a quality parameter for the doctoral thesis defence (with an impact factor according to the Journal Citation Report (2017), of 1,933, located in the second decile (21/140) within the category of Veterinary Sciences).

Objective 2: Evaluation of CD163 scavenger receptor expression in live cells isolated from BALF in infected-pigs with PRRSV-1 strains of different virulence ascertaining a possible association with viral replication and cell death phenomena.

Study 2: "Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells". This study has been published in the journal *Veterinary Microbiology*, 235, 101-109, https://doi.org/10.1016/j.vetmic.2019.06.011 and it is presented as a quality parameter for the doctoral thesis defence (with an impact factor according to the Journal Citation Report (2017), of 2,524, located in the first decile (8/140) within the category of Veterinary Sciences).

Study 3: "Activation of regulated cell death in the lung of piglets infected with virulent PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8". This manuscript was sent to *Veterinary Research* and it is under review.

Objective 3: Assessment of potential biomarkers expression such as sCD163, acute phase proteins o cytokines involved in the systemic or local inflammatory and immune response as potential biomarkers of infection in infected pigs with PRRSV-1 strains of different virulence.

Study 4: "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena" This study has been published in the journal *Veterinary Microbiology*, 246, 108-115, https://doi.org/10.1016/j.vetmic.2020.108744.

Study 5: "Characterisation of PRRSV-1-induced lung lesion using MALDI mass spectrometry imaging".

03

Estudios - Studies

Estudio 1 / Study 1

Objetivo 1 / Objective 1

Estudio 1:"Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence". Sánchez-Carvajal et al., 2019, *Veterinary research communications*, 43(3), 187-195.

Objetivo 1: Comparar la expresión, distribución y cinética del antígeno del PRRSV, del receptor CD163 y otras moléculas de interés en órganos diana a lo largo de una infección experimental con cepas de moderada y alta virulencia del PRRSV-1.

Objective 1: Evaluation of the expression, distribution, and kinetics of PRRSV antigen, the scavenger receptor CD163 and other potential molecule in target tissues along an experimental study with PRRSV-1 strains of different virulence.

Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence

José M. Sánchez-Carvajal[1,#], Irene M. Rodríguez-Gómez[1], Librado Carrasco[1], Inmaculada Barranco[1], Belén Álvarez[2], Javier Domínguez[2], Francisco J. Salguero[3,], Jaime Gómez-Laguna[1,*].*

[1]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, International Excellence Agrifood Campus 'ceiA3', Córdoba, Spain.

[2]Department of Biotechnology, National Institute for Agricultural and Food Research and Technology (INIA), Madrid, Spain.

[3]Department of Pathology. Public Health England. PHE Porton Down, Salisbury, United Kingdom.

*Both authors contributed equally

#Corresponding author at: Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain.

E-mail address: v42sancj@gmail.com (J.M. Sánchez-Carvajal).

Abstract

The emergence of virulent strains of porcine reproductive and respiratory syndrome virus (PRRSV), causing atypical and severe outbreaks, has been notified worldwide. This study assesses the expression, distribution and kinetics of PRRSV N-protein, CD163 and CD107a in the lung and tonsil from experimentally infected piglets with three different PRRSV-1 strains: a virulent PRRSV-1 subtype 3 strain (SU1-bel) and two low-virulent subtype 1 strains, Lelystad virus (LV) and 215-06. SU1-bel replicated more efficiently in the lungs and tonsils. The number of $CD163^+$ cells decreased in both organs from all infected groups at 7 dpi followed by an increase at the end of the study, displaying a negative correlation with the number of N-$protein^+$ infected cells. CD107a showed a significant increase in infected groups at 35 dpi but no differences were observed among them. Whereas the initial decrease of $CD163^+$ cells appears to be associated to virus replication and cell death, the later recovery of the $CD163^+$ population might be due to the induction of CD163 in immature cells, the recruitment of $CD163^+$ cells in the area of infection or both phenomena. These results highlight the capability of infected animals to recover their macrophage subpopulations and their potential biological functions one-month post-infection, being more pronounced for SU1-bel infected animals.

Keywords: PRRSV-1, CD163, CD107a, macrophages, lung, tonsil.

1. Intoduction

Porcine Reproductive and Respiratory Syndrome (PRRS) continues being one of the most important and costly diseases of the porcine industry worldwide (Nieuwenhuis et al., 2012). The causal agent, PRRS virus (PRRSV), is an enveloped, positive-stranded RNA virus belonging to genus Porartevirus within the family Arteriviridae, order Nidovirales. Recently, PRRSV-1 and PRRSV-2 have been considered as two distinct viral species (Adams et al., 2017) due to their high genetic variability. In the last decade, virulent PRRSV strains have emerged and caused atypical outbreaks linked to high mortality rates, severe clinical signs and lung lesions as well as an unusual immune response from the host (Han et al., 2006, Karniychuk et al., 2010, Xiao et al., 2010, Amarilla et al., 2015, Morgan et al., 2016, Canelli et al., 2017).

Pulmonary alveolar macrophages (PAMs) are the primary target cells for PRRSV replication, although the virus is able to replicate in other pulmonary macrophages subpopulations too (Duan et al., 1997). The expression of the scavenger receptor CD163 is restricted to the monocyte/macrophage lineage and is associated to macrophage differentiation, being expressed on the cell surface and within endosomal vesicles in PAMs (Sánchez et al., 1999). This receptor plays a key role in the internalization and disassembly of PRRSV, being considered as the essential receptor for PRRSV infection (Van Gorp et al., 2008) since macrophages from genome edited pigs lacking CD163 SRCR5 are resistant to the infection by several strains of PRRSV-1 and PRRSV-2 (Burkard et al., 2017).

CD107a, also known as lysosome associated membrane protein 1 (LAMP-1), is a glycoprotein mainly expressed in the lysosome and endosome surfaces of a high number of tissue cells before degranulation (Eskelinen, 2006). Cell surface expression of CD107a increases in activated macrophages (Min et al., 2013) and it is also a useful marker of cytotoxic CD8 T cell and NK cell activation. In vitro studies suggest a reduced capability of NK cells cytotoxicity after co-culture with PRRSV-1 infected PAMs (Cao et al., 2013), nevertheless, there are no previous studies deepening in the expression of CD107a in macrophages after PRRSV infection.

This study assesses the expression, distribution and kinetics of PRRSV antigen, the scavenger receptor CD163 and the molecule CD107a in the lung and tonsils of

piglets experimentally infected with three different PRRSV-1 strains: two low-virulent subtype 1 strains, Lelystad and 215-06 (British field strain) and a virulent PRRSV-1 subtype 3 strain (SU1-bel).

2. Material and methods

2.1. Viruses, animals and experimental design

The experimental design has been previously reported elsewhere by Morgan and co-authors (2013). Briefly, three PRRSV-1 strains of different virulence were used: Lelystad virus-Ter Huurne (LV; considered as prototype strain of PRRSV-1; at its 8th passage), strain 215-06 (a low virulent British field strain; at its 4th passage) and strain SU1-bel (a virulent subtype 3 strain isolated from a Belarusian swine farm in 2010; at its 4th passage).

Seventy-six Yorkshire cross Dutch Landrace 5-weeks old male piglets were obtained from an isolated, specific-pathogen-free pig farm in The Netherlands. All pigs were negative for porcine circovirus type 2, PRRSV and Mycoplasma hyopneumoniae by ELISA and PCR assays. Piglets were randomly distributed into four groups and housed in separate pens of a containment facility at the Animal and Plant Health Agency (APHA). Piglets were intranasally inoculated with 105 TCID50 of the respective viruses (LV, 215-06 or SU1-bel) in 1.5 ml of cRPMI. Control group was mock inoculated with 1.5 ml of naïve PAM cryolysate diluted in cRPMI.

All piglets were daily monitored to evaluate clinical outcomes and to determine rectal temperature from 3 days before infection onwards (Morgan et al., 2013, Weesendorp et al., 2013). At 3, 7 and 35 days post-infection (dpi), control and infected pigs were euthanised by administration of an intravenous lethal dose of pentobarbitone, followed by exsanguination. At necropsy, lung (apical, medial and caudal lobes from the right lung) and tonsil samples were collected and fixed in 10% neutral buffered formalin (Fisher Scientific Ltd., Loughborough, UK) and Zinc salts solution (Fisher Scientific Ltd.) for histopathological and immunohistochemical studies. This experiment was approved by the APHA Ethical Review Committee, and all procedures were carried out under the Animals (Scientific Procedures) Act, 1986, UK.

2.2. Immunohistochemistry

The Chemmate Dako Envision detection kit (Dako, Burlingame, CA, USA) was used to detect PRRSV nucleocapsid protein (Morgan et al., 2016) and the Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA) was used for the immunolabelling of CD163 and CD107a molecules (Bullido et al., 1997, Domenech et al., 2003). Table 1 summarizes the fixatives, antigen retrieval methods and primary antibodies used in each case. For negative controls, the primary antibody was replaced by an isotype control. The number of labelled cells was quantified using Fiji/ImageJ software (NIH, Bethesda, MD) in 20 non-overlapping selected high magnification fields of 0.2 mm2. Immunolabelled cells were morphologically identified, differentiating between PAMs, intravascular and interstitial macrophages in the lung.

2.3. Statistical analysis

The number of PRRSV N-protein, CD163 and CD107a positive cells was expressed as a mean ± SD of the score for each animal within each group. Values were evaluated for approximate normality of distribution by the D'Agostino & Pearson omnibus normality test followed by the Mann Whitney's U non-parametric mean comparisons test or by ANOVA test and Student's t-unpaired test. Correlations between the expression of PRRSV antigen, CD163 and CD107a labelled cells were assessed by the Pearson and Spearman tests. Differences with $p \leq 0.05$ were considered statistically significant. All analyses were run using the software GraphPad Prims version 7.0 (GraphPad Software, San Diego, USA).

Table 1. Summary of immunohistochemical methodology.

Specificity	Type of antibodies	Clone	Source	Fixative solution	Blocking solution	Dilution	Antigen retrieval
Anti-N protein of PRRSV	mAb	SDOW17	Rural Technologies, Brookings, SD, USA	Formalin 10%	BSA 1%	1:70	Protease
Anti-pig CD163	mAb	2A10/11	In house, INIA	Zinc salt solution	NGS 10%	1:100	Citrate pH 6
Anti-pig CD107a	mAb	4E9/11	In house, INIA	Zinc salt solution	BSA 1%	1:1.25	Citrate pH 6

mAb, monoclonal antibody; BSA, Bovine Serum Albumin; NGS, Normal Goat Serum; Protease, enzymatic digestion with trypsin (0.5%)/ chemotrypsin (0.5%) at 37°C for 10 minutes; Citrate pH 6, microwave heat treatment at 420W for 10 minutes; PRRSV, Porcine reproductive and respiratory syndrome virus.

3. Results

3.1. Immunohistochemical detection of PRRSV N-protein in the lung and tonsil

The expression of PRRSV N-protein in the lung and lymphoid tissues have been previously reported elsewhere (Morgan et al., 2016). Briefly, N-protein was detected in the lung of most infected piglets from all infected groups (SU1-bel, LV and 215-06) throughout the study. In tonsils, N-protein$^+$ cells were observed in all infected piglets at 7 dpi, but no positive cells were detected in most of the animals at 35 dpi (Table 2; Figure 1). No PRRSV N-protein labelling was found in any of control animals.

PRRSV N-protein was detected in alveolar, interstitial and intravascular macrophages from the lungs. All infected groups displayed a similar trend with a maximum in the number of N-protein$^+$ cells at 7 dpi, showing SU1-bel infected piglets the highest N-protein expression. Statistically significant differences were detected between infected groups (Figure 1A). In tonsils, the expression of N-protein was mainly detected in macrophages from the crypts and occasionally in dendritic-like cells. SU1-bel and 215-06 infected piglets showed the highest expression, following a similar kinetics than the one described for the lungs (Figure 1B).

3.2. CD163 tissue expression

Pulmonary immunolabelling of CD163 scavenger receptor was mainly observed in the cytoplasm and cell membrane of PAMs, with occasional labelling of interstitial and intravascular macrophages (Figures 2A-C). Infected groups showed a decrease in the number of CD163$^+$ cells at 7 dpi followed by an increase at 35 dpi with respect to control group. SU1-bel and LV infected piglets showed a significant increase in the number of CD163$^+$ macrophages at 35 dpi when compared with 215-06 ($p = 0.007$; $p = 0.041$, respectively) and control group ($p = 0.0002$; $p = 0.004$, respectively) (Figure 2D). Besides, LV infected piglets displayed a significant drop of CD163$^+$ cells at 7 dpi with respect to SU1-bel, 215-06 and control group ($p = 0,009$; $p = 0,0004$; $p = 0,002$; respectively) (Figure 2D). The frequency of CD163$^+$ cells remained relatively constant in 215-06 and control groups throughout the study.

In the tonsil, macrophages close to the crypts were the main CD163 immunolabelled cell population (Figures 3A-C), showing a similar kinetic pattern as the one reported for CD163 in the lung (Figure 3D). SU1-bel infected piglets showed a significantly increased number of $CD163^+$ macrophages at 35 dpi compared to LV and 215-06 groups ($p = 0.023$; $p = 0.024$, respectively).

3.3. CD107a tissue expression

CD107a immunolabelling was mainly observed in the cytoplasm of PAMs and secondly in interstitial and intravascular macrophages from lungs (Figures 4A-C). The kinetics of CD107a expression was similar in all infected groups, remaining at baseline or below control values at 3 and 7 dpi, with a significant increase at the end of the study (35 dpi). Thus, SU1-bel, LV and 215-06 infected piglets displayed a significantly increased number of $CD107a^+$ macrophages at 35 dpi when compared to control group (Figure 4D).

The expression of CD107a could not be evaluated in tonsils due to the immunolabelling was considered unspecific.

3.4. Correlation study

A significant positive correlation between CD163 and CD107a counts in the lung of infected piglets was found ($r = 0.58$, 0.67 and 0.79; $p = 0.008$, 0.002 and <0.0001, respectively, for 215-06, LV and SU1-bel). In addition, SU1-bel and LV infected pigs displayed a statistically significant negative correlation among the frequency of N-$protein^+$ cells antigen and $CD163^+$ cells in the lung ($r = -0.61$ and -0.60; $p = 0.029$ and 0.006, respectively). No statistical correlations were found in the tonsil.

Table 2. Number of pigs in which N-$protein^+$ was detected in the lungs and tonsils in relation to the total number of piglets per group.

Dpi	Lung				Tonsil			
	Control	215-06	LV	SU1-bel	Control	215-06	LV	SU1-bel
3	0/0	5/5	5/5	5/5	0/0	3/5	0/5	3/5
7	0/0	5/5	5/5	5/5	0/0	5/5	5/5	5/5
35	0/0	9/10	10/10	7/8	0/0	1/10	5/10	2/8

Figure 1

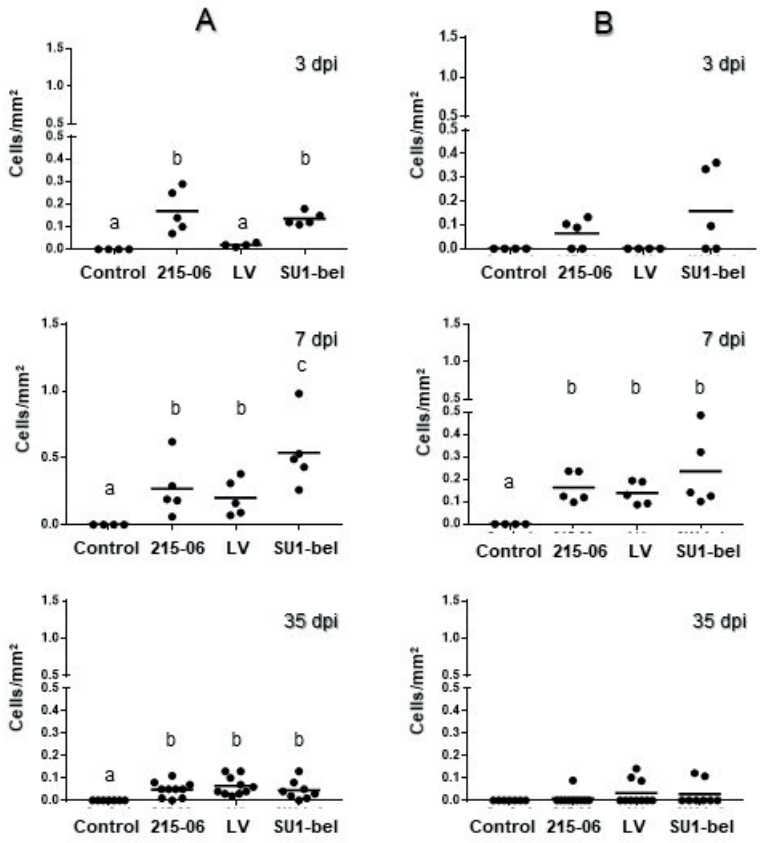

Figure 1. *Counts for cells expressing N-protein of PRRSV in the lung* ***(A)*** *and tonsil* ***(B)*** *of piglets infected with three different PRRSV-1 strains: 215-06, a British field strain, Lelystad virus (LV) and SU1-bel, a virulent PRRSV-1 subtype 3 strain. Single count for each piglet is showed with a black dot and the median is marked with a black line. Statistically significant differences ($p < 0.05$) among groups is indicated by different letters.*

Figure 2

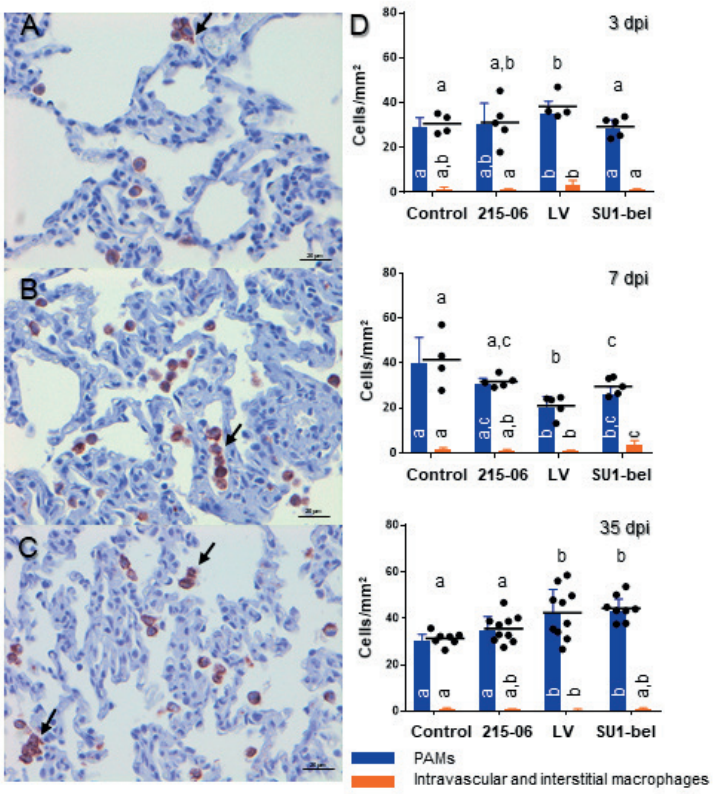

Figure 2. *Photomicrographs of the medial lung lobe from a pig belonging to SU1-bel-infected group euthanised at 3 dpi* ***(A),*** *control group euthanised at 7 dpi* ***(B)*** *and SU1-bel-infected group euthanised at 35 dpi* ***(C).*** *Immunolabelling of CD163 scavenger receptor was mainly observed in the cytoplasm and cell surface of PAMs (arrow) founds in the pulmonary alveolus (*). IHC. Bar, 50 µm. Counts for CD163⁺ PAMs, intravascular and interstitial macrophages in the lung* ***(D)*** *of pigs infected with three different PRRSV-1 strains: 215-06, a British field strain, Lelystad virus (LV) and SU1-bel, a virulent PRRSV-1 subtype 3 strain. The sum of PAMs, intravascular and interstitial macrophages counts for each piglet is showed with a black dot and the median is marked with a black line. Statistical significant differences ($p < 0.05$) among groups for each subset under study (total macrophages, PAMs, intravascular and interstitial macrophages) is indicated by different letters within each subset.*

Figure 3

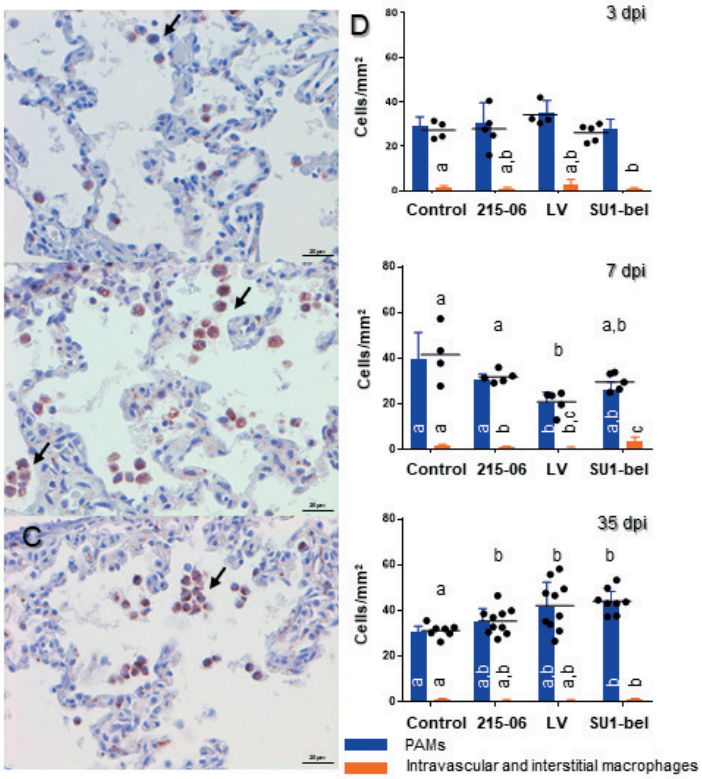

Figure 3. *Photomicrographs of the tonsils from one piglet of the SU1-bel-infected group euthanised at 3 dpi* ***(A),*** *control group euthanised at 7 dpi* ***(B)*** *and SU1-bel-infected group euthanised at 35 dpi* ***(C).*** *IHC. Bar, 100 µm. Inset: Immunolabelling details of CD163 scavenger receptor was mainly observed in the cytoplasm and cell surface of macrophages (arrow) nearby the crypts of the tonsil (*). IHC. Bar, 50 µm. Counts for CD163⁺ macrophages of the crypts and lymphoreticular tissue in the tonsils* ***(D)*** *of pigs infected with three different PRRSV-1 strains: 215-06, a British field strain, Lelystad virus (LV) and SU1-bel, a virulent PRRSV-1 subtype 3 strain. The sum of crypts and lymphoreticular tissue macrophages counts for each piglet is showed with a black dot and the median is marked with a black line. Statistical significant differences ($p < 0.05$) among groups for each subset under study (crypts macrophages and lymphoreticular tissue macrophages) is indicated by different letters within each subset.*

Figure 4

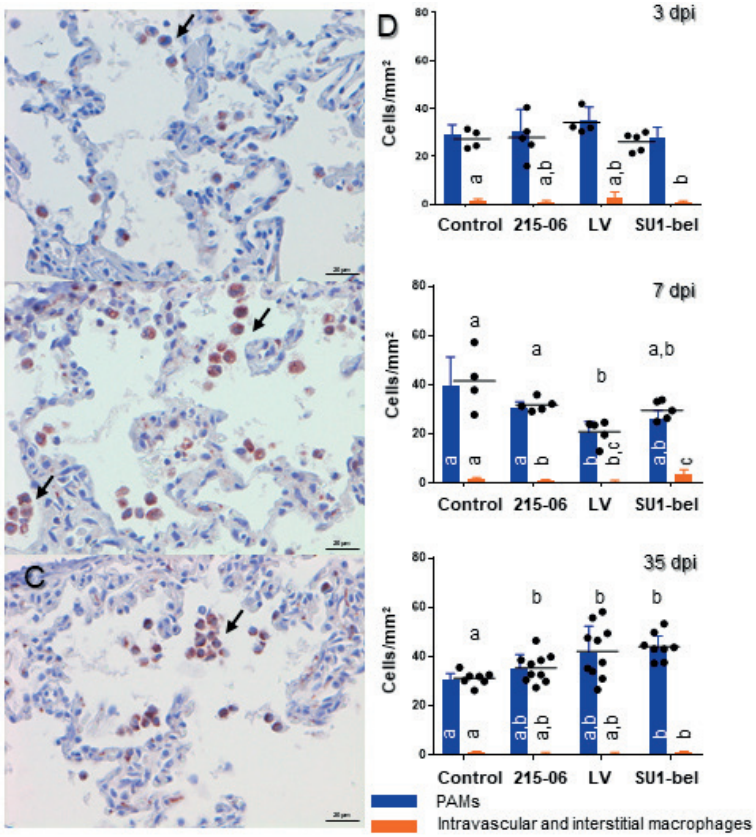

Figure 4. *Photomicrographs of the medial lung lobe of a piglet from the SU1-bel infected group euthanised at 3 dpi* ***(A),*** *control group euthanised at 7 dpi* ***(B)*** *and SU1-bel infected group euthanised at 35 dpi* ***(C).*** *Immunolabelling of CD107a in the cytoplasm of PAMs (arrow) and intravascular macrophages (head arrow) found in pulmonary alveolus (*). IHC. Bar, 50 µm. Counts for CD107a⁺ PAMs, intravascular and interstitial macrophages in the lung* ***(D)*** *of piglets infected with three different PRRSV-1 strains: 215-06, a British field strain, Lelystad virus (LV) and SU1-bel, a virulent PRRSV-1 subtype 3 strain.The sum of PAMs, intravascular and interstitial macrophages counts for each piglet is showed with a black dot and the median is marked with a black line. Statistical significant differences ($p < 0.05$) among groups for each subset under study (total macrophages, PAMs, intravascular and interstitial macrophages) is indicated by different letters within each subset.*

4. Discussion

Since PRRSV emerged, the virus has spread worldwide, undergone a rapid evolution, widened its genetic diversity and, in singular cases, increased its virulence. Virulent PRRSV strains are associated with severe clinical signs and pulmonary lesions, being also linked to high mortality and morbidity rates, and unusual host immune response (Xiao et al., 2010; Hu et al., 2013; Amarilla et al., 2015). Nowadays, the reasons for the differences in virulence among PRRSV strains are uncertain, increasing the level of concern within the swine industry.

Piglets infected with the virulent PRRSV strain SU1-bel showed the highest number of PRRSV N-protein$^+$ cells at 7 dpi in both lung and tonsil when compared with the other infected groups, which might be associated either to a higher replication efficiency of virulent strains in PAMs (Hu et al., 2013; Weesendorp et al., 2014) or to the use of alternative receptors (Frydas et al., 2013).

CD163 has been described as the most important host receptor for the entry and replication of PRRSV into PAMs (Van Gorp et al., 2008). However, in vitro and ex vivo studies support the existence of other potential receptors (Frydas et al., 2013). Despite its relevance, there are scarce in vivo studies analysing the expression of CD163 scavenger receptor along PRRSV infection. In the present study, the expression of CD163 was mainly detected in the cytoplasm and cell membrane of PAMs, interstitial and intravascular macrophages from the lung as well as macrophages nearby the crypts of the tonsils, which supports the higher replication of PRRSV in these macrophage subsets. This pattern of expression agrees with previous studies in snap frozen tissues and tissues fixed in formalin (Bullido et al., 1997, Gómez Laguna et al., 2010, 2013a).

A negative correlation was observed in the present study between the number of N-protein$^+$ cells and the expression of CD163 scavenger receptor. Thus, the initial drop of CD163$^+$ cells would be linked to virus replication and apoptosis phenomena, which have been widely associated to PRRSV infection both in PAMs and intravascular macrophages (Labarque et al., 2003; Wang et al., 2014) as well as in macrophages from lymphoid organs (Gómez-Laguna et al., 2013; Rodríguez-Gómez et al., 2014). In fact, a parallel study by Morgan and co-authors (2016) reported an increase in apoptotic

phenomena in the lung and tonsils in all infected groups when compared with control animals, being higher in SU1-bel infected group. On the other hand, the higher count of $CD163^+$ cells at 35 dpi could be associated with the induction of the expression of CD163 in negative immature cells, the recruitment of $CD163^+$ cells in the area of infection or both phenomena, suggesting that infected animals had the capability of recovering this macrophage subpopulation one-month post-infection with a higher intensity for SU1-bel infected animals.

The degranulation or cytotoxic potential of cells is associated to their content in lysosomes and the expression of associated proteins such as CD107a. The expression of CD107a was observed in the cytoplasm of PAMs, interstitial and intravascular macrophages (Domenech et al., 2003). The kinetics of CD107a expression observed in the present study was similar in all PRRSV-1 infected groups, showing a significant increase at the end of the study when compared to control group. Furthermore, the number of $CD107a^+$ cells was considerably higher in PAMs when compared to interstitial and intravascular macrophages. These results suggest a high lysosomal content in PAMs, which is linked to their main function as phagocytic cell of the alveolar space and its activated state (Min et al., 2013; Álvarez et al., 2014). Our findings provide evidence that the macrophage subpopulations of the lung may co-express both receptors, CD163 and CD107a, during PRRSV infection. Moreover, a statistically significant positive correlation between the number of $CD163^+$ and $CD107a^+$ cells in the lung was observed in piglets infected with any of the three PRRSV-1 strains, but not in the control group.

All in all, our results suggest that during the first week post-infection, PRRSV regulates by direct or indirect mechanisms the expression of CD163 scavenger receptor and CD107a, being able to actively replicate in their target cells, but after one month post-infection, the host is able to restore and to increase the number of cells expressing these receptors which may strengthen both local immune response by the activation of lung macrophages and sensitize the lung for new PRRSV re-infections as well as infection by other swine respiratory pathogens with a central role in the porcine respiratory disease complex.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

References

Adams MJ, Lefkowitz EJ, King AM, Harrach B, Harrison RL, Knowles NJ, Nibert, M (2017). Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2017). Archivesofvirology, 162(8),2505-2538. https://doi.org/10.1007/s 00705-017-3358-5

Álvarez B, Martínez, P, Yuste, M, Poderoso, T, Alonso, F, Domínguez, J, Revilla, C (2014). Phenotypic and functional heterogeneity of $CD169^{+}$ and $CD163^{+}$ macrophages from porcine lymph nodes and spleen. Developmental & Comparative Immunology, 44(1), 44-49. https://doi.org/10.1016/j.dci.2013.11.010

Amarilla SP, Gómez-Laguna J, Carrasco L, Rodríguez-Gómez IM, Ocerín, JMC, Morgan SB, Salguero, FJ (2015). A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: upregulation of IL-1α in highly pathogenic strain induced lesions. Veterinary immunology and immunopathology, 164(3-4),137-147. https://doi.org/10.1016/j.vetimm.2015.02.003

Bullido R, Moral MG, Alonso F, Ezquerra A, Zapata A, Sanchez C, Dominguez, J (1997). Monoclonal antibodies specific for porcine monocytes/macrophages: macrophage heterogeneity in the pig evidenced by the expression of surface antigens. Tissue antigens, 49(4), 403-413. https://doi.org/10.1111/j.1399-0039.1997.tb02769.x

Burkard C, Lillico SG, Reid E, Jackson B, Mileham AJ, Ait-Ali T, Archibald AL (2017). Precision engineering for PRRSV resistance in pigs: macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLoS pathogens, 13(2), e1006206. https://doi.org/10.1371/journal.ppat.1006206

Cao, J., Grauwet, K., Vermeulen, B., Devriendt, B., Jiang, P., Favoreel, H., Nauwynck, H. (2013). Suppression of NK cell-mediated cytotoxicity against PRRSV-infected porcine alveolar macrophages in vitro. Veterinary microbiology, 164(3-4), 261-269. https://doi.org/10.1016/j.vetmic.2013.03.001

Canelli E, Catella A, Borghetti P, Ferrari L, Ogno G, De Angelis E, Bonilauri P (2017). Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Veterinary microbiology, 210, 124-133. https://doi.org/10.1016/j.vetmic.2017.09.002

Domenech N, Rodríguez-Carreño MP, Filgueira P, Alvarez B, Chamorro S, Domínguez J (2003). Identification of porcine macrophages with monoclonal antibodies in formalin-fixed, paraffin-embedded tissues. Veterinary immunology and immunopathology, 94(1-2), 77-81. https://doi.org/10.1016/S0165-2427(03)00084-9

Duan X, Nauwynck HJ, Pensaert MB (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation

with porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary microbiology, 56(1-2), 9-19. https://doi.org/10.1016/S0378-1135(96)01347-8

Eskelinen EL (2006). Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. Molecular aspects of medicine, 27(5-6), 495-502. https://doi.org/10.1016/j.mam.2006.08.005

Frydas IS, Verbeeck M, Cao J, Nauwynck HJ (2013). Replication characteristics of porcine reproductive and respiratory syndrome virus (PRRSV) European subtype 1 (Lelystad) and subtype 3 (Lena) strains in nasal mucosa and cells of the monocytic lineage: indications for the use of new receptors of PRRSV (Lena). Veterinary research, 44(1), 73. http://doi.org/10.1186/1297-9716-44-73

Gómez-Laguna J, Salguero FJ, Fernández de Marco M, Barranco I, Rodríguez-Gómez IM, Quezada M, Carrasco L (2013). Type 2 porcine reproductive and respiratory syndrome virus infection mediated apoptosis in B and T cell areas in lymphoid organs of experimentally infected pigs. Transboundary and emerging diseases, 60(3), 273-278. https://doi.org/10.1111/j.1865-1682.2012.01338.x

Hu SP, Zhang Z, Liu YG, Tian ZJ, Wu DL, Cai XH, He XJ (2013). Pathogenicity and distribution of highly pathogenic porcine reproductive and respiratory syndrome virus in pigs. Transboundary and emerging diseases, 60(4), 351-359. https://doi.org/10.1111/j.1865-1682.2012.01354.x

Karniychuk UU, Geldhof M, Vanhee M, Van Doorsselaere J, Saveleva TA, Nauwynck HJ (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC veterinary research, 6(1), 30. https://doi.org/10.1186/1746-6148-6-30

Labarque G, Van Gucht S, Nauwynck H, Van Reeth K, Pensaert M (2003). Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Veterinary research, 34(3), 249-260. https://doi.org/10.1051/vetres:2003001

Min BK, Suk K, Lee WH (2013). Stimulation of CD107 affects LPS-induced cytokine secretion and cellular adhesion through the ERK signaling pathway in the human macrophage-like cell line, THP-1. Cellular immunology, 281(2), 122-128. https://doi.org/10.1016/j.cellimm.2013.02.003

Morgan SB, Graham SP, Salguero FJ, Cordon PS, Mokhtar H, Rebel JMJ, Frossard JP (2013). Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Veterinary microbiology, 163(1-2), 13-22. https://doi.org/10.1016/j.vetmic.2012.11.024

Morgan SB, Frossard JP, Pallares FJ, Gough J, Stadejek T, Graham SP, Salguero FJ (2016). Pathology and virus distribution in the lung and lymphoid tissues of pigs experimentally inoculated with three distinct type 1 PRRS virus isolates of varying pathogenicity. Transboundary and emerging diseases, 63(3), 285-295. https://doi.org/10.1111/tbed.12272

Nieuwenhuis N, Duinhof TF, Van Nes A (2012). Economic analysis of outbreaks of porcine reproductive and respiratory syndrome virus in nine sow herds. Veterinary Record-English Edition, 170(9), 225.http://dx.doi.org/10.1136/vr.100101

Rodríguez-Gómez IM, Barranco I, Amarilla SP, García-Nicolás O, Salguero FJ, Carrasco L, Gómez-Laguna J (2014). Activation of extrinsic-and Daxx-mediated pathways in lymphoid tissue of PRRSV-infected pigs. Veterinary microbiology, 172(1-2), 186-194. https://doi.org/10.1016/j.vetmic.2014.05.025

Sánchez C, Doménech N, Vázquez J, Alonso F, Ezquerra A, Domínguez J (1999). The porcine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. The Journal of Immunology, 162(9), 5230-5237. https://doi.org/10.1016/s0165-2427(96)05728-5

Van Gorp H, Van Breedam W, Delputte PL, Nauwynck HJ (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. Journal of General Virology, 89(12), 2943-2953. https://doi.org/10.1099/vir.0.2008/005009-0

Wang G, He Y, Tu Y, Liu Y, Zhou EM, Han Z, Cai X (2014). Comparative analysis of apoptotic changes in peripheral immune organs and lungs following experimental infection of piglets with highly pathogenic and classical porcine reproductive and respiratory syndrome virus. Virology journal, 11(1), 2. https://doi.org/10.1186/1743-422X-11-2

Weesendorp E, Morgan S, Stockhofe-Zurwieden N, Popma-De Graaf DJ, Graham SP, Rebel JM (2013). Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Veterinary microbiology, 163(1-2), 1-12. https://doi.org/10.1016/j.vetmic.2012.09.013

Weesendorp E, Rebel JM, Popma-De Graaf DJ, Fijten HP, Stockhofe-Zurwieden N (2014). Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Veterinary microbiology, 174(1-2), 127-138. https://doi.org/10.1016/j.vetmic.2014.09.010

Xiao S, Mo D, Wang Q, Jia J, Qin L, Yu X, Chen Y (2010). Aberrant host immune response induced by highly virulent PRRSV identified by digital gene expression tag profiling. BMC genomics, 11(1), 544. https://doi.org/10.1186/1471-2164-11-544

Estudio 2 / Study 2

Objetivo 2 / Objective 2

Estudio 2: "Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells". Rodríguez-Gómez et al., 2019. *Veterinary microbiology,* 235, 101-109.

Objetivo 2: Evaluar la cinética de expresión de la molécula CD163 en células obtenidas a partir de lavado broncoalveolar de cerdos infectados con cepas de distinta virulencia del PRRSV-1 y determinar su asociación con la replicación del virus y los fenómenos de muerte celular.

Objective 2: Evaluation of CD163 scavenger receptor expression in live cells isolated from BALF in infected-pigs with PRRSV-1 strains of different virulence ascertaining a possible association with viral replication and cell death phenomena.

Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells

Irene M. Rodríguez-Gómez[a,#], José M. Sánchez-Carvajal[a], Francisco J. Pallarés[b], Enric Mateu[c], Librado Carrasco[a], Jaime Gómez-Laguna[a]

[a]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, International Excellence Agrifood Campus 'ceiA3', Córdoba, Spain.

[b]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Murcia, Mare Nostrum Excellence Campus, Murcia, Spain.

[c]Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra, Barcelona, Spain.

[#] Corresponding author at: Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14071, Córdoba, Spain

E-mail: v22rogoi@uco.es

Abstract

Highly virulent porcine reproductive and respiratory syndrome virus (PRRSV) strains have increasingly overwhelmed Asia and Europe in recent years. This study aims to compare the clinical signs, gross and microscopic findings as well as the expression of CD163 within live pulmonary alveolar macrophages (PAMs) from bronchoalveolar lavage fluid (BALF) of pigs experimentally infected with two PRRSV strains of different virulence. Pigs were infected with either a subtype 1 PRRSV-1 3249 strain or a subtype 3 PRRSV-1 Lena strain and consecutively euthanized at 1, 3, 6, 8 and 13 days post-inoculation. Clinical signs were reported daily and BALF and lung tissue samples were collected at the different time-points and accordingly processed for their analysis. Pigs infected with Lena strain exhibited greater clinical signs as well as gross and microscopic lung scores compared to 3249-infected pigs. A decreased frequency of PAMs from BALF was observed early in pigs infected with Lena strain. Moreover, the frequency and median fluorescence intensity (MFI) of CD163 within PAMs were much lower in Lena-infected pigs than in 3249-infected pigs. This downregulation in CD163 was also observed in lung sections after the assessment of macrophages expressing CD163 by means of immunohistochemistry. This outcome may result from the effect of PRRSV replication, PRRSV-induced inflammation, the influx of immature macrophages to restore lung homeostasis and/or the evidence of CD163low cells after $CD163^{+}$ cells decrease in BALF.

Keywords: PRRSV-1, Lena, CD163, BALF, PAMs

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is still a significant problem for the global swine industry. Twenty years after the first description of PRRS virus (PRRSV) (Wensvoort et al., 1991; Benfield et al., 1992; Collins et al., 1992), a collection of severe outbreaks, by the so-called highly pathogenic-PRRSV (HP-PRRSV) strains, overwhelmed China and Southeast Asia in 2006 (Tian et al., 2007; Tong et al., 2007; Feng et al., 2008; Zhou et al., 2008). Shortly afterwards, PRRSV strains of increased virulence, although not comparable with the ones reported in Asia, emerged in Belarus, Belgium, Austria and Italy between 2007 and 2015 (Karniychuk et al., 2010; Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017).

Because of the high genetic and antigenic differences, PRRSV-1 and PRRSV-2 species have been recently renamed as Betaarterivirus suid 1 (formerly genotype 1) and Betaarterivirus suid 2 (formerly genotype 2) with different subtypes or lineages within them, respectively (Shi et al., 2010; Stoian and Rowland, 2019). In case of PRRSV-1, the phylogenetic diversity is quite broad, especially in Central Eastern Europe, where the transboundary movement of infected animals has contributed to the wide viral diversity (Balka et al., 2018). Up to now, four subtypes are described: Pan-European subtype 1, Eastern European subtypes 2 and 3 (Stadejek et al., 2006; 2008) and subtype 4 (Stadejek et al., 2013). Traditionally, virulent PRRSV-1 strains were restricted to subtype 3 strains (Lena strain -prototype- [Karniychuk et al., 2010] and SU1-bel strain [Morgan et al., 2013]); however, strains with similar characteristics have been identified within subtypes 1 (13V091 strain [Frydas et al., 2015], AUT15-33 strain [Sinn et al., 2016] and PR40/2014 [Canelli et al., 2017]) and 2 (BOR59 strain [Stadejek et al., 2017]) in recent years, being the hallmark between them a discontinuous deletion of amino acids in nonstructural protein 2 (Van Doorsselaere et al., 2012; Frydas et al., 2015; Canelli et al., 2017).

Virulent PRRSV-1 strains can induce high fever and cause increased mortality in both field and experimental conditions. Moreover, co-infections with bacterial pathogens which exacerbate clinical symptoms, especially in growing pigs, have been also reported (Karniychuk et al., 2010; Sinn et al., 2016; Canelli et al., 2017; Renson et al., 2017).

Previous studies have showed a stronger inflammatory response in lungs in the early phases of infection for virulent PRRSV-1 compared to animals infected with low-to-moderate virulent isolates (Amarilla et al., 2015). Overall, higher viral loads in serum, lower frequencies of IFN-γ secreting cells as well as PRRSV neutralizing antibodies have been reported, even though results were contradictory among studies (Karniychuk et al., 2010; Morgan et al., 2013; Weesendorp et al., 2013a; 2014; Frydas et al., 2015; Canelli et al., 2017; Renson et al., 2017; Stadejek et al., 2017). However, a minor proportion of studies have evaluated the impact of the infection with PRRSV strains of high virulence on its target cell and related molecules, such as CD163.

In vivo, PRRSV productively replicates in differentiated macrophages, being porcine alveolar macrophages (PAMs) the major target cells (Duan et al., 1997). CD163 has been identified as the essential receptor for PRRSV infection, playing an important role in viral uncoating and genome release (Van Goorp et al., 2008; Whitworth et al., 2016; Burkard et al., 2018). After infection with highly virulent PRRSV, a decrease in the frequency of mature macrophages from bronchoalveolar lavage fluid (BALF) and its phagocytic activity have been reported (Weesendorp et al., 2013b; Renson et al., 2017).

This study sets out to analyze the differences between the virulent PRRSV-1 subtype 3 Lena strain and the low virulent PRRSV-1 subtype 1 3249 strain, focusing on the clinical signs, lesional pattern as well as CD163 expression in live cells isolated from BALF.

2. Material and Methods

2.1. Animals and experimental design

A total of 70, male and female, four-week-old Landrace x Large White piglets were randomly distributed in three different groups and housed in three separate pens at the Biosafety Level 3 containment facilities of Centre de Recerca en Sanitat Animal (CReSA-IRTA, Cerdanyola del Vallès, Barcelona, Spain). Pigs were obtained from a high health historically PRRSV-negative farm and confirmed as negative for PRRSV, porcine circovirus type 2 and Mycoplasma hyopneumoniae (IDEXX PRRS X3 Ab Test, IDEXX Laboratorios, S.L., Barcelona, Spain; in-house PCR for porcine

circovirus type 2 and Mycoplasma hyopneumoniae detection based on previous publications - Mattsson et al., 1995; Sibila et al., 2004). After a 7-day acclimation period, pigs were intranasally inoculated as follows: (i) 3249 group, 26 pigs were inoculated with 2 ml (1.0 ml/nostril making use of MAD NasalTM Intranasal Mucosal Atomization Device, Teleflex, Alcalá de Henares, Madrid, Spain) of 1 x 105 TCID50 of the PRRSV-1 subtype 1 3249 strain (Gimeno et al., 2011); (ii) Lena group, 28 pigs were inoculated at same conditions with the PRRSV-1 subtype 3 Lena strain (Karniychuk et al., 2010); and (iii) control group, in which 16 pigs were inoculated with porcine alveolar macrophages cryolysate diluted in RPMI at same conditions. Three control pigs and 5 infected-pigs from each group were euthanized on days 1, 3, 6 and 8 post-inoculation (PI). At 13 days PI, 4 control pigs, 6 pigs from the 3249 group and 8 pigs from the Lena group were humanely killed. This experiment was performed according to the guidelines of the European Union (Directive 2010/63/EU) and approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413).

2.2. Clinical signs, gross pathology and histopathology of the lung

Pigs were monitored daily from one day prior to inoculation until the end of the study. Monitoring included measurement of rectal temperatures and development of clinical signs, such as abnormal behavior, anorexia, dyspnea and cough.

At necropsy, gross lung lesions were recorded by the same pathologist as described elsewhere (Halbur et al., 1998). Briefly, a percentage reflecting the approximate volume frequency of affected lung parenchyma with respect to the entire lung was assigned to each lung lobe. The sum of all frequencies was an estimation of the percentage of affected lung in a 0-100 scale. After that, the left lung was used to perform bronchoalveolar lavages. Thus, after the complete occlusion of the right primary bronchus by a mosquito forceps, the left lung was flushed with 100 ml of sterile PBS (ThermoFisher Scientific, Waltham, Massachusetts, USA). BALF was spun down (350 x g, 10 minutes [min], 4 °C), cell pellets were washed twice with PBS at same conditions, counted and finally resuspended in PBS containing 3 % of fetal bovine serum (FBS). Samples from all lung lobes of the right lung were collected and fixed in 10 % neutral buffered formalin for histopathological and immunohistochemical investigation.

For the histopathological examination, four-micron tissue sections were cut and stained with hematoxylin and eosin and blindly evaluated by two pathologists as previously described by Halbur et al. (1998) for the diagnosis of interstitial pneumonia. Accordingly: 0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia. In addition, following the same pattern, a similar score was developed considering the diagnosis of suppurative bronchopneumonia. Hence: 0, no microscopic lesions; 1, mild bronchopneumonia; 2, moderate multifocal bronchopneumonia; 3, moderate diffuse bronchopneumonia; and 4, severe bronchopneumonia. The final score comprised the sum of both, the interstitial pneumonia score and the bronchopneumonia score, being 8 points the maximum possible score. The Feulgen technique was also performed to demonstrate the presence of clumps of free chromatin within the alveoli (Feulgen and Rossenbeck, 1924).

2.3. Flow cytometry (FCM) staining and analysis

Freshly isolated cells from BALF were adjusted to 1.5 x 106 cells per sample in a final volume of 200 µl. Staining was performed in 96-well round-bottom plates. Firstly, cells were stained for CD163 (clone 2A10/11, IgG1, 10 µg/ml; Bio-Rad Laboratories, S.A., Alcobendas, Madrid, Spain). Thereafter, cells were washed twice with 200 µl of PBS and primary antibody was followed by a second incubation step with a fluorochrome-labelled isotype-specific secondary antibody (Alexa FluorTM 647 goat anti-mouse IgG1, 7 µg/ml; InvitrogenTM, Carlsbad, CA, USA) in combination with Live/DeadTM Fixable Aqua Dead Cell Stain (InvitrogenTM). Following surface labeling, cells were fixed and permeabilized with methanol overnight at -20 °C (VWR® International, Llinars del Vallès, Barcelona, Spain). Then, cells were incubated with anti-PRRSV-N-protein monoclonal antibody (clone 1CH5, IgG2b, 10 µg/ml; INGENASA, Madrid, Spain) and, after two washing steps, a secondary antibody was included (Alexa FluorTM 488 goat anti-mouse IgG2b, 5 µg/ml; InvitrogenTM). All incubation steps, except specified, took place for 30 min in the fridge. For all stainings, isotype-matched control samples were prepared.

FCM analysis was performed on a FACSCanto II (BD Biosciences, New Jersey, USA) flow cytometer equipped with three lasers (405, 488 and 633 nm). Between 5

x 105 and 1 x 106 cells per sample were recorded. By making use of FlowJo software version 10 (FLOWJO LLC, Ashland, Oregon, USA), cells were gated according to light scatter properties (FSC-A versus SSC-A) and subjected to doublet (FSC-H versus FSC-W and SSC-H versus SSC-W) and dead cell discrimination and further analyzed for the expression of CD163 and PRRSV-N-protein.

2.4. Immunohistochemistry in lung sections

Four µm formalin-fixed sections from lung were dewaxed in xylene for 30 min and rehydrated in descending grades of alcohol followed by endogenous peroxidase inhibition with 3 % H2O2 solution in methanol for 30 min. For antigen retrieval, enzymatic digestion with type XIV protease (Sigma-Aldrich, USA) at 37 °C for 8 min in water bath was used for PRRSV, in case of CD163, high temperature citrate buffer pH 3.2 (microwave 420W, 10 min) was used. Thereafter, sections were washed with PBS (pH 7.4, 0.01 M) and incubated for 1 hour at room temperature with 100 µl of bovine serum albumin 2 % (BSA) (Sigma-Aldrich, USA) as blocking solution in humid chamber. Monoclonal primary antibodies against PRRSV (diluted 1 in 500 in BSA; clone SDOW17; Rural Technologies, Brookings, SD, USA) and CD163 (undiluted; clone 2A10/11; kindly provided by Dr. J. Domínguez, INIA, Madrid, Spain) were incubated overnight at 4 °C in a humid chamber. Biotinylated secondary antibody was incubated for 30 min at room temperature. Then, avidin-biotin-peroxidase complex (ABC Vector Elite, Vector laboratories, USA) was applied for 1 hour at room temperature. Labeling was revealed by application of NovaRED™ substrate kit (Vector Laboratories, USA). Revealed sections were counterstained with Harris's haematoxylin, dehydrated in graded ascending ethylic alcohol and xylene and, finally, mounted. Antibody specificity was verified by substituting the primary antibody by isotype matched reagents of irrelevant specificity. Negative controls consisting on replacement of primary antibody by BSA blocking solution were included in each assay to confirm the lack of non-specific bindings. Labeled cells were counted on 25 non-overlapping consecutive high-magnification power fields of 0.2 mm2 (Olympus BX51, Olympus Iberia SAU, L'Hospitalet de Llobregat, Barcelona, Spain) and expressed as the number of cells per mm2. Positive cells were identified as PAMs, interstitial macrophages or intravascular macrophages.

2.5. Statistical analysis

Statistical analyses were performed using SPSS Statistics version 17.0.1 (IBM, Armonk, NY, USA). Data was evaluated for normality distribution by the Shapiro-Wilk test. Subsequently, data sets were analyzed using the Mann-Whitney U non-parametric test. Accordingly, P values below 0.05 were considered statistically significant and in accordance indicated (* P < 0.05; ** P < 0.01; *** = P < 0.001; **** = P <0.0001).

3. Results

3.1. PRRSV-infected pigs exhibited respiratory disease consisting of interstitial pneumonia and bronchopneumonia

PRRSV-infected pigs showed respiratory signs; however, differences between the infected groups were patent. Animals infected with strain 3249 exhibited mild dyspnea, especially visible from 9 days PI until the end of the study (5 pigs out 26); in contrast, most animals infected with Lena strain (22 pigs out of 28) displayed severe dyspnea with tachypnea, apathy, lethargy, even with prostration, and anorexia from the beginning of the study (2 days PI) (Figure 1A). As shown in figure 1B, these clinical signs were accompanied by pyrexia in both infected groups, but the difference was particularly noteworthy in pigs from the Lena group, with highest values (> 40.5 °C) from 2 to 13 days PI.

Grossly, lungs from infected pigs showed tan-mottled areas, atelectasis, rubbery consistency and areas of consolidation (Figure 2A). Macroscopic scores of 3249-infected pigs gradually increased alongside the study, with the highest score at 13 days PI due to the presence of consolidation, although remarkable individual differences between pigs took place. Lena-infected pigs reached severe macroscopic scores, mainly due to the presence of consolidation as well; however, the presentation of the lesions was earlier (from 6 days PI) and stronger than in 3249-infected pigs (Figure 2A, scatter diagram).

Histopathological lesions consisted of mild to moderate interstitial pneumonia characterized by thickening of the alveolar walls by infiltrating lymphocytes and macrophages in a lesser extent (Figure 2B). Syncytial cells were sporadically observed.

Of note, lung sections from pigs at 13 days PI for 3249 group and from 6 to 13 days PI for Lena group, showed neutrophils, cell debris and mucus filling the bronchial, bronchiolar and alveolar spaces, accompanied by secondary atelectasis. Edema of the interlobular septa as well as dilation of lymphatic blood vessels was also observed, confirming microscopically the co-existence of suppurative bronchopneumonia. In rare cases, the pleura was also affected and covered by fibrinous material. Additionally, clumps of free chromatin, demonstrated by the Feulgen technique, within the alveoli were identified (Figure 2B, inset). Histopathology scores of individual pigs are showed in Figure 2B. Findings were similar to that reported for gross pathology. Control animals did not show signs of disease throughout the study.

Figure 1

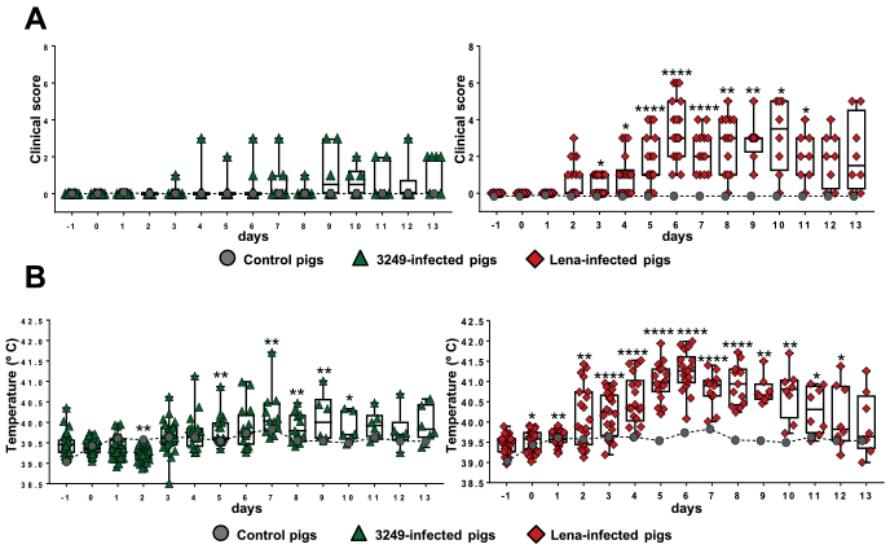

Figure 1. Clinical findings. *Pigs were monitored daily from 1 day prior to infection until 13 days PI for the appearance of clinical signs and pyrexia. Box plots show clinical score* ***(A)*** *and rectal temperature* ***(B)*** *for 3249-infected pigs (green triangles) and Lena-infected pigs (red diamonds). Gray circles indicate the mean average of pigs from the control group. Significant differences between 3249 vs. control group and Lena vs. control group are indicated (* = P < 0.05 ; ** = P < 0.01; **** = P < 0.0001).*

3.2. Downregulation and decrease of CD163$^+$ cells within live PAMs from BALF of PRRSV-infected pigs

CD163 expression was studied by FCM in cells isolated from BALF of control and PRRSV-infected pigs. In order to accurately analyze this molecule, only live cells from BALF were considered. Thereafter, live cells were subjected to doublet discrimination and further analyzed according to light and scatter properties. Data of one representative animal is showed in figure 3A. A homogeneous and stable subset of cells, compatible with PAMs because of size and granularity properties, was identified in all control animals alongside the study (Figure 3B, red circle). In contrast, in both infected groups, this subset proportionately decreased throughout the study (Figures 3B, red arrows and 3C). In the case of cells isolated from Lena-infected pigs, this drop occurred earlier (3 days PI vs. 8 days PI in 3249 group) and was more pronounced, reaching on average only ~16 % of live PAMs. Additionally, another population compatible with a mixture of neutrophils, monocytes and, to a lesser extent, lymphocytes was identified (Figure 3B, green circle). This population of cells was clearly observed in cells isolated from BALF at 13 days PI in 3249-infected pigs and from 6 days PI onwards in Lena-infected animals.

Next, as shown in Figure 4A, the frequency of CD163$^+$ cells within live PAMs was analyzed by FCM. CD163$^+$ cells remained stable in control animals (average >90 %) throughout the experiment (Figure 4B). Contrarily, a decrease in the proportion of CD163$^+$ cells took place from 8 days PI onwards in 3249-infected pigs, even though a high degree of variation was observed between individuals. The onset of this drop appeared earlier (from 3 days PI) and stronger in Lena-infected pigs, where four out of five animals had less than 10 % of live CD163$^+$ PAMs at 8 days PI (Figure 4B). Moreover, as illustrated in figure 4C, live PAMs for each individual animal were divided into PRRSV-N-protein$^-$ and PRRSV-N-protein$^+$ and the Median Fluorescence Intensity (MFI) of CD163 was analyzed for each subset of cells. MFI of control animals was set to 1 and fold-increase or fold-decrease were compared. In general, the average of the MFI of CD163 in infected pigs, independently of the strain, was lower than in control animals from 3 days PI onwards, reaching zero values at 13 days PI in most of the animals. At the end of the study, PRRSV-N-protein$^+$ PAMs showed a higher MFI than PRRSV-N-protein- of the same animal for both strains.

3.3. PRRSV-N-protein$^+$ cells were detected in BALF cells of infected pigs by FCM

The expression of PRRSV-N-protein was analyzed in live PAMs from BALF of control and PRRSV-infected pigs at the different time-points by means of FCM (Figure 5A). The frequency of PRRSV-N-protein$^+$ cells in pigs of the 3249-group increased alongside the study, showing the highest values at 13 days PI (Figure 5B). In the case of pigs from Lena group, infected-cells increased with time, reaching a peak at 6 days PI and gradually decreasing until the end of the study.

3.4. CD163$^+$ macrophages decreased in the lung of PRRSV-infected pigs whereas the number of PRRSV-N-protein$^+$ cells increased

Expression of scavenger receptor CD163 and PRRSV antigen were analyzed at strategic time points in the lung of control and infected pigs by immunohistochemistry, in order to match above described results for BALF. CD163 was mainly observed in the cell membrane and cytoplasm of PAMs and interstitial macrophages (Figure 6A, 1-3), and occasionally labelling intravascular macrophages as well. Infected groups showed a decrease in the number of CD163$^+$ cells at 6 and 8 days PI with respect to control group (Figure 6B). Lena-infected piglets displayed a pronounced drop in both time points compared to piglets from control and 3249 groups. CD163$^+$ intravascular and interstitial macrophages slightly increased in both infected groups with respect to control animals.

With regard to the expression of PRRSV antigen in lungs, positive cells were detected in all piglets from both infected groups at 6 and 8 days PI. No PRRSV-N-protein labelling was found in the control group. Staining was mainly observed in alveolar macrophages and in interstitial and intravascular macrophages in a lesser extent (Figure 6A, 4-6). Of note, clusters of PRRSV-N-protein$^+$ macrophages were observed in bronchopneumonic foci from Lena-infected piglets at 6 and 8 days PI. The number of PRRSV-N-protein$^+$ macrophages was higher at 8 days PI in 3249-infected pigs; this increase was stronger and earlier (6 days PI) in pigs belonging to the Lena group.

Figure 2

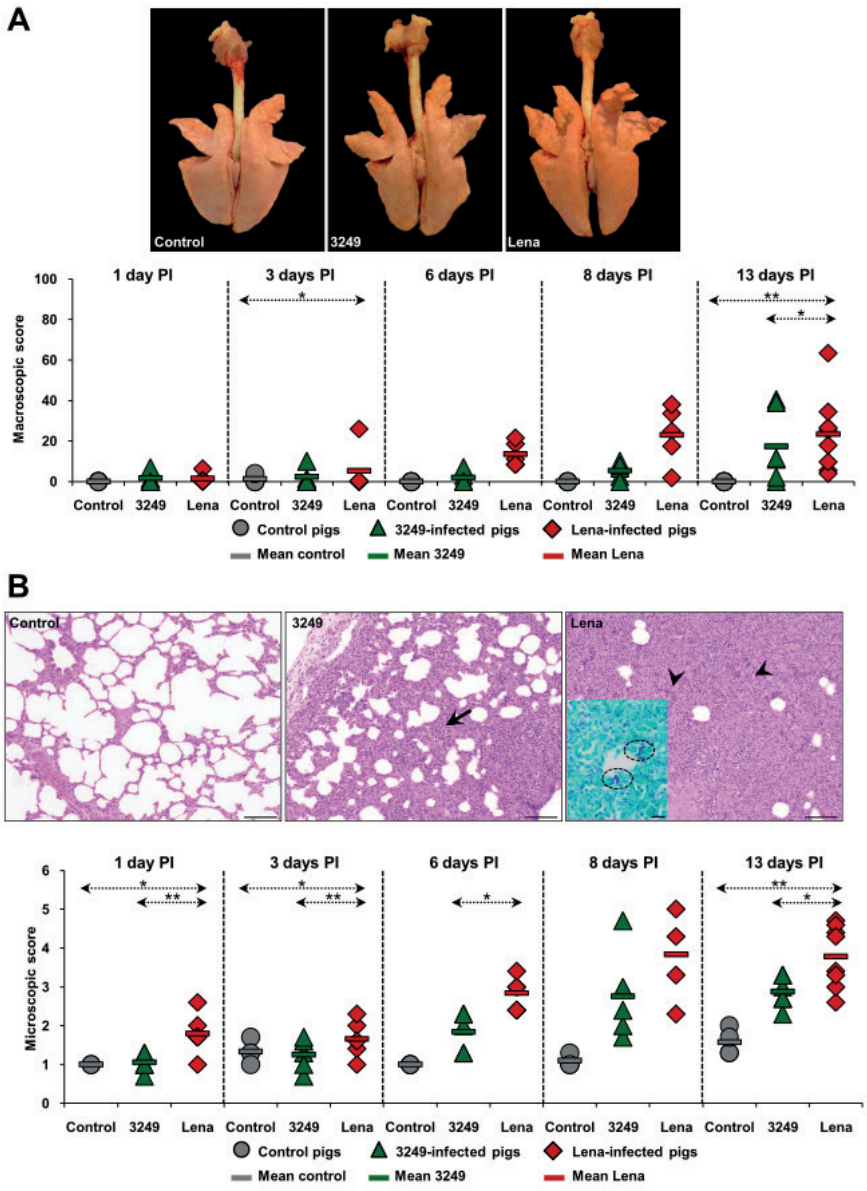
A
Control
3249
Lena
1 day PI
3 days PI
6 days PI
8 days PI
13 days PI
Macroscopic score
Control pigs
3249-infected pigs
Lena-infected pigs
Mean control
Mean 3249
Mean Lena
B
Control
3249
Lena
Microscopic score

Figure 2.A Gross and microscopic lung findings. *At necropsy, gross lung lesions were recorded and lung samples were collected and routinely processed for histopathology examination.* ***(A)*** *Pictures show the macroscopic lung appearance of a representative animal from the control (left), 3249 (middle) and Lena (right) group at 6 days PI. The scatter diagram show the macroscopic score (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point.* ***(B)*** *Representative microscopic pictures of the lung of a control (left), 3249-infected (middle) and Lena-infected pig (right) at 6 days PI (hematoxylin and eosin; bars, 100 micrometers). Black arrow indicates the thickening of the alveolar septa. Arrow heads point to neutrophils and cell debris filling the alveoli. The inset shows clumps of chromatin stained red-purple for Feulgen technique (black dashed circles; bar, 20 micrometers). The scatter diagram displays the microscopic score (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point.* ***(A+B)*** *Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are indicated. (* = P < 0.05 ; ** = P < 0.01).*

Figure 3

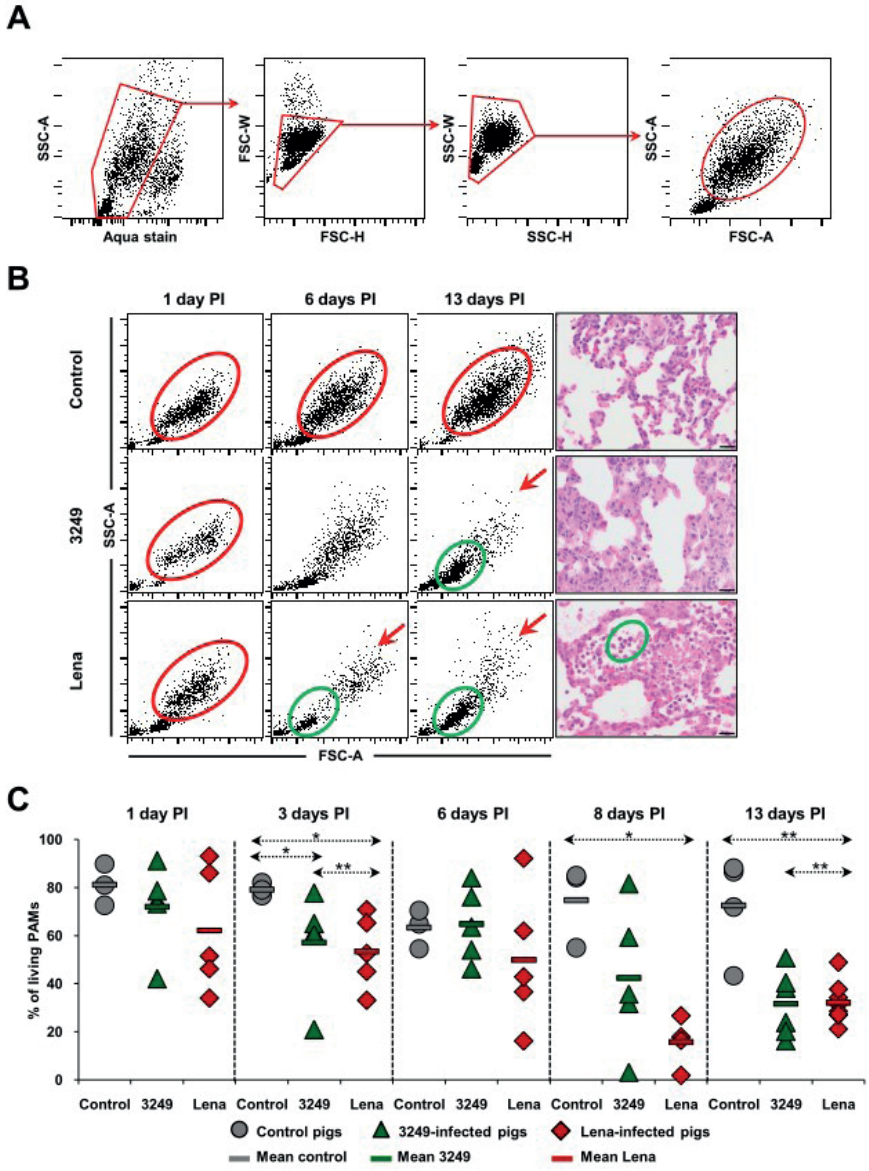
A
SSC-A
Aqua stain
FSC-W
FSC-H
SSC-W
SSC-H
SSC-A
FSC-A
B
1 day PI
6 days PI
13 days PI
Control
3249
Lena
SSC-A
FSC-A
C
1 day PI
3 days PI
6 days PI
8 days PI
13 days PI
% of living PAMs
100
80
60
40
20
0
Control
3249
Lena
Control pigs
3249-infected pigs
Lena-infected pigs
Mean control
Mean 3249
Mean Lena

Figure 3. Gating strategy and frequency of live PAMs. *BALF cells were stained to determine the viability of freshly isolated PAMs by FCM.* ***(A)*** *Gating strategy followed to analyze potential PAMs. Sequentially, exclusion of dead cells (Aqua stain vs. SSC-A) and doublets (FSC-H vs. FSC-W; SSC-H vs. SSC-W) were performed to identify living PAMs (red circle).* ***(B)*** *Black dot plots (FSC-A vs. SSC-A) from a representative pig of the control, 3249 and Lena-infected group at 1, 6 and 13 days PI are showed. Red circles indicate living potential PAMs according to light scatter properties (size and granularity). Red arrows refer to the decrease of the above mentioned subset in 3249-infected and Lena-infected pigs. Green circles indicate a mixture of neutrophils, monocytes and, in a lesser extent, lymphocytes, according to light scatter properties. Microscopic pictures of hematoxylin and eosin for each representative animal at 13 days PI are included to further support FCM findings. Bars, 20 micrometers.* ***(C)*** *Scatter diagram show the frequency of living potential PAMs (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are highlighted (* = P < 0.05; ** = P < 0.01).*

Figure 4

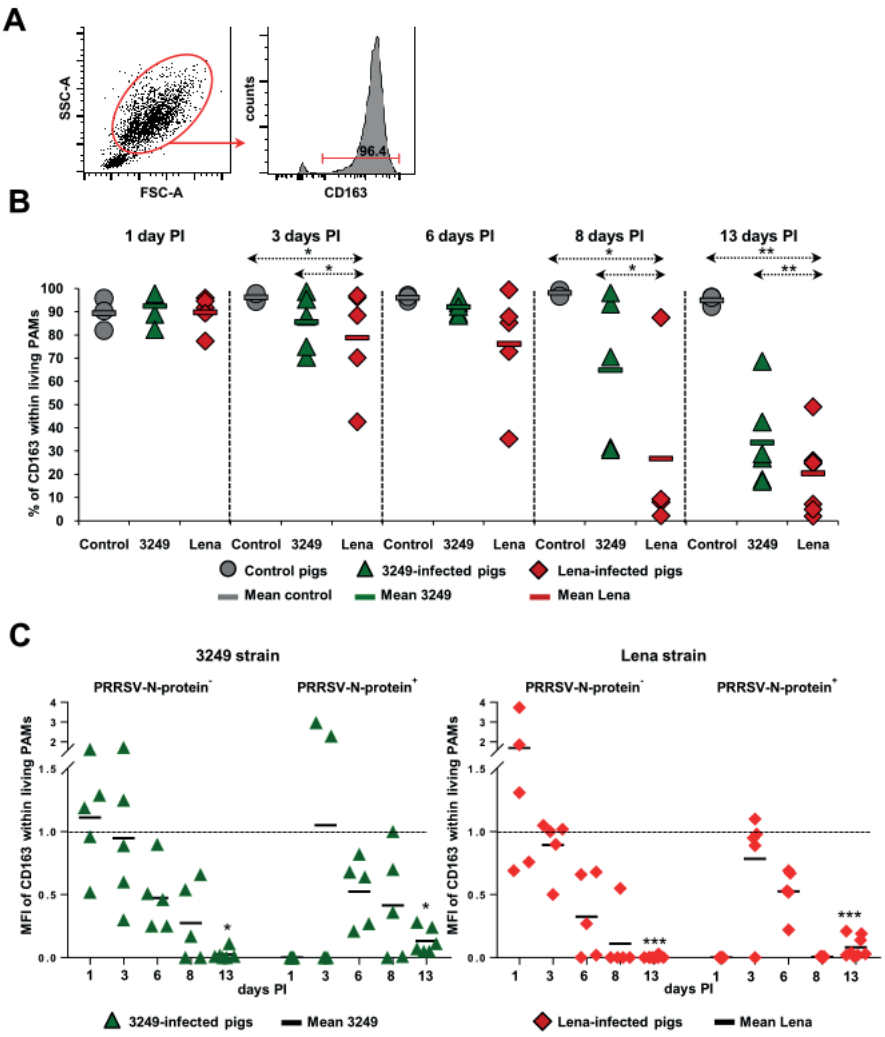
A
SSC-A
FSC-A
counts
96.4
CD163
B
1 day PI
3 days PI
6 days PI
8 days PI
13 days PI
% of CD163 within living PAMs
Control 3249 Lena
Control pigs
3249-infected pigs
Lena-infected pigs
Mean control
Mean 3249
Mean Lena
C
3249 strain
Lena strain
PRRSV-N-protein-
PRRSV-N-protein+
MFI of CD163 within living PAMs
days PI
3249-infected pigs
Mean 3249
Lena-infected pigs
Mean Lena

Figure 4. Frequency and MFI of live CD163⁺ PAMs. *Freshly isolated BALF cells from control and infected pigs were stained and analyzed for the expression of CD163 by FCM.* ***(A)*** *CD163 expression within living PAMs (red circle) was analyzed as indicated in subfigure 4A.* ***(B)*** *The scatter diagram shows the frequency of CD163 living PAMs (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are indicated (* = P < 0.05; ** = P < 0.01).* ***(C)*** *Scatter diagrams show the ratio of the MFI of CD163 (y-axis) within PRRSV-N-protein- and PRRSV-N-protein⁺ live PAMs of 3249-infected (green triangles) and Lena-infected pigs (red diamonds) for each day PI (x-axis). MFI of control animals were set to 1 and represented as black dashed line. Statistical differences between PRRSV-N-protein- and PRRSV-N-protein⁺ live PAMs are indicated (* = P < 0.05; *** = P < 0.001).*

Figure 5

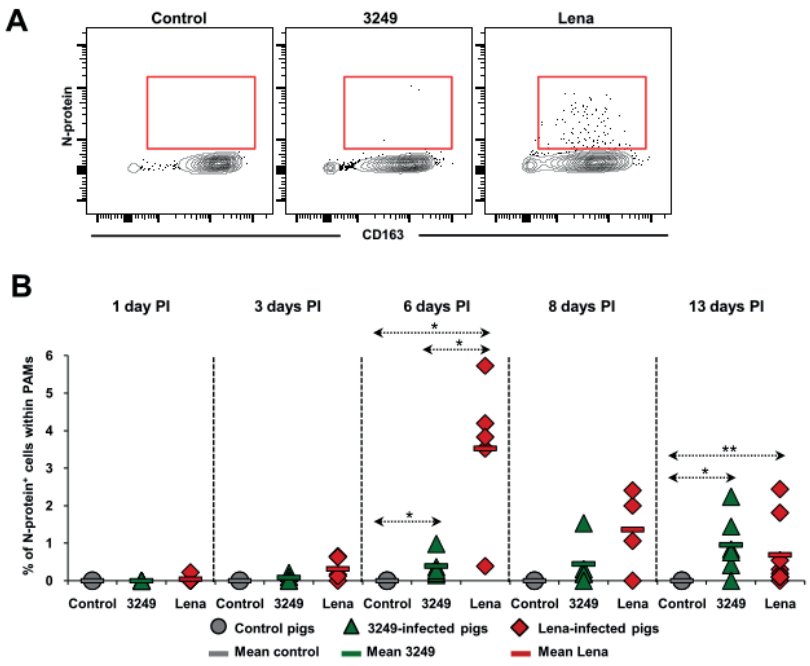

Figure 5. Frequency of PRRSV-infected PAMs. BALF cells were fixed and permeabilized with methanol and stained for the identification of PRRSV⁺ cells. (A) *Contour plots from a representative pig of the control (left), 3249-infected (middle) and Lena-infected (right) group at 6 days PI are showed.* ***(B)*** *Scatter diagram exhibits the frequency of PRRSV-N-protein⁺ cells within PAMs (y-axis) of each group (control, gray circles; 3249, green triangles; Lena, red diamonds) (x-axis) for each time point. Each symbol represents data of one individual pig. Colored bars indicate mean values for each group. Statistical differences between groups are indicated (* = P < 0.05; ** = P < 0.01).*

Figure 6

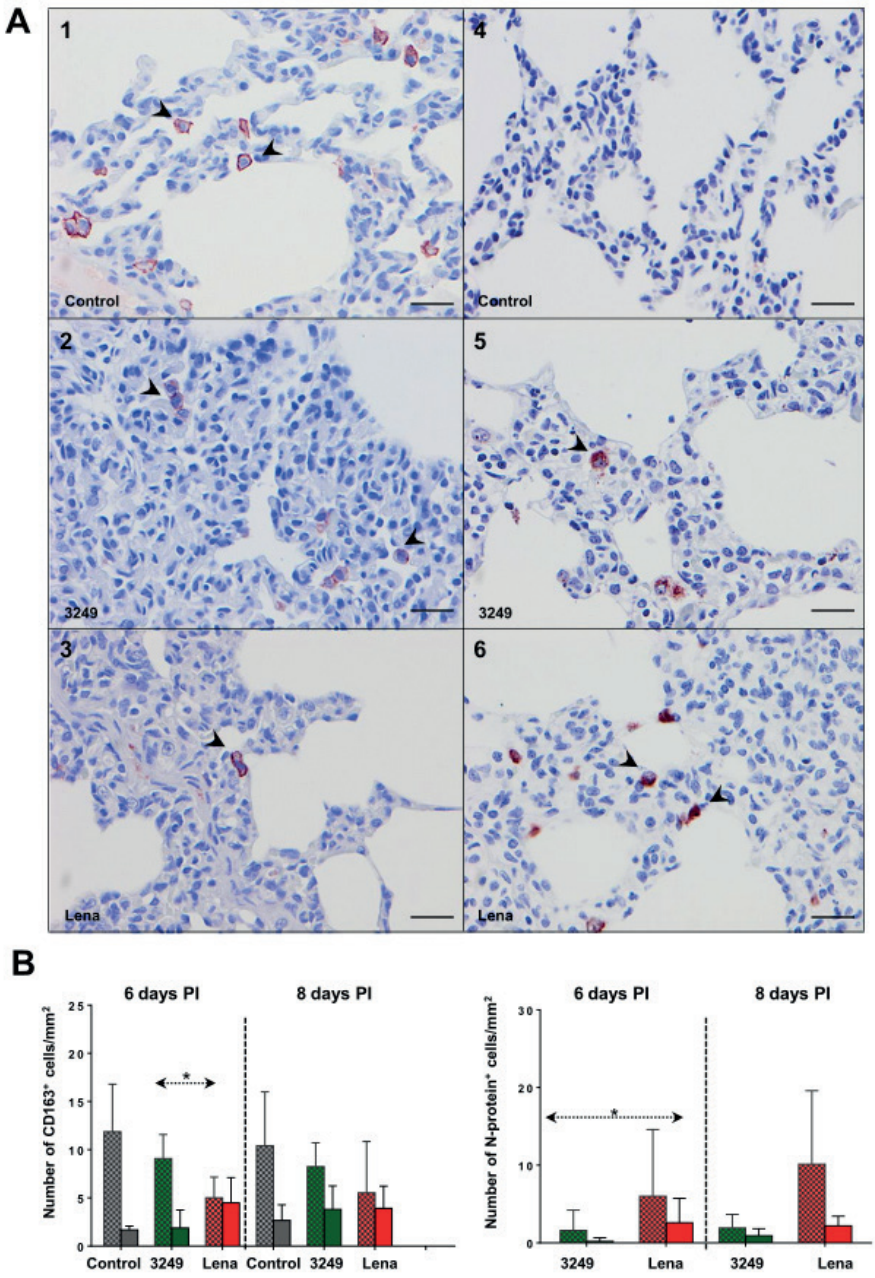
A
1
Control
4
Control
2
3249
5
3249
3
Lena
6
Lena
B
6 days PI
8 days PI
Number of CD163+ cells/mm2
Control
3249
Lena
Control
3249
Lena
6 days PI
8 days PI
Number of N-protein+ cells/mm2
3249
Lena
3249
Lena

Figure 6. Immunohistochemical expression of CD163 and PRRSV-N-protein in lung tissue at 6 and 8 days PI. *Lung tissue sections were accordingly processed and immunolabeled against CD163 and PRRSV antigen.* ***(A)*** *Pictures numbered as 1, 2 and 3 show the expression of CD163 molecule in a control, 3249-infected and Lena-infected representative pig at 6 days PI, respectively. Pictures 4, 5 and 6 exhibit the expression of PRRSV-N-protein in a control, 3249-infected and Lena-infected representative pig at 6 days PI, respectively. Arrow heads indicate immunolabelled PAMs or septal macrophages (Bars, 20 micrometers).* ***(B)*** *Bar charts display the number of $CD163^+$ cells per mm2 or the number of PRRSV-N-$protein^+$ cells per mm2 at 6 and 8 days PI. Tiled light colored bars refer to the number of positive PAMs, whereas dark colored bars comprise the number of positive interstitial and intravascular macrophages. Black dots represent the total number of macrophages for each pig and black lines the average of the different subsets of macrophages. Statistical differences between groups are indicated (* = $P < 0.05$).*

4. Discussion

The appearance of sudden and severe outbreaks caused by PRRSV strains of high virulence have increased in the last decade in China, Southeast Asia and Europe (Tian et al., 2007; Zhou et al., 2008; Karniychuk et al., 2010; Sinn et al., 2016). This fact has led to increase the consistent and continuous efforts of the scientific community to decipher the immunopathogenesis of PRRSV, especially by comparing the behavior of strains of different virulence (Morgan et al., 2013; Weesendorp et al., 2013a; Amarilla et al., 2015; Frydas et al., 2015; Canelli et al., 2017; Renson et al., 2017; Stadejek et al., 2017).

During the time course of our study, piglets infected with strain Lena showed severe clinical signs and developed pyrexia. Gross lung lesions were observed in both PRRSV-infected groups; however, the highest lung scores were found in pigs belonging to Lena group, due to an earlier onset of the typical interstitial pneumonia as well as the complication with suppurative bronchopneumonia, as microscopically confirmed. Our results reproduce the key clinical and lesional features described in the literature for pigs infected with the Lena strain (Karniychuk et al., 2010; Weesendorp et al., 2014; Renson et al., 2017) and other virulent PRRSV strains (Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017). In all cases, as soon as 2 to 3 days PI, severe general and respiratory clinical signs together with pyrexia were reported. Moreover, secondary findings such as catarrhal to suppurative bronchopneumonia, fibrinous pleuropneumonia and fibrinous pericarditis, among others, were also described (Karniychuk et al., 2010; Sinn et al., 2016; Canelli et al., 2017; Renson et al., 2017).

Subsequently, we analyzed the quantity of live PAMs within the cellular fraction of BALF from control and infected pigs. The frequency of PAMs decreased alongside the study in both PRRSV-infected groups. However, this decrease was much more evident in Lena-infected pigs. Besides this population, another subset of cells compatible with a mixture of mainly neutrophils and some monocytes, according to light scatter properties and histopathology observations, became obvious at 6 and 13 days PI in Lena- and 3249-infected pigs, respectively. This precisely coincides with the onset of the bronchopneumonia for the different infected groups and would be consistent with the development of secondary bacterial infections.

In addition, we analyzed the expression of the surface receptor CD163 within live PAMs from BALF of PRRSV-infected pigs. This receptor is widely known for being the key player in PRRSV uncoating and genome release (Calvert et al., 2007; Van Goorp et al., 2008; Van Breedam et al., 2010). The frequency of CD163 in live PAMs decreased in both infected groups, but more evidently in Lena-infected compared to 3249-infected piglets at the end of the study. This data matched with the expression of CD163 in the lung at 6 and 8 days PI by immunohistochemistry. Together, there was a decreased proportion of live PAMs and a decrease in the frequency of $CD163^+$ cells within live PAMs in the BALF and lung section of infected animals compared to controls. Weesendorp et al. (2013b) reported similar results with a lower frequency of bone marrow-derived dendritic cells expressing CD163 in vitro and $CD163^+$ PAMs in vivo after the infection with the strain Lena at 7 days PI. Likewise, Renson et al. (2017) described a reduced frequency of mature macrophages of phenotype $SWC3^+SWC7^+SWC8^-$ in the BALF of piglets infected with Lena strain. The replication of PRRSV may be one of the causes for the decrease of this subset, although more determinants such as induction of cell death, already reported in the lung and lymphoid organs of pigs infected with the virulent PRRSV-1 SU1-Bel strain (Morgan et al., 2016), could contribute to this fall.

Moreover, we wanted to analyze not only the frequency but also the MFI of CD163 within live PRRSV-N-protein- and PRRSV-N-$protein^+$ PAMs for each infected animal. PRRSV-N-$protein^+$ PAMs showed high levels of CD163 at the end of the study, although, in general, the MFI of CD163 within PRRSV-N-protein- and PRRSV-N-$protein^+$ PAMs decreased through the study with respect to control group. Apart from virus replication, this decreased frequency as well as expression of CD163 could be explained by different scenarios: (i) a downregulation of CD163 in the context of PRRSV-induced inflammation. Guo et al. (2014) demonstrated that the metalloprotease ADAM17 is directly activated during PRRSV infection of PAMs and MARC-145 cells in vitro decreasing the level of CD163. Thus, following inflammatory stimuli, such as cytokine production or LPS presence, metalloproteases like ADAM17 can be stimulated and cleaved CD163 (Etzerodt et al., 2010; Guo et al., 2014). (ii) The result of the influx of immature macrophages reaching the alveoli to restore the normal lung function. Due to the loss of $CD163^+$ PAMs, it is expected that new macrophages, still expressing low levels of CD163, replenish the lung to replace this subset. In this context, a clear increase of $CD14^+$ cells in the interstitium of PRRSV-

infected pigs, as a source for macrophages, has been previously described (Van Gucht et al., 2005). And, (iii) since the alveoli are devoid of $CD163^{+}$ PAMs, the frequency of $CD163^{low}$ cells becomes proportionally more noticeable in BALF cells.

5. Conclusion

In summary, the present study shows a decreased frequency and expression of the scavenger receptor CD163 in live PAMs isolated from the BALF of PRRSV-1 infected piglets, being this fact earlier and stronger when the viral strain was a virulent PRRSV-1 strain. This outcome may be due to the direct effect of the virus as well as other indirect mechanisms.

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Conflict of interest

The authors declare that they have no competing interest.

References

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad y Ocerín, J.M., Morgan, S.B., Graham, S.P., Frossard, J.P., Drew, T.W., Salguero, F.J., 2015. A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: upregulation of IL-1alpha in highly pathogenic strain induced lesions. Vet. Immunol. Immunopathol. 164, 137-147. doi:10.1016/j.vetimm.2015.02.003

Balka, G., Podgórska, K., Brar, M.S., Bálint, Á., Cadar, D., Celer, V., Dénes, L., Dirbakova, Z., Jedryczko, A., Márton, L., Novosel, D., Petrović, T., Sirakov, I., Szalay, D., Toplak, I., Leung, F.C., Stadejek, T., 2018. Genetic diversity of PRRSV 1 in Central Eastern Europe in 1994-2014: origin and evolution of the virus in the region. Sci. Rep. 8, 7811. doi:10.1038/s41598-018-26036-w

Benfield, D.A., Nelson, E., Collins, J.E., Harris, L., Goyal, S.M., Robison, D., Christianson, W.T., Morrison, R.B., Gorcyca, D., Chladek, D., 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4, 127-133. doi:10.1177/104063879200400202

Burkard, C., Opriessnig, T., Mileham, A.J., Stadejek, T., Ait-Ali, T., Lillico, S.G., Whitelaw, C.B.A., Archibald, A.L., 2018. Pigs lacking the scavenger receptor cysteine-rich domain 5 of CD163 are resistant to porcine reproductive and respiratory syndrome virus 1 infection. J. Virol. 92, e00415-e00418. doi:10.1128/JVI.00415-18

Calvert, J.G., Slade, D.E., Shields, S.L., Jolie, R., Mannan, R.M., Ankenbauer, R.G., Welch, S.K., 2007. CD163 expression confers susceptibility to porcine reproductive and respiratory syndrome viruses. J. Virol. 81, 7371-7379. doi:10.1128/JVI.00513-07

Canelli, E., Catella, A., Borghetti, P., Ferrari, L., Ogno, G., De Angelis, E., Corradi, A., Passeri, B., Bertani, V., Sandri, G., Bonilauri, P., Leung, F.C., Guazzetti, S., Martelli, P., 2017. Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Vet. Microbiol. 210, 124-133. doi:10.1016/j.vetmic.2017.09.002

Collins, J.E., Benfield, D.A., Christianson, W.T., Harris, L., Hennings, J.C., Shaw, D.P., Goyal, S.M., McCullough, S., Morrison, R.B., Joo, H.S., Gorcyca, D., Chladek, D., 1992. Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J. Vet. Diagn. Invest. 4, 117-126. doi:10.1177/104063879200400201

Duan, X., Nauwynck, H.J., Pensaert, M.B., 1997. Effects of origin and state of differentiation and activation of monocytes/macrophages on their susceptibility to porcine reproductive and respiratory syndrome virus (PRRSV). Arch. Virol. 142, 2483-2497.

Etzerodt, A., Maniecki, M.B., Moller, K., Moller, H.J., Moestrup, S.K., 2010. Tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. J. Leukoc. Biol. 88, 1201-1205. doi:10.1189/jlb.0410235

Feng, Y., Zhao, T., Nguyen, T., Inui, K., Ma, Y., Nguyen, T.H., Nguyen, V.C., Liu, D., Bui, Q.A., To, L.T., Wang, C., Tian, K., Gao, G.F., 2008. Porcine respiratory and reproductive syndrome virus variants, Vietnam and China, 2007. Emerg. Infect. Dis. 14, 1774-1776. doi:10.3201/eid1411.071676

Feulgen, R., Rossenbeck, H., 1924. Mikroskopischchemischer Nachweis einen Nucleinsaure von Typus der Thymonucleinsaure und die darauf berhende elective Farbung von Zellkernen in mikroskopischen Preparaten. Zeitschrift für Physiologische Chemie 135, 203.

Frydas, I.S., Trus, I., Kvisgaard, L.K., Bonckaert, C., Reddy, V.R., Li, Y., Larsen, L.E., Nauwynck, H.J., 2015. Different clinical, virological, serological and tissue tropism outcomes of two new and one old Belgian type 1 subtype 1 porcine reproductive and respiratory virus (PRRSV) isolates. Vet. Res. 46, 37. doi:10.1186/s13567-015-0166-3

Gimeno, M., Darwich, L., Diaz, I., de la Torre, E., Pujols, J., Martín, M., Inumaru, S., Cano, E., Domingo, M., Montoya, M., Mateu, E., 2011. Cytokine profile and phenotype regulation of antigen presenting cells by genotype-1 porcine reproductive and respiratory syndrome virus isolates. Vet. Res. 42, 9. doi:10.1186/1297-9716-42-9

Guo, L., Niu, J., Yu, H., Gu, W., Li, R., Luo, X., Huang, M., Tian, Z., Feng, L., Wang, Y., 2014. Modulation of CD163 expression by metalloprotease ADAM17 regulates porcine reproductive and respiratory syndrome virus entry. J. Virol. 88, 10448-10458. doi:10.1128/JVI.01117-14

Halbur, P.G., Paul, P.S., Frey, M.L., Landgraf, J., Eernisse, K., Meng, X.-J., Lum, M.A., Andrews, J.J., Rathje, J.A., 1998. Comparison of the pathogenicity of two US Porcine Reproductive and Respiratory Syndrome Virus isolates with that of the Lelystad Virus. Vet. Pathol. 32, 648-660. doi:10.1177/030098589503200606

Karniychuk, U.U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T.A., Nauwynck, H.J., 2010. Pathogenesis and antigenic characterization of new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Vet. Res. 6, 30. doi:10.1186/1746-6148-6-30

Mattsson, J.G., Bergström, K., Wallgren, P., Johansson, K.E., 1995. Detection of Mycoplasma hyopneumoniae in nose swabs from pigs by in vitro amplification of the 16S rRNA gene. J. Clin. Microbiol. 33, 893-897.

Morgan, S.B., Graham, S.P., Salguero, F.J., Sánchez-Cordón, P.J., Mokhtar, H., Rebel, J.M.J., Weesendorp, E., Bodman-Smith, K.B., Steinbach, F., Frossard, J.P., 2013. Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Vet. Microbiol. 163, 13-22. doi:10.1016/j.vetmic.2012.11.024

Morgan, S.B., Frossard, J.P., Pallarés, F.J., Gough, J., Stadejek, T., Graham, S.P., Steinbach, F., Drew, T.W., Salguero, F.J., 2016. Pathology and virus distribution in the lung and lymphoid tissues of pigs experimentally inoculated with three distinct type 1 PRRS virus isolates of varying pathogenicity. Transbound. Emerg. Dis. 63, 285-295. doi:10.1111/tbed.12272

Renson, P., Rose, N., Le Dimna, M., Mahé, S., Keranflec´h, A., Paboeuf, F., Belloc, C., Le Potier, M.F., Bourry, O., 2017. Dynamic changes in bronchoalveolar macrophages and cytokines during infection of pigs with a highly or low pathogenic genotype 1 PRRSV strain. Vet. Res. 48, 15. doi:10.1186/s13567-017-0420-y

Shi, M., Lam, T.T., Hon, C.C., Hui, R.K., Faaberg, K.S., Wennblom, T., Murtaugh, M.P., Stadejek, T., Leung, F.C., 2010. Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res. 154, 7-17. doi:10.1016/j.virusres.2010.08.014

Sibila, M., Calsamiglia, M., Segalés, J., Blanchard, P., Badiella, L., Le Dimma, M., Jestin, A., Domingo, M., 2004. Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. Am. J. Vet. Res. 65, 88-92.

Sinn, L.J., Klingler, E., Lamp, B., Brunthaler, R., Weissenböck, H., Rümenapf, T., Ladinig, A., 2016. Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria. Porcine Health Manag. 2, 28. doi:10.1186/s40813-016-0044-z

Stadejek, T., Oleksiewicz, M.B., Potapchuk, D., Podgorska, K., 2006. Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in Eastern Europe support the definition of new genetic subtypes. J. Gen. Virol. 87, 1835-1841. doi:10.1099/vir.0.81782-0

Stadejek, T., Oleksiewicz, M.B., Scherbakov, A.V., Timina, A.M., Krabbe, J.S., Chabros, K., Potapchuk, D., 2008. Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch. Virol. 153, 1479-1488. doi:10.1007/s00705-008-0146-2

Stadejek, T., Stankevicius, A., Murtaugh, M.P., Oleksiewicz, M.B., 2013. Molecular evolution of PRRSV in Europe: current state of play. Vet. Microbiol. 165, 21-28. doi:10.1016/j.vetmic.2013.02.029

Stadejek, T., Larsen, L.E., Podgórska, K., Botner, A., Botti, S., Dolka, I., Fabisiak, M., Heegaard, P.M.H., Hjulsager, C.K., Huc, T., Kvisgaard, L.K., Sapierzynski, R., Nielsen, J., 2017. Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs. Vet. Microbiol. 209, 13-19. doi:10.1016/j.vetmic.2017.05.011

Stoian, A.M.M., Rowland, R.R.R., 2019. Challenges for Porcine Reproductive and Respiratory Syndrome (PRRS) Vaccine Design: reviewing virus glycoprotein interactions with CD163 and targets of virus neutralization. Vet. Sci. 6, pii: E9. doi:10.3390/vetsci6010009

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J., Gao, G.F., 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. PloS One 2, e526. doi:10.1371/journal.pone.0000526

Tong, G.Z., Zhou, Y.J., Hao, X.F., Tian, Z.J., An, T.Q., Qiu, H.J., 2007. Highly pathogenic porcine reproductive and respiratory syndrome, China. Emerg. Infect. Dis. 13, 1434-1436.

doi:10.3201/eid1309.070399

Van Breedam, W., Delputte, P.L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X., Nauwynck, H.J., 2010. Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. J. Gen. Virol. 91, 1659-1667. doi:10.1099/vir.0.020503-0

Van Doorsselaere, J., Brar, M.S., Shi, M., Karniychuk, U., Leung, F.C., Nauwynck, H.J., 2012. Complete genome characterization of a East European Type 1 subtype 3 porcine reproductive and respiratory syndrome virus. Virus Genes 44, 51-54. doi:10.1007/s11262-011-0665-x

Van Goorp, H., Van Breedam, W., Delputte, P.L., Nauwynck, H.J., 2008. Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 89, 2943-2953. doi:10.1099/vir.0.2008/005009-0

Van Gucht, S., Van Reeth, K., Nauwynck, H., Pensaert, M., 2005. Porcine reproductive and respiratory syndrome virus infection increases CD14 expression and lypopolysccharide-binding protein in the lungs of pigs. Viral Immunol. 18, 116-126. doi:10.1089/vim.2005.18.116

Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., Popma-De Graaf, D.J., Grahm, S.P., Rebel, J.M., 2013a. Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Vet. Microbiol. 163, 1-12. doi:10.1016/j.vetmic.2012.09.013

Weesendorp, E., Stockhofe-Zurwieden, N., Popma-De Graaf, D.J., Fijten, H., Rebel, J.M., 2013b. Phenotypic modulation and cytokine profiles of antigen presenting cells by European subtype 1 and 3 porcine reproductive and respiratory syndrome virus strains in vitro and in vivo. Vet. Microbiol. 167, 638-650. doi:10.1016/j.vetmic.2013.09.021

Weesendorp, E., Rebel, J.M., Popma-De Graaf, D.J., Fijten, H.P., Stockhofe-Zurwieden, N., 2014. Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Vet. Microbiol. 174, 127-138. doi:10.1016/j.vetmic.2014.09.010

Wensvoort, G., Terpstra, C., Pol, J.M.A., ter Laak, E.A., Bloemraad, M., de Kluyver, E.P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J.M., Moonen, P.L.J.M., Zetstra, T., de Boer, E.A., Tibben, H.J., de Jong, M.F., van't Veld, P., Greenland, G.J.R., van Gennep, J.A., Voets, M.Th., Verheijden, J.H.M., Braamskamp, J., 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet. Q. 13, 121-130. doi:10.1080/01652176.1991.9694296

Whitworth, K.M., Rowland, R.R., Ewen, C.L., Trible, B.R., Kerrigan, M.A., Cino-Ozuna, A.G., Samuel, M.S., Lightner, J.E., McLaren, D.G., Mileham, A.J., Wells, K.D., Prather, R.S., 2016. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat. Biotechnol. 34, 20-22. doi:10.1038/nbt.3434

Zhou, Y.J., Hao, X.F., Tian, Z.J., Tong, G.Z., Yoo, D., An, T.Q., Zhou, T., Li, G.X., Qiu. H.J., Wei, T.C., Yuan, X.F., 2008. Highly virulent porcine reproductive and respiratory syndrome virus emerged in China. Transbound. Emerg. Dis. 55, 152-164. doi:10.1111/j.1865-1682.2008.01020.x

Estudio 3 / Study 3

Objetivo 2 / Objective 2

Estudio 3: "Activation of regulated cell death in lung during acute PRRSV-1 infection with strains of different virulence". Sanchez-Carvajal et al., 2020. Estudio enviado a *Veterinary Research* actualmente bajo revisión.

Objetivo 2: Evaluar la cinética de expresión de la molécula CD163 en células obtenidas a partir de lavado broncoalveolar de cerdos infectados con cepas de distinta virulencia del PRRSV-1 y determinar su asociación con la replicación del virus y los fenómenos de muerte celular.

Objective 2: Evaluation of CD163 scavenger receptor expression in live cells isolated from BALF in infected-pigs with PRRSV-1 strains of different virulence ascertaining a possible association with viral replication and cell death phenomena.

Activation of regulated cell death in the lung of piglets infected with virulent (Lena) PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8

José María Sánchez-Carvajal [1†*], Inés Ruedas-Torres [1†*], Librado Carrasco 1, Francisco José Pallarés [2], Enric Mateu [3,4], Irene Magdalena Rodríguez-Gómez [1§], Jaime Gómez-Laguna [1§]

[1] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain

[2] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Murcia, 30100, Murcia, Spain

[3] Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Autonomous University of Barcelona, 08193, Bellaterra, Spain

[4] IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.

[†] José María Sánchez-Carvajal and Inés Ruedas-Torres contributed equally as first authors.

[§] Irene Magdalena Rodríguez-Gómez and Jaime Gómez-Laguna contributed equally as last authors

[*]Corresponding authors at: Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain.

E-mail address: v42sancj@uco.es (J.M. Sánchez-Carvajal); iruedas@uco.es (I.Ruedas Torres).

Abstract

PRRSV-1 virulent strains, such as Lena strain, are characterised by high fever, severe respiratory disease and severe lesions in lung and lymphoid organs. Pulmonary alveolar macrophages (PAMs) and other macrophages subpopulationssupport PRRSV replication, impairing their basic functions. Regulated cell death (RCD), such as apoptosis, necroptosis and pyroptosis, is triggered by the host to interrupt viral replication and eliminate virus-infected cells and hence,it could play a role in the immunopathogenesis of PRRSV. The present study aimed to evaluate RCD events in the lung of pigs infected with PRRSV-1 strains of different virulence and their impact on pulmonary macrophages subpopulations as well as its contribution to lung lesion development. Conventional pigs were intranasally inoculated with the virulent subtype 3 Lena strain or the low virulent subtype 1 3249 strain and euthanised at 1, 3, 6, 8 and 13 dpi. Lena-infected piglets showed severe and early lung damage with a marked rise in the number of PRRSV-N-protein$^+$ cells, depletion of CD163$^+$ cells and high lung viral load. The frequency of TUNEL$^+$ cells was significantly higher than the frequency of cCasp3$^+$ cells in both PRRSV-1 infected piglets during the first week post-infection, being more evident in Lena group. cCasp8 and cCasp9 were activated by both PRRSV-1 strains after one week post-infection with a repletion of CD163$^+$ lung macrophages. These results highlight both PRRSV-1 strains induce non-apoptotic RCD during the first week post-infection followed by the activation of mainly extrinsic apoptosis with a self-repletion of CD163$^+$ macrophages as an potential attempt to restore pulmonary macrophages subpopulations lost during the early stages of the infection.

Keywords: PRRSV-1, virulent strain, regulated cell death, lung, cleaved caspase 8.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by PRRS virus (PRRSV), an enveloped, positive-stranded RNA virus, which belongs to the genus Betaarterivirus (family Arteriviridae, order Nidovirales) as two different viral species, Betarterivirus suid 1 (formerly PRRSV-1) and Betarterivirus suid 2 (formerly PRRSV-2) (Gorbalenya et al., 2018). PRRSV-1 exhibits a great phylogenetic diversity, presenting up to four different subtypes (Balka et al., 2018; Stadejek et al., 2006, 2008, 2013). In the last decade, PRRSV-1 virulent strains characterised by high mortality rate, fever, severe respiratory clinical signs and marked lung lesions with also affection of the primary lymphoid organs have been widely described (Canelli et al., 2017; Karniychuk et al., 2010; Sinn et al., 2016; Weesendorp et al., 2014; Yuzhakov et al., 2017). Among these PRRSV-1 virulent strains, the subtype 3 Lena strain is considered to be the prototype for PRRSV-1 virulent strains (Karniychuk et al., 2010).

PRRSV has a restricted tropism for cells of the monocyte/macrophage lineage (Duan et al., 1997), being the pulmonary alveolar macrophage (PAM) the target cell for viral replication and CD163 scavenger receptor the essential mediator for viral internalization and disassembly (Van Breedam et al., 2010; Van Gorp et al., 2010). PRRSV is also able to replicate in other pulmonary macrophages subpopulations such as interstitial and intravascular macrophages (PIMs) (Bordet et al., 2018; Duan et al., 1997; Gómez-Laguna et al., 2013). Whereas PAMs represent the first line of defence against pathogens invasion (De Baere et al., 2012; Du Manoir et al., 2002), PIMs and interstitial macrophages play a pivotal role in the production of pro-inflammatory cytokines (Amarilla et al., 2015; Gómez-Laguna et al., 2010; 2013). Thus, PRRSV replication in pulmonary macrophages subpopulations compromised their basic functions, including cytokine and chemokines production, polarization, phagocytosis and antigen presentation (Darwich et al., 2010; Gómez-Laguna et al., 2010; Jiang et al., 2013; Lu et al., 2012; Thanawongnuwech et al., 1998; Wang et al., 2017).

Regulated cell death (RCD) is considered a sophisticated molecular mechanism which can take place in physiological conditions, and then referred to as programmed cell death, or after extracellular or intracellular perturbations, comprising different modalities such as intrinsic and extrinsic apoptosis, necroptosis, pyroptosis and NETotic cell death, among others (Galluzzi et al., 2018). Thus, during the course of viral infections,

RCD can be activated to interrupt viral replication and eliminate virus-infected cells (Orzalli and Kagan, 2017). Apoptosis is an evolutionary conserved mechanism of RCD that can be started by either external or internal stimuli and requires the activation of cysteine-aspartic proteases known as caspases, which results in the degradation of the cellular components, DNA fragmentation and formation of apoptotic bodies, which are then mainly phagocytosed by macrophages (D'Arcy, 2019; Elmore, 2007; Kerr et al., 1972; Lockshin and Zakeri, 2004; Logue & Martin, 2008). In this cascade of events, caspase-8 and caspase-9 play a key role as initiator caspases, triggering off extrinsic or intrinsic apoptosis, respectively. In both cases, initiator caspases will ultimately activate the executioner caspases, such as caspase-3, for dismantling of dying cells (Galluzzi et al., 2018; Tait & Green, 2010; 2013).

In recent years, other mechanisms of RCD, such as necroptosis or pyroptosis (Galluzzi et al., 2017; 2018), which also play a pivotal role in antiviral responses, have been well described (Man et al., 2017; Nailwal and Chan, 2019; Orzalli and Kagan, 2017). Thus, when viruses try to manipulate RCD for its own advantage by inhibiting caspases-3, -8 and -9 signalling pathways, pyroptosis and necroptosis may be elicited by the host to destroy infective and non-infective cells, restricting viral replication and limiting reservoir cells for viruses (Galluzzi et al., 2016; Man et al., 2017). Thereby, there are many crosstalk between different RCD pathways and, as a result, innate immune signalling events are well activated even upon virus inhibitors (Nailwal and Chan, 2019; Orzalli and Kagan, 2017).

It is well-known that PRRSV elicits apoptosis, necrosis and necrosis like-apoptosis in lungs and lymphoid organs causing a detrimental effect of the host immune system (Costers et al., 2008; Gómez-Laguna et al., 2013; Labarque et al., 2003; Morgan et al., 2016; Rodríguez-Gómez et al., 2014; Sirinarumitr et al., 1998; Suárez, 2000; Sur et al., 1998). Nevertheless, the study of RCD in the lung associated with virulent strains is scarce and just focused on detection of cell death by TUNEL (Morgan et al., 2016; Wang et al., 2014). Therefore, the objective of this study was to evaluate RCD events in the lung of pigs infected with PRRSV-1 strains of different virulence and their impact on pulmonary macrophages subpopulations as well as its contribution to lung lesion development. We hypothesised that strain dependent induction of RCD might directly impact on the impairment of the innate and adaptive immune responses at lung level, decreasing pulmonary macrophages and T cells, hence increasing the susceptibility of infected pigs to secondary infections.

2. Material and methods

2.1. Animals and experimental design

Animals and samples used in this study belong to a large project carried out in order to investigate the pathogenesis of PRRSV-1 strains of different virulence (Rodríguez-Gómez et al., 2019). Briefly, a total of sixty-five 4-weeks-old piglets (Landrace x Large White) were assigned to three different experimental groups: (i) control group (n=15), (ii) 3249-infected group (n=25) and (iii) Lena-infected group (n=25). After a week of acclimation, 3249- and Lena-infected groups were intranasally inoculated with 2 ml of the low virulent 3249 strain or the virulent Lena strain (1x105 TCID50), respectively. The field isolate 3249 strain (subtype 1 PRRSV-1) was first isolated from the serum of a piglet with pneumonia belonging to a PRRSV-positive Spanish farm in 2005 (Gimeno et al., 2011). Lena strain (subtype 3 PRRSV-1) was isolated from a PRRSV-positive herd with a high mortality rate, reproductive failure and respiratory disorders in 2007 in Belarus (Karniychuk et al., 2010). Piglets from control group were inoculated with porcine alveolar macrophages supernatant diluted in RPMI similarly to the inoculum. All pigs included in the study were tested for relevant pathogens, such as PRRSV, porcine circovirus type 2 and Mycoplasma hyopneumoniae by ELISA and PCR assays (IDEXX PRRS X3 Ab Test, IDEXX Laboratorios, S.L., Barcelona, Spain; in-house PCR against porcine circovirus type 2 [Sibila et al., 2004] and Mycoplasma hyopneumoniae [Mattsson et al., 1995]), being negative for all of them at the beginning of the experiment. At 1, 3, 6, 8 and 13 days post-inoculation (dpi), 3 animals from the control group and 5 animals from both infected groups were humanely euthanised. At necropsy, gross lung lesions were recorded by the same pathologist as described elsewhere (Halbur et al., 1996). Samples from apical, medial and caudal lobes of the right lung were collected and conserved at -80 °C for the analysis of PRRSV viral load. In addition, samples from the medial lobe of the right lung were fixed in 10 % neutral buffered formalin for the histopathological and immunohistochemical analyses. This experiment was performed according to the guidelines of the European Union (Directive 2010/63/UE) and approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413).

2.2. PRRSV lung viral load

RNA was isolated and purified from a homogenate of lung tissue by using TRIzolTM LS Reagent and NucleoSpin® RNA virus columns kit according to manufacturer's protocols (Macherey-Nagel, Düren, Germany). Then, the number of viral genomic copies for either 3249 strain or Lena strain was quantified by RT-qPCR using VetMAX™ PRRSV EU/NA 2.0 kit (Thermo Fisher Scientific, Barcelona, Spain). RNA was amplified in the MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) under the following cycling conditions: 50 °C for 5 minutes (min), 95 °C for 10 min followed by 40 cycles of 95 °C for 3 seconds (s) and 60 °C for 30 s. For PRRSV genome quantification, an ORF7 RT-PCR product from both 3249 and Lena strains was firstly EtOH precipitated and purified using ExoSAP-ITTM (Thermo Fisher Scientific). The purified products were quantified using BioDrop (BioDrop, Cambridge, UK). Serial 10-fold dilutions of 3249 or Lena ORF7 RT-PCR products with known quantities, including from 108 to 102 genomic copies/ml were used as standard to generate a standard curve and, therefore, determine the PRRSV genomic copies in the lung. The RT-qPCR efficiency (E) was estimated for each strain by a linear regression model. The E value was calculated from the slope of the standard curve according to the equation: E=10(−1/slope) - 1. Also, a set of eight serial 10-fold dilutions of known TCID50/ml (starting at 106 TCID50/ml) was included in order to determine a relation between Ct-values, genomic copies/ml and TCID50/ml. Thus, the RT-qPCR presented an efficiency of 99 % and high linearity (r = 0.99) for determining viral load, with a slope of 3.34, a detection limit of 1 copy/µl and a slope-intercept of 39.5. An inter-run calibrator sample with a known number of PRRSV copies was introduced in each experiment to self-control inter-run variation. The area under the curve (AUC) for lung viral load was calculated using the trapezoidal approach (Greenbaum et al., 2001). Results of lung viral load are showed in equivalent TCID50 (eq TCID50) per ml.

2.3. Histopathology of lung tissue

Four-micron tissue sections were stained with haematoxylin and eosin (H&E) and blindly scored by two pathologists for the histopathological evaluation. The severity of lung lesions for the interstitial pneumonia was graded according to Halbur et al. (1996), as follows: no microscopic lesions, 0; mild interstitial pneumonia, 1;

moderate multifocal interstitial pneumonia, 2; moderate diffuse interstitial pneumonia, 3; and severe interstitial pneumonia, 4. Moreover, a similar score was conducted as previously described by Rodríguez-Gómez et al. (2019) to evaluate the suppurative bronchopneumonia: no microscopic lesions, 0; mild bronchopneumonia, 1; moderate multifocal bronchopneumonia, 2; moderate diffuse bronchopneumonia, 3; and severe bronchopneumonia, 4. The sum of both the interstitial pneumonia and the bronchopneumonia scores were considered the final score.

2.4. Immunohistochemistry assays

Terminal dUTP Nick End-Labelling (TUNEL) assay (In Situ Cell Death Detection Kit, POD, Roche, Mannheim, Germany) and cleaved Caspase-3 (cCasp3) (SignalStain Apoptosis Cleaved Caspase-3 IHC Detection Kit, Cell Signaling Technology, Inc., MA, USA) were performed by using commercial kits according to the manufacturer's protocols. In the case of TUNEL assay, sixty-one out of sixty-five animals were selected according to lung histopathology score as detailed in table 1.

The avidin-biotin-peroxidase complex technique was performed to detect PRRSV-N-protein, CD163, cleaved Caspase-8 (cCasp8) and cleaved Caspase-9 (cCasp9). Briefly, sections were dewaxed in xylene and rehydrated followed by endogenous peroxidase inhibition with 3 % H2O2 in methanol for 30 min in the darkness. Thereafter, slides were exposed to different antigen retrieval treatments as summarise in table 2. After PBS washes (pH 7.4) and incubation with 100 µl of 2 % bovine serum albumin (BSA), monoclonal primary antibodies against PRRSV-N-protein, CD163, cCasp8 and cCasp9 were incubated overnight at 4 °C in a humid chamber. Afterwards, sections were washed with PBS and incubated with a biotinylated goat anti-mouse secondary antibody (diluted 1 in 100 in 2 % BSA) (Dako, Glostrup, Denmark) for 30 min at room temperature. Then, the avidin-biotin-peroxidase complex (ABC Vector Elite, Vector Laboratories, Burlingame, CA, USA) was applied and incubated for 1 h at room temperature. Immunolabelling was visualized by application of the NovaREDTM substrate kit (Vector Laboratories). Sections were counterstained with Harris's haematoxylin, dehydrated in graded ascending alcohols and mounted. Antibody specificity was verified by substituting the primary antibody by isotype matched reagents of irrelevant specificity. For negative controls, the primary antibody was replaced by BSA blocking solution.

The labelled cells were counted in 25 non-overlapping selected high magnification fields of 0.2 mm2 (Olympus BX51, Olympus Iberia SAU, L'Hospitalet de Llobregat, Barcelona, Spain). The number of positive cells per mm2 (cells/ mm2) was expressed as the mean of the score for each animal within each group. Immunolabelled cells were morphologically identified as PAMs, PIMs, interstitial macrophages, lymphocytes or neutrophils. The AUC for cCasp3, cCasp8 and cCasp9 was calculated using the trapezoidal approach (Greenbaum et al., 2001).

Table 1. Number of pigs from each group subjected to TUNEL assay at 1, 3, 6, 8 and 13 days post-infection (dpi).

Dpi	Control	3249[a]	Lena[b]
1	3	4	4
3	3	5	5
6	2	5	5
8	2	5	5
13	3	5	5

[a]3249: field stran of low virulence

[b]Lena: virulent PRRSV-1 strain

2.5. Statistical analyses

Data analyses and figures were performed by using GraphPad Prism software version 7.0 (GraphPad software, San Diego, CA, USA) and InkScape software version 0.92.3 (InkScape, USA). Differences between groups were evaluated for approximate normality of distribution by the D'Agostino & Pearson omnibus normality test, followed by the Mann Whitney's U test. Correlation coefficients were assessed by the Spearman and Pearson tests and were considered relevant if $r > 0.6$ and $P < 0.05$. For all data, a P value lower than 0.05 was considered statistically significant and represented as * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

Table 2. Summary of immunohistochemical methodology.

Specificity	Type of antibody	Commercial origin	Blocking solution	Dilution	Antigen retrieval
TUNEL	N.A.	In Situ Cell Death Detection Kit, POD, Roche, Germany	N.A.	N.A.	Pronase K in heat incubator
cCasp3 (Asp175)	mAb	SignalStain Apoptosis Cleaved Caspase-3 IHC Detection Kit, Cell Signaling, USA	5 % NGS	1:500	pH 6 citrate buffer in microwave
PRRSV (SDOW 17)	mAb	Rural Technologies, Brookings, SD, USA	2 % BSA	1:500	Protease XIV in water bath
CD163* (2A10/11)	mAb	INIA, Madrid, Spain	2 % BSA	neat	pH 3.2 citrate buffer microwave
cCasp8 (Asp391)	mAb	Cell Signaling; Danvers, MA, USA	2 % BSA	1:100	Pronase K in heat incubator
cCasp9 (Asp330)	mAb	Cell Signaling; Danvers, MA, USA	2 % BSA	1:50	Pronase K in heat incubator

*TUNEL, Terminal dUTP Nick End-Labeling; cCasp3, cleaved Caspase-3; PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; cCasp8, cleaved Caspase-8; cCasp9, cleaved Caspase-9; N.A., not applicable; mAb, monoclonal antibody; pAb, polyclonal antibody; BSA, Bovine Serum Albumin; NGS, Normal Goat Serum; Pronase K (Roche, Basel, Switzerland), 15 min at 37° C in heat incubator; Protease Type XIV (Sigma-Aldrich, Taufkirchen, Germany), 8 min at 37° C in water bath. *CD163 was kindly provided by Dr. J. Domínguez, INIA, Madrid, Spain.*

3. Results

3.1. Lena-infected pigs displayed severe lung lesions compared to 3249-infected pigs

Gross lesions and histopathology were thoroughly described by Rodríguez-Gómez et al. (2019). At necropsy, tan-mottled areas, atelectasis, rubbery consistency and consolidated areas were observed in the lungs from both PRRSV-1-infected pigs with a marked increase from 6 dpi onwards (Fig. 1A-1C). From this time-point and until the end of the study, lung lesions were more intense in Lena- than in 3249-infected pigs and consisted of severe interstitial pneumonia and extensive consolidated areas in apical and medial lung lobes (Fig. 1A-1C). The main histopathological lesion in the lung of both infected groups consisted of a mild to moderate interstitial pneumonia characterised by thickening of the alveolar septa due to infiltrating lymphocytes and macrophages with occasional syncytial cells (Fig. 1B). This lesion had a stronger and earlier onset in pigs inoculated with virulent Lena strain (Fig. 1B-1C). Moreover, extensive foci of suppurative bronchopneumonia composed of neutrophils, cells debris and mucus filling the bronchial, bronchiolar and alveolar lumen were observed from 6 dpi onwards, particularly in those animals infected with Lena strain. Multifocal clumps of purple amorphous material which were proved to be free chromatin by Feulgen technique (Rodríguez-Gómez et al., 2019), were observed in the alveoli of some of the Lena-infected pigs with higher bronchopneumonia scores.

Figure 1

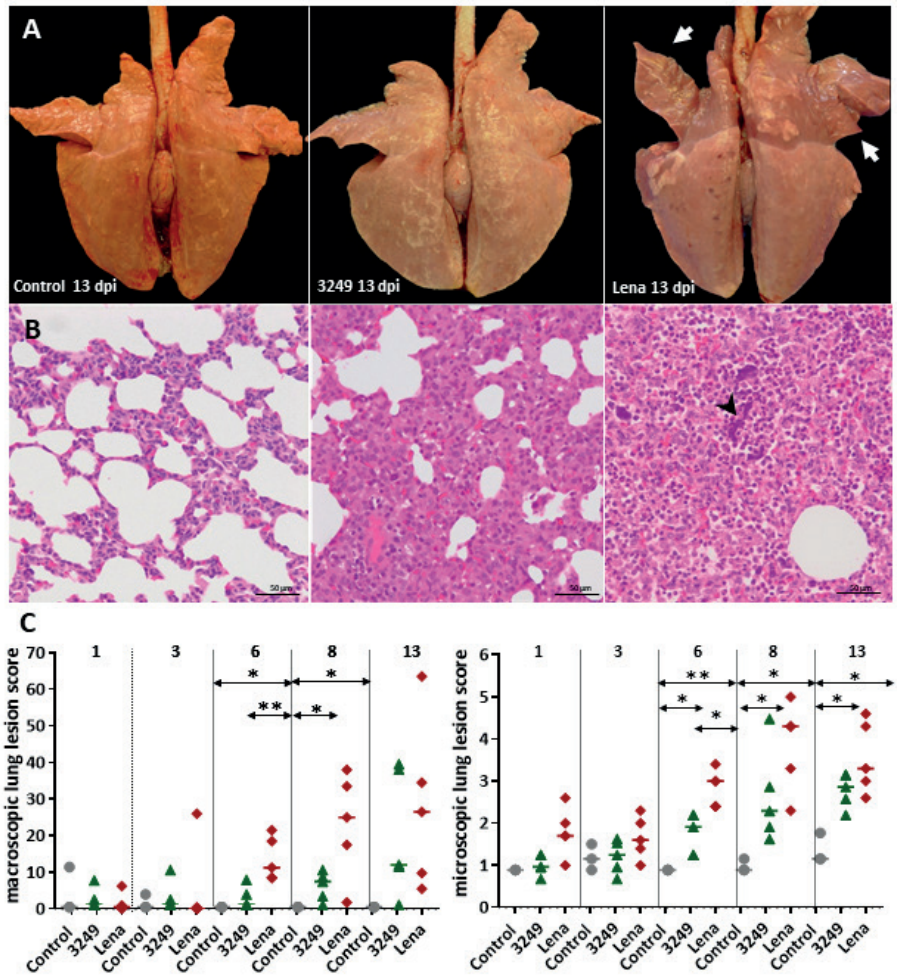
A
Control 13 dpi
3249 13 dpi
Lena 13 dpi
B
C
macroscopic lung lesion score
microscopic lung lesion score
1
3
6
8
13
Control
3249
Lena

Fig. 1. Macroscopic and microscopic lung findings. A. Pictures show the macroscopic lung appearance at necropsy of representative control (left), 3249 (middle) and Lena (right) animals euthanised at 13 dpi. Lungs from 3249 (middle) and Lena (right) infected pigs shown tan-mottled areas, atelectasis, rubbery consistency, but also consolidated areas are observed in apical and medial lung lobes in Lena-infected pigs (right) (white arrows). ***B.*** Photomicrographs of the medial lung lobe from control (left), 3249 (middle) and Lena (right) pigs euthanised at 13 dpi (H&E; bars 50 µm). Lung from 3249 (middle) and Lena (right) representative infected piglets show a moderate interstitial pneumonia characterised by thickening of the alveolar septa due to infiltrating lymphocytes and macrophages. Moreover, Lena-infected lung (right) exhibits extensive foci of suppurative bronchopneumonia with neutrophils, cellular debris, and multifocal chromatin clumps (arrowhead). Graphs display macroscopic ***(C)*** and microscopic ***(D)*** lung lesion score of each animal within each group, control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi. Bars indicate median values for each group. Statistical differences between groups are indicated as "*" for $P < 0.05$ and "**" for $P < 0.01$.

3.2. Lung viral load was higher in Lena-infected pigs

At day 0, all animals were negative by RT-qPCR, remaining pigs from control group negative throughout the study. As early as 1 dpi, two out of five piglets in both infected groups were PRRSV-1 positive; from this time-point onwards, viral copies were detected in all infected piglets. Viral load was higher in Lena- compared to 3249-infected pigs ($P < 0.01$ at 3; $P < 0.05$ at 6 and 8 dpi), reaching the peak at 6 dpi in Lena (1.9x107 eq TCID50/ml), whereas 3249 group peaked at 8 dpi (1.9x106 eq TCID50/ml). At the end of the study, viral load in the lung in each infected group was comparable (Fig. 2A). The AUC for lung viral load (median) in Lena and 3249 groups was 79 and 67.5, respectively. In Lena-infected group, statistical analysis revealed a positive significant correlation between lung viral load and the microscopic score ($r = 0.6$, $P < 0.01$).

3.3. Lena-infected pigs presented the highest number of PRRSV-N-protein$^+$ cells and the lowest number of CD163$^+$ cells

The expression of the scavenger receptor CD163 was detected in the cell membrane and cytoplasm of PAMs, interstitial macrophages and, occasionally, PIMs (Figs. 2E-2F). Control animals exhibited a steady frequency of CD163$^+$ cells during the whole study, whereas there was a continuous drop in the number of CD163$^+$ cells from the beginning until 8 dpi, followed by a sharp rise in the frequency of CD163$^+$ cells at 13 dpi in both PRRSV-1-infected groups (Fig. 2G). The decrease was more intense in Lena-infected animals when compared to 3249 (Fig. 2G). Of note, PAMs were the subpopulation of pulmonary macrophages which underwent the greatest reduction in the number of CD163$^+$ cells. Moreover, there was a significant negative correlation between PRRSV-N-protein$^+$ and CD163$^+$ cells ($r = 0.64$ and $P < 0.0005$) in Lena-infected piglets.

Figure 2

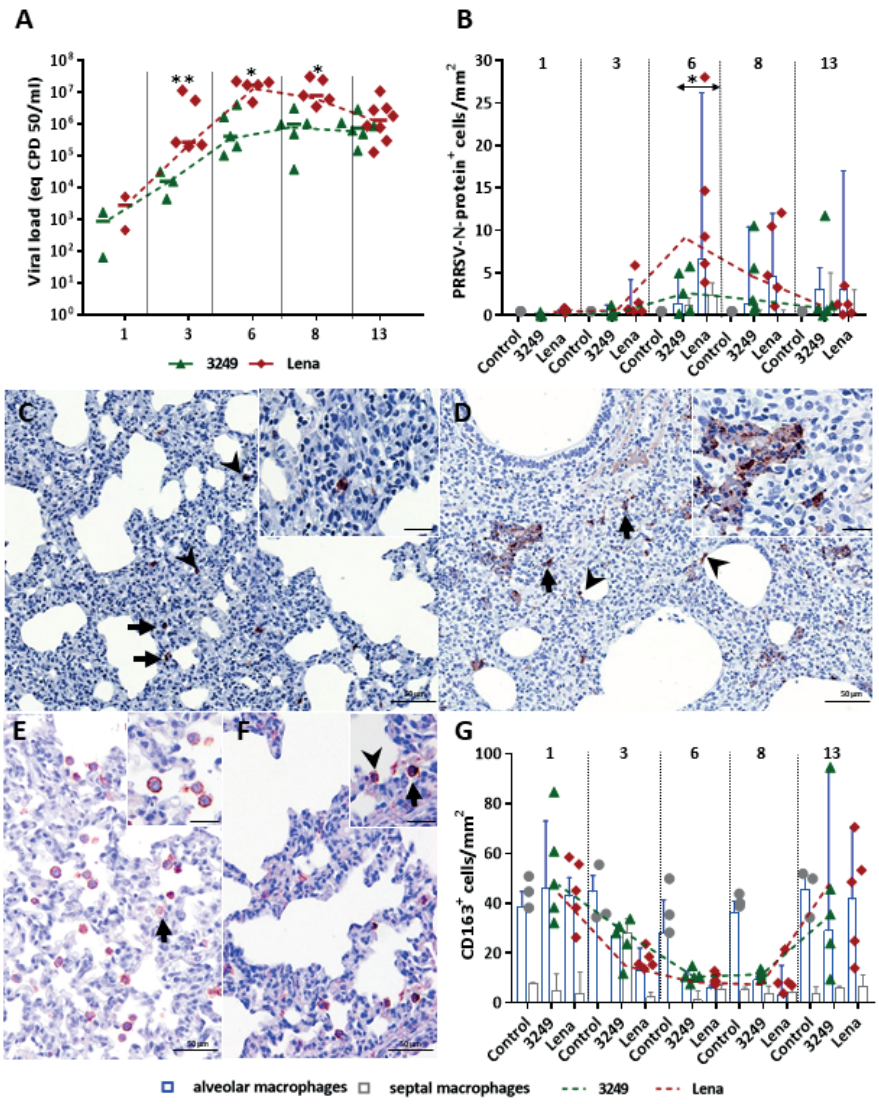
A
Viral load (eq CPD 50/ml)
3249
Lena
B
PRRSV-N-protein+ cells/mm2
Control
3249
Lena
C
D
E
F
G
CD163+ cells/mm2
alveolar macrophages
septal macrophages
3249
Lena

Fig. 2. PRRSV lung viral load and immunohistochemical expression of PRRSV-N-protein and CD163 in lung tissue along the experiment. A. *Graph displays lung viral load of 3249- and Lena-infected pigs along the infection. Individual values from each animal within 3249 (green triangles) and Lena-infected group (red diamonds) at 1, 3, 6, 8 and 13 dpi are shown. Bars indicate median values for each group. Dashed lines represent the median of each infected group, 3249 (green dashed line) and Lena (red dashed line) along the experiment. Statistical differences between groups at 3, 6 and 8 dpi are indicated as "*" for P < 0.05 and "**" for P < 0.01.* ***B.*** *Graph displays the number of PRRSV-N-protein cells^{+} per mm2 in the lung along the infection. Individual total values from each animal within control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi are represented. Columns show the median with range of PAMs (blue column) and septal macrophages (PIMS^{+} interstitial macrophages) (grey column) within each group. Dashed lines represent the median of each infected group, 3249 (green line) and Lena (red line) along the experiment. Statistical differences between groups are indicated as "*" for P < 0.05 at 6 dpi.* ***C.*** *PRRSV-N-protein expression in a representative 3249-infected pig infected euthanised at 6 dpi (bar, 50 µm). Black arrows show PIMs. Arrow heads show PAMs. Inset, detail of PRRSV-N-protein^{+} interstitial macrophage (bar, 20 µm).* ***D.*** *PRRSV-N-protein expression in a representative Lena-infected pig euthanised at 6 dpi (bar, 50 µm). Black arrows show PIMs. Arrow heads show PAMs. Inset, detail of PRRSV-N-protein^{+} PAMs and viral particles (bar, 20 µm).* ***E.*** *CD163 expression in a representative control pig euthanised at 6 dpi (bar, 50 µm). Black arrow shows an CD163^{+} interstitial macrophage. Inset, detail of CD163^{+} PAMs (bar, 20 µm).* ***F.*** *CD163 expression in a representative Lena-infected pig euthanised at 6 dpi (bar, 50 µm). Inset, detail of a CD163^{+} PAM (arrowhead) and interstitial macrophage (black arrow) (bar, 20 µm).* ***G.*** *Graph represents the number of CD163^{+} cells per mm2 in the lung along the infection. Individual values from each animal within each group, control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi are shown. Columns represent the median with range of PAMs (blue column) and septal macrophages (grey column) within each group. Dashed lines display the median of each infected group, 3249 (green line) and Lena (red line) along the experiment.*

3.4. TUNEL labelling was highly expressed in the lung of Lena-infected animals

TUNEL assay was conducted to detect in situ localization of DNA fragmentation. TUNEL labelling was detected in the nuclei and cytoplasm of PAMs, PIMs, interstitial macrophages, free apoptotic bodies and, occasionally, neutrophils, in both control and infected groups (Figs. 3A-3B). TUNEL$^+$ interstitial macrophages and PIMs were mainly observed in areas of interstitial pneumonia (Fig. 3A) whereas TUNEL$^+$ PAMs were identified in foci of suppurative bronchopneumonia with cell debris and mucus filling the lumen of bronchioles and alveoli (Fig. 3B). However, due to the aggregation of TUNEL$^+$ cells, mostly in areas of bronchopneumonia, it was not always possible to differentiate subsets of labelled cells, thus, in this case the results are expressed as the total number of TUNEL$^+$ cells. The frequency of TUNEL$^+$ cells was higher in Lena-infected pigs than in control animals from 3 dpi onwards ($P<0.05$), as well as compared with 3249-infected pigs at 3 ($P < 0.05$), 6 ($P < 0.01$), and 8 dpi ($P < 0.05$). For 3249-infected pigs, the rise in TUNEL$^+$ cells took place later at 8 and 13 dpi, as compared to control pigs ($P<0.05$). The highest expression was detected at 8 dpi in both PRRSV-1 infected groups (Fig. 4A). At 13 dpi a marked dropped in the total number of TUNEL$^+$ cells was observed in both infected groups. A negative correlation was revealed among TUNEL$^+$ cells and CD163$^+$ cells ($r = 0.57$ and $P < 0.003$) in Lena group.

3.5. cCasp3 followed a similar kinetics in Lena- and 3249-infected groups, being more intense for Lena group

cCasp3 was detected in the nuclei and cytoplasm of macrophages, lymphocytes, neutrophils and apoptotic bodies of both control and infected piglets (Fig. 3C-3D). cCasp3$^+$ interstitial macrophages, PIMs and in lesser extent lymphocytes were mainly located in areas of interstitial pneumonia, whereas cCasp3$^+$ PAMs and neutrophils were commonly observed infiltrating foci of bronchopneumonia (Fig. 3D). The kinetics of cCasp3$^+$ expression was similar in both PRRSV-1-infected groups, rising gradually from 3 dpi (Lena) or 6 dpi (3249) until the end of the study and showing a significant increase when compared to control group ($P < 0.05$ at 3, 6, 8 and 13 dpi) (Fig. 4B). This increase in cCasp3 cells was earlier, as above mentioned, and more intense in Lena-infected pigs showing a peak at 8 dpi, whereas the maximum expression in 3249-infected pigs was observed at 13 dpi (Fig. 4B). By contrast, the number of cCasp3$^+$

cells remained at low levels in the control group along the study. In both infected groups, a positive correlation between cCasp3 expression and lung viral load was found ($r = 0.61$ and $P < 0.01$, for 3249 group; and, $r = 0.60$ and $P < 0.001$, for Lena group). Moreover, in 3249-infected group, a significant correlation between cCasp3$^+$ cells and PRRSV-N-protein$^+$ cells was found ($r = 0.73$ and $P < 0.001$). The AUC for cCasp3 (median) in 3249 and Lena groups was 12.1 and 26.9, respectively (Figs 4E-4F).

3.6. A positive correlation between the frequency of cCasp8$^+$ cells and cCasp9$^+$ cells was observed in both PRRSV-1 infected groups

The immunolabelling of cCasp8 was primarily detected in the cytoplasm of interstitial macrophages as well as PIMs, and in a lesser extent in PAMs and lymphocytes (Figs. 5A-5B). The frequency of cCasp8$^+$ cells followed a bimodal curve in Lena-infected pigs with a first increase at 3 dpi followed by a second and higher peak at 8 dpi (Fig. 4C). This rise was significantly higher when compared with control group at 8 and 13 dpi ($P < 0.05$). By contrast, the number of cCasp8$^+$ cells in 3249 group remained at baseline or below control group with a significant ascent at 13 dpi ($P < 0.05$) (Fig. 4C). A wide individual variability was observed in both, Lena- and 3249-infected pigs.

Regarding cCasp9, its expression was mainly observed in the cytoplasm of interstitial macrophages and, occasionally, PIMs and PAMs (Fig. 5C-5D). Both PRRSV-1-infected groups displayed a similar kinetics for cCasp9$^+$ cells. Thus, there was a mild gradual rise from 8 dpi until the end of the study, reaching maximum frequencies at 13 dpi in both infected groups ($P < 0.05$) (Fig 4D). Lena virulent strain always induced a higher number of cCasp9$^+$ cells compared to 3249 strain. One pig infected with 3249 strain at 6 dpi was considered an outlier due to an extreme number of cCasp9$^+$ cells (Fig. 4D). The frequencies of cCasp8$^+$ and cCasp9$^+$ cells remained low and constant in control pigs during the whole study. In both PRRSV-1 infected groups, a positive correlation was found between the number of cCasp8$^+$ cells and the number of cCasp9$^+$ cells ($r = 0.86$ and $P < 0.001$, for 3249 group; and, $r = 0.6$ and $P < 0.01$, for Lena group). In addition, a positive correlation was observed in 3249-infected animals between the frequency of cCasp9$^+$ cells and lung viral load ($r = 0.60$, $P < 0.01$), PRRSV-N-protein$^+$ cells ($r = 0.60$, $P < 0.001$) and cCasp3$^+$ cells ($r = 0.68$, $P < 0.001$). The AUC for cCasp8 (median) in 3249 and Lena groups was 9 and 20.6, respectively, whereas for cCasp9 (median) was 5.5 in 3249 and 8.3 in Lena groups.

Figure 3

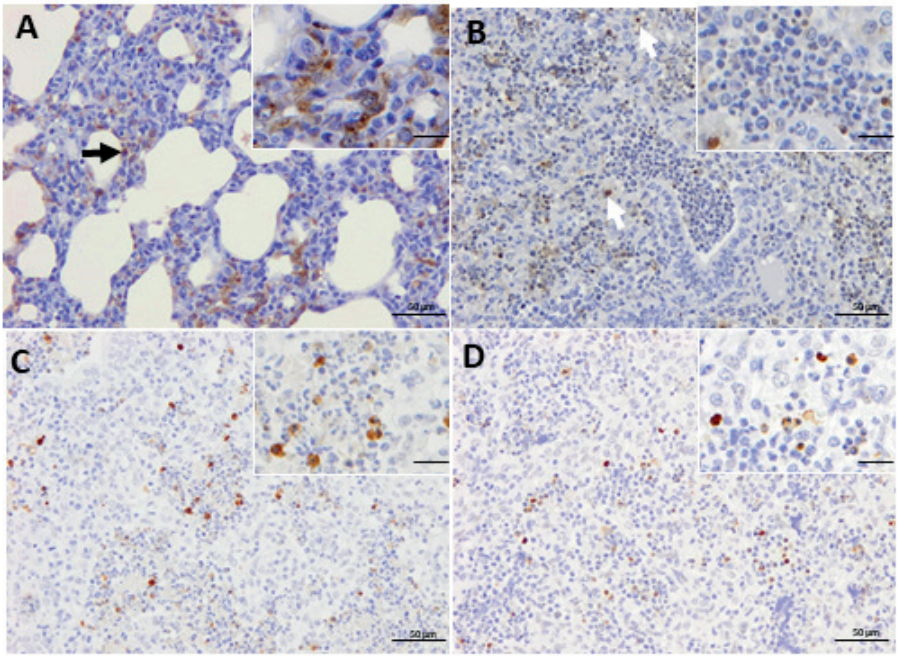

Fig. 3. Immunohistochemistry for TUNEL and cCasp3 in lung tissue. A. TUNEL expression in a representative 3249-infected pig euthanised at 8 dpi (bar, 50 μm). Black arrow shows an TUNEL^{+} PAM. Inset, detail of TUNEL^{+} interstitial macrophages (bar, 20 μm). ***B.*** TUNEL expression in a representative Lena-infected pig euthanised at 8 dpi (bar, 50 μm). White arrow shows TUNEL^{+} PAMs. Inset, black arrows show TUNEL^{-} neutrophils (bar, 20 μm). ***C.*** cCasp3 expression in a representative 3249-infected pig euthanised at 13 dpi (bar, 50 μm). Inset, detail of cCasp3^{+} PAMs (bar, 20 μm). ***D.*** cCasp3 expression in a representative Lena-infected pig euthanised at 13 dpi (bar, 50 μm). Inset, detail of cCasp3^{+} PAMs (bar, 20 μm).

Figure 4

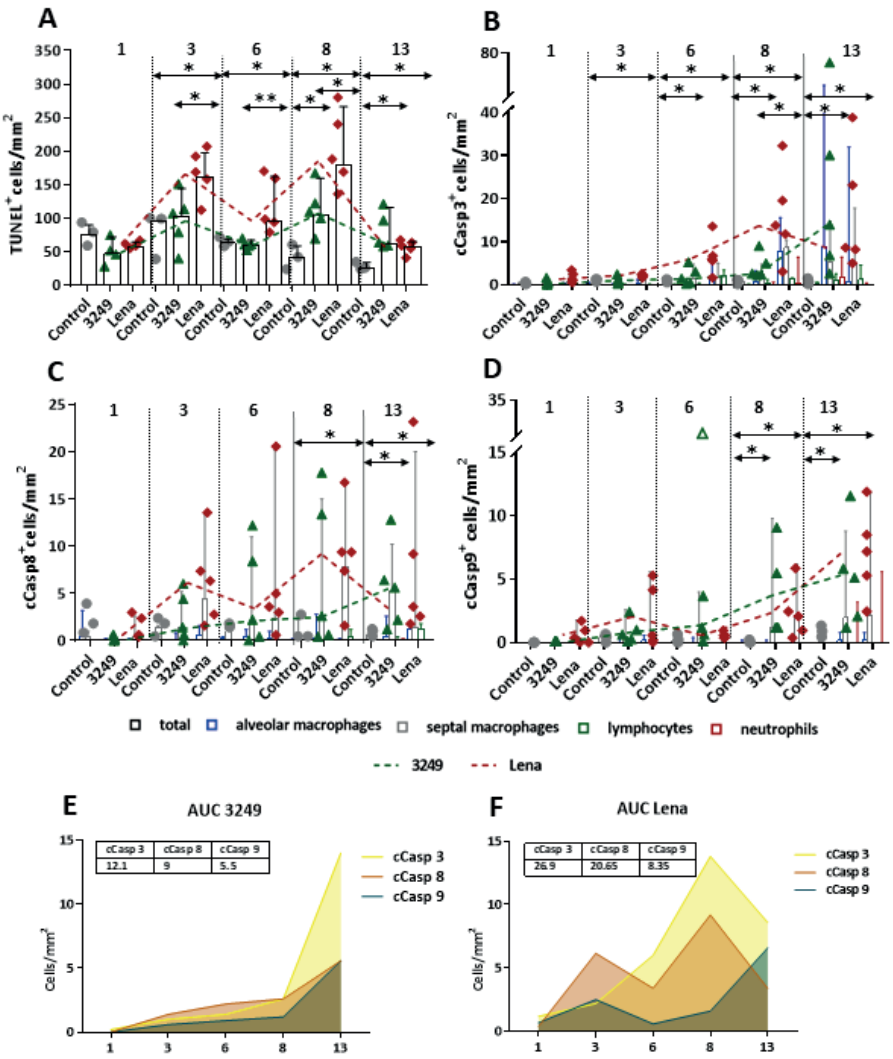
A
B
C
D
TUNEL+ cells/mm2
cCasp3+ cells/mm2
cCasp8+ cells/mm2
cCasp9+ cells/mm2
Control
3249
Lena
total
alveolar macrophages
septal macrophages
lymphocytes
neutrophils
E
AUC 3249
F
AUC Lena
cCasp 3
cCasp 8
cCasp 9
Cells/mm2

Fig. 4. Immunohistochemical expression of TUNEL, cCasp3, cCasp8 and cCasp9 in lung tissue along the experiment. A. *Graph displays the number of TUNEL$^+$ cells per mm2 in the lung along the experiment. Individual values from each animal within each group, control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi are shown. Due to the aggregation of TUNEL$^+$ cells black columns represent the median with range of total positive cells within each group. Dashed lines display the median of each infected group, 3249 (green line) and Lena (red line) along the experiment. Statistical differences between groups at 3, 6, 8 and 13 dpi are indicated as "*" for P < 0.05 and "**" for P < 0.01.* ***B.*** *Graph represents the number of cCasp3$^+$ cells per mm2 in the lung along the experiment. Individual values from each animal within each group, control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi are represented. Columns display the median with range of PAMs (blue column), septal macrophages (grey column), lymphocytes (green column) and neutrophils (red column) within each group. Dashed lines show the median of each infected group, 3249 (green line) and Lena (red line) along the experiment. Statistical differences between groups at 3, 6, 8 and 13 dpi are indicated as "*" for P < 0.05 and "**" for P < 0.01.* ***C.*** *Graph represents the number of cCasp8$^+$ cells per mm2 in the lung along the experiment. Individual values from each animal within each group, control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi are shown. Columns display the median with range of PAMs (blue column), septal macrophages (grey column), lymphocytes (green column) and neutrophils (red column) within each group. Dashed lines exhibit the median of each infected group, 3249 (green line) and Lena (red line) along the experiment. Statistical differences between groups at 8 and 13 dpi are indicated as "*" for P < 0.05 and "**" for P < 0.01.* ***D.*** *Graph shows the number of cCasp9$^+$ cells per mm2 in the lung along the experiment. Individual values from each animal within each group, control (grey circles), 3249 (green triangles) and Lena (red diamonds) at 1, 3, 6, 8 and 13 dpi are shown. One pig infected with 3249 strain at 6 dpi was considered an outlier (empty green triangle). Columns display the median with range of PAMs (blue column), septal macrophages (grey column), lymphocytes (green column) and neutrophils (red column) within each group. Dashed lines represent the median of each infected group, 3249 (green line) and Lena (red line) along the experiment. Statistical differences between groups at 8 and 13 dpi are indicated as "*" for P < 0.05 and "**" for P < 0.01.* ***E.*** *Graph displays the AUC (median) for the frequency of cCasp3 (yellow), cCasp8 (red) and cCasp9 positive cells (green) in 3249-infected group along the experiment.* ***F.*** *Graph represents the AUC (median) for the frequency of cCasp3 (yellow), cCasp8 (red) and cCasp9 positive cells (green) in Lena-infected group along the experiment.*

Figure 5

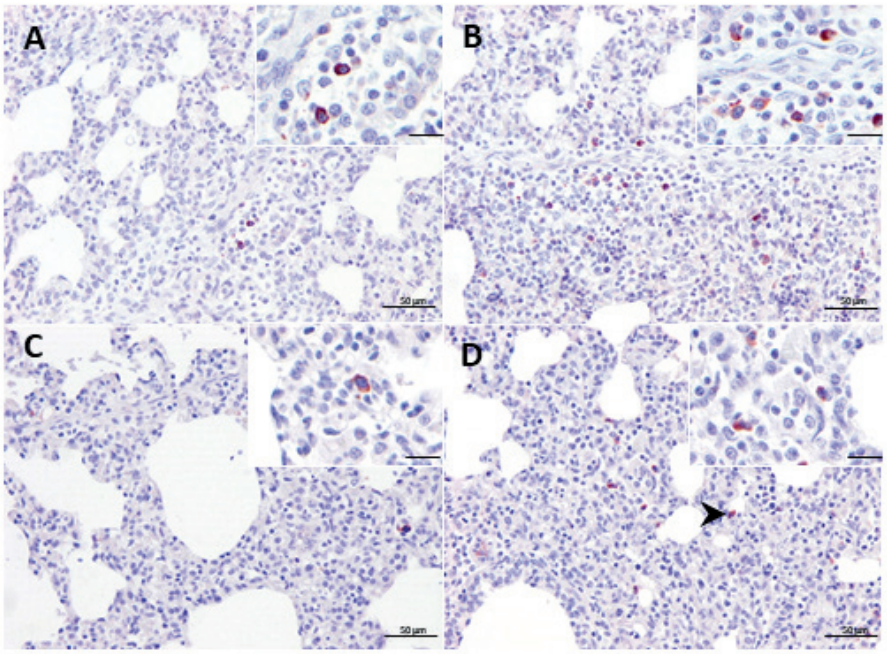

Fig. 5. Immunohistochemistry for cCasp8 and cCasp9 in lung tissue. A. cCasp8 expression in a representative 3249-infected pig euthanised at 8 dpi (bar, 50 μm). Inset, detail of cCasp8^{+} interstitial macrophages (bar, 20 μm). ***B.*** cCasp8 expression in a representative Lena-infected pig euthanised at 8 dpi (bar, 50 μm). Inset, detail of cCasp8^{+} macrophages (bar, 20 μm). ***C.*** cCasp9 expression in a representative 3249-infected pig euthanised at 13 dpi (bar, 50 μm). Inset, detail of cCasp9^{+} macrophages (bar, 20 μm). ***D.*** cCasp9 expression in a representative Lena-infected pig euthanised at 13 dpi (bar, 50 μm). Arrowhead shows a cCasp9^{+} PAM. Inset, detail of cCasp9^{+} interstitial macrophages (bar, 20 μm).

4. Discussion

RCD is a key player in the innate response to combat viral infection, disrupting replication and destroying infected cells from the host (Orzalli and Kagan, 2017). Therefore, PRRSV, as well as many other viruses, have developed strategies to obstruct or delay apoptosis in order to obtain a window of time in which viral replication, self-assembly and release can take place (Costers et al., 2008; Nailwal and Chan, 2019; Orzalli and Kagan, 2017; Yatim and Albert, 2011; Yuan et al., 2016). It has been showed that PRRSV-induced apoptosis is reliant on virulence-strain since PRRSV-1 virulent strains cause apoptosis and necrosis with a higher severity in lymphoid organs than low virulent strains (Amarilla et al., 2016; Morgan et al., 2016; Ruedas-Torres et al., 2020). However, the mechanisms involved in RCD phenomena used by virulent strains in the lung of PRRSV-1 infected pigs have not been elucidated yet.

In the present study, virulent Lena strain replicated more efficiently in the lung of infected pigs than 3249 strain, showing a significant correlation with the extent of lung lesion. PRRSV-N-protein$^+$ cells mainly consisted of PAMs and secondly PIMs and interstitial macrophages. Both PRRSV-1-infected piglets underwent a dramatic drop of CD163$^+$ pulmonary macrophages followed by the self-repletion of this subset at 13 dpi, which were particularly more intense in PAMs and in Lena-infected animals. The marked depletion in CD163$^+$ PAMs leads to a failure in the phagocytosis by these cells (Renson et al., 2017), which may affect the clearance of apoptotic bodies. Whereas in a previous study we were able to describe the replenishment of CD163$^+$ cells in the lung of SU1-bel infected pigs about one month post infection (Sánchez-Carvajal et al., 2019), this phenomenon was already observed at two week post-infection in the present study. This recruitment of CD163$^+$ PAMs coincided with the decrease in the number of PRRSV-N-protein$^+$ cells and the lung viral load and may represent an attempt to remove apoptotic cells and cellular debris, restoring tissue damage. It is well-established that the induction of CD163 is a feature of M2-alternative activated macrophages, which participate in tissue wound repair accelerating the resolution of inflammation (Hussell and Bell, 2014; García-Nicolás et al., 2014; Mantovani et al., 2004; Van Gorp et al., 2010).

Subsequently, TUNEL assay was carried out to identify in situ DNA fragmentation, a feature of both apoptotic as well as necrotic cells (Garrity et al., 2003). Besides, a cCasp3 immunolabelling method was used to demonstrate the activation of the caspase-dependent apoptotic pathway. In our study, the number of TUNEL$^+$ cells was significantly higher than the number of cCasp3$^+$ cells. This fact was observed not only in PRRSV-1-infected but also in non-infected piglets throughout the study. This result suggests that an independent cCasp3 pathway of RCD could be involved in both pulmonary cell homeostasis of pigs and clearance of virus-infected cells, since infected pigs exhibited higher frequencies of TUNEL$^+$ cells than control animals. Apoptosis, programmed necrosis (necroptosis) and pyroptosis are considered as distinct approaches of RCD, depending on the signalling pathway engaged. Thus, apoptosis is a non-lytic and immunologically silent manner of cell death; by contrast, programmed necrosis and pyroptosis are lytic and highly inflammatory processes (Galluzzi et al., 2018; Wallach et al., 2016). In vitro studies have evidenced the activation of anti-apoptotic pathways in PAMs and susceptible cell lines during the early infection with PRRSV, followed by the stimulation of apoptosis in late infection (Costers et al., 2008; Pujhari et al., 2014; Yuan et al., 2016). Accordingly, our results highlight a delayed activation of apoptosis phenomena up to one week post-infection, suggesting that other forms of RCD, such as necroptosis and pyroptosis, could be playing a role during the first week post-infection.

Pyroptosis may be initiated by inflammasomes, which in addition activate the secretion of proinflammatory cytokines (de Zoete et al., 2014). PRRSV can induce NLRP3 inflammasome in PAMs resulting in secretion of IL-1β and inflammatory pathologic responses (Bi et al., 2014; Li et al., 2015; Wang et al., 2015; Zhang et al., 2013), which is associated with marked lung lesion in piglets infected with PRRSV-1 virulent strains, such as Lena and SU1-bel strains (Amarilla et al., 2015; Renson et al., 2017; Weesendorp et al., 2014). In the present study, a significant rise in TUNEL$^+$ cells was observed in both PRRSV-1-infected pigs, although more pronounced in Lena-infected pigs, from the beginning of the study (3 dpi) and coinciding with the greater extent of lung injury (8 dpi). Therefore, the activation of RCD in PRRSV-1 virulent strain infection might play as a double-edged sword, in one way, trying to kill faster and earlier infected cells but on the other way also causing severe lung injury.

Curiously, nets of free chromatin were observed in the alveoli of some Lena-infected pigs, accompanied by a moderate neutrophil infiltrate in areas of suppurative bronchopneumonia. Many viruses, bacteria and fungi can stimulate the formation of neutrophils extracellular traps (NETs) in a process called NETosis, another type of RCD (de Buhr et al., 2019; Funchal et al., 2015; Galluzzi et al., 2018; Henthorn et al., 2018; Moorthy et al., 2013; Schönrich and Raftery et al., 2019; Storisteanu et al., 2017; Tripathi et al., 2014). These net-like structures are composed of chromatin and equipped with granule protein trapping and/or killing extracellular and intracellular microorganisms to prevent their local spreading (Schönrich and Raftery et al., 2019); however, bacteria can also take advantage of NETs as growth source (de Buhr et al., 2019; Henthorn et al., 2018). Since NETs and NET precursor neutrophils are described to be $TUNEL^-$ (Fuchs et al., 2007), and most neutrophils as well as the clumps of free chromatin observed in our study were $TUNEL^-$ and $cCasp3^-$, we hypothesised that these clumps could be associated with NETs triggered by neutrophils in foci of suppurative bronchopneumonia. NETs formation during infection with virulent PRRSV strains might be linked with the higher extent of the lung damage as well as with the onset of secondary bacterial infections and the PRDC.

There is mounting evidence of viruses activating extrinsic and/or intrinsic apoptosis (Orzalli and Kagan, 2017). In the present study, cCasp8 and cCasp9 were activated by Lena and 3249 strains after one week post-infection showing a similar kinetics and a correlation among them, which is consistent with the crosstalk between extrinsic and intrinsic apoptosis pathways already described in PRRSV infection (Lee and Kleiboeker, 2007; Yuan et al., 2016). Whereas Lena virulent strain induced a higher number of $cCasp8^+$ cells than $cCasp9^+$ cells, for 3249 strain, the expression of both caspases was quite similar. These results suggest that Lena virulent strain is able to induce a higher activation of $cCasp8^+$ in the lung, which is in agreement with a recent publication of our group which describes an unidirectional activation of apoptosis in the thymus of PRRSV-1 infected pigs through the activation of the extrinsic pathway induced by Fas/cCasp8 (Ruedas-Torres et al., 2020). These findings highlight the interest of examining different target organs to better understand the modulation of the host immune response.

The immunolabelling of PRRSV-N-protein was observed in PAMs and in a lesser extent in interstitial macrophages and PIMs. By contrast, apoptotic markers such as cCasp3, cCasp8 and cCasp9, were mainly observed in interstitial macrophages, PIMs, or lymphocytes located in lung interstitium and secondly in PAMs. Thus, whereas apoptosis was mainly activated in interstitial macrophages and PIMs, other mechanisms of RCD besides apoptosis, such as necroptosis and/or pyroptosis, might be involved in the cell death of PAMs. The enhancement of apoptosis phenomena in non-infected cells from the lung could be addressed by two different scenarios: (i) during PRRSV infection there is a strong influx of interstitial macrophages and monocytes mediated by chemotactic factors in an attempt to replace the lost cell population (Gómez-Laguna et al., 2010; Labarque et al., 2000; Van Gucht et al., 2005); overwhelming production of these chemotactic factors may lead to an excessive infiltration of cells and hence apoptosis is triggered as homeostatic mechanism to control cell population; (ii) apoptosis of uninfected bystander cells is a key element of PRRSV infection to destroy host immune cells (Labarque et al., 2003; Wang et al., 2014, 2015), which have been associated with the release of pro-inflammatory cytokines, such as TNF-α, IL-1 α, IL-β or IL-6, in the lung of pigs infected with both low virulent and virulent PRRSV-1 strains (Amarilla et al., 2015; Gómez-Laguna et al., 2010; Labarque et al., 2003; Weesendorp et al., 2014) as well as other porcine viruses (Salguero et al., 2005).

5. Conclusion

Taken together, the present study evaluated RCD in the lung upon infection with two PRRSV-1 strains of different virulence. Our results suggest the activation of non-apoptotic RCD during the first week post-infection followed by the activation of mainly extrinsic apoptosis, and in a lesser extent intrinsic apoptosis, during the second week post-infection. These phenomena were markedly elicited by Lena strain accompanied by a high viral load and severe depletion of $CD163^+$ PAMs, causing severe and early lung damage. Repletion of $CD163^+$ PAMs was observed at two weeks post-infection which point out an attempt to reconstitute the pulmonary macrophages subpopulations lost during the early stages of the infection. Further studies should be performed to decipher the role of other pathways of RCD during PRRSV infection.

Conflict of interest

The authors declare that they have no competing interest.

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References

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Graham, S. P., Frossard, J. P., Steinbach, F., & Salguero, F. J. (2016). Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains. Veterinary Microbiology, 188, 47–58. https://doi.org/10.1016/j.vetmic.2016.04.005

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Morgan, S. B., Graham, S. P., Frossard, J. P., Drew, T. W., & Salguero, F. J. (2015). A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: Upregulation of IL-1α in highly pathogenic strain induced lesions. Veterinary Immunology and Immunopathology, 164(3–4), 137–147. https://doi.org/10.1016/j.vetimm.2015.02.003

Balka, G., Podgórska, K., Brar, M. S., Bálint, Á., Cadar, D., Celer, V., Dénes, L., Dirbakova, Z., Jedryczko, A., Márton, L., Novosel, D., Petrović, T., Sirakov, I., Szalay, D., Toplak, I., Leung, F. C. C., & Stadejek, T. (2018). Genetic diversity of PRRSV 1 in Central Eastern Europe in 1994-2014: Origin and evolution of the virus in the region. Scientific Reports, 8(1), 1–12. https://doi.org/10.1038/s41598-018-26036-w

Barranco, I., Gómez-Laguna, J., Rodríguez-Gómez, I. M., Salguero, F. J., Pallarés, F. J., Bernabé, A., & Carrasco, L. (2011). Immunohistochemical detection of extrinsic and intrinsic mediators of apoptosis in porcine paraffin-embedded tissues. Veterinary Immunology and Immunopathology, 139(2–4), 210–216. https://doi.org/10.1016/j.vetimm.2010.10.017

Bordet, E., Maisonnasse, P., Renson, P., Bouguyon, E., Crisci, E., Tiret, M., Descamps, D., Bernelin-Cottet, C., Urien, C., Lefèvre, F., Jouneau, L., Bourry, O., Leplat, J. J., Schwartz-Cornil, I., & Bertho, N. (2018). Porcine alveolar macrophage-like cells are pro-inflammatory pulmonary intravascular macrophages that produce large titers of porcine reproductive and respiratory syndrome virus. Scientific Reports, 8(1), 10172. https://doi.org/10.1038/s41598-018-28234-y

Canelli, E., Catella, A., Borghetti, P., Ferrari, L., Ogno, G., De Angelis, E., Corradi, A., Passeri, B., Bertani, V., Sandri, G., Bonilauri, P., Leung, F. C., Guazzetti, S., & Martelli, P. (2017). Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Veterinary Microbiology, 210(July), 124–133. https://doi.org/10.1016/j.vetmic.2017.09.002

Chen, X. xin, Guo, Z., Jin, Q., Qiao, S., Li, R., Li, X., Deng, R., Feng, W. hai, & Zhang, G. P. (2018). Porcine reproductive and respiratory syndrome virus induces interleukin-1□ through MyD88/ERK/AP-1 and NLRP3 inflammasome in microglia. Veterinary Microbiology, 227, 82–89. https://doi.org/10.1016/j.vetmic.2018.10.030

Costers, S., Lefebvre, D. J., Delputte, P. L., & Nauwynck, H. J. (2008). Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. Archives of Virology, 153(8), 1453–1465. https://doi.org/10.1007/s00705-008-0135-5

D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. Cell Biology International, 43(6), 582–592. https://doi.org/10.1002/cbin.11137

Darwich, L., Díaz, I., & Mateu, E. (2010). Certainties, doubts and hypotheses in porcine reproductive and respiratory syndrome virus immunobiology. Virus Research, 154(1–2), 123–132. https://doi.org/10.1016/j.virusres.2010.07.017

De Baere, M. I., Van Gorp, H., Delputte, P. L., & Nauwynck, H. J. (2012). Interaction of the European genotype porcine reproductive and respiratory syndrome virus (PRRSV) with sialoadhesin (CD169/Siglec-1) inhibits alveolar macrophage phagocytosis. Veterinary Research, 43(1), 47. https://doi.org/10.1186/1297-9716-43-47

de Buhr, N., Bonilla, M. C., Pfeiffer, J., Akhdar, S., Schwennen, C., Kahl, B. C., Waldmann, K. H., Valentin-Weigand, P., Hennig-Pauka, I., & von Köckritz-Blickwede, M. (2019). Degraded neutrophil extracellular traps promote the growth of Actinobacillus pleuropneumoniae. Cell Death and Disease, 10(9), 1–16. https://doi.org/10.1038/s41419-019-1895-4

Du Manoir, J. M., Albright, B. N., Stevenson, G., Thompson, S. H., Mitchell, G. B., Clark, M. E., & Caswell, J. L. (2002). Variability of neutrophil and pulmonary alveolar macrophage function in swine. Veterinary Immunology and Immunopathology, 89(3–4), 175–186. https://doi.org/10.1016/S0165-2427(02)00207-6

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary Microbiology, 56(1–2), 9–19. https://doi.org/10.1016/S0378-1135(96)01347-8

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., & Zychlinsky, A. (2007). Novel cell death program leads to neutrophil extracellular traps. Journal of Cell Biology, 176(2), 231–241. https://doi.org/10.1083/jcb.200606027

Funchal, G. A., Jaeger, N., Czepielewski, R. S., Machado, M. S., Muraro, S. P., Stein, R. T., Bonorino, C. B. C., & Porto, B. N. (2015). Respiratory Syncytial Virus Fusion Protein Promotes TLR-4–Dependent Neutrophil Extracellular Trap Formation by Human Neutrophils. PLOS ONE, 10(4), e0124082. https://doi.org/10.1371/journal.pone.0124082

Galluzzi, L., Kepp, O., Ka-Ming Chan, F., & Kroemer, G. (2016). Necroptosis: Mechanisms and Relevance to Disease. Annu. Rev. Pathol. Mech. Dis. 2017, 12, 103–133. https://doi.org/10.1146/annurev-pathol-052016-100247

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., … Kroemer, G. (2018). Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. In Cell Death and Differentiation (Vol. 25, Issue 3, pp. 486–541). Nature Publishing Group. https://doi.org/10.1038/s41418-017-0012-4

Garrity, M. M., Burgart, L. J., Riehle, D. L., Hill, E. M., Sebo, T. J., & Witzig, T. (2003). Identifying and quantifying apoptosis: Navigating technical pitfalls. Modern Pathology, 16(4), 389–394. https://doi.org/10.1097/01.MP.0000062657.30170.92

Gimeno, M., Darwich, L., Diaz, I., De La Torre, E., Pujols, J., Martín, M., Inumaru, S., Cano, E., Domingo, M., Montoya, M., & Mateu, E. (2011). Cytokine profiles and phenotype regulation of antigen presenting cells by genotype-I porcine reproductive and respiratory syndrome virus isolates. Veterinary Research, 42(1), 9. https://doi.org/10.1186/1297-9716-42-9

Gómez-Laguna, J., Salguero, F. J., Barranco, I., Pallarés, F. J., Rodríguez-Gómez, I. M., Bernabé, A., & Carrasco, L. (2010). Cytokine Expression by Macrophages in the Lung of Pigs Infected with the Porcine Reproductive and Respiratory Syndrome Virus. Journal of Comparative Pathology, 142(1), 51–60. https://doi.org/10.1016/j.jcpa.2009.07.004

Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., & Carrasco, L. (2013). Immunopathogenesis of porcine reproductive and respiratory syndrome in the respiratory tract of pigs. The Veterinary Journal, 195(2), 148–155. https://doi.org/10.1016/j.tvjl.2012.11.012

Gorbalenya AE, Krupovic M, Siddell S, Varsani A, K. J. (2018). International Committee on Taxonomy of Viruses (ICTV). Retrieved 6 April 2019, from International Committee on Taxonomy of Viruses (ICTV) Website: Https://Talk.Ictvonline.Org/Taxonomy/p/Taxonomy-History?Taxnode_id=20186087. https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20186087

Greenbaum, E., Furst, A., Kiderman, A., Stewart, B., Levy, R., Schlesinger, M., Morag, A., & Zakay-Rones, Z. (2001). Area under the viraemia curve versus absolute viral load: Utility for predicting symptomatic cytomegalovirus infections in kidney transplant patients. Journal of Medical Virology, 65(1), 85–89. https://doi.org/10.1002/jmv.2005

Halbur, P. G., Paul, P. S., Meng, X. J., Lum, M. A., Andrews, J. J., & Rathje, J. A. (1996). Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. Journal of Veterinary Diagnostic Investigation, 8(1), 11–20. https://doi.org/10.1177/104063879600800103

He, Y., Wang, G., Liu, Y., Shi, W., Han, Z., Wu, J., Jiang, C., Wang, S., Hu, S., Wen, H., Dong, J., Liu, H., & Cai, X. (2012). Characterization of thymus atrophy in piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 160(3–4), 455–462. https://doi.org/10.1016/j.vetmic.2012.05.040

Henthorn, C. R., Chris Minion, F., & Sahin, O. (2018). Utilization of macrophage extracellular trap nucleotides by Mycoplasma hyopneumoniae. Microbiology, 164(11), 1394–1404. https://doi.org/10.1099/mic.0.000717

Jiang, Z., Zhou, X., Michal, J. J., Wu, X. L., Zhang, L., Zhang, M., Ding, B., Liu, B., Manoranjan, V. S., Neill, J. D., Harhay, G. P., Kehrli, M. E., & Miller, L. C. (2013). Reactomes of Porcine Alveolar Macrophages Infected with Porcine Reproductive and Respiratory Syndrome Virus. PLoS

ONE, 8(3). https://doi.org/10.1371/journal.pone.0059229

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A., & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Veterinary Research, 6(1), 30. https://doi.org/10.1186/1746-6148-6-30

Labarque, G., Nauwynck, H. J., Van Reeth, K., & Pensaert, M. B. (2000). Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. Journal of General Virology, 81(5), 1327–1334. https://doi.org/10.1099/0022-1317-81-5-1327

Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K., & Pensaert, M. (2003). Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Veterinary Research, 34(3), 249–260. https://doi.org/10.1051/vetres:2003001

Lee, S. M., & Kleiboeker, S. B. (2007). Porcine reproductive and respiratory syndrome virus induces apoptosis through a mitochondria-mediated pathway. Virology, 365(2), 419–434. https://doi.org/10.1016/j.virol.2007.04.001

Li, J., Hu, L., Liu, Y., Huang, L., Mu, Y., Cai, X., & Weng, C. (2015). DDX19A Senses Viral RNA and Mediates NLRP3-Dependent Inflammasome Activation. The Journal of Immunology, 195(12), 5732–5749. https://doi.org/10.4049/jimmunol.1501606

Li, Y., Wang, G., Liu, Y., Tu, Y., He, Y., Wang, Z., Han, Z., Li, L., Li, A., Tao, Y., & Cai, X. (2014). Identification of apoptotic cells in the thymus of piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Virus Research, 189, 29–33. https://doi.org/10.1016/j.virusres.2014.04.011

Logue, S. E., & Martin, S. J. (2008). Caspase activation cascades in apoptosis. Biochemical Society Transactions, 36(1), 1–9. https://doi.org/10.1042/BST0360001

Lu, Q., Bai, J., Zhang, L., Liu, J., Jiang, Z., Michal, J. J., He, Q., & Jiang, P. (2012). Two-dimensional liquid chromatography-tandem mass spectrometry coupled with isobaric tags for relative and absolute quantification (iTRAQ) labeling approach revealed first proteome profiles of pulmonary alveolar macrophages infected with porcine reproductive and respiratory syndrome virus. Journal of Proteome Research, 11(5), 2890–2903. https://doi.org/10.1021/pr201266z

Man, S. M., Karki, R., & Kanneganti, T.-D. (2017). Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. Immunological Reviews, 277(1), 61–75. https://doi.org/10.1111/imr.12534

Mattsson, J. G., Bergström, K., Wallgren, P., & Johansson, K.-E. E. (1995). Detection of Mycoplasma hyopneumoniae in nose swabs from pigs by in vitro amplification of the 16S rRNA gene. Journal of Clinical Microbiology, 33(4), 893–897. http://www.ncbi.nlm.nih.gov/

pubmed/7540629

Miller, L. C., & Fox, J. M. (2004). Apoptosis and porcine reproductive and respiratory syndrome virus. Veterinary Immunology and Immunopathology, 102(3), 131–142. https://doi.org/10.1016/j.vetimm.2004.09.004

Moorthy, A. N., Narasaraju, T., Rai, P., Perumalsamy, R., Tan, K. B., Wang, S., Engelward, B., & Chow, V. T. K. (2013). In vivo and in vitro studies on the roles of neutrophil extracellular traps during secondary pneumococcal pneumonia after primary pulmonary influenza infection. Frontiers in Immunology, 4(MAR). https://doi.org/10.3389/fimmu.2013.00056

Morgan, S. B., Frossard, J. P., Pallares, F. J., Gough, J., Stadejek, T., Graham, S. P., Steinbach, F., Drew, T. W., & Salguero, F. J. (2016). Pathology and Virus Distribution in the Lung and Lymphoid Tissues of Pigs Experimentally Inoculated with Three Distinct Type 1 PRRS Virus Isolates of Varying Pathogenicity. Transboundary and Emerging Diseases, 63(3), 285–295. https://doi.org/10.1111/tbed.12272

Nailwal, H., & Chan, F. K.-M. (2019). Necroptosis in anti-viral inflammation. Cell Death & Differentiation, 26(1), 4–13. https://doi.org/10.1038/s41418-018-0172-x

Orzalli, M. H., & Kagan, J. C. (2017). Apoptosis and Necroptosis as Host Defense Strategies to Prevent Viral Infection. Trends in Cell Biology, 27(11), 800–809. https://doi.org/10.1016/j.tcb.2017.05.007

Pujhari, S., Baig, T. T., & Zakhartchouk, A. N. (2014). Potential role of porcine reproductive and respiratory syndrome virus structural protein GP2 in apoptosis inhibition. BioMed Research International, 2014, 160505. https://doi.org/10.1155/2014/160505

Qiao, S., Feng, L., Bao, D., Guo, J., Wan, B., Xiao, Z., Yang, S., & Zhang, G. (2011). Porcine reproductive and respiratory syndrome virus and bacterial endotoxin act in synergy to amplify the inflammatory response of infected macrophages. Veterinary Microbiology, 149(1–2), 213–220. https://doi.org/10.1016/j.vetmic.2010.11.006

Rodríguez-Gómez, I. M., Barranco, I., Amarilla, S. P., García-Nicolás, O., Salguero, F. J., Carrasco, L., & Gómez-Laguna, J. (2014). Activation of extrinsic- and Daxx-mediated pathways in lymphoid tissue of PRRSV-infected pigs. Veterinary Microbiology, 172(1–2), 186–194. https://doi.org/10.1016/j.vetmic.2014.05.025

Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallarés, F. J., Mateu, E., Carrasco, L., & Gómez-Laguna, J. (2019). Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells. Veterinary Microbiology, 235, 101–109. https://doi.org/10.1016/j.vetmic.2019.06.011

Ruedas-Torres, I., Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallares, F. J., Barranco, I., Carrasco, L., & Gómez-Laguna, J. (2020). Activation of the extrinsic apoptotic pathway in the thymus of piglets infected with PRRSV-1 strains of different virulence. Veterinary Microbiology, 243, 108639. https://doi.org/10.1016/j.vetmic.2020.108639

Salguero, F. J., Sánchez-Cordón, P. J., Núñez, A., Fernández de Marco, M., & Gómez-Villamandos, J. C. (2005). Proinflammatory cytokines induce lymphocyte apoptosis in acute African swine fever infection. Journal of Comparative Pathology, 132(4), 289–302. https://doi.org/10.1016/j.jcpa.2004.11.004

Sibila, M., Calsamiglia, M., Segalés, J., Blanchard, P., Badiella, L., Le Dimma, M., Jestin, A., & Domingo, M. (2004). Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. American Journal of Veterinary Research, 65(1), 88–92. https://doi.org/10.2460/ajvr.2004.65.88

Sinn, L. J., Klingler, E., Lamp, B., Brunthaler, R., Weissenböck, H., Rümenapf, T., & Ladinig, A. (2016). Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria. Porcine Health Management, 2, 1–10. https://doi.org/10.1186/s40813-016-0044-z

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. Journal of General Virology, 94(10), 2141–2163. https://doi.org/10.1099/vir.0.056341-0

Stadejek, T., Oleksiewicz, M. B., Potapchuk, D., & Podgórska, K. (2006). Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes. Journal of General Virology, 87(7), 1835–1841. https://doi.org/10.1099/vir.0.81782-0

Stadejek, T., Oleksiewicz, M. B., Scherbakov, A. V., Timina, A. M., Krabbe, J. S., Chabros, K., & Potapchuk, D. (2008). Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Archives of Virology, 153(8), 1479–1488. https://doi.org/10.1007/s00705-008-0146-2

Stadejek, T., Stankevicius, A., Murtaugh, M. P., & Oleksiewicz, M. B. (2013). Molecular evolution of PRRSV in Europe: Current state of play. Veterinary Microbiology, 165(1–2), 21–28. https://doi.org/10.1016/j.vetmic.2013.02.029

Storisteanu, D. M. L., Pocock, J. M., Cowburn, A. S., Juss, J. K., Nadesalingam, A., Nizet, V., & Chilvers, E. R. (2017). Evasion of Neutrophil Extracellular Traps by Respiratory Pathogens. American Journal of Respiratory Cell and Molecular Biology, 56(4), 423–431. https://doi.org/10.1165/rcmb.2016-0193PS

Suárez, P. (2000). Ultrastructural pathogenesis of the PRRS virus. Veterinary Research, 31(1), 47–55. https://doi.org/10.1051/vetres:2000107

Suárez, P., Díaz-Guerra, M., Prieto, C., Esteban, M., Castro, J. M., Nieto, A., & Ortín, J. (1996). Open reading frame 5 of porcine reproductive and respiratory syndrome virus as a cause of virus-induced apoptosis. Journal of Virology, 70(5), 2876–2882. https://doi.org/10.1128/jvi.70.5.2876-2882.1996

Sur, J. H., Doster, A. R., Christian, J. S., Galeota, J. A., Wills, R. W., Zimmerman, J. J., & Osorio, F. A. (1997). Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. Journal of Virology, 71(12), 9170–9179. https://doi.org/10.1128/jvi.71.12.9170-9179.1997

Tait, S. W. G., & Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nature Reviews Molecular Cell Biology, 11(9), 621–632. https://doi.org/10.1038/nrm2952

Tait, S. W. G., & Green, D. R. (2013). Mitochondrial regulation of cell death. Cold Spring Harbor Perspectives in Biology, 5(9). https://doi.org/10.1101/cshperspect.a008706

Thanawongnuwech, R., Thacker, E. L., & Halbur, P. G. (1998). Influence of pig age on virus titer and bactericidal activity of porcine reproductive and respiratory syndrome virus (PRRSV)-infected pulmonary intravascular macrophages (PIMs). Veterinary Microbiology, 63(2–4), 177–187. https://doi.org/10.1016/S0378-1135(98)00245-4

Tripathi, S., Verma, A., Kim, E.-J., White, M. R., & Hartshorn, K. L. (2014). LL-37 modulates human neutrophil responses to influenza A virus. Journal of Leukocyte Biology, 96(5), 931–938. https://doi.org/10.1189/jlb.4a1113-604rr

Van Breedam, W., Delputte, P. L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X., & Nauwynck, H. J. (2010). Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. Journal of General Virology, 91(7), 1659–1667. https://doi.org/10.1099/vir.0.020503-0

Van Gorp, H., Delputte, P. L., & Nauwynck, H. J. (2010). Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. Molecular Immunology, 47(7–8), 1650–1660. https://doi.org/10.1016/j.molimm.2010.02.008

Van Gucht, S., Reeth, K. Van, Nauwynck, H., & Pensaert, M. (2005). Porcine Reproductive and Respiratory Syndrome Virus Infection Increases CD14 Expression and Lipopolysaccharide-Binding Protein in the Lungs of Pigs. Viral Immunology, 18(1), 116–126. https://doi.org/10.1089/vim.2005.18.116

Wallach, D., Kang, T.-B., Dillon, C. P., & Green, D. R. (2016). Programmed necrosis in inflammation: Toward identification of the effector molecules. Science, 352(6281), aaf2154–aaf2154. https://doi.org/10.1126/science.aaf2154

Wang, G., He, Y., Tu, Y., Liu, Y., Zhou, E. M., Han, Z., Jiang, C., Wang, S., Shi, W., & Cai, X. (2014). Comparative analysis of apoptotic changes in peripheral immune organs and lungs following experimental infection of piglets with highly pathogenic and classical porcine reproductive and respiratory syndrome virus. Virology Journal, 11(1), 2. https://doi.org/10.1186/1743-422X-11-2

Wang, G., Yu, Y., Tu, Y., Tong, J., Liu, Y., Zhang, C., Chang, Y., Wang, S., Jiang, C., Zhou, E. M., & Cai, X. (2015). Highly pathogenic porcine reproductive and respiratory syndrome virus infection

induced apoptosis and autophagy in thymi of infected piglets. PLoS ONE, 10(6), 1–12. https://doi.org/10.1371/journal.pone.0128292

Wang, L., Hu, S., Liu, Q., Li, Y., Xu, L., Zhang, Z., Cai, X., & He, X. (2017). Porcine alveolar macrophage polarization is involved in inhibition of porcine reproductive and respiratory syndrome virus (PRRSV) replication. Journal of Veterinary Medical Science, 79(11), 1906–1915. https://doi.org/10.1292/jvms.17-0258

Weesendorp, E., Rebel, J. M. J., Popma-De Graaf, D. J., Fijten, H. P. D., & Stockhofe-Zurwieden, N. (2014). Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Veterinary Microbiology, 174(1–2), 127–138. https://doi.org/10.1016/j.vetmic.2014.09.010

Yatim, N., & Albert, M. L. (2011). Dying to Replicate: The Orchestration of the Viral Life Cycle, Cell Death Pathways, and Immunity. Immunity, 35(4), 478–490. https://doi.org/10.1016/j.immuni.2011.10.010

Yuan, S., Zhang, N., Xu, L., Zhou, L., Ge, X., Guo, X., & Yang, H. (2016). Induction of Apoptosis by the Nonstructural Protein 4 and 10 of Porcine Reproductive and Respiratory Syndrome Virus. PLOS ONE, 11(6), e0156518. https://doi.org/10.1371/journal.pone.0156518

Yuzhakov, A. G., Raev, S. A., Skrylev, A. N., Mishin, A. M., Grebennikova, T. V., Verkhovsky, O. A., Zaberezhny, A. D., Trus, I., Nauwynck, H. J., & Aliper, T. I. (2017). Genetic and pathogenic characterization of a Russian subtype 2 PRRSV-1 isolate. Veterinary Microbiology, 211(June), 22–28. https://doi.org/10.1016/j.vetmic.2017.09.017

Zhang, K., Hou, Q., Zhong, Z., Li, X., Chen, H., Li, W., Wen, J., Wang, L., Liu, W., & Zhong, F. (2013). Porcine reproductive and respiratory syndrome virus activates inflammasomes of porcine alveolar macrophages via its small envelope protein E. Virology, 442(2), 156–162. https://doi.org/10.1016/j.virol.2013.04.007

Estudio 4 / Study 4

Objetivo 3 / Objective 3

Estudio 4: "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena". Sanchez-Carvajal et al., 2020. *Veterinary Microbiology*, 246, 108-115.

Objetivo 3: Evaluar la expresión de biomarcadores de interés, como CD163s, proteínas de fase aguda o citoquinas, relacionados con la respuesta inflamatoria y respuesta inmune a nivel sistémico y/o local como indicadores de la infección y evolución por cepas de distinta virulencia del virus del PRRS

Objective 3: Assessment of potential biomarkers expression such as sCD163, acute phase proteins o cytokines involved in the systemic or local inflammatory and immune response as potential biomarkers of infection in infected pigs with PRRSV-1 strains of different virulence.

Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of virulent (Lena) PRRSV-1 infection

Sánchez-Carvajal J.M.[a,], Rodríguez-Gómez I.M.[a], Ruedas-Torres I.[a], Larenas-Muñoz F.[a] Díaz I.[b], Revilla C.[c], Mateu E.[b,d], Domínguez J.[c], Martín-Valls G.[d], Barranco I.[a], Pallarés F.J.[e], Carrasco L.[a], Gómez-Laguna J.[a]*

[a]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain

[b]CRESA-IRTA, 08193, Bellaterra, Spain

[c]Department of Biotechnology, National Institute for Agricultural and Food Research and Technology (INIA), 28040, Madrid, Spain

[d]Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Autonomous University of Barcelona, 08193, Bellaterra, Spain

[e]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Murcia, 30100, Murcia, Spain

[*]Corresponding author at: Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain.

E-mail address: v42sancj@uco.es (J.M. Sánchez-Carvajal).

Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) plays a key role in porcine respiratory disease complex (PRDC) modulating the host immune response and favouring secondary bacterial infections. Pulmonary alveolar macrophages (PAMs) are the main cells supporting PRRSV replication in lung, with CD163 as the essential receptor for viral infection. Although interstitial pneumonia is by far the representative lung lesion, suppurative bronchopneumonia is described for PRRSV-1 virulent strains. This research explores the role of several immune makers potentially involved in the regulation of the lung inflammatory response and the sensitisation of lung to secondary bacterial exposure after infection with PRRSV-1 strains of different virulence. Pigs were infected with the virulent Lena strain or the low virulent 3249 strain and euthanised at 1, 3, 6 and 8 dpi. Lena-infected pigs exhibited more severe clinical signs, gross lung lesion score and viraemia associated with an increase of IL-6 and IFN-□ in sera compared to 3249-infected pigs. Extensive areas of lung consolidation corresponding with suppurative bronchopneumonia were observed in Lena-infected pigs. Lung viral load and PRRSV-N-protein$^+$ cells were always higher in Lena-infected animals. PRRSV-N-protein$^+$ cells were linked to a marked drop of CD163$^+$ macrophages. The number of CD14$^+$ and iNOS$^+$ cells gradually increased along PRRSV-1 infection, being more evident in Lena-infected pigs. The frequency of CD200R1$^+$ and FoxP3$^+$ cells peaked late in both PRRSV-1 strains, with a strong correlation between CD200R1$^+$ cells and lung injury in Lena-infected pigs. These results highlight the role of molecules involved in the earlier and higher extent of lung lesions in piglets infected with the virulent Lena strain, pointing out the activation of routes potentially involved in the restraint of the local inflammatory response.

Keywords: PRRSV-1, virulent strains, lung lesion, inflammatory response

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the agent of a highly contagious porcine disease and one of the costliest causes of economic losses affecting the modern swine industry worldwide (Holtkamp et al., 2013; Nathues et al., 2017). The disease caused by PRRSV is characterised by severe reproductive failure in pregnant sows and respiratory disorders in piglets and growing pigs (Meulenberg et al., 1993; Wensvoort et al., 1991).

PRRSV exhibits an extremely high mutation rate, which has caused a high genetic and antigenic variability. Thus, PRRSV has been recently reclassified within the novel genus Betaarterivirus (family Arteriviridae, order Nidovirales) as Betaarterivirus suid 1 and Betaarterivirus suid 2 (formerly, PRRSV-1 and PRRSV-2, respectively), with different subtypes or lineages included within each one, respectively (Gorbalenya et al., 2018). PRRSV-1, the most prevalent in Europe, displays a quite large phylogenetic diversity, mainly in Central and Eastern Europe, where the transboundary movements of infected animals, among other factors, have made the wide viral diversity easier (Balka et al., 2018). PRRSV-1 can be further divided into four subtypes: pan-European subtype 1, Eastern European subtypes 2 and 3 (which include virulent strains, such as Lena or SU1-bel) and a tentative subtype 4 with strains from Latvia and Belarus (Balka et al., 2018; Stadejek et al., 2013).

CD163 is the essential macrophage receptor supporting viral internalisation and disassembly interacting with GP2 and GP4 viral proteins (Das et al., 2010). Pulmonary alveolar macrophages (PAMs), that express high levels of CD163, are the main cellular target of PRRSV, although interstitial and intravascular macrophages can be infected too (Bordet et al., 2018; Duan et al., 1997; Gómez-Laguna et al., 2013). It is well established that PRRSV causes a mild to severe interstitial pneumonia which may be complicated to suppurative bronchopneumonia due to the increased lung susceptibility to bacterial infections associated to the damage of pulmonary macrophage subsets (PAMs, intravascular and interstitial macrophages) (Brockmeier et al., 2017; Thanawongnuwech et al., 2000; PRRS Virus-Induced Damage to Intravascular Macrophages)

In the last years, several contradictory results among studies have been reported about viraemia, tissue viral load, early virus clearance, low frequencies of PRRSV-specific IFN-γ secreting cells or PRRSV neutralizing antibodies (Canelli et al., 2017; Frydas et al., 2013; Geldhof et al., 2012; Karniychuk et al., 2010; Morgan et al., 2013; Renson et al., 2017; Stadejek et al., 2017; Weesendorp et al., 2013, 2014). However, there is consensus on the fact that some strains are more virulent than others. These virulent strains produce high fever, systemic signs and severe lesions in lungs and eventually other organs (Canelli et al., 2017; Karniychuk et al., 2010; Morgan et al., 2013, 2016; Sinn et al., 2016; Weesendorp et al., 2013). For PRRSV1, subtype 3 Lena seems to be the most virulent strain reported up to present (Karniychuk et al., 2010; Renson et al., 2017; Weesendorp et al., 2013). Nevertheless, the mechanisms and potential molecules involved in the interaction between PRRSV-1, opportunistic bacteria and lung tissue damage, contributing to the onset of the porcine respiratory disease complex (PRDC) have not been completely deciphered (Gómez-Laguna et al., 2013; Van Gucht et al., 2004). Some studies indicate an early overproduction of proinflammatory cytokines like IL-1β or IL-8-as the main source of pulmonary injury after infection with virulent PRRSV-1 strains (Amarilla et al., 2015; Morgan et al., 2013); however, we theorize other potential mechanisms which may sensitise the lung to secondary infections have been yet unexplored.

Therefore, the immunopathology of lung and systemic immune responses are evaluated in this study with the goal of exploring the role of molecules potentially involved either in the regulation of the lung inflammatory response and the pulmonary sensitisation to secondary bacteria after infection with a virulent PRRSV-1 strain (subtype 3, Lena strain) in comparison with a low virulent PRRSV-1 strain (subtype 1, 3249-strain).

2. Materials and methods

2.1. Porcine reproductive and respiratory syndrome viruses

The low virulent 3249 strain (subtype 1 PRRSV-1) was isolated from the serum of a piglet with pneumonia from a PRRSV-positive herd located in Spain in 2005 (Gimeno et al., 2011). The virulent Lena strain (subtype 3 PRRSV-1) is considered as the prototype of PRRSV-1 virulent strains. Lena strain isolation was performed

from lung homogenates belonging to weak born piglets from a PRRSV-positive herd from Belarus in 2007 with a high mortality rate, reproductive failure, and respiratory disorders (Karniychuk et al., 2010). Both viruses were propagated and titrated on PAMs.

2.2. Animals and experimental design

The animals and samples used in this study were part of a project to investigate the pathogenesis of the infection with PRRSV-1 strains of different virulence (Rodríguez-Gómez et al., 2019). Briefly, fifty-two 5-week-old piglets (Landrace x Large White crossbred) were obtained from a high health historically PRRSV-negative farm. All pigs were negative for porcine circovirus type 2, PRRSV and Mycoplasma hyopneumoniae by ELISA and PCR assays (Mattsson et al., 1995; Sibila et al., 2004).

Piglets were randomly assigned to three different groups and housed in separate pens: Lena group (n=20), 3249 group (n=20) and control group (n=12). After an acclimation period of seven days, piglets were intranasally inoculated with either the low virulent 3249 strain or the virulent Lena strain (both used at 1x105 TCID50/mL, 1 ml/nostril, using an atomiser). The control group was mock-inoculated with the PAM supernatant diluted with RPMI similarly to the inoculum. Three control pigs and five infected pigs from each group were euthanised on days 1, 3, 6 and 8 post-inoculation (dpi). This experiment was conducted according to the guidelines of the European Union (Directive 2010/63/EU) and approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413).

2.3. Clinical signs, gross pathology and histopathology of lung

Commencing 1 day prior to inoculation, piglets were daily monitored to evaluate clinical signs (unusual behaviour, anorexia, cough and dyspnoea) and rectal temperature. Rectal temperatures > 40.5 °C were considered hyperthermia. At necropsy, gross lung lesions were recorded and scored by the same pathologist (Halbur et al., 1998). Afterwards, samples from apical, medial and caudal lobes from the right lung were collected and fixed in 10 % neutral-buffered formalin (Fisher Scientific Ltd., Loughborough, UK) for histopathological and immunohistochemical studies.

Four-micron tissue sections were stained with haematoxylin and eosin and blindly graded by two pathologists for the histopathological evaluation. The severity of histopathological lesions in the lung was scored as previously described by Halbur et al. (1997): 0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia. In addition, a similar score was developed considering the diagnosis of suppurative bronchopneumonia (Rodríguez-Gómez et al., 2019): 0, no microscopic lesions; 1, mild bronchopneumonia; 2, moderate multifocal bronchopneumonia; 3, moderate diffuse bronchopneumonia; and 4, severe bronchopneumonia. Altogether, the final score included the total of both, the interstitial pneumonia score and the bronchopneumonia score, being 8 points the maximum possible score.

2.4. Viral genome quantification

RNA was isolated from sera using NucleoSpin® RNA virus (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. For lung, RNA was purified from tissue homogenate using a combined procedure with TRIzol™ (Thermo Fisher Scientific, Barcelona, Spain) and NucleoSpin® RNA Virus columns (Macherey-Nagel) according to manufacturer's instructions. Viral load for either 3249 strain or Lena strain was quantified by RT-qPCR using VetMAX™ PRRSV EU/NA 2.0 kit (Thermo Fisher Scientific, Barcelona, Spain). Amplifications were performed in duplicate from each animal in the QuantStudio 5 Real-time PCR System (Life Technologies, Carlsbad, CA, USA) for 5 minutes (min) at 50 °C, 10 min at 95 °C followed by 40 cycles of 3 seconds (s) at 95 °C and 30 s at 60 °C. For PRRSV genome quantification, an ORF7 RT-PCR product from both 3249 and Lena strains was firstly EtOH precipitated and purified using ExoSAP-ITTM (Thermo Fisher Scientific, Barcelona, Spain). The purified products were quantified using BioDrop (BioDrop, UK). Serial 10-fold dilutions of 3249 or Lena ORF7 RT-PCR products with known quantities, including from 108 to 102 genomic copies/ml were used as standard to generate a standard curve and, therefore, to determine the PRRSV genomic copies in sera and lung. The RT-qPCR efficiency (E) was estimated for each strain by a linear regression model. The E value was calculated from the slope of the standard curve according to equation: E=10(□1/slope) - 1. Also, a set of eight serial 10-fold dilutions of know TCID50/ml (starting at 106 TCID50/ml) was included in order to determine a relation between Ct-values, genomic copies/ml and TCID50/ml. An inter-run calibrator sample with a known number of PRRSV copies

was introduced in each experiment to self-control inter-run variation. The area under the curve (AUC) for viremia and lung viral load was calculated using the trapezoidal approach (Greenbaum et al., 2001). Results of viral load in sera and lung are showed in equivalent TCID 50 (eq TCID50) per mL.

2.5. Antibody and serological assessments

Specific antibodies against PRRSV were detected using IDEXX PRRS X3 ELISA test (IDEXX laboratories, Barcelona, Spain) following manufacturer's instructions. Levels of IFN-γ, IL-6 and IL-10 and the acute phase protein lipopolysaccharide binding protein (LBP) as well as the soluble form of swine CD163 (sCD163) were assessed in sera from all piglets. Different commercially available ELISA tests were used in accordance with manufacturer's guidelines (IFN-γ, IL-6, IL-10 [Invitrogen, Barcelona, Spain]; LBP, [Hycult Biotech, Uden, Netherlands]; sCD163 [Cusabio Biotech, Houston, USA]). Results were expressed in pg/mL for IFN-γ, IL-6 and IL-10, and ng/mL for LBP and sCD163. The minimum detectable concentrations were 2 pg/mL for IFN-γ, 45 pg/mL for IL-6, 3 pg/mL for IL-10, 1.6 ng/mL for LBP and 23.4 ng/ml for sCD163.

2.6. Immunohistochemistry in lung tissue

Four-micron sections from lung were dewaxed in xylene and rehydrated in descending grades of alcohol until distilled water. Then, endogenous peroxidase inhibition was performed in a 3% H2O2 solution in methanol for 30 min. Epitope demasking, primary antibodies dilutions and blocking of non-specific binding are detailed in Table 1. Monoclonal primary antibodies were incubated overnight at 4 °C in a humid chamber. Biotinylated secondary antibody was accordingly incubated for 30 min at room temperature. After washing in PBS, Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA) was applied and the immunolabelling was revealed by application of NovaRED™ substrate kit (Vector Laboratories). Sections were counterstained with Harris's haematoxylin, dehydrated and mounted. PBS (pH 7.4) and Tris buffered saline (pH 7.6) were used as wash and diluent buffers. Antibody specificity was verified by exchanging the primary antibody by isotype matched reagents of irrelevant specificity. One negative control which consisted of replacement of the primary antibody by BSA blocking solution was included in each immunohistochemical assay to confirm the lack of non-specific bindings.

Table 1. Summary of immunohistochemical methodology.

Specificity	Type of antibodies	Clone	Source	Blocking solution	Dilution	Antigen retrieval
PRRSV-N-protein	mAb	SDOW17	Rural Technologies, Brookings, SD, USA	BSA 2%	1:500	Protease XIV
CD163	mAb	2A10/11	In house, INIA	NGS 10%	neat	Citrate pH 6
CD200R1	mAb	PCT3	In house, INIA	BSA 2%	neat	Protease XIV
CD14	mAb	MIL2	Biorad, Hercules, CA	BSA 2%	1:100	Protease XIV
iNOS	pAb	NA	Neomarers, Fremont, CA, USA	BSA 2%	1:750	Citrate pH 6*
FoxP3	mAb	FJK-16s	eBioscience™, Bacelona, Spain	NGS 10%**	1:100	Citrate pH 6*

*PRRSV, Porcine reproductive and respiratory syndrome virus. mAb, iNOS, inducible nitric oxide synthase; CD200R1, CD200 Receptor 1; FoxP3, forkhead box protein 3; monoclonal antibody; pAb, polyclonal antibody; BSA, Bovine Serum Albumin; NGS, Normal Goat Serum; NA, not applicable; NGS 10%**: NGS diluted in phosphate buffer saline with 80% tween 20; Protease XIV, enzymatic digestion with protease type XIV (Sigma-Aldrich) at 38° C for 8 minutes; Citrate pH 6, microwave heat treatment at 420W for 10 minutes; Citrate pH 6*, autoclave treatment at 121° C for 10 min.*

2.7. Cell counting

The number of immunolabelled cells was quantified in 25 non-overlapping selected high magnification fields of 0.2 mm2 (Olympus BX51, Olympus Iberia SAU, L'Hospitalet de Llobregat, Barcelona, Spain). The number of immunolabelled cells per mm2 was expressed as a median of the score for each animal within each group. Labelled cells were morphologically identified by differentiating between PAMs, intravascular and interstitial macrophages.

2.8. Statistical analyses

Differences between groups were evaluated for approximate normality of distribution by the D'Agostino and Pearson omnibus normality test followed by the Mann Whitney's U non-parametric mean comparisons test. Correlation coefficients were assessed by the Spearman and Pearson tests and were considered relevant with $r > 0.6$ and $P < 0.05$. Data analyses and figures were performed by using GraphPad Prism 7.0 software (GraphPad Prism software 7.0, Inc., San Diego, CA, USA) and InkScape 0.92 software. A p value lower than 0.05 was considered statistically significant and represented as * $P \leq 0.05$, ** $P \leq 0.01$ *** $P \leq 0.001$ and **** $P \leq 0.0001$

*Table 2. Statistical correlations found in piglets infected with virulent Lena strain throughout the study. For all data, a P value lower than 0.05 was considered statistically significant and represented as * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 and ****P ≤ 0.0001.*

	Temperature	Clinical score	Viraemia	Lung viral Load	IFN-γ	IL-6	Gross lesion	Microscopic lesion	PRRSV-N-protein	CD163	CD200R1	FoxP3	CD14	iNOS
Temeprature	-	0.92****	0.79****	0.76****	0.67*	NS	0.62**	0.64**	0.78****	NS	NS	NS	NS	NS
Clinical score		.	0.84****	0.72****	0.72***	NS	0.68**	0.75****	0.78****	NS	-0.63**	NS	NS	NS
Viraemia			.	0.77****	0.71**	NS	NS	0.62**	0.74**	-0.78***	NS	NS	NS	NS
Lung viral load				.	0.71***	NS	0.72***	0.68**	0.85****	-0.71***	0.63**	NS	NS	NS
IFN-γ					-	NS	NS	0.62*	0.62*	NS	0.61**	0.70**	NS	NS
IL-6						-	NS	NS	0.55*	NS	0.56**	NS	0.84***	NS
Gross lesion							-	0.8***	NS	NS	0.73***	NS	NS	NS
Microscopic lesion								-	0.68***	NS	0.91****	NS	NS	NS
PRRSV-N-protein									-	-0.62**	0.63**	NS	NS	NS
CD163										-	NS	NS	NS	NS
CD200R1											-	NS	0.55*	NS
FoxP3												-	NS	NS
CD14													-	0.52*
iNOS														-

NS: not statistically significant or with r < 0.6. Correlation coefficients were considered relevant with r > 0.6 and P < 0.05.

3. Results

3.1. Acute suppurative bronchopneumonia and the highest rectal temperature and clinical scores were observed in Lena-infected pigs.

Clinical observations and gross pathology of lung were described thoroughly by Rodríguez-Gómez et al. (2019). In brief, piglets inoculated with virulent Lena strain had a long period of hyperthermia (mean rectal temperature above 40.5 °C) with a marked mean clinical score from 4 dpi onwards, both peaking at 6 dpi (Figs. 1A-1B, clinical signs and temperature). By contrast, an increase in rectal temperature below the hyperthermic threshold accompanied by mild clinical signs was observed in piglets infected with 3249 strain (Figs. 1A-1B). At necropsy, tan-mottled areas, atelectasis, rubbery consistency and consolidated areas were observed in the lungs from both PRRSV-1-infected pigs whose macroscopic score gradually increased throughout the study (Figs. 1C-1D). In particular, a stronger and earlier onset of the lung lesion (from 6 dpi onwards) associated with the presence of extensive consolidated areas in apical and medial lobes as well as more severe interstitial pneumonia were observed in Lena-infected pigs causing the highest macroscopic score compared to 3249-infected pigs (Figs. 1C-1D).

A mild to moderate interstitial pneumonia with thickening of the alveolar septa by infiltrate of lymphocytes and macrophages was caused by both strains, Lena and 3249 strains (Figs. 1E-1F). Furthermore, a suppurative bronchopneumonia characterised by neutrophils, cell debris and mucus filling the bronchial, bronchiolar and alveolar lumen was observed in lung section from Lena-infected pigs at 6 and 8 dpi (Figs. 1E-1F). A strong statistical correlation was found among temperature, clinical score, gross and microscopic lesions in Lena-infected pigs (Table 2 summarises the results of statistical analysis).

Figure 1

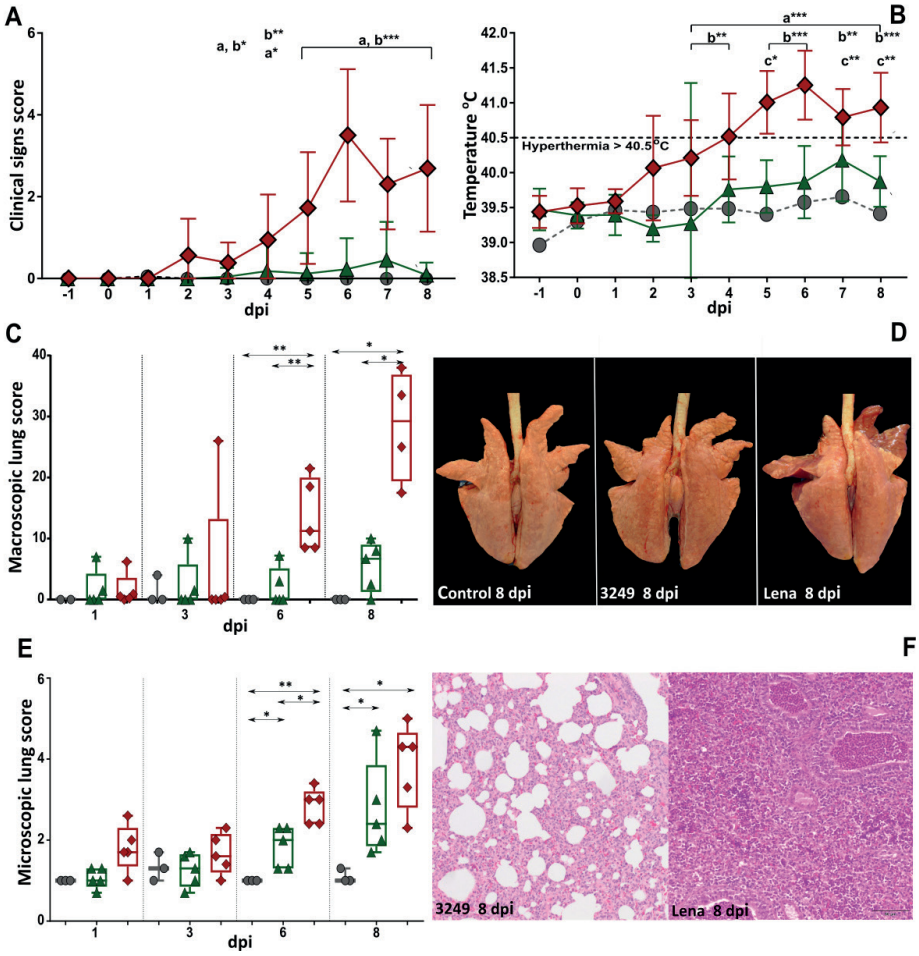
A
Clinical signs score
dpi
a, b*
b**
a*
a, b***
B
Temperature °C
Hyperthermia > 40.5°C
dpi
a***
b**
b***
b**
b***
c*
c**
c**
C
Macroscopic lung score
dpi
D
Control 8 dpi
3249 8 dpi
Lena 8 dpi
E
Microscopic lung score
dpi
F
3249 8 dpi
Lena 8 dpi

Fig. 1. Clinical, gross and microscopic lung outcomes. *Pigs were followed up from 1 day prior to infection until 8 dpi to evaluate clinical signs and hyperthermia (> 40.5°C). Plots show the mean average of clinical score* ***(A)*** *and rectal temperature* ***(B)*** *for control group (gray circles), 3249-infected group (green triangle) and Lena-infected group (red diamonds). "a" indicates a significant difference between the Lena and 3249 and control groups and "b" a significant difference between the 3249 and control groups. At necropsy, lungs were scored and processed for histological and immunohistochemical studies.* ***(C)*** *Box plots display the macroscopic lung score for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) for each time point.* ***(D)*** *Lung appearance of piglets from the control, 3249 and Lena group at 8 dpi are shown in the pictures.* ***(E)*** *Box plots show the microscopic lung score for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) for each time point. Each symbol represents individual data for each pig.* ***(F)*** *Photomicrographs of the medial lung lobe illustrate characteristic interstitial pneumonia in 3249-infected pig (left) and suppurative bronchopneumonia in Lena-infected pig (right) at 8 dpi. Haematoxylin and eosin. Bar, 100 µm. P value lower than 0.05 was considered statistically significant and represented as * $P \leq 0.05$ and ** $P \leq 0.01$.*

3.2. Viraemia and lung viral load followed a similar kinetics in PRRSV-1-infected pigs

Viraemia and lung viral load were determined by RT-qPCR (efficiency of 99 %; slope = 3.34; detection limit: 1 copy/µl; slope-intercept = 39.5; and high linearity, r = 0.99). All animals were negative by RT-qPCR at day 0 and control pigs remained so all throughout the experiment. In sera, four out of five 3249-infected pigs and all Lena-infected pigs were PRRSV positive as early as 1 dpi. Viraemia was always higher in Lena- than in 3249-infected pigs from 1 to 8 dpi ($P < 0.01$ at 1, 3, 6 dpi; $P<0.05$ at 8 dpi), reaching the highest viral load at 6 dpi (1.9x107 eq TCID50/mL). The AUC for viremia (mean) in Lena and 3249 group were 44.8 and 33.8 respectively (Fig. 2A). The viral load in the lung displayed a similar kinetics than that of serum for both infected groups, reaching the maximum lung viral loads at 6 dpi in Lena group (1.6x107 eq TCID50/mL), whereas 3249 group peaked at 8 dpi (1.9x106 eq TCID50/mL) (Fig. 2B). By contrast to sera, PRRSV-1 in lung was just detected in two out of five animals in both infected groups at 1 dpi, being positive all infected piglets from 3 dpi onwards. The AUC for lung viral load (mean) in Lena group was 45 and 36.2 for 3249 group (Fig. 2B). In Lena infected-group the statistical analysis revealed a positive correlation among viraemia, lung viral load, temperature, clinical score and the number of PRRSV-N-protein$^+$ cells in the lungs (Table 2 summarises the main results of statistical analysis). A correlation among lung viral load and viraemia and PRRSV-N-protein$^+$ cells was also observed in 3249-infected pigs ($r = 0.71$, $P < 0.0001$; and, $r = 0.60$, $P < 0.005$, respectively).

3.3. A significant increase in the serum concentration of IFN-γ was observed in Lena-infected pigs when compared with 3249-infected pigs

PRRSV-specific antibodies were first detected at 8 dpi in sera from both PRRSV-1-infected group (non-significant differences in S/P ratios) (data not shown). A significant increase in IFN-γ serum levels was detected after Lena infection (maximum mean level of 234 ± 100 pg/mL at 6 dpi) at 6 and 8 dpi compared to control ($P<0.05$) and 3249 ($P<0.01$) groups (Fig. 2C). Maximum IL-6 levels in serum of 3249 group were observed at 6 dpi (mean of 350 ± 220 pg/mL), whereas pigs belonging to Lena group reached the highest IL-6 levels at 8 dpi (mean of 480 ± 50 pg/mL) (Fig. 2D). IL-10, LBP or sCD163 were not detected in serum samples from both control and infected groups throughout the study. Both viraemia and lung viral load displayed a positive statistical correlation with IFN-γ levels, which in turn were also correlated with temperature and the clinical score in Lena infected-pigs (Table 2 summarises the main results of statistical analysis).

Figure 2

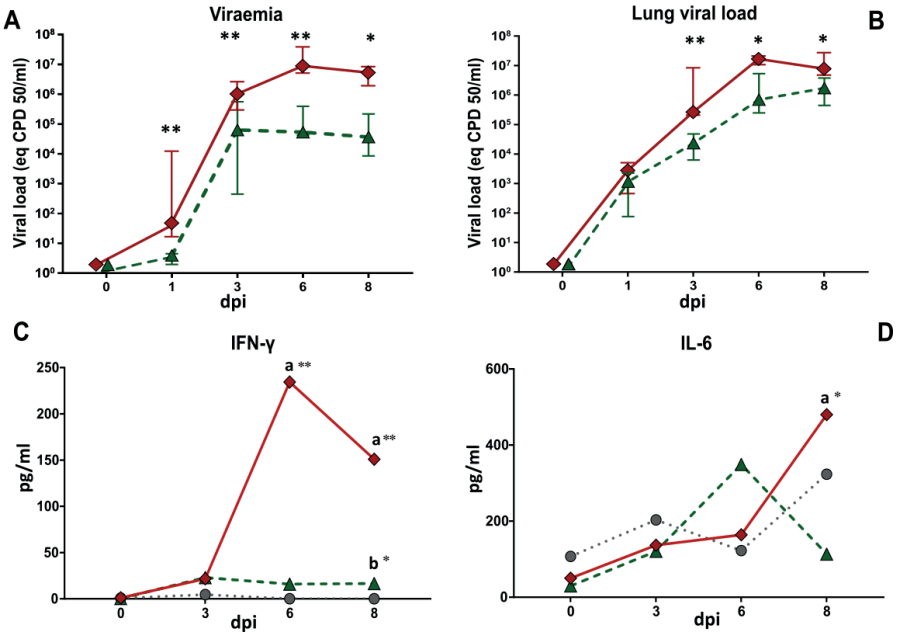

Fig. 2. PRRSV viraemia, lung viral load, IFN-γ and IL-6 level in sera. *PRRSV genome load was quantified in sera* ***(A)*** *and lung* ***(B)*** *by RT-qPCR. The concentration of IFN-γ* ***(C)*** *and IL-6* ***(D)*** *were assessed in sera by using ELISA. Diagrams* ***(A)*** *and* ***(B)*** *display the viral load (eq TCID50/mL) and* ***(C)*** *and* ***(D)*** *the concentration of the cytokines IFN-γ (pg/mL) and IL-6 (pg/mL), respectively, for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. All data are reported as the mean with range of results obtained for each group and time point. P value lower than 0.05 was considered statistically significant and represented as * P ≤ 0.05 and ** P ≤ 0.01. "a" indicates a significant difference between the Lena and 3249 and control groups and "b" a significant difference between the 3249 and control groups.*

3.4. The increase in the number of PRRSV-N-protein$^+$ cells was associated with a decrease of CD163$^+$ macrophages in the lung of PRRSV-infected pigs

The nucleocapsid protein N (PRRSV-N-protein) is the most abundant viral protein produced during PRRSV infection (Rowland et al., 1999). The labelling of PRRSV-N-protein was mainly observed in PAMs and in a lesser extent in interstitial and intravascular macrophages (Figs. 3A-3B). In Lena-infected pigs, clusters of PRRSV-N-protein$^+$ macrophages were observed within foci of bronchopneumonia at 6 and 8 dpi (Fig. 3B inset). A progressive increase in the number of PRRSV-N-protein$^+$ cells was detected throughout the study in both PRRSV-1-infected groups, reaching a peak at 6 and 8 dpi in Lena and 3249-infected piglets, respectively. This increase was significantly higher in Lena than in 3249 group ($P<0.05$ at 3, 6 y 8 dpi) (Fig. 3E, primary axis). No positive cells were detected in control pigs.

The expression of the scavenger receptor CD163 is restricted to the monocyte/macrophage lineage and it is considered as the essential receptor for PRRSV infection (Sánchez et al., 1999; Van Gorp et al., 2008). CD163 was detected in the cell membrane and cytoplasm of PAMs, interstitial macrophages and, occasionally, intravascular macrophages (Figs. 3C-3D, insets). A drop in the number of CD163$^+$ cells was observed from 3 to 8 dpi in both PRRSV-1-infected groups. This decrease was more intense in Lena-infected animals when compared to 3249 ($P<0.05$ at 3 dpi) and control groups ($P<0.05$ at 3 and 8 dpi) (Fig. 3E, secondary axis). By contrast, the frequency of CD163$^+$ cells remained constant in control pigs during the whole study. Of note, PAMs were the subset of pulmonary macrophages which underwent the strongest reduction in the number of CD163$^+$ cells. Furthermore, the frequency of CD163$^+$ cells showed a strong negative correlation with lung viral load and the number of PRRSV-N-protein$^+$ cells in Lena infected-pigs (Fig. 3E) (Table 2 summarises the main results of statistical analysis).

3.5. A strong influx of CD14$^+$ macrophages and monocytes infiltrating the interstitium was detected in Lena-infected pigs.

CD14, the primary lipopolysaccharide (LPS) receptor, is expressed on the membrane of monocytes, macrophages and, to a lesser extent, neutrophils. CD14 recognises several molecules from bacteria, initiating an inflammatory response against these organisms (Van Gucht et al., 2005; Zanoni and Granucci, 2013). Nevertheless, its contribution in viral infection remains largely to be defined. In our study, CD14 was mainly observed in the cell membrane and cytoplasm of monocytes, interstitial and intravascular macrophages and, occasionally, in PAMs (Figs. 4A-4B, insets). Whereas no changes were observed in the number of CD14$^+$ cells in the control group along the study, a gradual increase with maximum expression at 8 dpi was detected in both infected groups (Fig. 4E). Lena-infected pigs showed the highest frequency of CD14$^+$ cells when compared to control animals ($P<0.01$) in association with the presence of suppurative bronchopneumonia (Fig. 4B). CD14$^+$ interstitial and intravascular macrophages were observed infiltrating extensive areas of the interstitium, whereas almost no CD14$^+$ cells were present in the bronchial wall and alveolar lumen. Interestingly, the number of CD14$^+$ cells in Lena-infected piglets displayed a strong positive correlation with the concentration of IL-6 in sera (Table 2 summarises the main results of statistical analysis).

3.6. Lena virulent strain induced a strong increase of iNOS$^+$ cells associated with a higher microscopic lung lesion

Overproduction of nitric oxide (NO) is mainly triggered by inducible NO synthase (iNOS), which is mainly expressed by phagocytic cells, playing several roles in the pathogenesis of viral lung injury and inflammation (Akaike and Maeda, 2000). The granular intracytoplasmic immunostaining of iNOS was primarily observed in the cytoplasm of PAMs and interstitial macrophages in foci of interstitial pneumonia and bronchopneumonia (Figs. 4C-4D). The number of iNOS$^+$ cells followed a similar kinetics in both PRRSV-1-infected groups, with a progressive increase from 6 dpi onwards, reaching a significant increase by the end of the study (8 dpi) in Lena-infected pigs compared to 3249 ($P<0.01$) and control groups ($P<0.05$) (Fig. 4F).

Figure 3

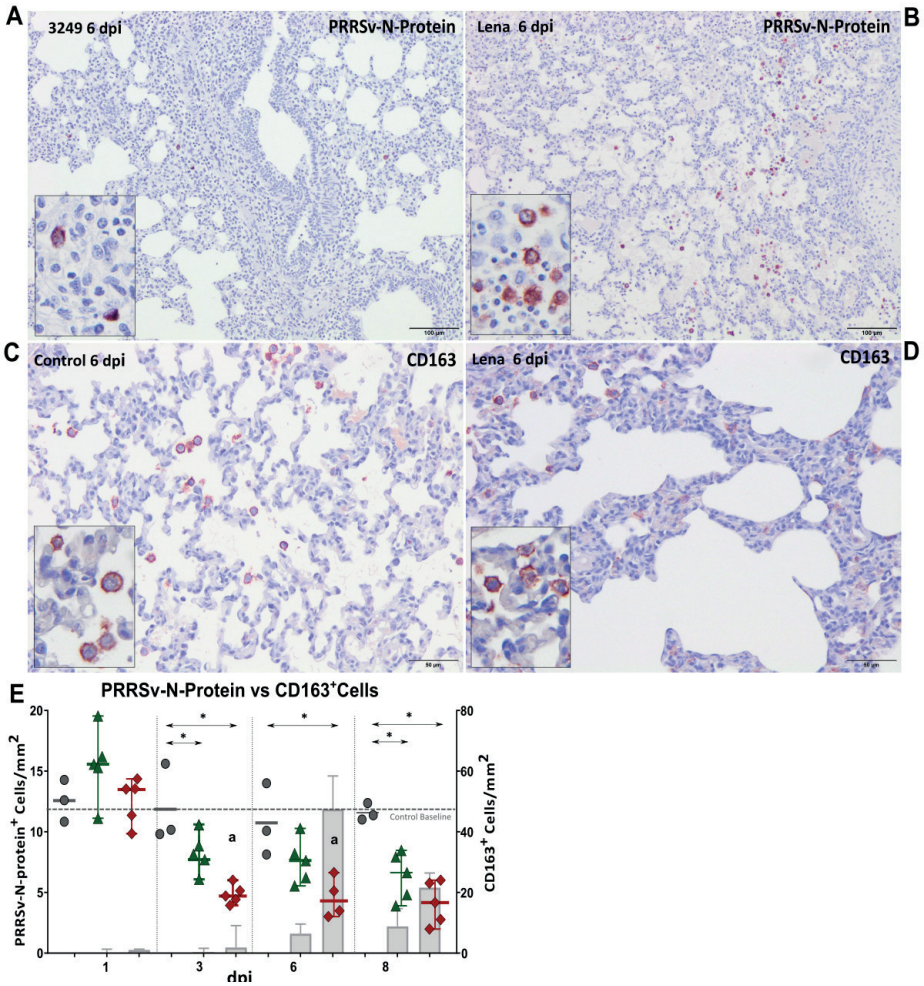
A
3249 6 dpi
PRRSv-N-Protein
B
Lena 6 dpi
PRRSv-N-Protein
C
Control 6 dpi
CD163
D
Lena 6 dpi
CD163
E
PRRSv-N-Protein vs CD163+Cells
PRRSv-N-protein+ Cells/mm2
CD163+ Cells/mm2
Control Baseline
dpi
1
3
6
8
a
a

Fig. 3. Immunohistochemical expression of PRRSV-N-protein and CD163 in lung tissue. *Lung tissue sections were immunolabelled against both antigens. Photomicrographs of the medial lung lobe illustrate the expression of PRRSV-N-protein in a 3249-infected* ***(A)*** *and Lena-infected* ***(B)*** *pig, which was mainly observed in PAMs (3A-3B, insets). IHC. Bar, 100 µm. Photomicrographs of the medial lung lobe illustrate the expression of CD163 in a control* ***(C)*** *and Lena-infected* ***(D)*** *pig. Immunolabelling of CD163 scavenger receptor was mainly observed in the cytoplasm and cell surface of PAMs found in the pulmonary alveolus and in a lesser extent in interstitial and intravascular macrophages (3C-3D, insets). IHC. Bar, 50 µm.* ***(E)*** *The diagram displays the number of PRRSV-N-protein$^+$ (primary axis), CD163$^+$ (secondary axis) cells/m2. Scatter dot plot shows the number of CD163$^+$ macrophages (secondary axis) for each animal (control, gray circles; 3249, green triangles; Lena, red diamonds) and coloured lines the average for each group and time point. Control baseline displays the mean of the number of positive cells along the study in control group. P value lower than 0.05 was considered statistically significant and represented as * $P \leq 0.05$ and ** $P \leq 0.01$. The grey bars display the average of total number of PRRSV-N-protein$^+$ macrophages (primary axis) for each group and time point. "a" indicates a significant difference between the Lena and 3249 groups.*

3.7. The increase of CD200R1$^+$ cells along the study was highly correlated with the course of lung injury

CD200R1 plays an important role inhibiting the production of proinflammatory cytokines (Vaine & Soberman, 2014). In pig, this receptor is mainly expressed on myeloid cells, such as monocytes, macrophages, granulocytes or dendritic cells, but also on B cell subsets (Poderoso et al., 2019). In the present study, CD200R1 labelling was detected in the cytoplasm of intravascular and interstitial macrophages located inside or surrounding bronchopneumonia foci, with occasional expression in PAMs and monocytes (Figs. 5A-5B, insets). Whereas the number of CD200R1$^+$ cells increased in Lena-infected pigs from 6 dpi onwards, this increase was just detected at 8 dpi in 3249-infected pigs. By contrast, control animals presented a scarce number of CD200R1$^+$ cells. This change was more marked in the Lena group with a significant increase in the number of CD200R1$^+$ cells in comparison to 3249 ($P<0.01$ at 6 dpi) and control ($P<0.05$ at 6 and 8 dpi) groups (Fig. 5E, primary axis). For Lena infected-pigs a strong positive correlation was observed among the frequency of CD200R1$^+$ cells and the microscopic lung lesion (Fig. 5E) (Table 2 summarises the main results of statistical analysis).

3.8. Both PRRSV-1 strains induced an increase of FoxP3$^+$ cells from 6 dpi onwards

The transcription factor forkhead box protein 3 (FoxP3) is the essential molecular marker of regulatory T cells (Tregs) (Käser et al., 2008). Tregs are specialised in the silencing of exuberant immune response to infectious and non-infectious agents and the management of self-tolerance homeostasis (Pereira et al., 2017). In our study, FoxP3 yielded a nuclear immunolabelling in lymphocytes mainly located in areas of atelectasis and interstitial pneumonia (Figs. 5C-5D, insets). Although two Lena-infected pigs exhibited a higher number of FoxP3$^+$ cells at 1 dpi, the kinetics of positive cells against this molecule showed a gradual increase along the study in both Lena- and 3249-infected animals, reaching the maximum at 6 dpi (Fig. 5F). There were no significant differences in the number of FoxpP3$^+$ cells among infected groups. However, a significant increase of FoxP3$^+$ cells was detected at 6 and 8 dpi in Lena-infected pigs compared to control animals ($P<0.05$).

Figure 4

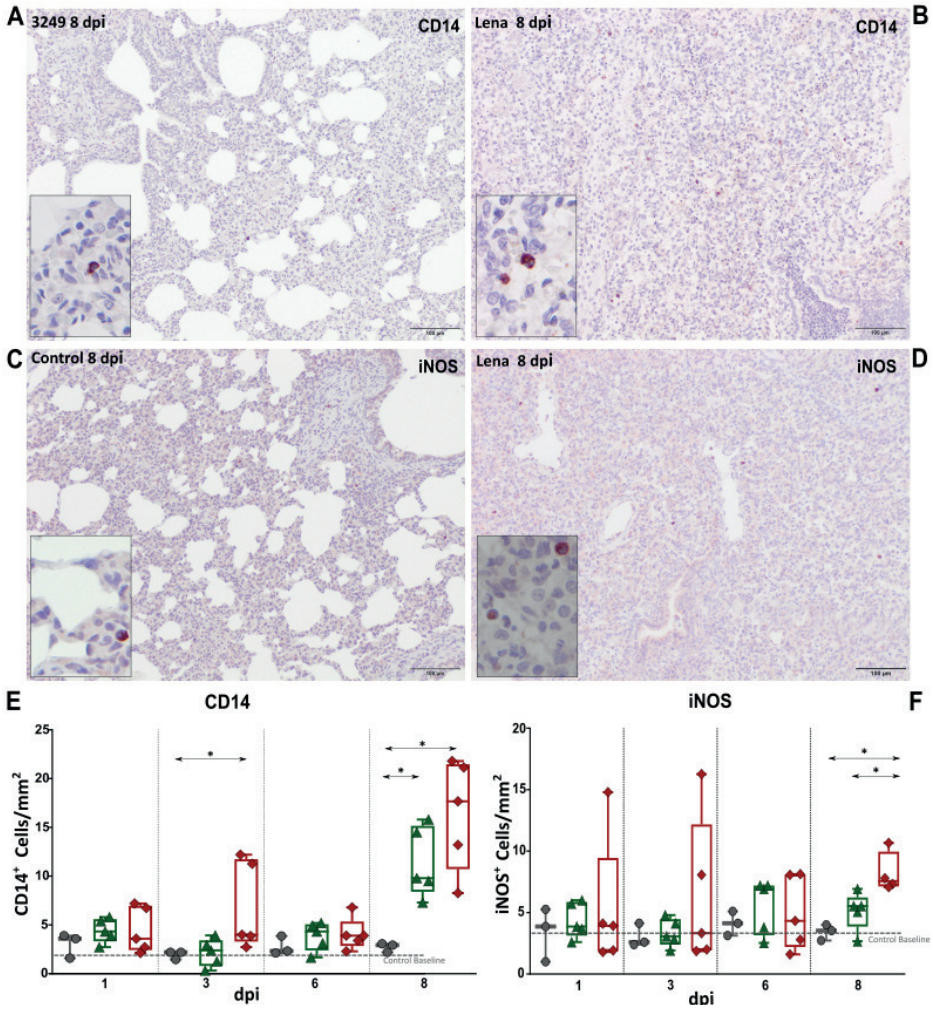
A
3249 8 dpi
CD14
B
Lena 8 dpi
CD14
C
Control 8 dpi
iNOS
D
Lena 8 dpi
iNOS
E
CD14
CD14⁺ Cells/mm²
dpi
Control Baseline
F
iNOS
iNOS⁺ Cells/mm²
dpi
Control Baseline

Fig. 4. Immunohistochemical expression of CD14 and iNOS in lung tissue. *Lung tissue sections were immunolabelled against both antigens. Photomicrographs of the medial lung lobe illustrate the expression of CD14 in a 3249-infected* ***(A)*** *and Lena-infected pig* ***(B)****, which was mainly expressed on the cell membrane and cytoplasm of monocytes, interstitial and intravascular macrophages (4A-4B, insets). IHC. Bar, 100 µm. Photomicrographs of the medial lung lobe illustrate the expression of iNOS in a control* ***(C)*** *and Lena-infected* ***(D)*** *pig. iNOS was observed primarily in the cytoplasm of PAMs and interstitial macrophages detected in foci of interstitial pneumonia and bronchopneumonia (4C-4D, insets). IHC. Bar, 100 µm.* ***(E)*** *Box plots shows the number of $CD14^+$ cells for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. (F) Box plots display the number of $iNOS^+$ cells for each group (Control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. Control baseline displays the mean of the number of positive cells along the study in control group. P value lower than 0.05 was considered statistically significant and represented as * $P \leq 0.05$ and ** $P \leq 0.01$.*

Figure 5

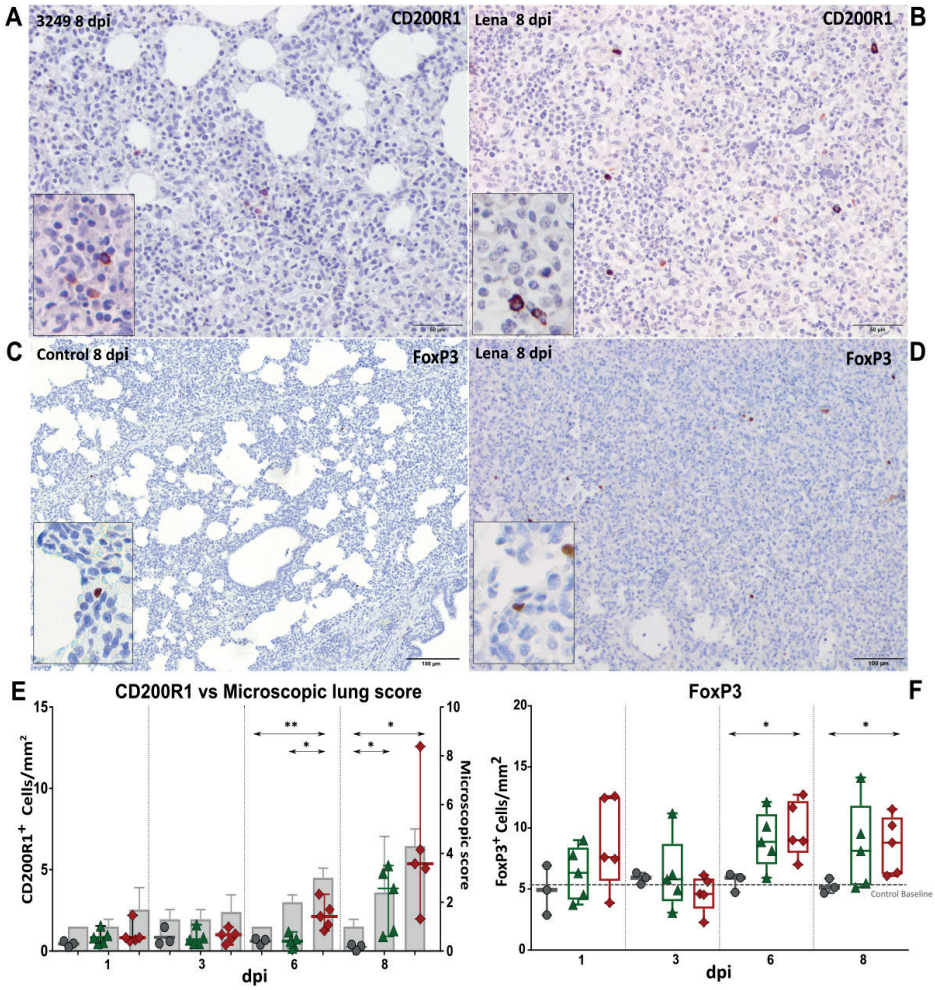
A
3249 8 dpi
CD200R1
B
Lena 8 dpi
CD200R1
C
Control 8 dpi
FoxP3
D
Lena 8 dpi
FoxP3
E
CD200R1 vs Microscopic lung score
CD200R1+ Cells/mm2
Microscopic score
dpi
F
FoxP3
FoxP3+ Cells/mm2
Control Baseline
dpi

Fig. 5. Immunohistochemical expression of CD200R1 and FoxP3 in lung tissue. *Lung tissue sections were immunolabelled for both antigens. Photomicrographs of the medial lung lobe illustrate the expression of CD200R1 in a 3249-infected* ***(A)*** *and Lena-infected* ***(B)*** *pig, which was mainly detected in the cytoplasm of intravascular and interstitial macrophages located inside or surrounding bronchopneumonia foci (5A-5B, insets). IHC. Bar, 50 µm. Photomicrographs of the medial lung lobe illustrate the expression of FoxP3 in a control* ***(C)*** *and Lena-infected* ***(D)*** *pig. FoxP3 yielded a nuclear immunolabelling in lymphocytes mainly located in areas of atelectasis and interstitial pneumonia (5C-5D, insets). IHC. Bar, 100 µm.* ***(E)*** *The diagram displays the number of CD200R1$^+$ cells/m2 versus the microscopic score of lungs. Scatter dot plot shows the number of CD200R1$^+$ cells (primary axis) for each animal (control, gray circles; 3249, green triangles; Lena, red diamonds) and coloured lines the average for each group and day point. The gray bar display the microscopic lung score (secondary axis) for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) at each time point.* ***(F)*** *Box plots shows the number of FoxP3$^+$ cells for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. Control baseline displays the mean of the number of positive cells along the study in control group. P value lower than 0.05 was considered statistically significant and represented as * $P \leq 0.05$ and ** $P \leq 0.01$.*

4. Discussion

PRRSV plays a pivotal role in PRDC, modulating the host immune response and favouring secondary bacterial infections (Gómez-Laguna et al., 2013; Van Gucht et al., 2004). Virulent PRRSV-1 strains cause more severe clinical signs, higher mortality rates as well as marked lung injury with a higher incidence of bronchopneumonia as opposed to low virulent strains (Amarilla et al., 2015; Canelli et al., 2017; Frydas et al., 2013; Gómez-Laguna et al., 2010; Morgan et al., 2013; Renson et al., 2017; Rodríguez-Gómez et al., 2019; Stadejek et al., 2017; Weesendorp et al., 2013). Accordingly, it has been hypothesised that severe pulmonary lesions observed along infection with virulent PRRSV-1 strains might be associated with a higher decrease in the amount of PAMs as well as an imbalance between anti- and pro-inflammatory responses with different molecules potentially involved in this process.

As previously described (Canelli et al., 2017; Renson et al., 2017; Weesendorp et al., 2013), severe systemic and respiratory symptoms as well as hyperthermia were observed in animals infected with virulent Lena strain, whereas low virulent 3249 strain only caused mild clinical signs and a slightly increase of rectal temperature. Furthermore, virulent Lena strain caused an earlier and stronger onset of lung lesions due to extensive consolidated areas in the apical and medial lobes which were microscopically linked to suppurative bronchopneumonia as well as severe characteristic interstitial pneumonia. On the other hand, PRRSV virulence has been associated with higher virus titre and antibody response in vivo (Brockmeier et al., 2012; Liu et al., 2010). Therefore, Lena strain virulence might be speculated to induce a stronger antibody response associated with a higher replication rate; however, no differences were observed in the antibody response among both Lena and 3249 strains in the early phase of infection. The high virus titre observed in both infected groups in our study may be responsible for the lack of differences in the antibody response among them during the acute phase of the infection.

PRRSV infection with virulent strains usually induces a strong inflammatory immune response compared with low virulent strains (Amarilla et al., 2015; Liu et al., 2010; Morgan et al., 2013; Renson et al., 2017; Weesendorp et al., 2013). In our study, high levels of IL-6 and IFN-γ were detected in the sera of Lena-infected pigs at 8 and 6 dpi, respectively. Increased concentration of IL-6 in plasma is associated

with both systemic and respiratory symptoms (Van Reeth and Nauwynck, 2000) and could play a dual role during virus infection: (i) protecting the host from infection and (ii) inducing inflammation and tissue damage when it is overexpressed (Liu et al., 2010; Lunney et al., 2010). IFN-γ is mostly produced by activated T cells and NK cells, which participate in regulating the immune and inflammatory responses (Van Reeth and Nauwynck, 2000). In our study, animals infected with the virulent Lena strain presented a marked increase in the serum concentration of IFN-γ. When analysed together, viraemia and lung viral load were significantly correlated with the serum level of IFN-γ in Lena-infected animals, which may point to an attempt of the host immune response in controlling virus replication. The enhanced serum concentration of IL-6 could mirror the stronger systemic inflammatory response induced by Lena during the acute phase response contributing to the fever and more severe clinical signs as well as lung lesion that specifically arise in virulent PRRSV-1 strains (Amarilla et al., 2015; Renson et al., 2017).

In order to evaluate the kinetics of PRRSV-1 in the lung, we analysed the expression of PRRSV-N-protein. A progressive increase in the number of PRRSV-N-protein$^+$ cells was observed throughout the infection in both PRRSV-1-infected groups, with Lena-infected piglets presenting the highest number of PRRSV-N-protein$^+$ cells. Interestingly, both the number of PRRSV-N-protein$^+$ cells and lung viral load showed a solid negative correlation with the frequency of CD163 scavenger receptor, the essential host receptor for PRRSV infection (Burkard et al., 2017; Van Gorp et al., 2008). The drop in the number of pulmonary CD163$^+$ cells was detected in both PRRSV-1 infected groups, being more intense in Lena-infected animals. This reduction in the number of CD163$^+$ cells has been already reported in live PAMs from the BALF of Lena-infected piglets (Renson et al., 2017; Rodríguez-Gómez et al., 2019) and from lung tissue sections of piglets infected with SU1-bel virulent strain (Sánchez-Carvajal et al., 2019). This finding could be due to the direct cytopathic effect of the virus in its target cell but also to an indirect induction of cell death phenomena in infected and non-infected cells, which has been broadly described in the lung and lymphoid organs of pigs infected with virulent PRRSV-1 strains (Morgan et al., 2013; Ruedas-Torres et al., 2020; Sánchez-Carvajal et al., 2019). In our study, the decrease of CD163$^+$ macrophages, which is an important cell subset to tackle bacterial infections due to the sensor role of this molecule (Fabriek et al., 2009), points out to a mechanism involved in the impairment in the local pulmonary immune response which potentially may favour the co-infection with secondary commensal microorganisms leading to bronchopneumonia.

Besides the role of CD163 molecule, other potential mechanisms involved in the onset of the bronchopneumonia along PRRSV-1 infection were evaluated. CD14 and iNOS are involved in lung inflammation after infection with PRRSV (Van Gucht et al., 2004, 2005; Yan et al., 2017) Upregulation of CD14, as the primary LPS receptor, after infection with PRRSV sensitises the lungs for the production of proinflammatory cytokines and respiratory signs upon exposure to bacterial lipopolysaccharides (LPS) (Van Gucht et al., 2005). For its part, iNOS is mainly expressed in response to different stimuli, such as cytokines and LPS, playing a role in tissue injury upon production of NO (Wink et al., 2011). In this study, an increase in the number of $CD14^+$ cells was observed after PRRSV-1 infection in association with suppurative bronchopneumonia, which was more evident in Lena-infected piglets at 6 – 8 dpi. This increase was mainly due to $CD14^+$ monocytes, interstitial and intravascular macrophages infiltrating extensive areas of the interstitium. The influx of $CD14^+$ immature macrophages and monocyte may be explained by an attempt to replenish the loss of $CD163^+$ macrophages restoring the normal lung function. On the other hand, the increase of $CD14^+$ cells implies a higher availability of the LPS-LBP complex receptor, which is likely to sensitise the lung to future secondary bacterial infections making the onset of PRDC easier (Van Gucht et al., 2005). In the case of iNOS, a significant increase in the number of $iNOS^+$ cells, mainly because of PAMs and interstitial macrophages, was observed in areas of interstitial pneumonia as well as bronchopneumonia in Lena-infected pigs. The induction of iNOS has been associated with both a direct effect of the viral replication or viral components and an indirect effect mediated by cytokines, such as IFN-γ, or by LPS (Akaike and Maeda, 2000). Of note, the peak of iNOS in Lena-infected animals appeared in our study just after the peak of PRRSV replication in the lung as well as after the peak of serum IFN-γ, being associated with the maximum lung injury and bronchopneumonia lesion. These factors may play a role in the regulation of iNOS expression along PRRSV infection and its role in lung injury development (Yan et al., 2017).

After a cascade of proinflammatory events, the host is able to trigger off the release of anti-inflammatory or regulatory mediators to restrain the extent of the injury. Thus, the role of CD200R1 and FoxP3 as molecules of interest was examined in the present study. A strong positive correlation was detected among the frequency of $CD200R1^+$ cells and the microscopic score which was mainly associated with a higher severity of typical interstitial pneumonia and suppurative bronchopneumonia in Lena-infected

pigs. Interestingly, CD200R1, which has been involved in reducing the expression of proinflammatory cytokines in a wide range of inflammatory diseases (Vaine & Soberman, 2014), was mostly detected in intravascular and interstitial macrophages located inside or surrounding bronchopneumonia foci. Likewise, an increase of frequency of $CD200R1^{+}$ cells and $FoxP3^{+}$ cells between 6 and 8 dpi was triggered by both PRRSV-1 strains when the lung injury was higher. To the authors' knowledge, the role of CD200R1 in viral diseases of swine is largely unknown. Previous studies in a murine model reported that virus influenza infection induced the upregulation of CD200R1 in macrophages, decreasing their responsiveness and increasing the sensitivity to bacterial infection and finally severe lung injury (Snelgrove et al., 2008; Vaine & Soberman, 2014). In contrast, CD200/CD200R1 signalling pathway limited type I IFN production during coronavirus infection protecting the host from cytokine storm (Vaine & Soberman, 2014). FoxP3 has been reported as a potential inhibitor of the cell-mediated immune response in pigs and along PRRSV infection (Ferrarini et al., 2015; Silva-Campa et al., 2009; 2010); however, its role in the immunopathogenesis of PRRSV-induced lung injury is unexplored. Immune checkpoints and other regulatory molecules, such as FoxP3, have been reported to play a dual role along viral infections, regulating immunopathology and tissue repair during acute viral infection, but leading to exhaustion and suppression of antiviral immune responses in the chronic infection (Arpaia et al., 2015; Schönrich and Raftery, 2019; Wang et al., 2018). Taken together, our results highlight the upregulation of CD200R1 and FoxP3 as mechanisms involved in the constraint and recovery of lung injury during acute PRRSV infection. Further studies are needed to determine the mechanisms involved in the activation of these molecules during acute PRRSV as well as their role and related signalling pathways along the persistent infection.

5. Conclusion

The present study dissects the immunopathology of lung injury along acute infection with PRRSV-1 strains of different virulence, revealing a drop in the number of $CD163^+$ cells together with an enhancement in the expression of CD14 and iNOS as mechanisms involved in the earlier and higher extent of lung lesion in Lena-infected piglets. These changes could sensitise the lung to future secondary bacterial infections. In addition, the increase in the number of $CD14^+$ cells is likely to respond to an attempt to replenish the $CD163^+$ macrophages subset lost along the infection with both PRRSV-1 strains. On the other hand, the increase in the expression of CD200R1 and FoxP3 represents potential pathways activated to contain the inflammatory response.

Conflict of interest

The authors declare that they have no competing interest.

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References

Akaike, T., & Maeda, H. (2000). Nitric oxide and virus infection. Immunology, 101(3), 300–308. https://doi.org/10.1046/j.1365-2567.2000.00142.x

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Morgan, S. B., Graham, S. P., Frossard, J. P., Drew, T. W., & Salguero, F. J. (2015). A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: Upregulation of IL-1α in highly pathogenic strain induced lesions. Veterinary Immunology and Immunopathology, 164(3–4), 137–147. https://doi.org/10.1016/j.vetimm.2015.02.003

Arpaia, N., Green, J. A., Moltedo, B., Arvey, A., Hemmers, S., Yuan, S., Treuting, P. M., & Rudensky, A. Y. (2015). A Distinct Function of Regulatory T Cells in Tissue Protection. Cell, 162(5), 1078–1089. https://doi.org/10.1016/j.cell.2015.08.021

Balka, G., Podgórska, K., Brar, M. S., Bálint, Á., Cadar, D., Celer, V., Dénes, L., Dirbakova, Z., Jedryczko, A., Márton, L., Novosel, D., Petrović, T., Sirakov, I., Szalay, D., Toplak, I., Leung, F. C. C., & Stadejek, T. (2018). Genetic diversity of PRRSV 1 in Central Eastern Europe in 1994-2014: Origin and evolution of the virus in the region. Scientific Reports, 8(1), 1–12. https://doi.org/10.1038/s41598-018-26036-w

Bordet, E., Maisonnasse, P., Renson, P., Bouguyon, E., Crisci, E., Tiret, M., Descamps, D., Bernelin-Cottet, C., Urien, C., Lefèvre, F., Jouneau, L., Bourry, O., Leplat, J. J., Schwartz-Cornil, I., & Bertho, N. (2018). Porcine alveolar macrophage-like cells are pro-inflammatory pulmonary intravascular macrophages that produce large titers of porcine reproductive and respiratory syndrome virus. Scientific Reports, 8(1), 10172. https://doi.org/10.1038/s41598-018-28234-y

Brockmeier, S. L., Loving, C. L., Nelson, E. A., Miller, L. C., Nicholson, T. L., Register, K. B., Grubman, M. J., Brough, D. E., & Kehrli, M. E. (2012). The presence of alpha interferon at the time of infection alters the innate and adaptive immune responses to porcine reproductive and respiratory syndrome virus. Clinical and Vaccine Immunology, 19(4), 508–514. https://doi.org/10.1128/CVI.05490-11

Brockmeier, S. L., Loving, C. L., Palmer, M. V., Spear, A., Nicholson, T. L., Faaberg, K. S., & Lager, K. M. (2017). Comparison of Asian porcine high fever disease isolates of porcine reproductive and respiratory syndrome virus to United States isolates for their ability to cause disease and secondary bacterial infection in swine. Veterinary Microbiology, 203, 6–17. https://doi.org/10.1016/j.vetmic.2017.02.003

Burkard, C., Lillico, S. G., Reid, E., Jackson, B., Mileham, A. J., Ait-Ali, T., Whitelaw, C. B. A., & Archibald, A. L. (2017). Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLoS Pathogens, 13(2), e1006206. https://

doi.org/10.1371/journal.ppat.1006206

Canelli, E., Catella, A., Borghetti, P., Ferrari, L., Ogno, G., De Angelis, E., Corradi, A., Passeri, B., Bertani, V., Sandri, G., Bonilauri, P., Leung, F. C., Guazzetti, S., & Martelli, P. (2017). Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Veterinary Microbiology, 210(July), 124–133. https://doi.org/10.1016/j.vetmic.2017.09.002

Das, P. B., Dinh, P. X., Ansari, I. H., De Lima, M., Osorio, F. A., & Pattnaik, A. K. (2010). The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor CD163. Journal of Virology, 84(4), 1731–1740. https://doi.org/10.1128/JVI.01774-09

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary Microbiology, 56(1–2), 9–19. https://doi.org/10.1016/S0378-1135(96)01347-8

Fabriek, B. O., Bruggen, R. Van, Deng, D. M., Ligtenberg, A. J. M., Nazmi, K., Schornagel, K., Vloet, R. P. M., Dijkstra, C. D., & Van Den Berg, T. K. (2009). The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. Blood, 113(4), 887–892. https://doi.org/10.1182/blood-2008-07-167064

Ferrarini, G., Borghetti, P., De Angelis, E., Ferrari, L., Canelli, E., Catella, A., Di Lecce, R., & Martelli, P. (2015). Immunoregulatory signal FoxP3, cytokine gene expression and IFN-γ cell responsiveness upon porcine reproductive and respiratory syndrome virus (PRRSV) natural infection. Research in Veterinary Science, 103, 96–102. https://doi.org/10.1016/j.rvsc.2015.09.018

Frydas, I. S., Verbeeck, M., Cao, J., & Nauwynck, H. J. (2013). Replication characteristics of porcine reproductive and respiratory syndrome virus (PRRSV) European subtype 1 (Lelystad) and subtype 3 (Lena) strains in nasal mucosa and cells of the monocytic lineage: Indications for the use of new receptors of PRRSV (Lena). Veterinary Research, 44(1), 73. https://doi.org/10.1186/1297-9716-44-73

Geldhof, M. F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U. U., & Nauwynck, H. J. (2012). Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. BMC Veterinary Research, 8(1), 182. https://doi.org/10.1186/1746-6148-8-182

Gómez-Laguna, J., Salguero, F. J., Barranco, I., Pallarés, F. J., Rodríguez-Gómez, I. M., Bernabé, A., & Carrasco, L. (2010). Cytokine Expression by Macrophages in the Lung of Pigs Infected with the Porcine Reproductive and Respiratory Syndrome Virus. Journal of Comparative Pathology, 142(1), 51–60. https://doi.org/10.1016/j.jcpa.2009.07.004

Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., & Carrasco, L. (2013). Immunopathogenesis of porcine reproductive and respiratory syndrome in the respiratory tract of pigs. The Veterinary Journal, 195(2), 148–155. https://doi.org/10.1016/j.tvjl.2012.11.012

Gorbalenya AE, Krupovic M, Siddell S, Varsani A, K. J. (2018). International Committee on Taxonomy of Viruses (ICTV). Retrieved 6 April 2019, from International Committee on Taxonomy of Viruses (ICTV) Website: Https://Talk.Ictvonline.Org/Taxonomy/p/Taxonomy-History?Taxnode_id=20186087.https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20186087

Greenbaum, E., Furst, A., Kiderman, A., Stewart, B., Levy, R., Schlesinger, M., Morag, A., & Zakay-Rones, Z. (2001). Area under the viraemia curve versus absolute viral load: Utility for predicting symptomatic cytomegalovirus infections in kidney transplant patients. Journal of Medical Virology, 65(1), 85–89. https://doi.org/10.1002/jmv.2005

Holtkamp, D. J., Kliebenstein, J. B., Neumann, E. J., Zimmerman, J. J., Rotto, H. F., Yoder, T. K., Wang, C., Yeske, P. E., Mowrer, C. L., & Haley, C. A. (2013). Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. Journal of Swine Health and Production, 21(2), 72–84.

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A., & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Veterinary Research, 6(1), 30. https://doi.org/10.1186/1746-6148-6-30

Käser, T., Gerner, W., Hammer, S. E., Patzl, M., & Saalmüller, A. (2008). Detection of Foxp3 protein expression in porcine T lymphocytes. Veterinary Immunology and Immunopathology, 125(1–2), 92–101. https://doi.org/10.1016/j.vetimm.2008.05.007

Liu, Y., Shi, W., Zhou, E., Wang, S., Hu, S., Cai, X., Rong, F., Wu, J., Xu, M., Xu, M., & Li, Q. (2010). Dynamic changes in inflammatory cytokines in pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Clinical and Vaccine Immunology, 17(9), 1439–1445. https://doi.org/10.1128/CVI.00517-09

Lunney, J. K., Fritz, E. R., Reecy, J. M., Kuhar, D., Prucnal, E., Molina, R., Christopher-Hennings, J., Zimmerman, J., & Rowland, R. R. R. (2010). Interleukin-8, interleukin-1beta, and interferon-gamma levels are linked to PRRS virus clearance. Viral Immunology, 23(2), 127–134. https://doi.org/10.1089/vim.2009.0087

Mattsson, J. G., Bergström, K., Wallgren, P., & Johansson, K.-E. E. (1995). Detection of Mycoplasma hyopneumoniae in nose swabs from pigs by in vitro amplification of the 16S rRNA gene. Journal of Clinical Microbiology, 33(4), 893–897. http://www.ncbi.nlm.nih.gov/pubmed/7540629

Meulenberg, J. J. M., Hulst, M. M., De Meijer, E. J., Moonen, P. L. J. M., Den Besten, A., De Kluyver, E. P., Wensvoort, G., & Moormann, R. J. M. (1993). Lelystad virus, the causative agent of porcine

epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology, 192(1), 62–72. https://doi.org/10.1006/viro.1993.1008

Morgan, S. B., Frossard, J. P., Pallares, F. J., Gough, J., Stadejek, T., Graham, S. P., Steinbach, F., Drew, T. W., & Salguero, F. J. (2016). Pathology and Virus Distribution in the Lung and Lymphoid Tissues of Pigs Experimentally Inoculated with Three Distinct Type 1 PRRS Virus Isolates of Varying Pathogenicity. Transboundary and Emerging Diseases, 63(3), 285–295. https://doi.org/10.1111/tbed.12272

Morgan, S. B., Graham, S. P., Salguero, F. J., Sánchez Cordón, P. J., Mokhtar, H., Rebel, J. M. J., Weesendorp, E., Bodman-Smith, K. B., Steinbach, F., & Frossard, J. P. (2013). Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Veterinary Microbiology, 163(1–2), 13–22. https://doi.org/10.1016/j.vetmic.2012.11.024

Nathues, H., Alarcon, P., Rushton, J., Jolie, R., Fiebig, K., Jimenez, M., Geurts, V., & Nathues, C. (2017). Cost of porcine reproductive and respiratory syndrome virus at individual farm level – An economic disease model. Preventive Veterinary Medicine, 142, 16–29. https://doi.org/10.1016/j.prevetmed.2017.04.006

Pathogenesis and antigenic characterization of a new East European Subtype 3 PRRSV isolate. (2010). 1–10.

Poderoso, T., Martínez de la Riva, P., Uenishi, H., Alvarez, B., Toki, D., Nieto-Pelegrín, E., Alonso, F., Domínguez, J., Ezquerra, A., & Revilla, C. (2019). Analysis of the expression of porcine CD200R1 and CD200R1L by using newly developed monoclonal antibodies. Developmental & Comparative Immunology, 100(June), 103417. https://doi.org/10.1016/j.dci.2019.103417

Renson, P., Rose, N., Le Dimna, M., Mahé, S., Keranflec'h, A., Paboeuf, F., Belloc, C., Le Potier, M. F., & Bourry, O. (2017). Dynamic changes in bronchoalveolar macrophages and cytokines during infection of pigs with a highly or low pathogenic genotype 1 PRRSV strain. Veterinary Research, 48(1), 15. https://doi.org/10.1186/s13567-017-0420-y

Rijkers, E. S. K., de Ruiter, T., Baridi, A., Veninga, H., Hoek, R. M., & Meyaard, L. (2008). The inhibitory CD200R is differentially expressed on human and mouse T and B lymphocytes. Molecular Immunology, 45(4), 1126–1135. https://doi.org/10.1016/j.molimm.2007.07.013

Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallarés, F. J., Mateu, E., Carrasco, L., & Gómez-Laguna, J. (2019). Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells. Veterinary Microbiology, 235, 101–109. https://doi.org/10.1016/j.vetmic.2019.06.011

Rowland, R. R. R., Steffen, M., Ackerman, T., & Benfield, D. A. (1999). The evolution of porcine reproductive and respiratory syndrome virus: Quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. Virology, 259(2), 262–266. https://doi.org/10.1006/viro.1999.9789

Ruedas-Torres, I., Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallares, F. J., Barranco, I., Carrasco, L., & Gómez-Laguna, J. (2020). Activation of the extrinsic apoptotic pathway in the thymus of piglets infected with PRRSV-1 strains of different virulence. Veterinary Microbiology, 243, 108639. https://doi.org/10.1016/j.vetmic.2020.108639

Sánchez-Carvajal, J. M., Rodríguez-Gómez, I. M., Carrasco, L., Barranco, I., Álvarez, B., Domínguez, J., Salguero, F. J., & Gómez-Laguna, J. (2019). Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence. Veterinary Research Communications, 43(3), 187–195. https://doi.org/10.1007/s11259-019-09755-x

Sánchez, C., Doménech, N., Alonso, F., Ezquerra, A., Domínguez, J., & Vázquez, J. (1999). The porcine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. Journal of Immunology, 162(9), 5230–5237.

Schönrich, G., & Raftery, M. J. (2019). The PD-1/PD-L1 axis and virus infections: A delicate balance. In Frontiers in Cellular and Infection Microbiology (Vol. 9, p. 207). Frontiers Media S.A. https://doi.org/10.3389/fcimb.2019.00207

Shi, M., Lam, T.T.Y., Hon, C. C., Hui, R. K. H., Faaberg, K. S., Wennblom, T., Murtaugh, M. P., Stadejek, T., & Leung, F. C. C. (2010). Molecular epidemiology of PRRSV: A phylogenetic perspective. Virus Research, 154(1–2), 7–17. https://doi.org/10.1016/j.virusres.2010.08.014

Sibila, M., Calsamiglia, M., Segalés, J., Blanchard, P., Badiella, L., Le Dimma, M., Jestin, A., & Domingo, M. (2004). Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. American Journal of Veterinary Research, 65(1), 88–92. https://doi.org/10.2460/ajvr.2004.65.88

Silva-Campa, E., Cordoba, L., Fraile, L., Flores-mendoza, L., Montoya, M., & Hernández, J. (2010). European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology, 396(2), 264–271. https://doi.org/10.1016/j.virol.2009.10.024

Silva-Campa, E., Flores-Mendoza, L., Reséndiz, M., Pinelli-Saavedra, A., Mata-Haro, V., Mwangi, W., & Hernández, J. (2009). Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. Virology, 387(2), 373–379. https://doi.org/10.1016/j.virol.2009.02.033

Sinn, L. J., Klingler, E., Lamp, B., Brunthaler, R., Weissenböck, H., Rümenapf, T., & Ladinig, A. (2016). Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria. Porcine Health Management, 2, 1–10. https://doi.org/10.1186/s40813-016-0044-z

Snelgrove, R. J., Goulding, J., Didierlaurent, A. M., Lyonga, D., Vekaria, S., Edwards, L., Gwyer, E., Sedgwick, J. D., Barclay, A. N., & Hussell, T. (2008). A critical function for CD200 in lung

immune homeostasis and the severity of influenza infection. Nature Immunology, 9(9), 1074–1083. https://doi.org/10.1038/ni.1637

Stadejek, T., Larsen, L. E., Podgórska, K., Bøtner, A., Botti, S., Dolka, I., Fabisiak, M., Heegaard, P. M. H., Hjulsager, C. K., Huć, T., Kvisgaard, L. K., Sapierzyński, R., & Nielsen, J. (2017). Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs. Veterinary Microbiology, 209(April), 13–19. https://doi.org/10.1016/j.vetmic.2017.05.011

Stadejek, T., Stankevicius, A., Murtaugh, M. P., & Oleksiewicz, M. B. (2013). Molecular evolution of PRRSV in Europe: Current state of play. Veterinary Microbiology, 165(1–2), 21–28. https://doi.org/10.1016/j.vetmic.2013.02.029

PRRS Virus-Induced Damage to Intravascular Macrophages, 43 (testimony of R. Thanawongnuwech, Patrick G Halbur, R. Royer, & J. Bruna). https://lib.dr.iastate.edu/swinereports_1997/43

Thanawongnuwech, R., Halbur, P. G., & Thacker, E. L. (2000). The role of pulmonary intravascular macrophages in porcine reproductive and respiratory syndrome virus infection. In Animal health research reviews / Conference of Research Workers in Animal Diseases (Vol. 1, Issue 2, pp. 95–102). https://doi.org/10.1017/S1466252300000086

Vaine, C. A., & Soberman, R. J. (2014). The CD200-CD200R1 Inhibitory Signaling Pathway. Immune Regulation and Host-Pathogen Interactions. In Advances in Immunology (Vol. 121, pp. 191–211). Academic Press Inc. https://doi.org/10.1016/B978-0-12-800100-4.00005-2

Van Gorp, H., Van Breedam, W., Delputte, P. L., & Nauwynck, H. J. (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. Journal of General Virology, 89(12), 2943–2953. https://doi.org/10.1099/vir.0.2008/005009-0

Van Gucht, S., Labarque, G., & Van Reeth, K. (2004). The combination of PRRS virus and bacterial endotoxin as a model for multifactorial respiratory disease in pigs. Veterinary Immunology and Immunopathology, 102(3), 165–178. https://doi.org/10.1016/j.vetimm.2004.09.006

Van Gucht, S., Reeth, K. Van, Nauwynck, H., & Pensaert, M. (2005). Porcine Reproductive and Respiratory Syndrome Virus Infection Increases CD14 Expression and Lipopolysaccharide-Binding Protein in the Lungs of Pigs. Viral Immunology, 18(1), 116–126. https://doi.org/10.1089/vim.2005.18.116

Van Reeth, K., & Nauwynck, H. (2000). Proinflammatory cytokines and viral respiratory disease in pigs. Veterinary Research, 31(2), 187–213. https://doi.org/10.1051/vetres:2000113

Wang, L., Wang, X., Tong, L., Wang, J., Dou, M., Ji, S., Bi, J., Chen, C., Yang, D., He, H., Bai, C., Zhou, J., & Song, Y. (2018). Recovery from acute lung injury can be regulated via modulation of regulatory T cells and Th17 cells. Scandinavian Journal of Immunology, 88(5), e12715. https://doi.org/10.1111/sji.12715

Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., Graaf, D. J. P. De, Graham, S. P., & Rebel, J. M. J. (2013). Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of

differing virulence. Veterinary Microbiology, 163(1–2), 1–12. https://doi.org/10.1016/j.vetmic.2012.09.013

Weesendorp, E., Rebel, J. M. J., Popma-De Graaf, D. J., Fijten, H. P. D., & Stockhofe-Zurwieden, N. (2014). Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Veterinary Microbiology, 174(1–2), 127–138. https://doi.org/10.1016/j.vetmic.2014.09.010

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., & Wagenaar, F. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. The Veterinary Quarterly, 13(3), 121–130. https://doi.org/10.1080/01652176.1991.9694296

Wink, D. A., Hines, H. B., Cheng, R. Y. S., Switzer, C. H., Flores-Santana, W., Vitek, M. P., Ridnour, L. A., & Colton, C. A. (2011). Nitric oxide and redox mechanisms in the immune response. Journal of Leukocyte Biology, 89(6), 873–891. https://doi.org/10.1189/jlb.1010550

Wright, G. J., Cherwinski, H., Foster-Cuevas, M., Brooke, G., Puklavec, M. J., Bigler, M., Song, Y., Jenmalm, M., Gorman, D., McClanahan, T., Liu, M.-R., Brown, M. H., Sedgwick, J. D., Phillips, J. H., & Barclay, A. N. (2003). Characterization of the CD200 Receptor Family in Mice and Humans and Their Interactions with CD200. The Journal of Immunology, 171(6), 3034–3046. https://doi.org/10.4049/jimmunol.171.6.3034

Yan, M., Hou, M., Liu, J., Zhang, S., Liu, B., Wu, X., & Liu, G. (2017). Regulation of iNOS-Derived ROS Generation by HSP90 and Cav-1 in Porcine Reproductive and Respiratory Syndrome Virus-Infected Swine Lung Injury. Inflammation, 40(4), 1236–1244. https://doi.org/10.1007/s10753-017-0566-9

Estudio 5 / Study 5

Objetivo 3 / Objective 3

Estudio 5: "Characterisation of PRRSV-1-induced lung lesion using MALDI mass spectrometry imaging".

Objetivo 3: Evaluar la expresión de biomarcadores de interés, como CD163s, proteínas de fase aguda o citoquinas, relacionados con la respuesta inflamatoria y respuesta inmune a nivel sistémico y/o local como indicadores de la infección y volución por cepas de distinta virulencia del virus del PRRS

Objective 3: Assessment of potential biomarkers expression such as sCD163, acute phase proteins o cytokines involved in the systemic or local inflammatory and immune response as potential biomarkers of infection in infected pigs with PRRSV-1 strains of different virulence.

Characterisation of PRRSV-1-induced lung lesion using MALDI mass spectrometry imaging

Jose María Sánchez-Carvajal[1], Irene Magdalena Rodríguez-Gómez[1], Inés Ruedas-Torres[1], Francisco José Pallarés[2], Librado Carrasco[1], Eduardo Chicano-Gálvez[3§], Jaime Gómez-Laguna [1§]

[1] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain

[2] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Murcia, 30100, Murcia, Spain

[3] Proteomics Unit, IMIBIC/UCO/HURS, Cordoba, Spain

[§] Eduardo Chicano-Gálvez and Jaime Gómez-Laguna share the senior authorship

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is considered one of the costliest viral diseases for the modern pig industry worldwide (Nathues et al., 2017; Valdes-Donoso et al., 2018). PRRS has two clinical presentations, which may differ among herds, reproductive failure in sows or gilts and respiratory disorders in growing pigs (Meulenberg et al., 1993; Wensvoort et al., 1991). PRRS virus (PRRSV), the aetiological agent of PRRS, is included within the genus Betaarterivirus (family Arteriviridae, order Nidovirales) as two different virus species, Betarterivirus suid 1 (formerly PRRSV-1) and Betarterivirus suid 2 (formerly PRRSV-2) (Gorbalenya et al., 2018).

PRRSV is well known to modulate the host immune response by inducing an impairment of both innate and adaptive immune responses (Butler et al., 2014; Huang et al., 2015; Mair et al., 2014). Although PRRSV replicates mainly in the lung (Duan et al., 1997), it can also replicate in other organs eliciting a dissimilar immune response depending on both the virulence of the strain and the infected tissue (Amarilla et al., 2016; Barranco et al., 2012; Inés Ruedas-Torres et al., 2020). Therefore, the evaluation of target organs turns into essential to decipher the immunopathogenesis of PRRSV infection with strains of different virulence.

Different outbreaks caused by virulent PRRSV-1 strains have been reported across Europe since 2006 inducing distinctive clinical features, such as high mortality rate, fever and severe respiratory disorders (Canelli et al., 2017; Karniychuk et al., 2010; Morgan et al., 2013; Sinn et al., 2016; Weesendorp et al., 2014; Yuzhakov et al., 2017). These clinical signs have been associated with an earlier and exacerbated inflammatory response which leads to a severe acute damage in the lung and primary lymphoid organs (Amarilla et al., 2015, 2016; Ogno et al., 2019; Renson et al., 2017; I. Ruedas-Torres et al., 2020; Sánchez-Carvajal et al., 2020; Weesendorp et al., 2014). PRRSV triggers a typical interstitial pneumonia as a result of the thickening of the alveolar septa with infiltrate of lymphocytes and macrophages (Halbur et al., 1995, 1996); however, the proliferation of secondary commensal bacteria is frequently observed after infection with virulent PRRSV strains leading to the onset of suppurative bronchopneumonia (Brockmeier et al., 2017; Karniychuk et al., 2010; Renson et al., 2017; Rodríguez-Gómez et al., 2019; Sinn et al., 2016). To date, little is known about

what proteins might be involved in the development of these lesions as well as their role in the pathogenesis of the disease.

Several studies have analysed changes in cytokine mRNA expression in both BALF and lung tissue after infection with PRRSV-1 virulent strains (Renson et al., 2017; Weesendorp et al., 2014). Nonetheless, certain differences among mRNA abundance and actual protein expression level may be evident, according to posttranscriptional regulatory processes which control steady-state protein abundance (Fortelny et al., 2017; Liu et al., 2016; Vogel and Marcotte, 2012). In this sense, knowledge on protein changes upon PRRSV infection might be relevant to figure out the host response against the virus and indeed to characterise lung damage.

Proteomics, in particular MALDI-MS imaging, has been successfully applied in an increasing number of pathogenesis studies, biomarker identification and protein-protein interaction studies in human diseases (Aichler and Walch, 2015; Neagu, 2019; Schöne et al., 2013). This approach provide information comparable to immunohistochemistry but with the advantage of measuring hundreds of analytes simultaneously as well as liquid-based proteomics does but gaining also information about spatial distribution pattern (Norris and Caprioli, 2013a, 2013b).

Currently, most proteomic approaches and efforts have been mainly focused on detecting differentially expressed proteins by means of in vitro studies upon PRRSV infection (Ding et al., 2012; Y. Li et al., 2017; Xiao et al., 2010; Zhang et al., 2009; Zhao et al., 2016; Y.-J. Zhou et al., 2014), however, we hypothesise that determining peptide molecular signature in lung tissue upon PRRSV infection would be critical to elucidate the pathogenesis of PRRSV-1, making easier the detection of potential biomarkers as well as the characterisation of the lesion.

2. Materials and methods

2.1. Porcine reproductive and respiratory syndrome strains, animals and experimental design

The virulent Lena strain (subtype 3 PRRSV-1), consider as the prototype of PRRSV-1 virulent strains (Karniychuk et al., 2010), as well as the low virulent 3249 strain (subtype 1 PRRSV-1) (Gimeno et al., 2011) were used in this study. Viral stocks were produced from the 4th passage of each strain on PAMs, titrated by means of immunoperoxidase monolayer assay and expressed as tissue culture infectious doses 50 (TCID50)/mL (3249 strain: 105.79 TCID50/mL; Lena strain: 105.66 TCID50/mL).

The animals and samples employed in this study are part of a project to investigate the pathogenesis of the infection with PRRSV-1 strains of different virulence (Rodríguez-Gómez et al., 2019). Briefly, fifty-two 4-week-old piglets (Landrace x Large White crossbred) were randomly assigned to three different groups and housed in separate pens in biosafety level III containment facilities: Lena group (n=20), 3249 group (n=20) and control group (n=12). At the beginning of the study, all pigs were negative for porcine circovirus type 2 (PCV2), PRRSV and Mycoplasma hyopneumoniae by ELISA and PCR assays (Mattsson et al., 1995; Sibila et al., 2004). Piglets were challenged with virulent Lena strain or low virulent 3249 strain (1 mL/nostril, 1x105 TCID50/mL) after one week of acclimation by intranasal inoculation with a mucosal atomiser (MAD Nasal™ Intranasal Mucosal Atomization Device, Teleflex, Alcalá de Henares, Madrid, Spain). The control group was mock inoculated with porcine alveolar macrophages supernatant diluted in culture medium (RPMI 1640 Medium, Thermo Fisher Scientific, Barcelona, Spain). Clinical symptoms and rectal temperature were daily recorded from two days after inoculation to 8 days post-inoculation (dpi). Three control pigs and five infected pigs from each group were necropsied at 1, 3, 6 and 8 dpi recording gross lung lesions by the same pathologist as described by Halbur et al. (1996). Parallel samples from the right lung were collected and immediately frozen at -80 °C for the analysis of lung viral load or fixed in 10 % neutral buffered formalin for histopathological evaluation followed by proteomic analysis by MALDI-MS imaging. All animal procedures were performed according to the guidelines of the European Union (Directive 2010/63/EU) and approved by the IRTA Ethics Committee and by the Catalan Autonomous Government (Project 3647; FUE-2017-00533413).

2.2. PRRSV lung viral load

A homogenate of lung tissue was made to carry out RNA isolation and purification by using TRIzolTM LS Reagent (Thermo Fisher Scientific) followed by NucleoSpin® RNA virus columns kit according to manufacturer's protocols (Macherey-Nagel, Düren, Germany). PRRSV RNA quantification was conducted using specific primers of target genes (Lena strain ORF7: forward primer AGCGCCAATTCAGAAAGAAA, reverse primer TGGATCGATTGCAGACAGAG; 3249 strain ORF7: forward primer GGCAAACGAGCTGTTAAACG, reverse primer AATTTCGGTCACATGGTTCC; Darwich et al., 2011). The ORF7 RT-PCR product from both 3249 and Lena strains was firstly ethanol precipitated and purified using ExoSAP-ITTM (Thermo Fisher Scientific). The purified products were quantified using BioDrop (BioDrop, UK). Then, serial 10-fold dilutions of 3249 or Lena ORF7 RT-PCR products with known quantities, ranging from 108 to 102 genomic copies/mL were used as standards generating a standard curve to determine PRRSV genomic copies in the lung. The RT-qPCR efficiency was of 99 % and high linearity (r = 0.99) for determining viral load, with a slope of 3.34, a detection limit of 1 copy/µL and a slope-intercept of 39.5. Then, viral load for either Lena- or 3249-infected piglets was quantified by RT-qPCR using VetMAX™ PRRSV EU/NA 2.0 kit (Thermo Fisher Scientific). For each animal, amplifications were run in duplicate in a MyiQ™2 Two-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Cyclin conditions consisted of 50 °C for 5 minutes (min), 95 °C for 10 min followed by 40 cycles of 95 °C for 3 seconds (s) and 60 °C for 30 s. An inter-run calibrator sample with a known number of PRRSV copies was introduced in each experiment to self-control inter-run variation.

2.3. Histopathological evaluation of lung tissue

Four-micron lung tissue sections were routinely processed for haematoxylin and eosin (H&E) staining. The histopathological findings were evaluated and graded by two different pathologists. The severity of lung lesions for the interstitial pneumonia was scored as previously described by Halbur et al. (1996): no microscopic lesions, 0; mild interstitial pneumonia, 1; moderate multifocal interstitial pneumonia, 2; moderate diffuse interstitial pneumonia, 3; and severe interstitial pneumonia, 4. In order to score the suppurative bronchopneumonia, a similar score was done as previously described by Rodríguez-Gómez et al. (2019): no microscopic lesions, 0; mild bronchopneumonia,

1; moderate multifocal bronchopneumonia, 2; moderate diffuse bronchopneumonia, 3; and severe bronchopneumonia, 4. The final score for each piglet was calculated as a sum of both the interstitial pneumonia and the bronchopneumonia scores.

2.4. Lung tissue preparation for MALDI-MS Imaging

Three animals were selected from each experimental group at 6 and 8 dpi according to their clinical signs, gross and microscopic lesions, and viral load to perform MALDI-MS Imaging analysis. Four-micron formalin-fixed paraffin-embedded lung tissue sections were mounted onto slides with indium tin oxide slides (Bruker Daltonics GmbH, Bremen, Germany) previously coated with poly-L-lysine (Sigma Aldrich, Steinheim, Germany) for downstream MALDI-MS imaging. Tissue sections were dewaxed in xylene for 20 min and rehydrated in descending grades of alcohol followed by an additional clean washing with ammonium bicarbonate 10mM (NH_4HCO_3) for 10 min. Then, a heat-induced antigen retrieval with Tris 100mM buffer pH 9 (98 °C, 30 min) was conducted. Slides were washed again with NH_4HCO_3 10 mM and dried under vacuum for 15 min before an on-tissue digestion step.

On-tissue tryptic digestion was performed by spraying a 0.1 µg/µL solution of trypsin (V5111, Promega, Madison, WI, USA) in NH_4HCO_3 25 mM and 10 % v/v trifluoroethanol (Sigma Aldrich) over the tissue sections and incubating them overnight at 37 °C into a saturated humid chamber. Then, slides were dried under vacuum. Trypsin deposition was made by SunCollect sprayer (Sunchrom, Friedrichsdorf, Germany) at a constant flow of 10 µL/min.

Following this step, a MALDI matrix solution consisting of 10 mg/mL alpha-cyano-4-hydroxycinnamic acid (HCCA, Sigma Aldrich) in 60 % ACN and 0.2 % TFA (Trifluoroacetic acid, Sigma Aldrich) was sprayed onto digested lung sections using the SunCollect sprayer. In addition, internal calibrants such as BradikyninF1-7, Angiotensin2 and Glu-Fibrinopeptide (Sigma Aldrich) were added to matrix solution. After matrix deposition, slides were dried under vacuum for 30 minutes.

2.5. MALDI-MS Imaging

Previously to run the acquisition, each slide was fixed with copper conductive tape on a special adapter plate for imaging mass spectrometry (HTX imaging, Chapel Hill, USA).

MALDI imaging data was acquired on AB-Sciex 5800 MALDI TOF/TOF mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with a Nd:YAG laser (355 nm) in positive ionization mode. Spectra were measured in the mass range of 650-1,800 m/z since at least 80 % of visualized peptides for these samples were in that range (data not shown). Laser intensity was adjusted to 3,300, delay extraction was set to 310 ns and a total of 150 shots per pixel were used. External calibration was made with four different references peaks (BradykininF-1,7: 757.3998 [M+H]+; Trypsin autolysis peak: 842.508 [M+H]+; Angiotensin II: 1046.5420 [M+H] +; and Glu-Fibrinopeptide: 1570.6770 [M+H] +). Peak assignments were performed using a tolerance of 25 ppm with a maximum mass deviation of 0.01 ppm and a minimum signal-to-noise ratio (S/N) of 100. In addition, deflector parameters were adjusted for each sample in order to ensure a resolution (FWHW) > 15,000 at reference mass peak of Glu-Fibrinopeptide. All datasets were processed by TOF/TOFTM Imaging Acquisition Software (ABSciex) using a lateral resolution of 150 µm for all samples. Tissue samples were randomly analysed to prevent any possible bias due to a variation in mass spectrometer sensitivity or a matrix influence.

2.6. Gene ontology (GO) terms enrichment and pathway analysis

The proteomic pathway tool String 11.0 (https://string-db.org/) was used for protein-protein network analysis and visualisation. Functional enrichment analysis of the upregulated proteins upon PRRSV-1 was conducted with ClueGO (version 2.3.3) and CluePedia (version 1.3.3), plugins for Cytoscape version 3.8, describing biological processes and reactome pathways integrated with immune system processes GO.

2.7. Data analysis

Differences between groups for rectal temperature, clinical signs, macroscopic and microscopic lung scores, as well as lung viral load, were evaluated for approximate

normality of distribution by the D'Agostino and Pearson omnibus normality test followed by the Mann Whitney's U non-parametric mean comparisons test. Data analyses and figures were performed by using GraphPad Prism 7.0 software (GraphPad Prism software 7.0, Inc., San Diego, CA, USA) and InkScape 0.92 software. A P value lower than 0.05 was considered statistically significant.

For MALDI-MS Imaging, acquired data were first exported into the common imzML open format, processed, and analysed in R (v.3.6.0) using Cardinal package (v1.10.0). Data files were normalised by total ion current (TIC) and spectra were peak picked to reduce dimensionality. In addition, all exported spectra underwent baseline subtraction to remove noise, resampling to lower data dimensionality and smoothing to remove tissue and measurement artefacts. Samples were spatially segmented using unsupervised clustering analysis by KMeans algorithm (k = 15). After clustering, lung tissue sections were "virtually microdissected", cleaning the dataset from histological artifacts or poor resolved areas. Then, a mean spectrum for each tissue was extracted for statistical analysis.

Statistical analysis was performed with MetaboAnalyst 4.0 software (http://www.metaboanalyst.ca/) using a one-way Analysis of Variance (ANOVA) test. Data sets were structured according to developer's instructions and limited to a m/z range from 700 to 1,800 with a total of 1,024 peaks. Datasets were previously normalised including sample median normalisation, logarithmic transformation and auto-scaling.

Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was used to represent the separation between the groups. A correlation analysis was made in order to discover plausible associated peaks that might define a progression between the different analysed conditions. Besides, a hierarchical clustering and heatmap that allow a visualization of the MALDI-MS analysis was also performed. All software used in our analysis have an open access from their corresponding authors repositories.

3. Results

3.1. PRRSV-1 infected piglets exhibited interstitial pneumonia and acute suppurative bronchopneumonia with the highest microscopic lung score at 6 and 8 dpi

Clinical observations and lung lesions have been thoroughly described by Rodríguez-Gómez et al. (2019). Briefly, both PRRSV-1-infected piglets exhibited clinical signs associated with respiratory disease, in addition, Lena-infected pigs presented a long period of hyperthermia (mean above 40.5 °C) with marked clinical signs score, both peaking at 6 dpi (Fig. 1-A). At necropsy, the main lung lesion consisted of tan-mottled areas, with a rubbery consistency, failure to collapse, atelectasis and consolidated areas. Lung macroscopic score gradually increased throughout the study in both PRRSV-1 infected groups, reaching the highest score in Lena group associated with more severe interstitial pneumonia and extensive areas of pulmonary consolidation in apical and medial lobes (Fig. 1-B). Supporting gross lesion, histopathological evaluation proved a mild to moderate interstitial pneumonia and emphysema with thickening of alveolar septa by infiltrating lymphocytes, macrophages, and occasional syncytial cells. Particularly, Lena-infected piglets developed suppurative bronchopneumonia characterised by extensive foci of neutrophils, cells debris and mucus filling the bronchial, bronchiolar and alveolar lumen at 6 and 8 dpi. Microscopic lung lesion severity increased during the course of PRRSV-1 infection reaching the maximum score at 8 dpi in both infected groups (Fig. 1-B) with a stronger and earlier onset in pigs inoculated with virulent Lena strain. Control animals showed neither clinical signs minimal lung lesion throughout the study.

3.2. Lung viral load peaks were reached at 6 dpi and 8 dpi in Lena- and 3249-infected pigs, respectively

At day 0, all animals were negative by RT-qPCR with pigs from control group remaining negative throughout the study. As early as 1 dpi, two out of five piglets in both infected groups were PRRSV-1 positive detecting viral RNA copies from 3 dpi onwards in all infected piglets. Virulent Lena strain induced higher lung tissue viral titres compared to low virulent 3249 strain ($P < 0.01$ at 3 dpi; $P < 0.05$ at 6 and 8 dpi) (Fig. 1-C), showing a peak at 6 dpi in Lena group (1.9x107 eq TCID50/mL) and at 8 dpi in 3249 group (1.9x106 eq TCID50/mL) (Fig. 1-C).

Figure 1

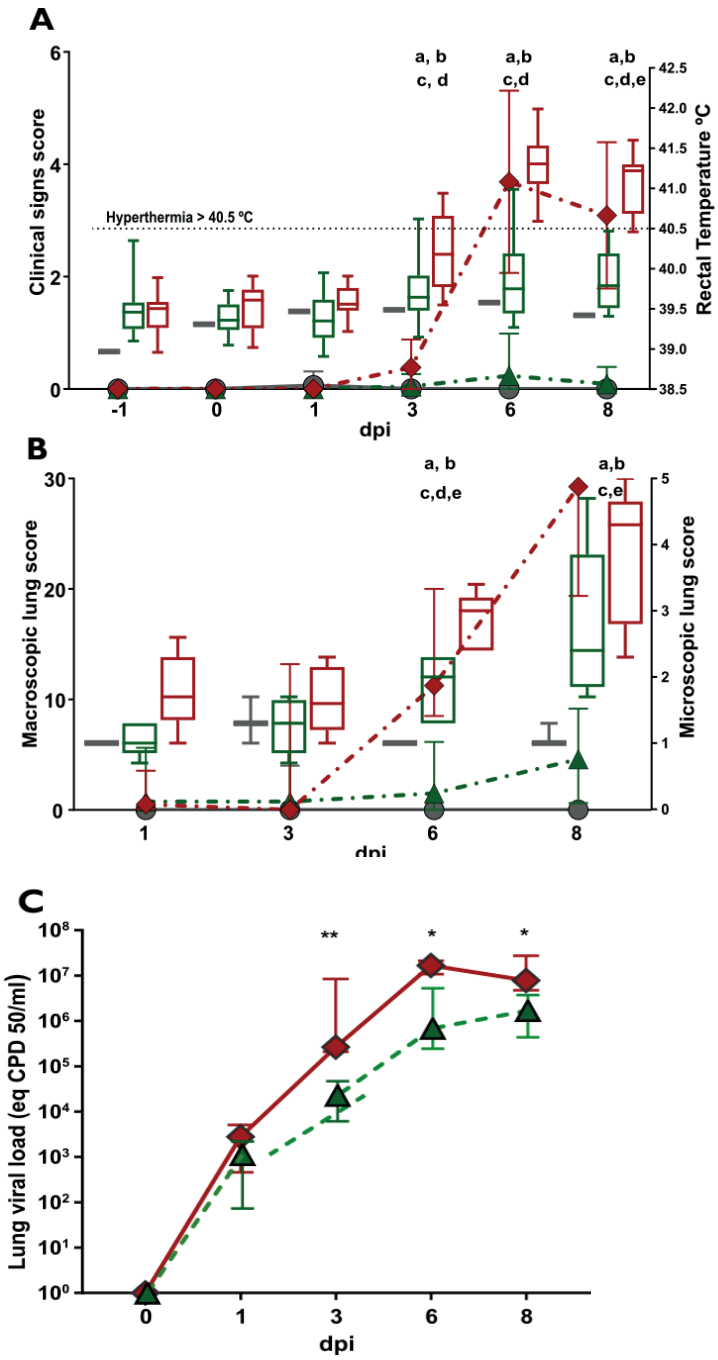
A
Clinical signs score
Rectal Temperature ºC
Hyperthermia > 40.5 ºC
a, b
c, d
a,b
c,d
a,b
c,d,e
dpi
B
Macroscopic lung score
Microscopic lung score
a, b
c,d,e
a,b
c,e
dpi
C
Lung viral load (eq CPD 50/ml)
**
*
*
dpi

Figure 1. Clinical signs score, rectal temperature, macroscopic and microscopic lung score as well as lung viral load. *(A) Clinical signs and rectal temperature were evaluated one-week after inoculation, no changes were detected among experimental groups. The diagram displays clinical signs score (primary axis) and rectal temperature (secondary axis). Line plots shows the mean clinical score with SD (primary axis) for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. Box plots with mix/max whiskers display the mean rectal temperature (secondary axis) for each group (control, gray boxes; 3249, green boxes; Lena, red boxes) and time point. A rectal temperature higher than 40.5 °C was considered as hyperthermia. (B) The diagram represents macroscopic (primary axis) and microscopic lung score (secondary axis). Line plots shows the median macroscopic score with range (primary axis) for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. Box plots with mix/max whiskers display the median rectal temperature (secondary axis) for each group (control, gray boxes; 3249, green boxes; Lena, red boxes) and time point. P value lower than 0.05 was considered statistically significant. For clinical sings score and macroscopic lung score "a" indicates a significant difference between Lena and control groups, "b" a significant difference between Lena and 3249 groups. For rectal temperature and microscopic lung score "c" indicates a significant difference between Lena and control groups, "d" a significant difference between Lena and 3249 groups, "e" a significant difference between 3249 and control groups (C) PRRSV lung viral load was quantified using RT-qPCR (efficiency: 99 %; slope=3.34; detection limit: 1 copy/µl; slope-intercept= 39.5; high linearity, r=0.99). Diagram displays the lung viral load (eq TCID50/mL) as the median with range of results obtained for each group (control, gray circles; 3249, green triangles; Lena, red diamonds) and time point. P value lower than 0.05 was considered statistically significant and represented as * P≤ 0.05 and ** P≤ 0.01. Standard deviation: SD. Days post-inoculation: dpi*

3.3. Identification of differentially expressed peptides induced by PRRSV-1 infection at 6 and 8 dpi

MALDI-MS imaging analyses were performed on selected animals (3 animals/group) from 6 and 8 dpi. These time-points were selected according to the detection of marked clinical sings, gross and microscopic lung lesion scores as well as viral load in Lena- and 3249-infected piglets. Differentially peptide profiles and their putative associated proteins, when comparing both infected groups with control group, are represented in the heat map and network of Figure 2. The sPLS-DA clustering of all lung tissues based on their peptide mass intensity profile showed an almost perfect distribution according to experimental group as well as time point (Fig 2-C). After ANOVA analysis, a total of fifty-four differentially expressed peaks were tentatively identified among PRRSV-1-infected and control groups. Thirty-eight of these putative proteins were upregulated at 6 dpi including proteins, such as heat shock protein beta-1 (HSPB1) , interferon-induced GTP-binding protein Mx1, bcl-2 homologous antagonist/killer (BAK1), apolipoprotein A-IV (APOA4), annexin A6 (ANXA6), matrix metallopeptidase 9 (MMP9), vimentin, apolipoprotein A-I (APOA1), ras-related protein Rap-1b (RAP1B) or nucleolysin TIA-1 isoform p40 (TIA1) (Tables 1 and 2). At 8 dpi, 19 upregulated proteins were tentatively identified, such as heat shock protein 72, complement C3 (C3), filamin-A (FLNA), annexin IV (ANXA6), cytoskeleton-associated protein 4 (CKAP4), ribosomal protein L13 (RPL13A), L-lactate dehydrogenase B chain, tropomyosin 1 (TPM1) or histone H4 (H4C1) (Tables 1 and 2).

Figure 2

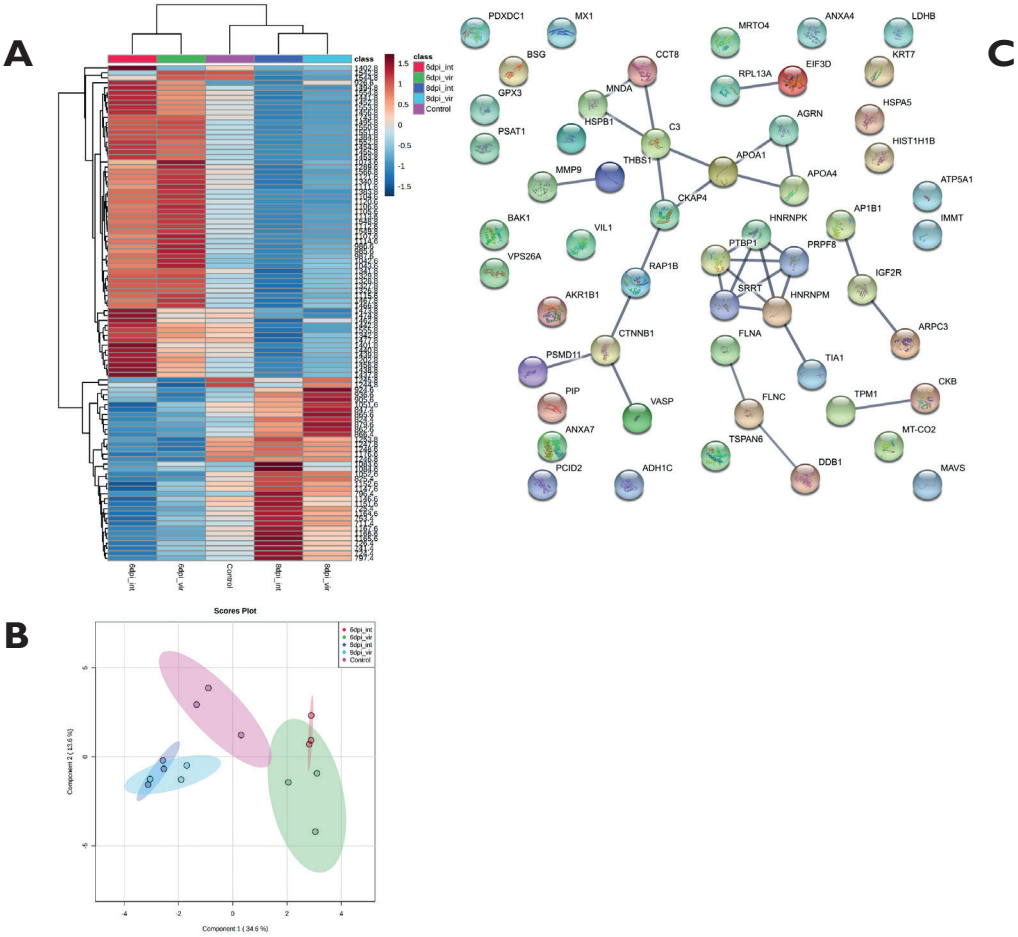

Figure 2. Identification of differentially expressed putative proteins induced by PRRSV-1 infection at 6 and 8 dpi. *(A) Data set of significant proteins expressed in the lungs is represented in a heat-map comparing both Lena and 3249 strains with control group at 6 and 8 dpi. (B) Score plot from sPLS-DA is shown based on differentially expressed peaks among Lena (green dots for 6 dpi; sky blue dots for 8 dpi) and 3249 (red dots for 6 dpi; dark blue for 8 dpi) infected groups and control group (pink dots). (C) Protein-protein interaction network was generated and visualised with STRING 11.0 for significant proteins detected (network enrichment P-value: 0.00108). These proteins are represented as sphere-shaped nodes; the association between proteins is represented with edges (lines) connecting them, thus thicker edges represent stronger associations.*

Table 1. *All significant peptide profiles (m/z) and their putative associated proteins (and gene names) upon PRRSV-1 infection in the lung grouped by Go terms (Biological processes).*

GO Term	Genes	Putative Proteins	m/z
Terpenoid metabolic process	ADH1C	Alcohol dehydrogenase 1C	796,45
	AGRN	Agrin	1,051.594
	AKR1B1	Aldo-keto reductase family 1 member B1	1,112.595
	APOA1	Apolipoprotein A-I	1,467.792
	APOA4	Apolipoprotein A-IV	1,439.801
Cell junction organization	AGRN	Agrin	1,051.594
	BSG	Basigin	1,073.595
	C3	Complement C3	1,152.605
	CTNNB1	Catenin beta 1	1,083.598
	FLNA	Filamin A	724.388
	FLNC	Filamin C	986.599
	RAP1B	Ras-related protein Rap-1b	1,494.795
	THBS1	Thrombospondin 1	1,544.792
	VASP	Vasodilator-stimulated phosphoprotein	1,342.794
Response to metal ion	ANXA7	Annexin A7	1,743.804
	BSG	Basigin	1,073.595
	HSPA5	Endoplasmic reticulum chaperone BiP	1,552.793
	MMP9	Matrix metalloproteinase 9	1,440.817
	THBS1	Thrombospondin 1	1,544.792
Regulated exocytosis	APOA1	Apolipoprotein A-I	1,467.792
	C3	Complement C3	1,152.605
	CCT8	T-complex protein 1 subunit theta	1,453.790
	CKAP4	Cytoskeleton-associated protein 4	1,146.600
	FLNA	Filamin-A	724.388
	IGF2R	Cation-independent mannose-6-phosphate receptor	1,176.594
	MMP9	Matrix metalloproteinase 9	1,440.817
	MNDA	Myeloid cell nuclear differentiation antigen	1,165.604
	PSMD11	26S proteasome non-ATPase regulatory subunit 11	1,341.799
	RAP1B	Ras-related protein Rap-1b	1,494.795
	THBS1	Thrombospondin-1	1,544.792
Positive regulation of cytokine production	C3	Complement C3	1,152.605
	CTNNB1	Catenin beta 1	1,083.598
	HSPB1	Heat shock protein beta 1	987,61
	MAVS	Mitochondrial antiviral-signalling protein	1,441.780
	MNDA	Myeloid cell nuclear differentiation antigen	1,165.604
	THBS1	Thrombospondin 1	1,544.792
	TSPAN6	Tetraspanin 6	1,555.800

GO Term	Genes	Putative Proteins	m/z
Regulation of body fluid levels	ANXA7	Annexin A7	1,743.804
	AKR1B1	Aldo-keto reductase family 1 member B1	1,112.595
	BSG	Basigin	1,073.595
	FLNA	Filamin-A	724.388
	HSPB1	Heat shock protein beta 1	987,61
	MT-CO2	Cytochrome c oxidase subunit 2	1,152.601
	PCID2	PCI domain-containing protein 2	1,202.758
	THBS1	Thrombospondin-1	1,544.792
Response to oxidative stress	APOA4	Apolipoprotein A-IV	1,439.801
	BAK1	Bcl-2 homologous antagonist/killer	1,550.796
	BSG	Basigin	1,073.595
	CTNNB1	Catenin beta 1	1,083.598
	GPX3	Glutathione peroxidase 3	1,554.801
	HNRNPM	Heterogeneous nuclear ribonucleoprotein M	1,542.801
	HSPB1	Heat shock protein beta 1	987,61
	MMP9	Matrix metalloproteinase 9	1,440.817
	TPM1	Tropomyosin alpha-1 chain	1,429.79
Posttranscriptional regulation of gene expression	EIF3D	Eukaryotic translation initiation factor 3 subunit D	1,442.801
	HNRNPM	Heterogeneous nuclear ribonucleoprotein M	1,542.801
	H4C1	Histone H4	1,167.601
	PSMD11	26S proteasome non-ATPase regulatory subunit 11	1,341.799
	PCID2	PCI domain-containing protein 2	1,202.758
	PTBP1	Polypyrimidine tract-binding protein 1	1,107.605
	SRRT	Serrate RNA effector molecule homolog	1,245.793
	RPL13A	60S ribosomal protein L13a	726,393
	THBS1	Thrombospondin 1	1,544.792
	TIA1	Nucleolysin TIA-1 isoform p40	1,553.778
Positive regulation of organelle organization	AGRN	Agrin	1,051.594
	AKR1B1	Aldo-keto reductase family 1 member B1	1,112.595
	APOA1	Apolipoprotein A-I	1,467.792
	ARPC3	Actin-related protein 2/3 complex subunit 3	725398
	BAK1	Bcl-2 homologous antagonist/killer	1,550.796
	C3	Complement C3	1,152.605
	CCT8	T-complex protein 1 subunit theta	1,453.790
	CTNNB1	Catenin beta 1	1,083.598
	DDB1	DNA damage-binding protein 1	1,114.593
	FLNA	Filamin-A	724.388
	H1-5	Histone H1.5	1,167.601
	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	1,340.804
	HSPA5	Endoplasmic reticulum chaperone BiP	1,552.793
	MMP9	Matrix metalloproteinase 9	1,440.817
	RAP1B	Ras-related protein Rap1b	1,494.795
	TPM1	Tropomyosin alpha-1 chain	1,429.79
	VASP	Vasodilator-stimulated phosphoprotein	1,342.794
	VIL1	Villin 1	1,166.605

GO Term	Genes	Putative Proteins	m/z
Regulation of cell death	AKR1B1	Aldo-keto reductase family 1 member B1	1,112.595
	ANXA4	Annexin A4	847,416
	BAK1	Bcl-2 homologous antagonist/killer	1,550.796
	BSG	Basigin	1,073.595
	CTNNB1	Catenin beta 1	1,083.598
	DDB1	DNA damage-binding protein 1	1,114.593
	FLNA	Filamin A	724.388
	GPX3	Glutathione peroxidase 3	1,554.801
	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	1,340.804
	HSPB1	Heat shock protein beta 1	987,61
	HSPA5	Endoplasmic reticulum chaperone BiP	1,552.793
	IGF2R	Cation-independent mannose-6-phosphate receptor	1,176.594
	MMP9	Matrix metalloproteinase 9	1,440.817
	MNDA	Myeloid cell nuclear differentiation antigen	1,165.604
	MT-CO2	Cytochrome c oxidase subunit 2	1,152.601
	PDXDC1	Pyridoxal-dependent decarboxylase domain-containing protein 1	1,495.787
	PCID2	PCI domain-containing protein 2	1,202.758
	PIP	Prolactin-inducible protein	1,329.814
	PTBP1	Polypyrimidine tract-binding protein 1	1,107.605
	QARS1	Glutamine--tRNA ligase	1,383.799
	THBS1	Thrombospondin 1	1,544.792
	TIA1	Nucleolysin TIA-1 isoform p40	1,553.778
	VIL1	Villin 1	1,166.605

Table 2. *All significant peptide profiles (m/z) and their putative associated proteins (and gene names) upon PRRSV-1 infection in the lung grouped by Go terms (Reactome and immunological processes).*

GO Term	Genes	Putative Proteins	m/z
Signaling by receptor tyrosine kinases	CTNNB1 HNRNPM HSPB1 MMP9 PTBP1 RAP1B THBS1 TIA1	Catenin beta 1 Heterogeneous nuclear ribonucleoprotein M Heat shock protein beta 1 Matrix metalloproteinase 9 Polypyrimidine tract-binding protein 1 Ras-related protein Rap-1b Thrombospondin 1 Nucleolysin TIA-1 isoform p40	Catenin beta 1 Heterogeneous nuclear ribonucleoprotein M Heat shock protein beta 1 Matrix metalloproteinase 9
Platelet activation, signaling and aggregation	APOA1 FLNA HSPA5 RAP1B THBS1	Apolipoprotein A-I Filamin-A Endoplasmic reticulum chaperone BiP Ras-related protein Rap-1b Thrombospondin-1	1,467.792 724.388 1,552.793 1,494.795 1,544.792
Cellular responses to external stimuli	GPX3 H1-5 H4C1 PSMD11 RPL13A	Glutathione peroxidase 3 Histone H1.5 Histone H4 26S proteasome non-ATPase regulatory subunit 11 60S ribosomal protein L13a	1,554.801 1,167.601 1,167.601 1,341.799 726,393
Neutrophil degranulation	C3 CCT8 CKAP4 IGF2R MMP9 MNDA PSMD11 RAP1B	Complement C3 T-complex protein 1 subunit theta Cytoskeleton-associated protein 4 Cation-independent mannose-6-phosphate receptor Matrix metalloproteinase 9 Myeloid cell nuclear differentiation antigen 26S proteasome non-ATPase regulatory subunit 11 Ras-related protein Rap-1b	1,152.605 1,453.790 1,146.600 1,176.594 1,440.817 1,165.604 1,341.799 1,494.795
mRNA Splicing - Major Pathway	HNRNPM HNRNPK HSPB1 PTBP1 RPL13A SRRT	Heterogeneous nuclear ribonucleoprotein M Heterogeneous nuclear ribonucleoprotein K Heat shock protein beta 1 Polypyrimidine tract-binding protein 1 60S ribosomal protein L13a Serrate RNA effector molecule homolog	1,542.801 1,340.804 987,61 1,107.605 726,393 1,245.793

3.4. Functional ontology classification of differentially regulated putative proteins in PRRSV-1 infected piglets

The proteomic pathway tool String 11.0 was used to illustrate the interactions between all regulated proteins. Figure 2-C shows protein–protein interactions by spatial positioning and linear connections with thicker edges (lines) indicating stronger associations between them. In addition, in order to provide gene ontology (GO) and biological process of the above proteins, functional analyses were performed. The ClueGo and the CluePedia plugins of Cytoscape were used to determine the enriched biological processes, reactome pathways and immune system processes GO of the 54 overexpressed proteins in the lung of PRRSV-1-infected piglets. Within biological processes 49 specific terms connected by 550 edges were found (Fig 3), the majority of upregulated proteins were involved in "regulation of cell death" (adjusted P-value = 1,92E-07), "positive regulation of cellular component organization" (adjusted P-value = 9,19E-07), "posttranscriptional regulation of gene expression" (adjusted P-value = 3,93E-05) and "response to oxidative stress" (adjusted P-value = 1,85E-05) (Table 1). Among reactome pathways and immune system processes (Fig 4), 11 specific terms connected by 94 edges were observed with mRNA "splicing-major pathway" (adjusted P-value = 0,02), neutrophil degranulation (adjusted P-value = 0.02), cellular responses to external stimuli (adjusted P-value = 0.03), platelet signalling and aggregation (adjusted P-value = 0.02), and signalling by receptor tyrosine kinases (adjusted P-value = 0.01) grouping the most of overexpressed proteins (Table 2).

Figure 3

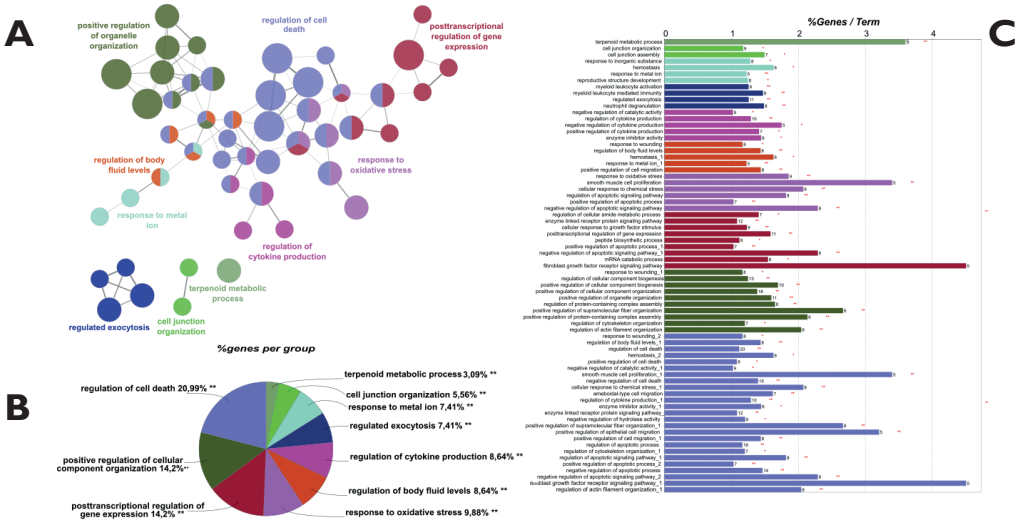

Figure 3. Biological process of differentially expressed putative proteins in the lung upon PRRSV-1 infection at 6 and 8 dpi. *(A) Functionally grouped networks associated with overexpressed proteins based on GO terms. Only the statistically significant terms (P < 0.05) in each group are represented using ClueGO. Terms are displayed as nodes (filled circle) linked by edges (lines) based on their κ score level (≥ 0.4), where only the label of the most significant term per group is shown. (B) Overview pie chart summarises the number of terms per group for differentially expressed proteins. (C) GO/pathway terms specific for overexpressed proteins. The bars represent the number of proteins associated with the terms. The percentage of proteins per term is shown as bar label. The same key colour used in the pie charts applies in these charts. P < 0.05 was used as a threshold for GO categories enrichment. Single (*) or double (**) asterisk indicate significant enriched GO terms at the P < 0.05 and P < 0.01 statistical levels, respectively.*

Figure 4

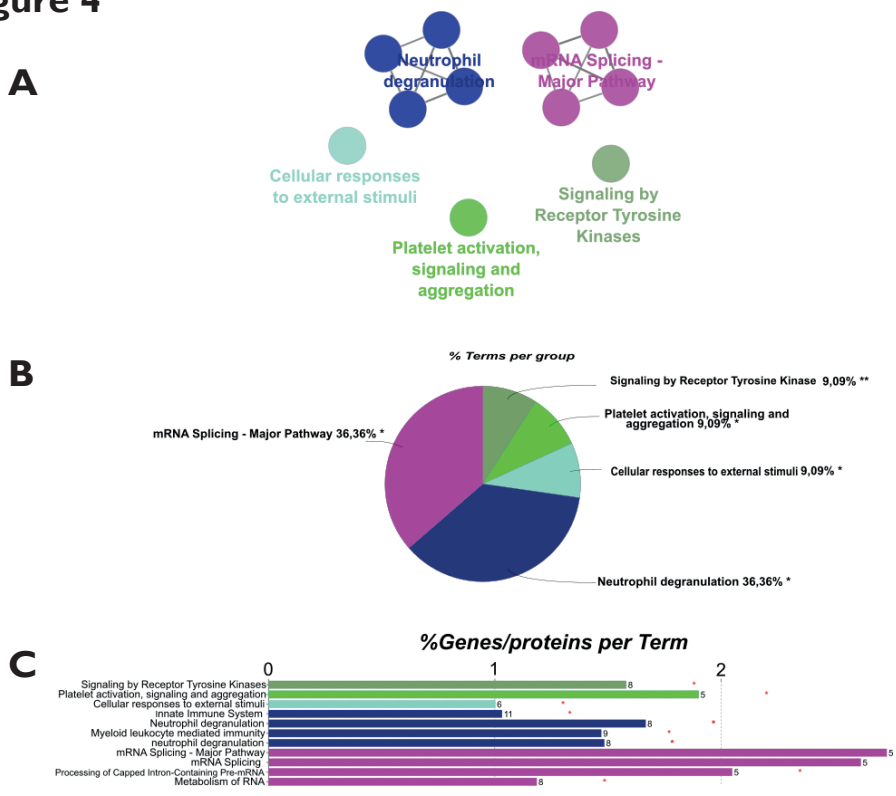

Figure 4. Reactome pathways and immune system processes of differentially expressed putative proteins in the lung upon PRRSV-1 infection at 6 and 8 dpi. (A) Functionally grouped networks associated with overexpressed proteins based on GO terms. Only the statistically significant terms ($P < 0.05$) in each group are represented using ClueGO. Terms are displayed as nodes (filled circle) linked by edges (lines) based on their κ score level (≥ 0.4), where only the label of the most significant term per group is shown. (B) Overview pie chart summarises the number of terms per group for differentially expressed proteins. (C) GO/pathway terms specific for overexpressed proteins. The bars represent the number of proteins associated with the terms. The percentage of proteins per term is shown as bar label. The same key colour used in the pie charts applies in these charts. $P < 0.05$ was used as a threshold for gene ontology categories enrichment. Single (*) or double (**) asterisk indicate significant enriched GO terms at the $P < 0.05$ and $P < 0.01$ statistical levels, respectively.

4. Discussion

PRRSV-1 negatively impacts on the lung local immune response by modulating the basic functions of pulmonary macrophages subsets, inducing regulated cell death of infected and non-infected immune cells as well as impairing secretion of pro- and anti-inflammatory cytokines (Gómez-Laguna et al., 2010, 2013; Labarque et al., 2002, 2003; Rodríguez-Gómez et al., 2019; Sánchez-Carvajal et al., 2019, 2020). These changes usually drive to an interstitial pneumonia which may lead to a severe acute lung damage associated with suppurative bronchopneumonia when virulent PRRSV-1 virulent strains are involved in (Amarilla et al., 2016; Morgan et al., 2016; Sánchez-Carvajal et al., 2020). Thus, our study focuses on determining the peptide molecular signature in lung tissue upon PRRSV-1 infection with strains of different virulence to favour the detection of potential biomarkers as well as the characterisation of lung lesion.

In our study, both PRRSV-1 strains caused a progressive increase of lung lesion scores throughout the study, with severe interstitial pneumonia as well as suppurative bronchopneumonia being observed in Lena-infected pigs. These pathological findings were supported by severe respiratory disorders and a long period of hyperthermia compared with 3249 strain. In addition, Lena virulent strain replicated more efficiently than 3249 strain, detecting higher titres in the lung of this group. These findings are consistent with the characteristic clinical and pathological features caused by PRRSV-1 virulent strains (Canelli et al., 2017; Karniychuk et al., 2010; Morgan et al., 2016; Renson et al., 2017; Weesendorp et al., 2014).

In order to conduct a comprehensive analysis of the molecular profile of the lung tissue, three piglets per group were selected from 6 and 8 dpi according to severity of clinical and lesional features and lung viral load and subjected to MALDI-MS imaging analyses. Fifty-four differentially expressed peptide profiles and their putative associated proteins were identified among PRRSV-1-infected and control piglets, with thirty-eight of them overexpressed at 6 dpi and nineteen at 8 dpi. Differentially expressed proteins were assigned with GO terms which were classified into functional groups under the categories Biological processes and Reactome with immunological processes.

Within Biological processes, regulation of cell death, positive regulation of cellular component organization, posttranscriptional regulation of gene expression and response

to oxidative stress were the major GO terms. In this sense, the majority of putative upregulated proteins were related to the regulation of cell death functionality GO group. Some of them, such as HSPB1 (Xiao et al., 2010; Zhou et al., 2014), endoplasmic reticulum chaperone BiP (HSPA5) (Chen et al., 2018; Jiang et al., 2013) and MMP9 (Lee and Kleiboeker, 2007; Xiao et al., 2010) have been previously described in in vitro studies with PRRSV-2 strains of different virulence. Interestingly, new peptides not previously associated with PRRSV infection, such as aldo-keto reductase family 1 member B1 (AKR1B1), BAK1, cation-independent mannose-6-phosphate receptor (IGF2R), cytochrome c oxidase subunit 2 (MT-CO2) or Nucleolysin TIA-1 isoform p40 (TIA1), were also identified. Regulated cell death plays a key role in host immune system upon virus infection, however, several viruses, as PRRSV do, manipulate cell death signalling for viral spread (Costers et al., 2008; Orzalli & Kagan, 2017; Yuan et al., 2016). Thus, our results highlight the complexity of cell death pathways activated upon PRRSV-1 infection in the lung of infected animals with differently expressed putative proteins playing a role as anti- or pro-apoptotic mediators.

MMP9 is an endopeptidase, which may act as an anti- or pro-apoptotic mediator regulating inflammatory response and acute lung injury (Marchant et al., 2014). Upon Influenza or Respiratory Syncytial Virus infection, overproduction of MMP9 induced marked lung damage because of an increased cell death an extracellular matrix degradation, but also was involved in neutrophil recruitment (Dabo et al., 2015; Kuhn et al., 2016; Rojas-Quintero et al., 2018). Other putative peptides directly connected with initial or advanced stages of apoptosis, such as Annexin IV, AKR1B1, PDXDC1 and THB1, were identified together with specific pro-apoptotic members of intrinsic (BAK1, MT-CO2) or extrinsic (TIA1) pathways of apoptosis. Thus, BAK1 protein works as pro-apoptotic regulator inducing the release of cytochrome c and other pro-apoptotic proteins, activating caspase 9 which in turn activate effector caspases (Alibek et al., 2014; Chittenden et al., 1995; Dewson et al., 2010). TIA1 protein is suggested to regulate cellular proliferation and inflammation (Reyes et al., 2009), tumour growth (Izquierdo et al., 2011) and apoptosis (Sánchez-Jiménez et al., 2015). Moreover, an increased expression of the putative protein MNDA, which has been previously associated with apoptosis of neutrophils (Milot et al., 2012), was also observed in our study. This protein may play a central role in those cases associated with suppurative bronchopneumonia. In addition, negative regulators of apoptosis were also identified (BSG, FLNA, PCID2, QARS1), with some of them identified as specific inhibitors of the granzyme (GPX3, HNRNPK, IGF2R) or the intrinsic (VIL1, PTBP1) pathways of apoptosis. Finally, an upregulation of the putative heat shock proteins

HSPB1 (also known as HSP27) and HSPA5 were detected in our study. Heat shock proteins are mainly involved in cellular protective response to stress, but they also induce an enhanced innate and adaptive immune response when interact with viral proteins (Bolhassani & Agi, 2019; Ikwegbue et al., 2018). In addition, HSPB1 has been suggested to play a role inhibiting either procaspase-8 or Daxx apoptotic protein, and in the activation of proteasome (Charette et al., 2000; Parcellier et al., 2003; Sarto et al., 2000), as well as HSPA5 as regulator of ferroptosis, other form of RCD, in cancer cells (Zhu et al., 2017). These findings add more complexity to the regulation of cell death in the lung of infected animals.

In addition, among reactome pathways and immune system processes mRNA splicing-major pathway, neutrophil degranulation, cellular responses to external stimuli, platelet signalling, and aggregation were the top four GO terms. Differentially expressed putative proteins involved in mRNA splicing-major pathway, such as heterogeneous nuclear ribonucleoprotein M (HNRNPM), heterogeneous nuclear ribonucleoprotein K (HNRNPK), polypyrimidine tract-binding protein 1 (PTBP1) and HSPB1 (Xiao et al., 2010; Zhou et al., 2014) have been previously connected with PRRSV infection (Chen et al., 2018), as well as Serrate RNA effector molecule homolog (SRRT) which not have been yet described associated with these viral infection. These proteins are involved in alternative RNA splicing, an important post-transcriptional mechanism by which precursor mRNA is transformed into mature mRNA and in turn translated into protein (Li et al., 2016). Alternative RNA splicing has been associated with several infectious diseases (Chauhan et al., 2019; De Maio et al., 2016; Hu et al., 2017; Kalam et al., 2017), in particular, viruses hijack host alternative splicing machinery for viral proteins production (Zhou et al., 2018).

In conclusion, this study confirms that proteomic approach with MALDI-MS Imaging enables the detection of new peptides potentially associated with PRRSV-1infection. Further studies are being conducted to confirm the peptides profile as well as their spatial distribution according to the virulence of the strain and the lesional pattern associated to each strain.

References

Aichler, M., & Walch, A. (2015). MALDI Imaging mass spectrometry: Current frontiers and perspectives in pathology research and practice. Laboratory Investigation, 95(4), 422–431. https://doi.org/10.1038/labinvest.2014.156

Alibek, K., Irving, S., Sautbayeva, Z., Kakpenova, A., Bekmurzayeva, A., Baiken, Y., Imangali, N., Shaimerdenova, M., Mektepbayeva, D., Balabiyev, A., & Chinybayeva, A. (2014). Disruption of Bcl-2 and Bcl-xL by viral proteins as a possible cause of cancer. In Infectious Agents and Cancer (Vol. 9, Issue 1, pp. 1–13). BioMed Central Ltd. https://doi.org/10.1186/1750-9378-9-44

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Graham, S. P., Frossard, J. P., Steinbach, F., & Salguero, F. J. (2016). Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains. Veterinary Microbiology, 188, 47–58. https://doi.org/10.1016/j.vetmic.2016.04.005

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Morgan, S. B., Graham, S. P., Frossard, J. P., Drew, T. W., & Salguero, F. J. (2015). A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: Upregulation of IL-1α in highly pathogenic strain induced lesions. Veterinary Immunology and Immunopathology, 164(3–4), 137–147. https://doi.org/10.1016/j.vetimm.2015.02.003

Barranco, I., Gómez-Laguna, J., Rodríguez-Gómez, I. M., Quereda, J. J., Salguero, F. J., Pallarés, F. J., & Carrasco, L. (2012). Immunohistochemical expression of IL-12, IL-10, IFN-α and IFN-γ in lymphoid organs of porcine reproductive and respiratory syndrome virus-infected pigs. Veterinary Immunology and Immunopathology, 149(3–4), 262–271. https://doi.org/10.1016/j.vetimm.2012.07.011

Bolhassani, A., & Agi, E. (2019). Heat shock proteins in infection. In Clinica Chimica Acta (Vol. 498, pp. 90–100). Elsevier B.V. https://doi.org/10.1016/j.cca.2019.08.015

Brockmeier, S. L., Loving, C. L., Palmer, M. V., Spear, A., Nicholson, T. L., Faaberg, K. S., & Lager, K. M. (2017). Comparison of Asian porcine high fever disease isolates of porcine reproductive and respiratory syndrome virus to United States isolates for their ability to cause disease and secondary bacterial infection in swine. Veterinary Microbiology, 203, 6–17. https://doi.org/10.1016/j.vetmic.2017.02.003

Butler, J. E., Lager, K. M., Golde, W., Faaberg, K. S., Sinkora, M., Loving, C., & Zhang, Y. I. (2014). Porcine reproductive and respiratory syndrome (PRRS): An immune dysregulatory

pandemic. Immunologic Research, 59(1–3), 81–108. https://doi.org/10.1007/s12026-014-8549-5

Canelli, E., Catella, A., Borghetti, P., Ferrari, L., Ogno, G., De Angelis, E., Corradi, A., Passeri, B., Bertani, V., Sandri, G., Bonilauri, P., Leung, F. C., Guazzetti, S., & Martelli, P. (2017). Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Veterinary Microbiology, 210(July), 124–133. https://doi.org/10.1016/j.vetmic.2017.09.002

Charette, S. J., Lavoie, J. N., Lambert, H., & Landry, J. (2000). Inhibition of Daxx-Mediated Apoptosis by Heat Shock Protein 27. Molecular and Cellular Biology, 20(20), 7602–7612. https://doi.org/10.1128/mcb.20.20.7602-7612.2000

Chauhan, K., Kalam, H., Dutt, R., & Kumar, D. (2019). RNA Splicing: A New Paradigm in Host–Pathogen Interactions. In Journal of Molecular Biology (Vol. 431, Issue 8, pp. 1565–1575). Academic Press. https://doi.org/10.1016/j.jmb.2019.03.001

Chen, Z., Liu, S., Zhang, S., Zhang, Y., Yu, J., Sun, W., Chen, L., Du, Y., Wang, J., Li, Y., & Wu, J. (2018). Porcine Reproductive and Respiratory Syndrome Virus strains with Higher Virulence Cause Marked Protein Profile Changes in MARC-145 Cells. Scientific Reports, 8(1), 1–14. https://doi.org/10.1038/s41598-018-32984-0

Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., & Lutz, R. J. (1995). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. The EMBO Journal, 14(22), 5589–5596. https://doi.org/10.1002/j.1460-2075.1995.tb00246.x

Costers, S., Lefebvre, D. J., Delputte, P. L., & Nauwynck, H. J. (2008). Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. Archives of Virology, 153(8), 1453–1465. https://doi.org/10.1007/s00705-008-0135-5

Dabo, A. J., Cummins, N., Eden, E., & Geraghty, P. (2015). Matrix metalloproteinase 9 exerts antiviral activity against respiratory syncytial virus. PLoS ONE, 10(8). https://doi.org/10.1371/journal.pone.0135970

De Maio, F. A., Risso, G., Iglesias, N. G., Shah, P., Pozzi, B., Gebhard, L. G., Mammi, P., Mancini, E., Yanovsky, M. J., Andino, R., Krogan, N., Srebrow, A., & Gamarnik, A. V. (2016). The Dengue Virus NS5 Protein Intrudes in the Cellular Spliceosome and Modulates Splicing. PLOS Pathogens, 12(8), e1005841. https://doi.org/10.1371/journal.ppat.1005841

Dewson, G., Kluck, R. M., & Kluck, R. (2010). Cell Health and Cytoskeleton Bcl-2 family-regulated apoptosis in health and disease. In Cell Health and Cytoskeleton (Vol. 2, Issue 9). https://www.dovepress.com/

Ding, Z., Li, Z. J., Zhang, X. D., Li, Y. G., Liu, C. J., Zhang, Y. P., & Li, Y. (2012). Proteomic alteration

of Marc-145 cells and PAMs after infection by porcine reproductive and respiratory syndrome virus. Veterinary Immunology and Immunopathology, 145(1–2), 206–213. https://doi.org/10.1016/j.vetimm.2011.11.005

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary Microbiology, 56(1–2), 9–19. https://doi.org/10.1016/S0378-1135(96)01347-8

Fortelny, N., Overall, C. M., Pavlidis, P., & Freue, G. V. C. (2017). Can we predict protein from mRNA levels? In Nature (Vol. 547, Issue 7664, pp. E19–E20). Nature Publishing Group. https://doi.org/10.1038/nature22293

Gimeno, M., Darwich, L., Diaz, I., De La Torre, E., Pujols, J., Martín, M., Inumaru, S., Cano, E., Domingo, M., Montoya, M., & Mateu, E. (2011). Cytokine profiles and phenotype regulation of antigen presenting cells by genotype-I porcine reproductive and respiratory syndrome virus isolates. Veterinary Research, 42(1), 9. https://doi.org/10.1186/1297-9716-42-9

Gómez-Laguna, J., Salguero, F. J., Barranco, I., Pallarés, F. J., Rodríguez-Gómez, I. M., Bernabé, A., & Carrasco, L. (2010). Cytokine Expression by Macrophages in the Lung of Pigs Infected with the Porcine Reproductive and Respiratory Syndrome Virus. Journal of Comparative Pathology, 142(1), 51–60. https://doi.org/10.1016/j.jcpa.2009.07.004

Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., & Carrasco, L. (2013). Immunopathogenesis of porcine reproductive and respiratory syndrome in the respiratory tract of pigs. The Veterinary Journal, 195(2), 148–155. https://doi.org/10.1016/j.tvjl.2012.11.012

Halbur, P. G., Miller, L. D., Paul, P. S., Meng, X.-J., Huffman, E. L., & Andrews, J. J. (1995). Immunohistochemical Identification of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Antigen in the Heart and Lymphoid System of Three-week-old Colostrum-deprived Pigs. Veterinary Pathology, 32(2), 200–204. https://doi.org/10.1177/030098589503200218

Halbur, P. G., Paul, P. S., Meng, X.-J., Lum, M. A., Andrews, J. J., & Rathje, J. A. (1996). Comparative Pathogenicity of Nine US Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Isolates in a Five-Week-Old Cesarean-Derived, Colostrum-Deprived Pig Model. Journal of Veterinary Diagnostic Investigation, 8(1), 11–20. https://doi.org/10.1177/104063879600800103

Hu, B., Huo, Y., Yang, L., Chen, G., Luo, M., Yang, J., & Zhou, J. (2017). ZIKV infection effects changes in gene splicing, isoform composition and lncRNA expression in human neural progenitor cells. Virology Journal, 14(1), 1–11. https://doi.org/10.1186/s12985-017-0882-6

Huang, C., Zhang, Q., & Feng, W. hai. (2015). Regulation and evasion of antiviral immune responses by porcine reproductive and respiratory syndrome virus. Virus Research, 202,

101–111. https://doi.org/10.1016/j.virusres.2014.12.014

Ikwegbue, P. C., Masamba, P., Oyinloye, B. E., & Kappo, A. P. (2018). Roles of heat shock proteins in apoptosis, oxidative stress, human inflammatory diseases, and cancer. In Pharmaceuticals (Vol. 11, Issue 1). MDPI AG. https://doi.org/10.3390/ph11010002

Izquierdo, J. M., Alcalde, J., Carrascoso, I., Reyes, R., & Ludeña, M. D. (2011). Knockdown of T-cell intracellular antigens triggers cell proliferation, invasion and tumour growth. Biochemical Journal, 435(2), 337–344. https://doi.org/10.1042/BJ20101030

Jiang, Z., Zhou, X., Michal, J. J., Wu, X. L., Zhang, L., Zhang, M., Ding, B., Liu, B., Manoranjan, V. S., Neill, J. D., Harhay, G. P., Kehrli, M. E., & Miller, L. C. (2013). Reactomes of Porcine Alveolar Macrophages Infected with Porcine Reproductive and Respiratory Syndrome Virus. PLoS ONE, 8(3). https://doi.org/10.1371/journal.pone.0059229

Kalam, H., Fontana, M. F., & Kumar, D. (2017). Alternate splicing of transcripts shape macrophage response to Mycobacterium tuberculosis infection. PLOS Pathogens, 13(3), e1006236. https://doi.org/10.1371/journal.ppat.1006236

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A., & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Veterinary Research, 6(1), 30. https://doi.org/10.1186/1746-6148-6-30

Kuhn, J. H., Lauck, M., Bailey, A. L., Shchetinin, A. M., Vishnevskaya, T. V., Bào, Y., Ng, T. F. F., LeBreton, M., Schneider, B. S., Gillis, A., Tamoufe, U., Diffo, J. L. D., Takuo, J. M., Kondov, N. O., Coffey, L. L., Wolfe, N. D., Delwart, E., Clawson, A. N., Postnikova, E., … Goldberg, T. L. (2016). Reorganization and expansion of the nidoviral family Arteriviridae. Archives of Virology, 161(3), 755–768. https://doi.org/10.1007/s00705-015-2672-z

Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K., & Pensaert, M. (2003). Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and associations with the production of apoptogenic cytokines. Veterinary Research, 34(3), 249–260. https://doi.org/10.1051/vetres:2003001

Labarque, G., Van Reeth, K., Van Gucht, S., Nauwynck, H., & Pensaert, M. (2002). Porcine reproductive-respiratory syndrome virus infection predisposes pigs for respiratory signs upon exposure to bacterial lipopolysaccharide. Veterinary Microbiology, 88(1), 1–12. https://doi.org/10.1016/S0378-1135(02)00104-9

Lee, S. M., & Kleiboeker, S. B. (2007). Porcine reproductive and respiratory syndrome virus induces apoptosis through a mitochondria-mediated pathway. Virology, 365(2), 419–434. https://doi.org/10.1016/j.virol.2007.04.001

Li, Y. I., Van De Geijn, B., Raj, A., Knowles, D. A., Petti, A. A., Golan, D., Gilad, Y., & Pritchard, J.

K. (2016). RNA splicing is a primary link between genetic variation and disease. Science, 352(6285), 600–604. https://doi.org/10.1126/science.aad9417

Li, Y., Wu, Z., Liu, K., Qi, P., Xu, J., Wei, J., Li, B., Shao, D., Shi, Y., Qiu, Y., & Ma, Z. (2017). Proteomic Analysis of the Secretome of Porcine Alveolar Macrophages Infected with Porcine Reproductive and Respiratory Syndrome Virus. Proteomics, 17(21). https://doi.org/10.1002/pmic.201700080

Liu, Y., Beyer, A., & Aebersold, R. (2016). On the Dependency of Cellular Protein Levels on mRNA Abundance. In Cell (Vol. 165, Issue 3, pp. 535–550). Cell Press. https://doi.org/10.1016/j.cell.2016.03.014

Mair, K. H., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., Saalmüller, A., Summerfield, A., Gerdts, V., Wilson, H. L., & Meurens, F. (2014). The porcine innate immune system: An update. In Developmental and Comparative Immunology (Vol. 45, Issue 2, pp. 321–343). Elsevier Ltd. https://doi.org/10.1016/j.dci.2014.03.022

Marchant, D. J., Bellac, C. L., Moraes, T. J., Wadsworth, S. J., Dufour, A., Butler, G. S., Bilawchuk, L. M., Hendry, R. G., Robertson, A. G., Cheung, C. T., Ng, J., Ang, L., Luo, Z., Heilbron, K., Norris, M. J., Duan, W., Bucyk, T., Karpov, A., Devel, L., … Overall, C. M. (2014). A new transcriptional role for matrix metalloproteinase-12 in antiviral immunity. Nature Medicine, 20(5), 493–502. https://doi.org/10.1038/nm.3508

Mattsson, J. G., Bergström, K., Wallgren, P., & Johansson, K.-E. E. (1995). Detection of Mycoplasma hyopneumoniae in nose swabs from pigs by in vitro amplification of the 16S rRNA gene. Journal of Clinical Microbiology, 33(4), 893–897. http://www.ncbi.nlm.nih.gov/pubmed/7540629

Meulenberg, J. J. M., Hulst, M. M., De Meijer, E. J., Moonen, P. L. J. M., Den Besten, A., De Kluyver, E. P., Wensvoort, G., & Moormann, R. J. M. (1993). Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology, 192(1), 62–72. https://doi.org/10.1006/viro.1993.1008

Milot, E., Fotouhi-Ardakani, N., & Filep, J. G. (2012). Myeloid nuclear differentiation antigen, neutrophil apoptosis and sepsis. In Frontiers in Immunology (Vol. 3, Issue DEC). Front Immunol. https://doi.org/10.3389/fimmu.2012.00397

Morgan, S. B., Frossard, J. P., Pallares, F. J., Gough, J., Stadejek, T., Graham, S. P., Steinbach, F., Drew, T. W., & Salguero, F. J. (2016). Pathology and Virus Distribution in the Lung and Lymphoid Tissues of Pigs Experimentally Inoculated with Three Distinct Type 1 PRRS Virus Isolates of Varying Pathogenicity. Transboundary and Emerging Diseases, 63(3), 285–295. https://doi.org/10.1111/tbed.12272

Morgan, S. B., Graham, S. P., Salguero, F. J., Sánchez Cordón, P. J., Mokhtar, H., Rebel, J. M. J.,

Weesendorp, E., Bodman-Smith, K. B., Steinbach, F., & Frossard, J. P. (2013). Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Veterinary Microbiology, 163(1–2), 13–22. https://doi.org/10.1016/j.vetmic.2012.11.024

Nathues, H., Alarcon, P., Rushton, J., Jolie, R., Fiebig, K., Jimenez, M., Geurts, V., & Nathues, C. (2017). Cost of porcine reproductive and respiratory syndrome virus at individual farm level – An economic disease model. Preventive Veterinary Medicine, 142, 16–29. https://doi.org/10.1016/j.prevetmed.2017.04.006

Neagu, A. N. (2019). Proteome Imaging: From Classic to Modern Mass Spectrometry-Based Molecular Histology. In Advances in experimental medicine and biology (Vol. 1140, pp. 55–98). NLM (Medline). https://doi.org/10.1007/978-3-030-15950-4_4

Norris, J. L., & Caprioli, R. M. (2013a). Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research. In Chemical Reviews (Vol. 113, Issue 4, pp. 2309–2342). Chem Rev. https://doi.org/10.1021/cr3004295

Norris, J. L., & Caprioli, R. M. (2013b). Imaging mass spectrometry: A new tool for pathology in a molecular age. Proteomics - Clinical Applications, 7(11–12), 733–738. https://doi.org/10.1002/prca.201300055

Ogno, G., Rodríguez-Gómez, I. M., Canelli, E., Ruedas-Torres, I., Álvarez, B., Domínguez, J., Borghetti, P., Martelli, P., & Gómez-Laguna, J. (2019). Impact of PRRSV strains of different in vivo virulence on the macrophage population of the thymus. Veterinary Microbiology, 232, 137–145. https://doi.org/10.1016/j.vetmic.2019.04.016

Orzalli, M. H., & Kagan, J. C. (2017). Apoptosis and Necroptosis as Host Defense Strategies to Prevent Viral Infection. Trends in Cell Biology, 27(11), 800–809. https://doi.org/10.1016/j.tcb.2017.05.007

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantôme, A., Plenchette, S., Khochbin, S., Solary, E., & Garrido, C. (2003). HSP27 Is a Ubiquitin-Binding Protein Involved in I-κBα Proteasomal Degradation. Molecular and Cellular Biology, 23(16), 5790–5802. https://doi.org/10.1128/mcb.23.16.5790-5802.2003

Renson, P., Rose, N., Le Dimna, M., Mahé, S., Keranflec'h, A., Paboeuf, F., Belloc, C., Le Potier, M. F., & Bourry, O. (2017). Dynamic changes in bronchoalveolar macrophages and cytokines during infection of pigs with a highly or low pathogenic genotype 1 PRRSV strain. Veterinary Research, 48(1), 15. https://doi.org/10.1186/s13567-017-0420-y

Reyes, R., Alcalde, J., & Izquierdo, J. M. (2009). Depletion of T-cell intracellular antigen proteins promotes cell proliferation. Genome Biology, 10(8), R87. https://doi.org/10.1186/gb-2009-

10-8-r87

Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallarés, F. J., Mateu, E., Carrasco, L., & Gómez-Laguna, J. (2019). Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells. Veterinary Microbiology, 235, 101–109. https://doi.org/10.1016/j.vetmic.2019.06.011

Rojas-Quintero, J., Wang, X., Tipper, J., Burkett, P. R., Zuñiga, J., Ashtekar, A. R., Polverino, F., Rout, A., Yambayev, I., Hernández, C., Jimenez, L., Ramírez, G., Harrod, K. S., & Owen, C. A. (2018). Matrix metalloproteinase-9 deficiency protects mice from severe influenza A viral infection. JCI Insight, 3(24). https://doi.org/10.1172/jci.insight.99022

Ruedas-Torres, I., Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallares, F. J., Barranco, I., Carrasco, L., & Gómez-Laguna, J. (2020). Activation of the extrinsic apoptotic pathway in the thymus of piglets infected with PRRSV-1 strains of different virulence. Veterinary Microbiology, 243. https://doi.org/10.1016/j.vetmic.2020.108639

Ruedas-Torres, Inés, Rodríguez-Gómez, I. M. I. M., Sánchez-Carvajal, J. M. J. M., Pallares, F. J. F. J., Barranco, I., Carrasco, L., & Gómez-Laguna, J. (2020). Activation of the extrinsic apoptotic pathway in the thymus of piglets infected with PRRSV-1 strains of different virulence. Veterinary Microbiology, 243, 108639. https://doi.org/10.1016/j.vetmic.2020.108639

Sánchez-Carvajal, J. M., Rodríguez-Gómez, I. M., Carrasco, L., Barranco, I., Álvarez, B., Domínguez, J., Salguero, F. J., & Gómez-Laguna, J. (2019). Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence. Veterinary Research Communications, 43(3), 187–195. https://doi.org/10.1007/s11259-019-09755-x

Sánchez-Carvajal, J. M., Rodríguez-Gómez, I. M., Ruedas-Torres, I., Larenas-Muñoz, F., Díaz, I., Revilla, C., Mateu, E., Domínguez, J., Martín-Valls, G., Barranco, I., Pallarés, F. J., Carrasco, L., & Gómez-Laguna, J. (2020). Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena. Veterinary Microbiology, 246. https://doi.org/10.1016/j.vetmic.2020.108744

Sánchez-Jiménez, C., Ludeña, M. D., & Izquierdo, J. M. (2015). T-cell intracellular antigens function as tumor suppressor genes. Cell Death and Disease, 6(3), e1669–e1669. https://doi.org/10.1038/cddis.2015.43

Sarto, C., Binz, P., & Mocarelli, P. (2000). Heat shock proteins in human cancer. ELECTROPHORESIS, 21(6), 1218–1226. https://doi.org/10.1002/(SICI)1522-2683(20000401)21:6<1218::AID-ELPS1218>3.0.CO;2-H

Schöne, C., Höfler, H., & Walch, A. (2013). MALDI imaging mass spectrometry in cancer research: Combining proteomic profiling and histological evaluation. In Clinical Biochemistry (Vol. 46,

Issue 6, pp. 539–545). Elsevier. https://doi.org/10.1016/j.clinbiochem.2013.01.018

Sibila, M., Calsamiglia, M., Segalés, J., Blanchard, P., Badiella, L., Le Dimma, M., Jestin, A., & Domingo, M. (2004). Use of a polymerase chain reaction assay and an ELISA to monitor porcine circovirus type 2 infection in pigs from farms with and without postweaning multisystemic wasting syndrome. American Journal of Veterinary Research, 65(1), 88–92. https://doi.org/10.2460/ajvr.2004.65.88

Sinn, L. J., Klingler, E., Lamp, B., Brunthaler, R., Weissenböck, H., Rümenapf, T., & Ladinig, A. (2016). Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria. Porcine Health Management, 2, 1–10. https://doi.org/10.1186/s40813-016-0044-z

Valdes-Donoso, P., Alvarez, J., Jarvis, L. S., Morrison, R. B., & Perez, A. M. (2018). Production Losses From an Endemic Animal Disease: Porcine Reproductive and Respiratory Syndrome (PRRS) in Selected Midwest US Sow Farms. Frontiers in Veterinary Science, 5(MAY), 102. https://doi.org/10.3389/fvets.2018.00102

Vogel, C., & Marcotte, E. M. (2012). Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nature Reviews Genetics, 13(4), 227–232. https://doi.org/10.1038/nrg3185

Weesendorp, E., Rebel, J. M. J., Popma-De Graaf, D. J., Fijten, H. P. D., & Stockhofe-Zurwieden, N. (2014). Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Veterinary Microbiology, 174(1–2), 127–138. https://doi.org/10.1016/j.vetmic.2014.09.010

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., & Wagenaar, F. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. The Veterinary Quarterly, 13(3), 121–130. https://doi.org/10.1080/01652176.1991.9694296

Xiao, S., Wang, Q., Jia, J., Cong, P., Mo, D., Yu, X., Qin, L., Li, A., Niu, Y., Zhu, K., Wang, X., Liu, X., & Chen, Y. (2010). Proteome changes of lungs artificially infected with H-PRRSV and N-PRRSV by two-dimensional fluorescence difference gel electrophoresis. Virology Journal, 7, 107. https://doi.org/10.1186/1743-422X-7-107

Yuan, J., Najafov, A., & Py, B. F. (2016). Roles of Caspases in Necrotic Cell Death. In Cell (Vol. 167, Issue 7, pp. 1693–1704). Cell Press. https://doi.org/10.1016/j.cell.2016.11.047

Yuzhakov, A. G., Raev, S. A., Skrylev, A. N., Mishin, A. M., Grebennikova, T. V., Verkhovsky, O. A., Zaberezhny, A. D., Trus, I., Nauwynck, H. J., & Aliper, T. I. (2017). Genetic and pathogenic characterization of a Russian subtype 2 PRRSV-1 isolate. Veterinary Microbiology, 211(June), 22–28. https://doi.org/10.1016/j.vetmic.2017.09.017

Zhang, H., Guo, X., Ge, X., Chen, Y., Sun, Q., & Yang, H. (2009). Changes in the cellular proteins of pulmonary alveolar macrophage infected with porcine reproductive and respiratory syndrome virus by proteomics analysis. Journal of Proteome Research, 8(6), 3091–3097. https://doi.org/10.1021/pr900002f

Zhao, F., Fang, L., Wang, D., Song, T., Wang, T., Xin, Y., Chen, H., & Xiao, S. (2016). SILAC-based quantitative proteomic analysis of secretome of Marc-145 cells infected with porcine reproductive and respiratory syndrome virus. Proteomics, 16(20), 2678–2687. https://doi.org/10.1002/pmic.201500486

Zhou, C., Liu, S., Song, W., Luo, S., Meng, G., Yang, C., Yang, H., Ma, J., Wang, L., Gao, S., Wang, J., Yang, H., Zhao, Y., Wang, H., & Zhou, X. (2018). Characterization of viral RNA splicing using whole-transcriptome datasets from host species. Scientific Reports, 8(1), 1–14. https://doi.org/10.1038/s41598-018-21190-7

Zhou, Y.-J., Zhu, J.-P., Zhou, T., Cheng, Q., Yu, L.-X., Wang, Y.-X., Yang, S., Jiang, Y.-F., Tong, W., Gao, F., Yu, H., Li, G.-X., & Tong, G.-Z. (2014). Identification of Differentially Expressed Proteins in Porcine Alveolar Macrophages Infected with Virulent/Attenuated Strains of Porcine Reproductive and Respiratory Syndrome Virus. PLoS ONE, 9(1), e85767. https://doi.org/10.1371/journal.pone.0085767

Zhu, S., Zhang, Q., Sun, X., Zeh, H. J., Lotze, M. T., Kang, R., & Tang, D. (2017). HSPA5 regulates ferroptotic cell death in cancer cells. Cancer Research, 77(8), 2064–2077. https://doi.org/10.1158/0008-5472.CAN-16-1979

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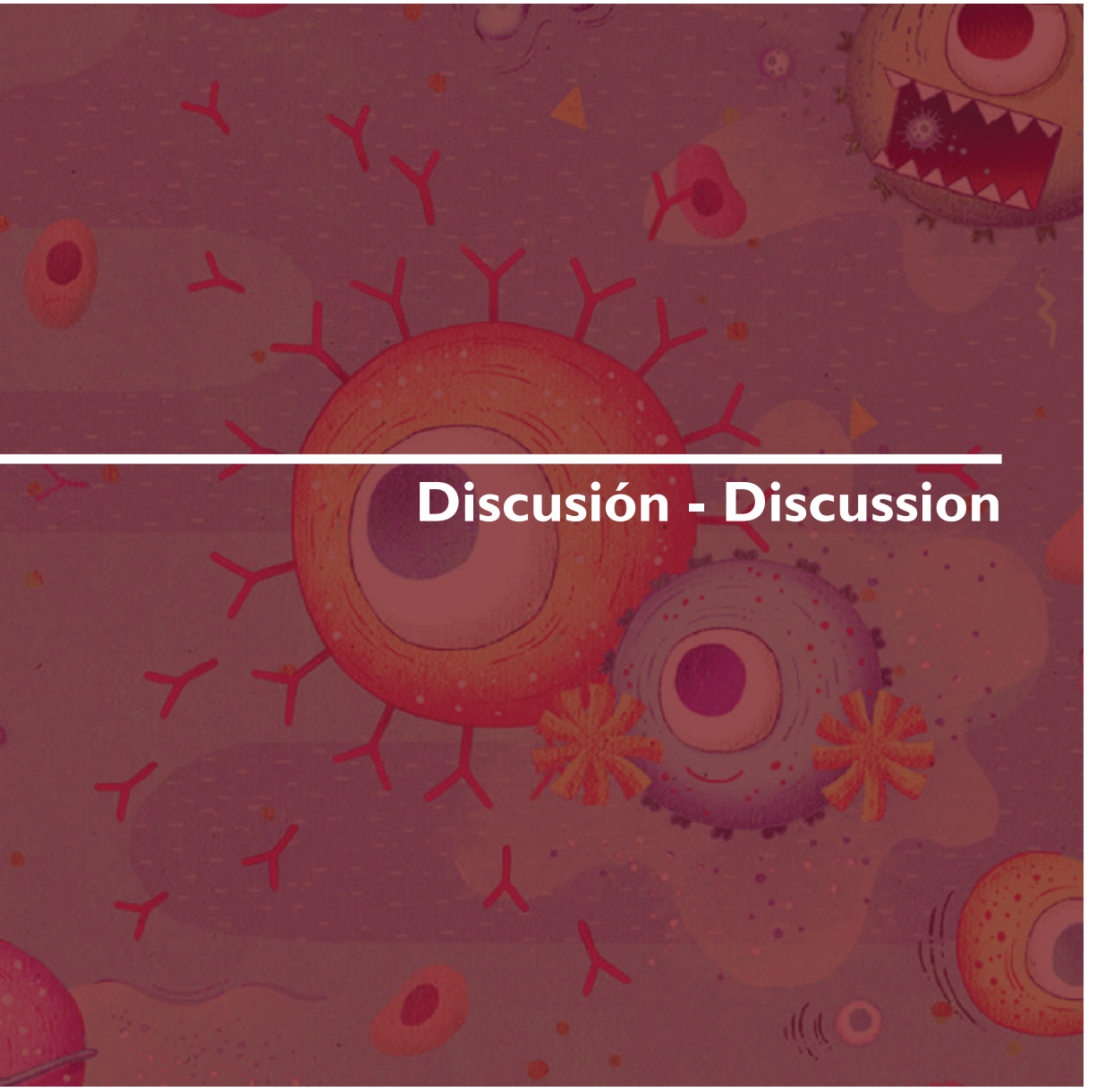

Discusión - Discussion

Imagen: Julia Yellow

DISCUSIÓN

El PRRSV se caracteriza por su capacidad para modular, e incluso evadir, la respuesta inmune del hospedador, favoreciendo su replicación, distribución y latencia en el organismo, así como el desarrollo de infecciones bacterianas secundarias propias del PRDC. Algunos de los principales mecanismos implicados en la homeostasis del sistema inmune como la producción y señalización de IFNs, la modulación de la expresión de diferentes citoquinas, la inducción de muerte celular en células del sistema inmune y la regulación de la inmunidad adaptativa se ven alterados como consecuencia de la infección por el PRRSV (Butler et al., 2014; Han et al., 2017; Huang et al., 2015; Lunney et al., 2016; Mateu and Diaz, 2008; Mair et al., 2014). Además, la respuesta inmune inducida por el PRRSV varía en función del órgano afectado y de la virulencia de la cepa, siendo necesario evaluar in situ los distintos tejidos de interés para descifrar la inmunopatogenia de la infección con diferentes cepas del PRRSV.

Teniendo en cuenta estos precedentes, en la presente tesis doctoral hemos evaluado el cuadro lesional, la muerte celular (mediante la expresión de diferentes caspasas) y la expresión de diferentes marcadores inmunológicos de potencial interés, especialmente el receptor CD163, a nivel pulmonar así como la respuesta inmune a nivel sistémico con el objetivo de profundizar en la inmunopatogenia de la forma respiratoria del PRRSV-1 con cepas de diferente virulencia durante la fase aguda de la infección.

En nuestro estudio, coincidiendo con artículos previos (Renson et al., 2017; Weesendorp et al., 2013), la cepa virulenta Lena provocó un cuadro clínico grave a nivel sistémico y respiratorio, así como hipertermia, en comparación con la cepa de baja virulencia 3249 que causó un cuadro clínico leve acompañado de un ligero incremento de la temperatura rectal. Además, en los animales infectados con la cepa virulenta Lena se detectaron niveles de viremia mucho más altos que se asociaron tanto con el cuadro clínico como con un incremento en los niveles séricos de IFN-γ, sugiriendo un intento de la respuesta innata del hospedador por controlar la replicación vírica. Por otro lado, conviene recordar que la infección con cepas de elevada virulencia del PRRSV-1 se ha asociado con una fuerte respuesta inflamatoria comparado con cepas de baja virulencia (Amarilla et al., 2015; Ferrari et al., 2018; Morgan et al., 2013; Renson et al., 2017; Weesendorp et al., 2013a, 2013b). En nuestro

estudio, la cepa Lena provocó un fuerte incremento en la concentración sérica de IL-6 a los 8 dpi, citoquina que podría tener un papel dicotómico en la infección con el PRRSV: (I) protegiendo al hospedador de la infección, e (II) induciendo una fuerte respuesta inflamatoria y daño tisular cuando se produce una liberación excesiva (Liu et al., 2010; Van Reeth & Nauwynck, 2000). Este incremento en los niveles de IL-6 podría relacionarse con la fiebre, la sintomatología clínica y el daño pulmonar mucho más intenso observado en infecciones con cepas virulentas del PRRSV-1 (Amarilla et al., 2015; Renson et al., 2017). Estos resultados ponen de manifiesto una activación más intensa de la respuesta inmune acorde con la virulencia de las cepas, y destaca el papel tanto del IFN-γ como de la IL-6 en la monitorización de la infección por estas cepas.

Ambas cepas del PRRSV-1, Lena y 3249, independientemente de su virulencia, dieron lugar al desarrollo de neumonía intersticial, lesión característica de la forma respiratoria del PRRS. Además, en los animales infectados con la cepa virulenta Lena estas lesiones se instauraron de forma más temprana e intensa, observándose áreas extensas de consolidación en los lóbulos apical y medial que se correspondieron a nivel microscópico con focos de bronconeumonía catarro-purulenta. Estos rasgos lesionales observados en los animales infectados con la cepa Lena son muy similares a los descritos para otras cepas virulentas del PRRSV-1 (Canelli et al., 2017; Morgan et al., 2013; Sinn et al., 2016), explicando la marcada sintomatología respiratoria que se describe asociada a la infección con estas cepas. Sin embargo, hasta el momento no se ha llevado a cabo una exhaustiva caracterización de la patogenia de estas lesiones ocasionadas por cepas virulentas del PRRSV-1, siendo necesario identificar marcadores inmunes que potencialmente jueguen un papel clave y permitan entender mejor cómo o por qué se desarrollan dichas lesiones.

En este sentido, a nivel del parénquima pulmonar se analizó la frecuencia de células PRRSV-N-protein^{+} y la carga viral, observándose una correlación negativa entre ambos parámetros y la frecuencia de células CD163^{+}, receptor esencial para la infección por el PRRSV (Burkard et al., 2017; Van Gorp et al., 2008; Whitworth et al., 2016). Aunque en nuestro estudio hemos observado una disminución en la frecuencia de células CD163^{+} tanto con cepas de baja virulencia como de alta virulencia del PRRSV-1, este descenso fue mucho más intenso y temprano en los animales infectados con la cepa virulenta Lena tanto a nivel tisular como en PAMs

vivos aislados del lavado broncoalveolar, lo que conduciría a un colapso en el proceso de fagocitosis mediado por estas células (Renson et al., 2017). Esta disminución en el número y en la expresión del receptor scavenger CD163 y por tanto de la capacidad fagocítica de PAMs, conllevaría igualmente una disminución de la eliminación de cuerpos apoptóticos y favorecería la proliferación secundaria de bacterias comensales conduciendo al desarrollo de bronconeumonía (Fabriek et al., 2009).

Esta disminución del número de células $CD163^+$ podría estar relacionada con el efecto citopático del virus sobre las células diana, pero también debido a los fenómenos de RCD tanto en células infectadas como no infectadas, lo que ha sido ampliamente descrito en el PRRS tanto en el pulmón como en órganos linfoides (Amarilla et al., 2016; Morgan et al., 2013; Ruedas-Torres et al., 2020). En este sentido, la marcada depleción en el número de PAMs $CD163^+$ coincidió con un incremento en la frecuencia de células $TUNEL^+$ que fue significativamente superior a la frecuencia de células $cCasp3^+$ en los animales infectados con ambas cepas del PRRSV-1 durante la primera semana postinfección. Sin embargo, pasado este periodo evidenciamos una activación de la cCasp3, cCasp8 y cCasp9, más temprana e intensa y derivada hacia la activación de la vía extrínseca en Lena, lo que sugiere que ambas cepas del PRRSV-1 provocan un retraso en la activación de la apoptosis durante la primera semana postinfección en línea con descripciones previas realizadas en aproximaciones in vitro (Costers et al., 2008; Pujhari et al., 2014; Yuan et al., 2016). Como consecuencia, en las primeras fases de la infección se produciría la activación de vías de señalización de RCD independientes de la cCasp3, como necroptosis y piroptosis, que podrían jugar un papel importante tanto en la homeostasis de las células pulmonares como en la eliminación de células infectadas, ya que los animales infectados presentaron un número mayor de células $TUNEL^+$ que los animales control.

Además, en los animales infectados con la cepa Lena se produjo un incremento significativo en la frecuencia de células $TUNEL^+$ a los 3 dpi y a los 8 dpi, así como de células $iNOS^+$ a los 8 dpi, coincidiendo con el mayor grado de lesión pulmonar. En este sentido, tanto la liberación de NO (Chen et al., 2014; Lee and Kleiboeker, 2005; Yan et al., 2017) como la activación de la RCD en la infección con la cepa virulenta Lena podría ser un arma de doble filo, ya que por un lado, contribuiría a una eliminación más rápida y temprana de las células infectadas, pero por otro lado provocaría un mayor daño pulmonar (Bi et al., 2014; de Zoete et al., 2014; Li et al., 2015; Wang et al., 2015;

Zhang et al., 2013). Curiosamente, en los alvéolos de algunos animales infectados con la cepa Lena también se observaron a los 8 dpi y 13 dpi redes de cromatina libre acompañadas por un infiltrado moderado de neutrófilos TUNEL-/cCasp3-, los cuales podrían estar asociados con la formación de trampas extracelulares de neutrófilos (NETs, del inglés Neutrophils Extracellular Traps) (Fuchs et al., 2007), como parte de un proceso denominado NETosis, otro tipo de RCD (de Buhr et al., 2019; Funchal et al., 2015; Galluzzi et al., 2018; Henthorn et al., 2018; Moorthy et al., 2013; Schönrich and Raftery et al., 2019; Storisteanu et al., 2017; Tripathi et al., 2014). Estos resultados abren nuevos campos de investigación, como las vías de señalización de RCD activadas en las fases tempranas de la infección del PRRSV que sin duda juegan un papel clave en la modulación de las poblaciones de macrófagos a nivel pulmonar.

Una hipótesis alternativa a la disminución en la frecuencia de células $CD163^{+}$ sería la pérdida del dominio extracelular de CD163 de la superficie celular como consecuencia de la activación de los macrófagos tanto por efecto directo del virus como de forma secundaria a la activación vírica (Møller, 2012). Sin embargo, no parece probable que la disminución en la expresión del CD163 observada en nuestros estudios se deba a este fenómeno, ya que, por un lado, observamos una disminución en la frecuencia de PAMs vivos en el lavado broncoalveolar a lo largo del estudio en ambos grupos infectados y, por otro lado, no se observaron cambios en la concentración sérica de sCD163 en la infección con PRRSV-1, en contraste, por ejemplo, a lo observado previamente con cepas virulentas de Glaesserella parasuis que indujeron un incremento en los niveles plasmáticos de sCD163 asociado con la reducción en la expresión de CD163 en la superficie celular de PAMs (Costa-Hurtado et al., 2013).

Curiosamente, nuestros estudios revelan que a partir de la segunda semana postinfección, se produce un incremento en el número de células $CD163^{+}$ que contrarresta así la marcada depleción observada en esta subpoblación durante la primera semana, coincidiendo con una disminución en el número de células PRRSV-N-protein^{+} y de la carga viral a nivel pulmonar. Además, también se observó una disminución en la MFI (del inglés Median Fluorescence Intensity) del CD163 dentro de la subpoblación de PAMs vivos PRRSV-N-protein- al final del estudio en comparación con el grupo control que estaría asociado, entre otras posibilidades, a la presencia de macrófagos inmaduros $CD163^{low}$ en el lavado broncoalveolar. De acuerdo con

nuestros resultados, el hospedador sería capaz de recuperar niveles normales de células $CD163^+$ a nivel pulmonar entorno a un mes después de la infección. Esta repleción se iniciaría a partir de los 6 – 8 dpi asociada a un incremento en el número de células $CD14^+$ que consisten principalmente en monocitos y macrófagos inmaduros que infiltraron áreas extensas del intersticio pulmonar en los animales infectados con PRRSV-1, hallazgos que también han sido descritos previamente por otros autores (Van Gucht et al., 2005). Esta repoblación de células $CD163^+$ a partir de monocitos y macrófagos inmaduros $CD14^+$, coincide igualmente con el aumento en la frecuencia de células $CD107a^+$, glicoproteína identificada en la superficie de lisosomas y endosomas previa a la degranulación y cuya expresión aumenta en PAMs activados, lo que indicaría un mayor contenido lisosomal en esta subpoblación de macrófagos y, por tanto, una mayor capacidad para la fagocitosis. Todos estos resultados representan un intento del sistema inmune del hospedador por recuperar la homeostasis a nivel pulmonar, contribuyendo a la eliminación de residuos celulares y células apoptóticas, ayudando así a restaurar el daño tisular y resolver el proceso inflamatorio (Hussell & Bell, 2014; García-Nicolás et al., 2014; Mantovani et al., 2004; Van Gorp et al., 2010). En este contexto, también hay que tener en cuenta que un aumento en la frecuencia de células $CD14^+$ y por tanto de la disponibilidad de CD14, receptor principal para el complejo LPS-LBP, sensibilizaría el pulmón frente a futuras infecciones bacterianas secundarias, facilitando así el desarrollo del PRDC (Van Gucht et al., 2004, 2005).

Simultáneamente a esta cascada de eventos proinflamatorios y de muerte celular, a nivel pulmonar se inicia una respuesta antiinflamatoria o reguladora, en la cual estarían implicados marcadores como el CD200R1 y el FoxP3. Nuestros resultados muestran un incremento en la frecuencia de células $CD200R1^+$ y $FoxP3^+$ entre los 6 y 8 dpi, coincidiendo con el mayor grado de lesión pulmonar, guardando una correlación positiva la frecuencia de células $CD200R1^+$ y la lesión pulmonar en los animales infectados con la cepa Lena. CD200R1 se ha relacionado con una disminución en la expresión de citoquinas proinflamatorias en una amplia variedad de enfermedades inflamatorias (Vaine & Soberman, 2014), mientras que el FoxP3, es un marcador utilizado para detectar linfocitos Tregs (Käser et al., 2008), los cuales podrían estar implicados en la supresión de la respuesta celular mediada en cerdos tras la infección por PRRSV (Ferrarini et al., 2015; Silva-Campa et al., 2009; 2010; Nedumpun et al., 2018). En este contexto, nuestros resultados sugieren que el incremento en la frecuencia de células $CD200R1^+$ y $FoxP3^+$ sería un mecanismo potencial para contener y favorecer

la recuperación del daño pulmonar durante la fase aguda de la infección, aunque son necesarios futuros estudios para descifrar el papel de estas células tanto en las fases tempranas como en las fases más avanzadas de la inmunopatogenia del PRRSV.

Teniendo en cuenta estudios in vitro donde se ha llevado a cabo la polarización de macrófagos derivados de monocitos (García-Nicolás et al., 2014; Wang et al., 2017) y la gran plasticidad y capacidad que tienen los macrófagos pulmonares para adaptarse al microambiente en el que se encuentran (Guth et al., 2009; Hussell & Bell, 2014) podemos hipotetizar que la llegada de monocitos y macrófagos a un microambiente proinflamatorio como el que se produce en la infección por cepas virulentas del PRRSV-1 a nivel pulmonar, favorecería la activación alternativa de estos como macrófagos M2, macrófagos con capacidad antiinflamatoria cuya activación no interfiere en la replicación del PRRSV pero que sí influye en una mayor expresión de CD163 (García-Nicolás et al., 2014; Gordon & Martinez, 2010; Mosser, 2003; Mosser & Edwards, 2008; Wang et al., 2017), participando en la reparación tisular y la resolución del proceso inflamatorio (Hussell & Bell, 2014; García-Nicolás et al., 2014; Mantovani et al., 2004; Van Gorp et al., 2010), complementado así la acción previamente iniciada por las células CD200R1^{+} y linfocitos Tregs.

DISCUSSION

PRRSV is able to manipulate, even to evade, the host immune response, facilitating its replication and distribution. This state can lead to persistent infection as well as favouring secondary bacterial infections, which plays a central role in PRCD. PRRSV infection impairs different mechanisms involve in the homeostasis of the swine immune system by supressing type I IFN signalling and production, modulating cytokine expression as well as the host adaptive immune response (Butler et al., 2014; Han et al., 2017; Huang et al., 2015; Lunney et al., 2016; Mateu and Diaz, 2008; Mair et al., 2014). Furthermore, PRRSV elicits a distinct immune response depending on both the virulence of the strain and the infected tissue, therefore the study of target organs are encouraged in order to decipher the immunopathogenesis of PRRSV infection with strain of different virulence.

Bearing in mind these previous statements, the present PhD dissertation has been focused on the evaluation of the lesion patterns, cell death phenomena (by means of the expression of different caspases) and the expression of several immune markers of potential interest, especially the scavenger receptor CD163, in the lung of infected animals as well as the systemic immune response with the aim to dissect the immunopathogenesis of the respiratory disease caused by PRRSV-1 strains of different virulence in the acute phase of infection.

As previously described (Renson et al., 2017; Weesendorp et al., 2013), our results showed severe systemic and respiratory clinical signs and hyperthermia associated with the virulent Lena strain, by contrast low virulent 3249 strain induced mild symptoms and a slight increase of rectal temperature. Besides, viraemia levels were higher in Lena-infected piglets associated with both clinical sings and an increase in the sera levels of IFN-γ, suggesting an attempt of the host immune response to control viral replication. On the other hand, it is well established that PRRSV infection with virulent strains induces a strong inflammatory immune response when compared with low virulent strains (Amarilla et al., 2015; Ferrari et al., 2018; Morgan et al., 2013; Renson et al., 2017; Weesendorp et al., 2013a, 2013b). Thus, in our study, virulent Lena strain elicited a marked increase in sera levels of IL-6 at 8 dpi. It is reported that IL-6 is a pro-inflammatory cytokine which could play a dual role along PRRSV infection:

(I) by protecting the host from infection, and (II) inducing inflammation and tissue damage when it is overexpressed (Liu et al., 2010; Van Reeth & Nauwynck, 2000). Therefore, it seems likely that the increase of IL-6, observed in the sera of Lena-infected animals in our study, would be linked to the fever, clinical signs and severe lung damage, which has been broadly reported in piglets infected with PRRSV-1 virulent strains (Amarilla et al., 2015; Renson et al., 2017). These results point out to a more intense activation of the immune response according to the strain virulence, highlighting not only the role of IFN-γ but also of IL-6 to monitor the infection with PRRSV virulent strains.

Regardless of strain virulence, both Lena and 3249 strains caused the typical interstitial pneumonia described for the respiratory disease of PRRS. Furthermore, virulent Lena strain triggered an earlier and stronger onset of lung lesions due to extensive consolidated areas in the apical and medial lung lobes which corresponded microscopically with foci of suppurative bronchopneumonia. These key lesional features observed in Lena-infected piglets are in line with similar results described for other virulent PRRSV-1 strains (Canelli et al., 2017; Morgan et al., 2013; Sinn et al., 2016), giving an explanation to the severe respiratory disorders observed upon infection with virulent strains. However, an exhaustive characterisation of the lesions caused by virulent PRRSV-1 strains has not been carried out yet, being essential to find out potential immune markers involved in lesion development.

Accordingly, the frequency of PRRSV-N-protein$^+$ cells and viral load were evaluated in the lung tissue, showing a solid negative correlation with the frequency of CD163$^+$ cells, the essential host receptor for PRRSV infection (Burkard et al., 2017; Van Gorp et al., 2008; Whitworth et al., 2016). Although, the frequency of CD163$^+$ cells plummeted in both PRRSV-1-infected groups, this finding was earlier and more intense in the pulmonary parenchyma as well as in live PAMs from the BALF of Lena-infected piglets, leading to a failure in the phagocytosis by these cells (Renson et al., 2017). This reduction in both the surface expression and the frequency of the scavenger receptor CD163, and hence in the phagocytic capacity of PAMs, could negatively affect the clearance of apoptotic bodies and favour the co-infection with secondary commensal microorganisms driving to suppurative bronchopneumonia (Fabriek et al., 2009).

The decrease in the frequency of $CD163^+$ cells is likely to be due to the direct cytopathic effect of the virus in its target cell, but also related to the induction of RCD in infected and non-infected cells, which has been broadly described in the lung and lymphoid organs of pigs infected with virulent PRRSV-1 strains (Amarilla et al., 2016; Morgan et al., 2013; Ruedas-Torres et al., 2020). In this sense, the marked depletion of $CD163^+$ PAMs coincided with an increase in the frequency of $TUNEL^+$ cells, which was significantly higher than the frequency of $cCasp3^+$ cells in both Lena- and 3249-infected piglets during the first week post-infection. Interestingly, an increase in cCasp3, cCasp8 and cCasp9 was triggered in PRRSV-1-infected piglets during the second week post-infection. This event which was earlier and more intense in Lena-infected piglets was followed by the activation of the extrinsic apoptotic pathway. These facts suggest that both PRRSV-1 strains, Lena and 3249, induce a delayed activation of apoptotic signalling pathways up to one week post-infection, in line with the evidences of previous in vitro studies (Costers et al., 2008; Pujhari et al., 2014; Yuan et al., 2016). Since infected pigs exhibited higher frequencies of $TUNEL^+$ cells than control animals, it is, therefore, likely that a cCasp3-independent RCD pathway, such as pyroptosis or necroptosis, would be triggered during the first week post-infection, playing a relevant role in pulmonary cell homeostasis and virus-infected cell clearance.

Futhermore, a significant increase in the frequency of $TUNEL^+$ cells was observerd in Lena-infected piglets at 3 and 8 dpi together with a rise in the number of $iNOS^+$ cells at 8 dpi, being associated both findings with the peak of lung damage. In this sense, the relase of NO (Chen et al., 2014; Lee and Kleiboeker, 2005; Yan et al., 2017) but also the activaction of RCD in virulent Lena strain infection may act as a double-edged sword, in one way, trying to kill fast and early infected cells but, on the other way, also causing severe lung injury (Bi et al., 2014; de Zoete et al., 2014; Li et al., 2015; Wang et al., 2015; Zhang et al., 2013). Interestingly, nets of free chromatin were observed in the alveoli of several Lena-infected pigs at 8 and 13 dpi with a moderate infiltrate of $TUNEL^-$/$cCasp3^-$ neutrophils in foci of suppurative bronchopneumonia. This finding might be linked to the formation of neutrophils extracellular traps (NETs), as a part of a process called NETosis, another kind of RCD (de Buhr et al., 2019; Funchal et al., 2015; Galluzzi et al., 2018; Henthorn et al., 2018; Moorthy et al., 2013; Schönrich and Raftery et al., 2019; Storisteanu et al., 2017; Tripathi et al., 2014). These results open new lines of research into the RCD pathways engaged in the acute phase of PRRSV infection and the impairment of pulmonary macrophage subpopulations.

One alternative hypothesis to explain the decrease in the frequency of $CD163^+$ cells could be the shedding of the extracellular ectodomain of CD163 leading to the release of sCD163 due to macrophage activation either by a direct effect of the virus or secondary to viral infection (Møller, 2012). However, it seems unlikely in our study, since a decrease in the frequency of $CD163^+$ cells within live PAMs was observed in the BALF of both PRRSV-1-infected groups throughout the study. Moreover, no changes were detected in the sera concentration of sCD163 in piglets infected with the virulent Lena strain. In contrast, virulent strains of Glaesserella parasuis have been reported to increase serum levels of sCD163 linked to a reduction in CD163 surface expression in PAMs (Costa-Hurtado et al., 2013).

Interestingly, we have also observed an increase in the frequency of $CD163^+$ cells in the lung of PRRSV-1 infected animals at two weeks post-infection counteracting the marked depletion induced during the first week post-infection. This recruitment of $CD163^+$ macrophages occurred together with a decrease in the number of PRRSV-N-$protein^+$ cells and lung viral load. In addition, the MFI (Median Fluorescence Intensity) of CD163 within PRRSV-N-$protein^-$ subsets of live PAMs from BALF declined along the study with respect to control group, which could be associated with, among other plausible explanations, the presence of immature macrophages $CD163^{low}$ in the BALF of PRRSV-1-infected piglets. Our findings point to the ability of the host to restore, and even to increase, the frequency of $CD163^+$ macrophages after one month post-infection. This replenishment of $CD163^+$ cells would be initiated by the influx of $CD14^+$ cells, mainly monocytes and immature macrophages, infiltrating extensive areas of the lung interstitium at 6 – 8 dpi in agreement with Van Gucht et al. (2005). Besides, an increase in the frequency of $CD107a^+$ cells, a glycoprotein primarily expressed on the surface of lysosomes and endosomes prior to degranulation and whose expression is higher in activated PAMs, was also observed. These facts suggest that after replenishment, PAMs have a high lysosomal content and hence, a better phagocytic capacity. All in all, these findings suggest an attempt of the host immune system to reconstitute the pulmonary immune homeostasis, contributing to apoptotic cell and cellular debris removal, restoring tissue damage as well as accelerating the resolution of inflammation (Hussell and Bell, 2014; García-Nicolás et al., 2014; Mantovani et al., 2004; Van Gorp et al., 2010). In this context, it should be also considered that an increase in the frequency of $CD14^+$ cells and, thus, a higher availability of CD14 in the pulmonary parenchyma, as the primary LPS-LBP complex receptor, is likely to

sensitise the lung to future secondary bacterial infections making the onset of PRDC easier (Van Gucht et al., 2004, 2005).

Simultaneously with this cascade of pro-inflammatory and RCD events in the lung, an anti-inflammatory and/or regulatory immune response is launched, in which CD200R1 and FoxP3 immune markers might play a role. Our results show a rise in the frequency of $CD200R1^+$ and $FoxP3^+$ cells at 6 and 8 dpi, coinciding with the highest severity of lung damage. Likewise, a strong positive correlation was detected among the frequency of $CD200R1^+$ cells and the microscopic lung score in Lena-infected pigs. CD200R1 has been involved in reducing the expression of pro-inflammatory cytokines in a wide range of inflammatory diseases (Vaine and Soberman, 2014), nevertheless, to the best of the authors' knowledge, the role of CD200R1 in viral diseases of swine is largely unknown. In addition, FoxP3 is the marker to detect Tregs, which have been reported as potential inhibitors of the cell-mediated immune response in pigs upon PRRSV infection (Ferrarini et al., 2015; Silva-Campa et al., 2009; 2010; Nedumpun et al., 2018). Therefore, our results highlight the upregulation of $CD200R1^+$ and $FoxP3^+$ cells as mechanisms involved in the constraint and recovery of lung injury during acute PRRSV infection; however, further studies are encouraged to decipher the role of these cells in the immunopathogenesis of the acute and chronic PRRSV infection.

In light of this framework, and considering in vitro studies on monocytes-derived macrophages upon polarization (García-Nicolás et al., 2014; Wang et al., 2017), as well as the high functional plasticity of pulmonary macrophages and their ability to adapt to different microenvironments (Guth et al., 2009; Hussell & Bell, 2014), we theorise that the influx of monocytes and macrophages, which replenish the lung resident macrophages after PRRSV-1 infection, are bound to alternatively activate M2 macrophages as a consequence of the pro-inflammatory microenvironment induced by virulent PRRSV-1strains in the lung. In addition, it is known that M2 macrophages have anti-inflammatory functions, do not interfere with PRRSV replication and express elevated levels of the scavenger receptor CD163 (García-Nicolás et al., 2014; Gordon & Martinez, 2010; Mosser, 2003; Mosser & Edwards, 2008; Wang et al., 2017). Thus, it seems that M2 macrophages take part in protecting the host from an overwhelming inflammatory response, restoring tissue and supporting the resolution of inflammation (Hussell & Bell, 2014; García-Nicolás et al., 2014; Mantovani et al., 2004; Van Gorp et al., 2010), and indeed, enhancing the process previously initiated by $CD200R1^+$ and Tregs.

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Conclusiones - Conclusions

Imagen: Gettyimages

CONCLUSIONES GENERALES

Primera conclusión: PRRSV-1 regula la expresión del receptor scavenger CD163 y del CD107a mediante mecanismos directos e indirectos durante la primera semana postinfección, siendo el hospedador capaz de restaurar e incluso incrementar el número de células que expresan ambos marcadores en el periodo de un mes tras la infección. Aunque esta restauración de las poblaciones de macrófagos podría reforzar la respuesta inmune local, también predispondría al pulmón frente a futuras reinfecciones con PRRSV así como frente a otros patógenos respiratorios (correspondiente al estudio "Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence").

Segunda conclusión: la frecuencia y expresión de superficie del receptor scavenger CD163 disminuye en PAMs vivos aislados del lavado broncoalveolar de cerdos infectados con PRRSV-1 produciéndose esta disminución de forma más temprana e intensa en el caso de la cepa virulenta Lena (correspondiente al estudio "Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells").

Tercera conclusión: PRRSV-1 induce muerte celular regulada sin activación de las vías de señalización de la apoptosis durante la primera semana postinfección, activando principalmente la vía extrínseca de la apoptosis, y en menor medida la vía intrínseca, durante la segunda semana postinfección. En este sentido, la cepa virulenta Lena induce una activación más intensa de estos fenómenos de muerte celular regulada asociada a una replicación más eficiente a nivel pulmonar y una marcada disminución de los PAMs $CD163^{+}$, causando daño pulmonar de forma más intensa y temprana (correspondiente al estudio "Activation of regulated cell death in the lung of piglets infected with virulent (Lena) PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8").

Cuarta conclusión: la infección por PRRSV-1 provoca una repoblación de PAMs $CD163^{+}$ PAMs a las dos semanas postinfección, probablemente a partir de células $CD14^{+}$, indicando un intento del hospedador de recuperar las poblaciones de macrófagos destruidas durante la fase inicial de la infección, contribuyendo además a la resolución del proceso inflamatorio a nivel pulmonar (correspondiente a los estudios

"Activation of regulated cell death in the lung of piglets infected with virulent (Lena) PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8" y "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena").

Quinta conclusión: la cepa virulenta PRRSV-1 Lena induce un incremento en la concentración sérica de IL-6 e IFN-γ asociada a una viremia más intensa, destacando por un lado un marcador de interés para la monitorización de la virulencia y daño pulmonar a nivel sistémico (IL-6) y por otro lado un intento de la respuesta inmune innata del hospedador por controlar la replicación del virus (IFN-γ) (correspondiente al estudio "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena").

Sexta conclusión: la respuesta inflamatoria a nivel pulmonar con la cepa virulenta PRRSV-1 Lena está regulada por un incremento en la expresión del CD14 y de iNOS, mecanismos involucrados en el desarrollo más temprano e intenso de dicha lesión y que además pueden predisponer al pulmón frente a infecciones bacterianas secundarias. Por otro lado, el incremento en la expresión del CD200R1 y el FoxP3 representa la activación de un mecanismo potencial involucrado en la contención de la respuesta inflamatoria (correspondiente al estudio "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena").

Séptima Conclusión: La aproximación con MALDI-MS Imaging permite la detección de una firma molecular de péptidos a nivel pulmonar tras la infección por PRRSV-1 con sus proteínas putativas asociadas a una serie de vías de señalización, destacando entre ellas la regulación de la muerte celular, la ruta principal de señalización de splicing del ARNm y la degranulación de neutrófilos.

GENERAL CONCLUSIONS

First conclusion: PRRSV-1 regulates the expression of CD163 scavenger receptor and CD107a through either a direct or indirect mechanism during the first week-postinfection, with the host being able to restore and to increase the number of cells expressing these receptors after one-month post-infection. This effect may strengthen both local immune response by the activation of lung macrophages and prime the lung against future PRRSV re-infections, as well as infections by other swine respiratory pathogens (corresponding to the study "Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different").

Second conclusion: the frequency and surface expression of the scavenger receptor CD163 decrease in live PAMs isolated from the BALF of PRRSV-1 infected piglets, being this fact earlier and stronger for the virulent Lena strain (corresponding to the study "Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells").

Third conclusion: PRRSV-1 induces the activation of non-apoptotic RCD during the first week post-infection followed by the activation of mainly extrinsic apoptosis, and in a lesser extent intrinsic apoptosis, during the second week post-infection. These events are markedly elicited by Lena strain associated with a high viral load and severe depletion of $CD163^{+}$ PAMs, causing severe and early lung damage (corresponding to the study "Activation of regulated cell death in the lung of piglets infected with virulent (Lena) PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8").

Fourth conclusion: Upon PRRSV-1 infection a repletion of $CD163^{+}$ PAMs is elicited at two weeks post-infection, probably from $CD14^{+}$ cells, which point out an attempt to reconstitute the pulmonary macrophages subpopulations lost during the early stages of the infection, contributing to resolution of pulmonary inflammation (corresponding to the study "Activation of regulated cell death in the lung of piglets infected with virulent (Lena) PRRSV-1 strain occurs earlier and is mediated by cleaved caspase 8" and "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena").

Fifth conclusion: Lena virulent PRRSV-1 strain induces a rise in sera concentration of IL-6 and IFN-γ associated with a higher level of viremia, pointing out to a marker of interest for monitoring virulence and lung injury (IL-6) as well as an attempt of the host innate immune response in controlling virus replication (IFN-**γ**) (corresponding to the study "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena").

Sixth conclusion: Virulent PRRSV-1 Lena strain-induced lung inflammatory response is regulated by the expression of CD14 and iNOS, mechanisms involved in the earlier and higher extent of lung lesion which may sensitise the lung to future secondary bacterial infections. On the other hand, the increase in the expression of CD200R1 and FoxP3 represents potential pathways activated to contain the inflammatory response (corresponding to the study "Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena").

Seventh conclusion: MALDI-MS Imaging approach enables the detection of peptide molecular signature in lung tissue upon PRRSV-1 infection with putative proteins associated with a number of pathways that might be regulated, highlighting among them the regulation of cell death, mRNA splicing-major pathway and neutrophil degranulation.

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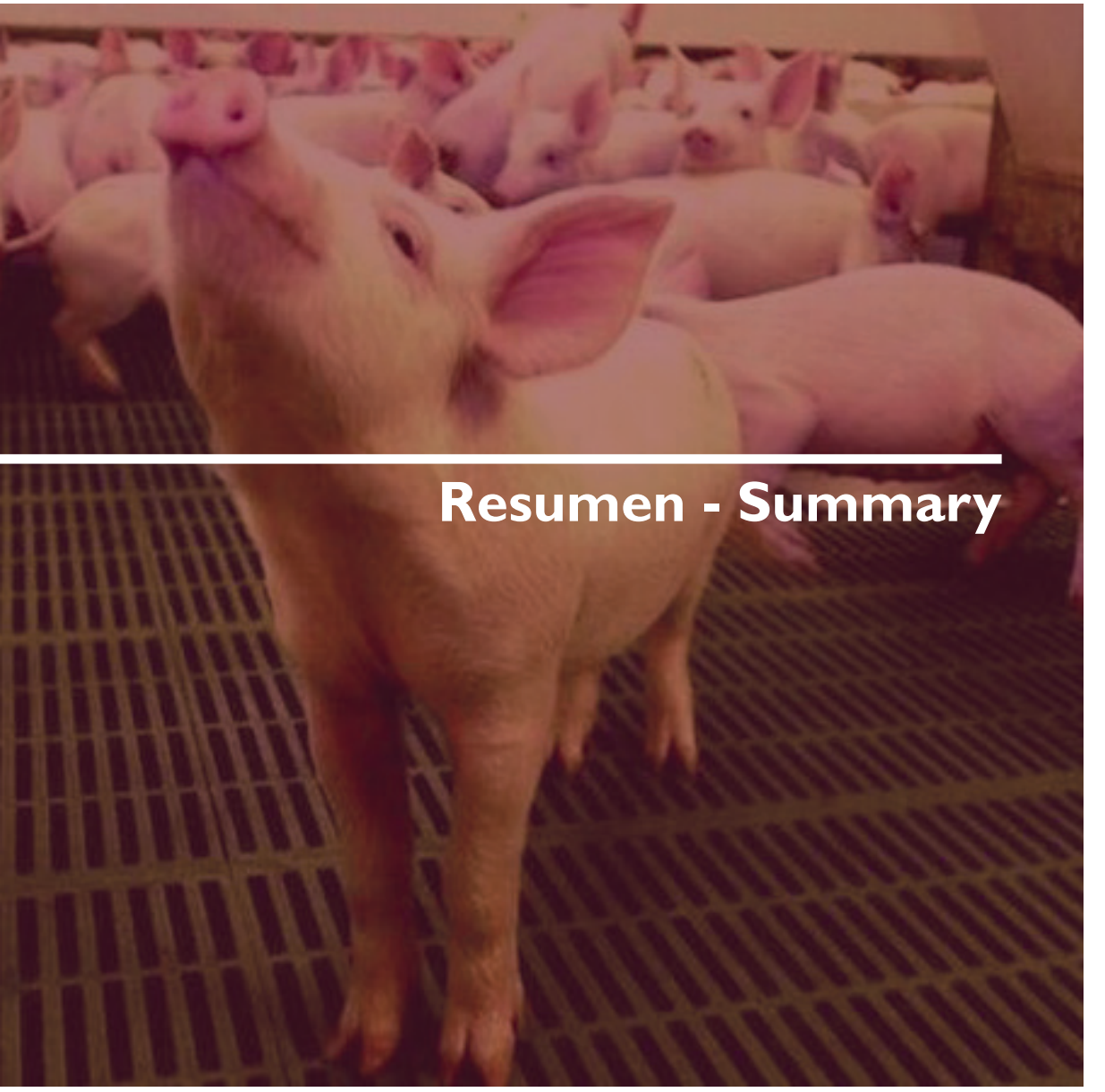


Resumen - Summary

El síndrome reproductivo y respiratorio porcino (PRRS, del inglés Porcine Reproductive and Respiratory Syndrome) es una de las enfermedades víricas más importantes para el sector porcino a nivel mundial debido a las enormes pérdidas económicas que genera. El virus del PRRS (PRRSV, del inglés Porcine Reproductive and Respiratory Syndrome Virus), agente etiológico del PRRS, juega un papel importante en el complejo respiratorio porcino (PRDC, del inglés Porcine Respiratory Disease Complex) provocando una modulación de la respuesta inmune que favorece su persistencia en el organismo, así como infecciones bacterianas secundarias.

La replicación del PRRSV tiene lugar principalmente en el pulmón, siendo los macrófagos alveolares pulmonares (PAMs, del inglés Pulmonary Alveolar Macrophages) la célula diana del virus y el receptor scavenger CD163, el cual se expresa mayoritariamente en los PAMs, esencial para que ocurra una infección productiva. EL PRRSV provoca una neumonía intersticial de moderada a grave, así como una modulación y detrimento de las poblaciones de macrófagos pulmonares, lo que complica este cuadro respiratorio favoreciendo el desarrollo de una bronconeumonía purulenta. Desde el año 2006, se han descrito diferentes brotes asociados a cepas de elevada virulencia del PRRSV-1 a lo largo del continente europeo. Estos brotes presentan un cuadro clínico lesional muy particular caracterizado por una tasa de mortalidad elevada, fiebre y una sintomatología respiratoria grave asociada con una respuesta inflamatoria más intensa y temprana, así como un marcado daño a nivel pulmonar y de los órganos linfoides. Además, el PRRSV induce una respuesta inflamatoria que varía en función de la cepa del virus y del órgano infectado, por lo que la evaluación in situ de cada uno de los órganos diana del virus es esencial para descifrar la inmunopatogenia de la infección con cepas de diferente virulencia del PRRSV.

Teniendo en cuenta estos antecedentes, la presente tesis doctoral se centra en la valoración del cuadro lesional, la evaluación de la muerte celular y la expresión de diferentes marcadores inmunológicos de potencial interés, especialmente el receptor scavenger CD163, a nivel pulmonar, así como la respuesta inmune a nivel sistémico. Por tanto, el principal objetivo de esta tesis consiste en profundizar en la inmunopatogenia de la forma respiratoria del PRRSV-1 con cepas de diferente virulencia durante la fase aguda de la infección.

El primer estudio de esta tesis evalúa la expresión, distribución y cinética de células PRRSV-N-protein$^+$, CD163$^+$ y CD107a$^+$ en el pulmón y la tonsila de cerdos infectados experimentalmente con 3 cepas del PRRSV-1: la cepa virulenta del subtipo 3 SU1-bel, y dos cepas de baja virulencia del subtipo 1, LV y 215-06. Los animales fueron eutanasiados a los 3, 7 y 35 días post-inoculación (dpi). La cepa SU1-bel se replicó de forma más eficiente en el pulmón y la tonsila. El número de células CD163$^+$ disminuyó tanto en la tonsila como en el pulmón de todos los animales infectados a los 7 dpi seguido por un incremento al final del estudio, destacando una correlación negativa con la frecuencia de células PRRSV-N-protein$^+$. Además, se observó un incremento en el número de células CD107a$^+$ en todos los grupos infectados a los 35 dpi sin diferencias entre grupos. Mientras que la disminución inicial en el número de células CD163$^+$ parece estar asociada tanto a la replicación del virus como a la activación de procesos de muerte celular, la recuperación de la población de macrófagos CD163$^+$ al final del estudio podría deberse tanto al influjo de células CD163 inmaduras, al reclutamiento de células CD163$^+$ en la zona de infección, o a ambos procesos. Estos resultados sugieren que el hospedador es capaz de restaurar esta subpoblación de macrófagos, así como sus potenciales funciones biológicas después de un mes tras la infección con cepas del PRRSV-1, con una especial relevancia en los animales infectados con la cepa virulenta SU1-bel.

En el segundo estudio, nuestro objetivo se centra en comparar los signos clínicos, las lesiones macroscópicas y microscópicas, así como la expresión del receptor CD163 dentro de la subpoblación de PAMs vivos del lavado broncoalveolar (BALF, del inglés BronchoAlveolar Lavage Fluid). Para ello, cerdos convencionales libres de PRRSV se inocularon por vía intranasal con dos cepas del PRRSV-1 de diferente virulencia, la cepa virulenta Lena perteneciente al subtipo 3, o la cepa de baja virulencia 3249, perteneciente al subtipo 1, y que se sacrificaron consecutivamente a los 1, 3, 6, 8 y 13 dpi. Los signos clínicos más graves, así como el mayor grado de lesión a nivel macroscópico y microscópico se observaron en los lechones infectados con la cepa virulenta Lena, en comparación con el grupo 3249. Asimismo, se detectó un descenso temprano en la frecuencia de PAMs en el BALF, asociado a una disminución de la frecuencia y la intensidad de fluorescencia media (MFI, del inglés Median Fluorescence Intensity) del CD163 dentro de estos PAMs que fueron mucho más bajas en los cerdos infectados con Lena que en los cerdos infectados con la cepa 3249. Estos resultados podrían deberse a distintos factores como el efecto de la replicación del PRRSV, la inflamación

inducida por el propio virus, la llegada de macrófagos inmaduros para restaurar la homeostasis pulmonar y/o la presencia de células CD163low tras la disminución sufrida por la subpoblación de células CD163$^+$ en el BALF.

En el tercer estudio, nos centramos en evaluar los eventos de muerte celular regulada (RCD, del inglés Regulated Cell Death) mediante la expresión de una serie de caspasas tras la infección por ambas cepas del PRRSV-1, Lena y 3249. Las muestras de tejido pulmonar se seleccionaron a partir del experimento anterior. La cepa virulenta Lena indujo una frecuencia significativamente mayor de células TUNEL$^+$ comparado con la frecuencia de las células cCasp3$^+$ durante la primera semana post-infección. Además, ambas cepas de PRRSV-1 activaron la cCasp8 y, en menor medida, la cCasp9 después de la primera semana post-infección junto con un restablecimiento de la frecuencia de macrófagos pulmonares CD163$^+$. Estos resultados evidencian la inducción de diferentes vías de RCD además de la apoptosis, como la necroptosis y la piroptosis, durante la primera semana post-infección, junto con una activación posterior de la vía extrínseca de la apoptosis principalmente durante la segunda semana post-infección. Además, el restablecimiento de los macrófagos CD163$^+$ durante la segunda semana postinfección representa un intento de restaurar las subpoblaciones perdidas de macrófagos pulmonares durante las primeras etapas de la infección.

El objetivo del cuarto estudio es explorar el papel de varios marcadores inmunes potencialmente involucrados en la regulación de la respuesta inflamatoria y en la sensibilización del pulmón a infecciones bacterianas secundarias durante la primera semana post-infección con dos cepas del PRRSV-1 de diferente virulencia, Lena y 3249. Para ello se recogieron muestras de suero y tejido pulmonar, que posteriormente fueron procesados para sus correspondientes análisis. Los títulos de viremia y los signos clínicos fueron significativamente más altos en los lechones infectados con la cepa Lena, junto con un aumento en los niveles séricos de IFN-γ e IL-6 en comparación con los cerdos infectados con la cepa 3249. Lena provocó la aparición de las lesiones pulmonares de forma mucho más temprana e intensa, como consecuencia de extensas áreas de consolidación en los lóbulos pulmonares apical y medial, las cuales se correspondieron microscópicamente con focos de bronconeumonía catarro purulenta. La carga viral y la frecuencia de células PRRSV-N-protein$^+$ a nivel pulmonar siempre fueron superiores en los animales infectados con la cepa Lena, que se asociaron a una marcada disminución en la frecuencia de macrófagos CD163$^+$. El número de células CD14$^+$ e iNOS$^+$ aumentó

gradualmente durante la infección por PRRSV-1, siendo este incremento mucho más intenso en los cerdos infectados con Lena. La frecuencia de las células $CD200R1^+$ y $FoxP3^+$ alcanzó su pico a los 8 dpi en los animales infectados con ambas cepas del PRRSV-1, observándose además una fuerte correlación entre el número de células $CD200R1^+$ y la lesión pulmonar en los cerdos infectados con Lena. Estos resultados ponen en evidencia el papel que estas moléculas parecen jugar en el desarrollo más temprano de las graves lesiones pulmonares observadas en los animales infectados con la cepa virulenta Lena, sugiriendo la activación de rutas potencialmente involucradas en la restricción de la respuesta inflamatoria a nivel local.

Finalmente, nuestro quinto estudio consiste en una aproximación a la histopatología molecular mediante MALDI-MS Imaging con el objetivo de identificar nuevos péptidos involucrados en la inmunopatogénesis del PRRSV-1 así como diferentes patrones de lesión pulmonar. Los análisis se realizaron en 3 animales de cada grupo seleccionados a los 6 y 8 dpi utilizando como criterios la gravedad de los signos clínicos, el grado de lesión pulmonar a nivel macroscópico y microscópico, así como la carga viral en cerdos infectados con las cepas Lena y 3249, en base a los estudios previos. El estudio MALDI-MS Imaging detectó un perfil de 54 péptidos sobreexpresados diferencialmente, así como las proteínas putativas asociadas al comparar ambos grupos infectados con PRRSV-1 con el grupo control a los 6 y 8 dpi. Algunas de estas potenciales proteínas se han descrito previamente en la infección por PRRSV, como la proteína de choque térmico beta-1, la proteína de choque térmico 72, la proteína de unión a GTP inducida por interferón Mx1, la variante de proteína proapoptótica BAKM, la apolipoproteína A-IV, la anexina A6, matrix metalopeptidasa 9, vimentina, complemento C3, filamina A, anexina IV o la proteína asociada al citoesqueleto 4. Además, otras proteínas potenciales sobreexpresadas como la apolipoproteína AI, la proteína relacionada con ras Rap-1b, la proteína ribosómica L13, L-lactato deshidrogenasa B, la tropomiosina 1 o la histona H4 se identificaron en nuestro estudio, aunque no se habían relacionado previamente con la infección por PRRSV. A partir de las proteínas sobreexpresadas se generó una red de grupos funcionales utilizando las herramientas bioinformáticas Cluego y Cluepedia, la cual mostró, que la mayoría de estas proteínas putativas estuvieron involucradas principalmente en la "regulación de la muerte celular" dentro de la categoría de ontología génica de procesos biológicos, mientras que se agruparon en la "vía principal del splicing de ARNm" y "desgranulación de neutrófilos" dentro de la categoría de reactoma y procesos del sistema inmune.

Porcine reproductive and respiratory syndrome (PRRS) is still considered one of the costliest viral diseases impacting on the modern pig industry worldwide. PRRS virus (PRRSV), the causative agent of PRRS, is also a key player in porcine respiratory disease complex (PRDC), modulating the host immune response, leading to persistent infection, and favouring secondary bacterial infections.

PRRSV replicates primarily in the lung, being the pulmonary alveolar macrophages (PAMs) the target cell for viral replication and CD163 scavenger receptor the essential mediator for viral internalisation and disassembly. PRRSV induces a mild to severe interstitial pneumonia, with modulation and impairment of pulmonary macrophage subpopulations, which may be complicated to suppurative bronchopneumonia. Since 2006, outbreaks of virulent PRRSV-1 strains have been described across Europe. They were characterised by high mortality rates, fever and severe respiratory disorders associated with an earlier and exacerbated inflammatory response with a marked tissue injury in the lung and lymphoid organs. Furthermore, PRRSV elicits a distinct immune response depending on both the virulence of the strain and the infected tissue, therefore the evaluation of target organs may pave the way to decipher the immunopathogenesis of PRRSV infection with strain of different virulence.

In the light of the above statements, the present PhD dissertation focuses on the assessment of the lesion patterns, cell death phenomena and the expression of several immune markers of potential interest, particularly the scavenger receptor CD163, in the lung of infected animals as well as the systemic immune response. Thus, this thesis aims to dissect the immunopathogenesis of the respiratory disease caused by PRRSV-1 strains of different virulence in the acute phase of infection.

The first study of this PhD Thesis aims to compare the expression, distribution and kinetics of PRRSV-N-protein, CD163 and CD107a positive cells in the lung and tonsil from experimentally-infected piglets with three different PRRSV-1 strains: a virulent PRRSV-1 subtype 3 strain (SU1-bel) and two low virulent subtype 1 strains, LV and 215–06. Piglets were sequentially euthanised at 3, 7 and 35 dpi. SU1-bel strain replicated more efficiently in the lungs and tonsils. The number of $CD163^{+}$ cells decreased in both tissues from all infected groups at 7 dpi, followed by an increase at the end of the study, highlighting a negative correlation with the frequency of PRRSV-N-$protein^{+}$ cells. A significant increase in $CD107a^{+}$ cells was observed in all infected groups at 35 dpi but

no differences were observed among them. Whereas the initial decrease of $CD163^+$ cells appears to be associated to virus replication and cell death, the later recovery of the $CD163^+$ population may be due to either the induction of CD163 in immature cells, the recruitment of $CD163^+$ cells in the area of infection, or both. These results suggest that the host is able to restore its macrophage subpopulations as well as their potential biological functions after one-month post-infection with PRRSV-1 strains, finding the greatest recovery in SU1-bel-infected animals.

In the second study, our goal was to compare the clinical signs, gross and microscopic lesions as well as the expression of CD163 within live PAM subset from BALF. Conventional piglets were intranasally inoculated with two PRRSV-1 strains of different virulence, the virulent subtype 3 Lena strain or the low virulent subtype 1 3249 strain and euthanised at 1, 3, 6, 8 and 13 dpi. More severe clinical signs as well as higher macroscopic and microscopic lung scores were observed in Lena-infected piglets compared to 3249-infected pigs. A decreased frequency of PAMs in BALF was detected earlier in Lena group. Besides, the frequency and median fluorescence intensity of CD163 within PAMs were much lower in Lena-infected pigs than in 3249-infected pigs. This outcome may result from the effect of PRRSV replication, PRRSV-induced inflammation, the influx of immature macrophages to restore lung homeostasis and/or the evidence of CD163low cells after $CD163^+$ cells decrease in BALF.

In the third study, we focused on evaluating Regulated Cell Death (RCD) events by means of the expression of caspases after Lena and 3249 strains infection. Lung tissue samples from the previous experiment were collected at 1, 3, 6, 8 and 13 dpi and hence, processed for their analysis. Lena virulent strain induced a significantly higher frequency of $TUNEL^+$ cells when comparing with the frequency of $cCasp3^+$ cells during the first week post-infection. In addition, both PRRSV-1 strains triggered cCasp8 and in a lesser extent cCasp9 after one-week post-infection together with a replenishment of $CD163^+$ pulmonary macrophages. These results highlight the induction of several RCD pathways beyond apoptosis, such as necroptosis and pyroptosis, during the first week post-infection followed by activation of, mainly, the extrinsic apoptosis pathway during the second week post-infection. Moreover, the recovery of $CD163^+$ macrophages at second week post-infection represents an attempt to restore pulmonary macrophage subpopulations lost during the early stages of the infection.

The goal of the fourth study was to explore the role of several immune markers potentially involved during the first week post-infection in the regulation of the inflammatory response and sensitisation of lung to secondary bacterial infections upon two PRRSV-1 strains of different virulence, Lena and 3249 strains. Sera and lung tissue samples were collected and accordingly processed for their corresponding analyses. Viraemia titres and clinical signs were higher in Lena-infected piglets with an increase in the sera levels of IFN-γ and IL-6 compared to 3249-infected pigs. Lena strain triggered an earlier and stronger onset of lung lesions due to extensive consolidated areas in the apical and medial lung lobes which corresponded microscopically with foci of suppurative bronchopneumonia. Lung viral load and PRRSV-N-protein$^+$ cells were always higher in Lena-infected animals with PRRSV-N-protein$^+$ cells associated with a marked drop in CD163$^+$ macrophages. The number of CD14$^+$ and iNOS$^+$ cells gradually increased upon PRRSV-1 infection, being more intense in Lena-infected pigs. The frequency of CD200R1$^+$ and FoxP3$^+$ cells peaked in both PRRSV-1 strains at 8 dpi, highlighting a strong correlation among CD200R1$^+$ cells and lung injury in Lena-infected pigs. These results point out the role of molecules involved in the earlier and higher extent of lung lesions in piglets infected with the virulent Lena strain, underlining the activation of routes potentially involved in the restraint of the local inflammatory response.

Finally, the fifth study consisted in an approach to molecular histopathology by MALDI-MSI with the goal of identifying new peptides involved in PRRSV-1 immunopathogenesis and lung lesion patterns. Analyses were conducted in 3 selected animals per group at 6 and 8 dpi. These time-points were selected according to the detection of marked clinical sings, gross and microscopic lung lesion scores as well as viral load in Lena- and 3249-infected piglets after previous studies. MALDI-MSI study revealed a profile of 54 differentially overexpressed peptides and their putative associated proteins when comparing both infected PRRSV-1-infected groups with control group at 6 and 8 dpi. Some of these putative proteins have been previously described upon PRRSV infection, such as heat shock protein beta-1, heat shock protein 72, interferon-induced GTP-binding protein Mx1, pro-apoptotic protein BAKM variant, apolipoprotein A-IV, annexin A6, matrix metallopeptidase 9, vimentin, complement C3, filamin-A, annexin IV or cytoskeleton-associated protein 4. But also other overexpressed putative proteins, such as apolipoprotein A-I, ras-related protein Rap-1b, ribosomal protein L13, L-lactate dehydrogenase B chain, tropomyosin 1 or histone H4 were also identified in our studies but not previously associated with PRRSV infection. A functionality grouped network

of the overexpressed proteins created by Cluego and Cluepedia tools, showed that the majority of these putative proteins were mainly involved in "regulation of cell death" within biological processes, and they were in "mRNA splicing-major pathway" and "neutrophil degranulation" within reactome pathways and immune system processes.

07

Indicios de calidad - Quality evidences

INDICIOS DE CALIDAD DE LA TESIS APORTADOS POR EL DOCTORANDO

APELLIDOS	NOMBRE
Sánchez Carvajal	*José María*

TÍTULO DE LA TESIS

El papel del CD163 y otros biomarcadores de interés en la inmunopatogenia del PRRSV-1 a nivel pulmonar

Role of CD163 and other biomarkers of interest in the immunopathogenesis of PRRSV-1 at lung level

PROGRAMA DE DOCTORADO

Biociencias y Ciencias Agroalimentarias

X ESPECIFICAR LA PUBLICACIÓN QUE APORTA COMO INDICIOS DE CALIDAD DE LA TESIS, ESTABLECIDOS EN EL ARTº. 25.a DE LAS ACTUALES NORMAS REGULADORAS DE LOS ESTUDIOS DE DOCTORADO (el Doctorando figurará como primer autor, o segundo si el director es el primero)

- Título: ***Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence***
- Autores (p.o. de firma): *José M. Sánchez-Carvajal, Irene M. Rodríguez-Gómez, Librado Carrasco, Inmaculada Barranco, Belén Álvarez, Javier Domínguez, Francisco J. Salguero, Jaime Gómez-Laguna*
- Revista (año vol.,pág.): *Veterinary research communications (2019), 43(3), 187-195.*
- Base de Datos Internacional o Nacional (caso de CC.JJ., CC.SS. Y Humanidades) en las que está indexada: ISI Web of Citation.
- Área temática en la Base de Datos de referencia: Veterinary Sciencies.
- Índice de impacto de la revista en el año de publicación del Artículo: 1.013
- Lugar que ocupa/Nº de revistas del Área temática: 70/140

- Título: ***Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells***
- Autores (p.o. de firma): *Irene M. Rodríguez-Gómez, José M. Sánchez-Carvajal, Francisco J. Pallarés, Enric Mateu, Librado Carrasco, Jaime Gómez-Laguna*
- Revista (año vol., pág.): *Veterinary microbiology (2019), 235, 101-109.*
- Base de Datos Internacional o Nacional (caso de CC.JJ., CC.SS. Y Humanidades) en las que está indexada: ISI Web of Citation
- Área temática en la Base de Datos de referencia: Veterinary Sciencies.
- Índice de impacto de la revista en el año de publicación del Artículo: 2.791
- Lugar que ocupa/Nº de revistas del Área temática: 7/141

- Título ***Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena***
- Autores (p.o. de firma): *J.M. Sánchez-Carvajal, I.M. Rodríguez-Gómez, I. Ruedas-Torres, F. Larenas-Muñoz, I. Díaz, C. Revilla, E. Mateu, J. Domínguez, G. Martín-Valls, I. Barranco, F.J. Pallarés, L. Carrasco, J. Gómez-Laguna.*
- Revista (año vol., pág.): *Veterinary Microbiology (2020), 108744*
- Base de Datos Internacional o Nacional (caso de CC.JJ., CC.SS. Y Humanidades) en las que está indexada: ISI Web of Citation
- Área temática en la Base de Datos de referencia: Veterinary Sciencies.
- Índice de impacto de la revista en el año de publicación del Artículo: 2.791
- Lugar que ocupa/Nº de revistas del Área temática: 7/141

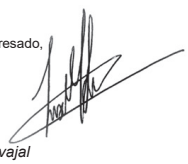
X INTERNACIONALIZACIÓN TESIS: PRESENTA LA TESIS CON MENCION INTERNACIONAL Y APORTA CONTRIBUCIONES CIENTIFICAS NO INCLUIDAS COMO PUBLICACIONES

SOLICITA:

Sea autorizada la tramitación de la tesis doctoral, una vez que sean constatados los indicios de calidad que se adjuntan a esta solicitud.

Córdoba, a 16 de junio de 2020

Firma del interesado,



Fdo.: *José María Sánchez Carvajal*

SR/A. COORDINADOR/A DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Institute of Virology and Immunology IVI
Sensemattstrasse 293, 3147 Mittelhäusern
Switerland

June 12, 2019

To whom it may concern,

I hereby confirm that Mr. Jose Maria Sanchez Carvajal has been a guest PhD student at Institute of Virology and Immunology from 15/ 03/ 2019 to 15/ 06/ 2019 under my supervision.
Jose has been conducting experiments to determine the impact of monocyte activation by various ligands on PRRSV infection and replication in monocyte-derived macrophages. His work was also directed towards identification of ligands which "train" monocytes for higher innate responses following secondary stimulation. The ligands included TLR2/1, TLR4 and TLR7/8 ligands as well as dectin-1 ligands. This included preparation of monocytes from porcine blood using magnetic cell sorting, generation of monocyte-derived macrophages, flow cytometry-based analysis of virus infection and cytokine ELISAs. The results generated are very interesting and worth following up.

Jose has been very dedicated to his work and productive during this short stay. He has very rapidly learned the new techniques and his experiments have been successful. He was well integrated into our team and it was a great pleasure to have him with us.
I wish him all the best for the continuation of his career.

Sincerely,

Institut für Virologie und Immunologie IVI
Prof. Dr. Artur Summerfield
Länggassstrasse 122, 3012 Bern
Tel. +41 31 631 2423, Fax +41 31 634 2534
Artur.Summerfield@ivi.admin.ch
Artur.Summerfield@vetsuisse.unibe.ch

Professor Artur Summerfield
Department of Infectious Diseases and Pathobiology, Vetsuisse Faculty, University of Bern
Head of the Host Pathogen Interaction Research Priority Focus of the Vetsuisse Faculty
Head of the Immunology and member of the Board of Directors of the Institute of Virology and Immunology
Contact: artur.summerfield@vetsuisse.unibe.ch; tel. +41 31 631 24 73 or 058 469 9377

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SHORT COMMUNICATION

Kinetics of the expression of CD163 and CD107a in the lung and tonsil of pigs after infection with PRRSV-1 strains of different virulence

Jose M. Sánchez-Carvajal[1] · Irene M. Rodríguez-Gómez[1] · Librado Carrasco[1] · Inmaculada Barranco[1] · Belén Álvarez[2] · Javier Domínguez[2] · Francisco J. Salguero[3] · Jaime Gómez-Laguna[1]

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Abstract
The emergence of virulent strains of porcine reproductive and respiratory syndrome virus (PRRSV), causing atypical and severe outbreaks, has been notified worldwide. This study assesses the expression, distribution and kinetics of PRRSV N-protein, CD163 and CD107a in the lung and tonsil from experimentally-infected piglets with three different PRRSV-1 strains: a virulent PRRSV-1 subtype 3 strain (SU1-bel) and two low-virulent subtype 1 strains, Lelystad virus (LV) and 215–06. SU1-bel replicated more efficiently in the lungs and tonsils. The number of $CD163^+$ cells decreased in both tissues from all infected groups at 7 dpi, followed by an increase at the end of the study, highlighting a negative correlation with the number of N-$protein^+$-infected cells. A significant increase in CD107a was observed in all infected groups at 35 dpi but no differences were observed among them. Whereas the initial decrease of $CD163^+$ cells appears to be associated to virus replication and cell death, the later recovery of the $CD163^+$ population may be due to either the induction of CD163 in immature cells, the recruitment of $CD163^+$ cells in the area of infection, or both. These results highlight the ability of macrophage subpopulations in infected animals to recover and restore their potential biological functions at one-month post-infection, with the greatest improvement observed in SU1-bel-infected animals.

Keywords PRRSV-1 · CD163 · CD107a · Macrophages · Lung · Tonsil

Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) continues being one of the most important and costly diseases of the porcine industry worldwide (Nieuwenhuis et al. 2012). The causal agent, PRRS virus (PRRSV), is an enveloped, positive-stranded RNA virus belonging to genus *Betaartevirus* within the family *Arteriviridae*, order Nidovirales. Recently, PRRSV-1 and PRRSV-2 have been considered as two distinct viral species, *Betaartevirus suid 1* and *Betaarterivirus suid 2*, respectively (Gorbalenya et al. 2018), due to their high genetic variability. In the last decade, virulent PRRSV strains have emerged and caused atypical outbreaks; these have been linked to high mortality rates, severe clinical signs and lung lesions, and an unusual immune response from the host (Han et al. 2006, Karniychuk et al. 2010, Xiao et al. 2010, Amarilla et al. 2015, Morgan et al. 2016, Canelli et al. 2017).

Pulmonary alveolar macrophages (PAMs) are the primary target cells for PRRSV replication, although the virus is able to replicate in other pulmonary macrophages subpopulations too (Duan et al. 1997). The expression of the scavenger receptor CD163 is restricted to the monocyte/macrophage lineage and is associated with macrophage differentiation being expressed on the cell surface and within endosomal vesicles in PAMs (Sánchez et al. 1999). This receptor plays a key role in the

Francisco J. Salguero and Jaime Gómez-Laguna contributed equally to this work.

✉ Jose M. Sánchez-Carvajal
jmsc919@gmail.com

[1] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, International Excellence Agrifood Campus 'ceiA3', 14071 Córdoba, Spain

[2] Department of Biotechnology, National Institute for Agricultural and Food Research and Technology (INIA), Madrid, Spain

[3] Department of Pathology, Public Health England, PHE Porton Down, Salisbury, UK

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Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells

Irene M. Rodríguez-Gómez[a,*], José M. Sánchez-Carvajal[a], Francisco J. Pallarés[b], Enric Mateu[c], Librado Carrasco[a], Jaime Gómez-Laguna[a]

[a] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, International Excellence Agrifood Campus 'ceiA3', Córdoba, Spain
[b] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Murcia, Mare Nostrum Excellence Campus, Murcia, Spain
[c] Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra, Barcelona, Spain

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ABSTRACT

Highly virulent porcine reproductive and respiratory syndrome virus (PRRSV) strains have increasingly overwhelmed Asia and Europe in recent years. This study aims to compare the clinical signs, gross and microscopic findings as well as the expression of CD163 within live pulmonary alveolar macrophages (PAMs) from bronchoalveolar lavage fluid (BALF) of pigs experimentally infected with two PRRSV strains of different virulence. Pigs were infected with either a subtype 1 PRRSV-1 3249 strain or a subtype 3 PRRSV-1 Lena strain and consecutively euthanized at 1, 3, 6, 8 and 13 days post-inoculation. Clinical signs were reported daily and BALF and lung tissue samples were collected at the different time-points and accordingly processed for their analysis. Pigs infected with Lena strain exhibited greater clinical signs as well as gross and microscopic lung scores compared to 3249-infected pigs. A decreased frequency of PAMs from BALF was observed early in pigs infected with Lena strain. Moreover, the frequency and median fluorescence intensity (MFI) of CD163 within PAMs were much lower in Lena-infected pigs than in 3249-infected pigs. This downregulation in CD163 was also observed in lung sections after the assessment of macrophages expressing CD163 by means of immunohistochemistry. This outcome may result from the effect of PRRSV replication, PRRSV-induced inflammation, the influx of immature macrophages to restore lung homeostasis and/or the evidence of $CD163^{low}$ cells after $CD163^{+}$ cells decrease in BALF.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is still a significant problem for the global swine industry. Twenty years after the first description of PRRS virus (PRRSV) (Wensvoort et al., 1991; Benfield et al., 1992; Collins et al., 1992), a collection of severe outbreaks, by the so-called highly pathogenic-PRRSV (HP-PRRSV) strains, overwhelmed China and Southeast Asia in 2006 (Tian et al., 2007; Tong et al., 2007; Feng et al., 2008; Zhou et al., 2008). Shortly afterwards, PRRSV strains of increased virulence, although not comparable with the ones reported in Asia, emerged in Belarus, Belgium, Austria and Italy between 2007 and 2015 (Karniychuk et al., 2010; Morgan et al., 2013; Frydas et al., 2015; Sinn et al., 2016; Canelli et al., 2017; Stadejek et al., 2017).

Because of the high genetic and antigenic differences, PRRSV-1 and PRRSV-2 species have been recently renamed as *Betaarterivirus suid 1* (formerly genotype 1) and *Betaarterivirus suid 2* (formerly genotype 2) with different subtypes or lineages within them, respectively (Shi et al., 2010; Stoian and Rowland, 2019). In case of PRRSV-1, the phylogenetic diversity is quite broad, especially in Central Eastern Europe, where the transboundary movement of infected animals has contributed to the wide viral diversity (Balka et al., 2018). Up to now, four subtypes are described: Pan-European subtype 1, Eastern European subtypes 2 and 3 (Stadejek et al., 2006, 2008) and subtype 4 (Stadejek et al., 2013). Traditionally, virulent PRRSV-1 strains were restricted to subtype 3 strains (Lena strain -prototype- [Karniychuk et al., 2010] and SU1-Bel strain [Morgan et al., 2013]); however, strains with similar characteristics have been identified within subtypes 1 (13V091 strain [Frydas et al., 2015], AUT15-33 strain [Sinn et al., 2016] and PR40/2014 [Canelli et al., 2017]) and 2 (BOR59 strain [Stadejek et al., 2017]) in recent years, being the hallmark between them a discontinuous deletion of amino acids in nonstructural protein 2 (Van Doorsselaere et al.,

* Corresponding author at: Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14071, Córdoba, Spain.
E-mail address: v22rogoi@uco.es (I.M. Rodríguez-Gómez).

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Activation of pro- and anti-inflammatory responses in lung tissue injury during the acute phase of PRRSV-1 infection with the virulent strain Lena

J.M. Sánchez-Carvajal[a,*], I.M. Rodríguez-Gómez[a], I. Ruedas-Torres[a], F. Larenas-Muñoz[a], I. Díaz[b], C. Revilla[c], E. Mateu[b,d], J. Domínguez[c], G. Martín-Valls[d], I. Barranco[a], F.J. Pallarés[e], L. Carrasco[a], J. Gómez-Laguna[a]

[a] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, 14014, Córdoba, Spain
[b] Institut de Recerca i Tecnologia Agroalimentàries - Centre de Recerca en Sanitat Animal (IRTA-CReSA), Campus de la Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain
[c] Department of Biotechnology, National Institute for Agricultural and Food Research and Technology (INIA), 28040, Madrid, Spain
[d] Department of Animal Health and Anatomy, Faculty of Veterinary Medicine, Autonomous University of Barcelona, 08193, Bellaterra, Spain
[e] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Murcia, 30100, Murcia, Spain

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) plays a key role in porcine respiratory disease complex modulating the host immune response and favouring secondary bacterial infections. Pulmonary alveolar macrophages (PAMs) are the main cells supporting PRRSV replication, with CD163 as the essential receptor for viral infection. Although interstitial pneumonia is by far the representative lung lesion, suppurative bronchopneumonia is described for PRRSV virulent strains. This research explores the role of several immune markers potentially involved in the regulation of the inflammatory response and sensitisation of lung to secondary bacterial infections by PRRSV-1 strains of different virulence. Conventional pigs were intranasally inoculated with the virulent subtype 3 Lena strain or the low virulent subtype 1 3249 strain and euthanised at 1, 3, 6 and 8 dpi. Lena-infected pigs exhibited more severe clinical signs, macroscopic lung score and viraemia associated with an increase of IL-6 and IFN-γ in sera compared to 3249-infected pigs. Extensive areas of lung consolidation corresponding with suppurative bronchopneumonia were observed in Lena-infected pigs. Lung viral load and PRRSV-*N*-protein$^+$ cells were always higher in Lena-infected animals. PRRSV-*N*-protein$^+$ cells were linked to a marked drop of CD163$^+$ macrophages. The number of CD14$^+$ and iNOS$^+$ cells gradually increased along PRRSV-1 infection, being more evident in Lena-infected pigs. The frequency of CD200R1$^+$ and FoxP3$^+$ cells peaked late in both PRRSV-1 strains, with a strong correlation between CD200R1$^+$ cells and lung injury in Lena-infected pigs. These results highlight the role of molecules involved in the earlier and higher extent of lung lesions in piglets infected with the virulent Lena strain, pointing out the activation of routes potentially involved in the restraint of the local inflammatory response.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) encompasses two species, *Betaarterivirus suid 1* and *Betaarterivirus suid 2* (formerly, PRRSV-1 and PRRSV-2, respectively) (Gorbalenya et al., 2018), which present a wide inter- and intra-species viral diversity (Balka et al., 2018; Shi et al., 2010; Stadejek et al., 2017). Since 2006, different outbreaks characterised by high morbidity and mortality rates, fever, haemorrhages, severe lesions in lung and, eventually, in other organs such as thymus or lymph nodes, have been reported associated with virulent PRRSV-1 strains (Canelli et al., 2017; Karniychuk et al., 2010; Morgan et al., 2013, 2016; Ogno et al., 2019; Sinn et al., 2016; Weesendorp et al., 2013). Several contradictory results about viraemia, tissue viral load, early virus clearance, low frequencies of PRRSV-specific IFN-γ secreting cells or PRRSV neutralizing antibodies have been reported after infection with PRRSV-1 virulent strains. However, there is consensus on the fact that some strains are more virulent than others (Canelli et al., 2017; Ferrari et al., 2018; Frydas et al., 2013; Geldhof et al., 2012; Morgan et al., 2013; Renson et al., 2017; Stadejek et al., 2017; Weesendorp et al., 2013, 2014).

* Corresponding author.
E-mail address: v42sancj@uco.es (J.M. Sánchez-Carvajal).

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Referencias - References

Imagen: Infobae

Aichler, M., & Walch, A. (2015). MALDI Imaging mass spectrometry: Current frontiers and perspectives in pathology research and practice. Laboratory Investigation, 95(4), 422–431. https://doi.org/10.1038/labinvest.2014.156

Albina, E., Carrat, C., & Charley, B. (1998). Interferon-α response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. Journal of Interferon and Cytokine Research, 18(7), 485–490. https://doi.org/10.1089/jir.1998.18.485

Allende, R., Laegreid, W. W., Kutish, G. F., Galeota, J. A., Wills, R. W., & Osorio, F. A. (2000). Porcine Reproductive and Respiratory Syndrome Virus: Description of Persistence in Individual Pigs upon Experimental Infection. Journal of Virology, 74(22), 10834–10837. https://doi.org/10.1128/jvi.74.22.10834-10837.2000

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Graham, S. P., Frossard, J. P., Steinbach, F., & Salguero, F. J. (2016). Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains. Veterinary Microbiology, 188, 47–58. https://doi.org/10.1016/j.vetmic.2016.04.005

Amarilla, S. P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I. M., Caridad y Ocerín, J. M., Morgan, S. B., Graham, S. P., Frossard, J. P., Drew, T. W., & Salguero, F. J. (2015). A comparative study of the local cytokine response in the lungs of pigs experimentally infected with different PRRSV-1 strains: Upregulation of IL-1α in highly pathogenic strain induced lesions. Veterinary Immunology and Immunopathology, 164(3–4), 137–147. https://doi.org/10.1016/j.vetimm.2015.02.003

An, T. Q., Tian, Z. J., Leng, C. L., Peng, J. M., & Guang-Zhi, T. (2011). Highly pathogenic porcine reproductive and respiratory syndrome virus, asia. In Emerging Infectious Diseases (Vol. 17, Issue 9, pp. 1782–1784). Centers for Disease Control and Prevention (CDC). https://doi.org/10.3201/eid1709.110411

Atkin-Smith, G. K., Duan, M., Chen, W., & Poon, I. K. H. (2018). The induction and consequences of Influenza A virus-induced cell death. In Cell Death and Disease (Vol. 9, Issue 10, pp. 1–11). Nature Publishing Group. https://doi.org/10.1038/s41419-018-1035-6

Balka, G., Ladinig, A., Ritzmann, M., Saalmüller, A., Gerner, W., Käser, T., Jakab, C., Rusvai, M., & Weißenböck, H. (2013). Immunohistochemical characterization of type II pneumocyte proliferation after challenge with type I porcine reproductive and respiratory syndrome virus. Journal of Comparative Pathology, 149(2–3), 322–330. https://doi.org/10.1016/j.jcpa.2012.12.006

Balka, G., Podgórska, K., Brar, M. S., Bálint, Á., Cadar, D., Celer, V., Dénes, L., Dirbakova, Z., Jedryczko, A., Márton, L., Novosel, D., Petrović, T., Sirakov, I., Szalay, D., Toplak, I., Leung, F. C. C., & Stadejek, T. (2018). Genetic diversity of PRRSV 1 in Central Eastern Europe in 1994-2014: Origin and evolution of the virus in the region. Scientific Reports, 8(1), 1–12. https://doi.org/10.1038/s41598-018-26036-w

Banadyga, L., Veugelers, K., Campbell, S., & Barry, M. (2009). The Fowlpox Virus BCL-2 Homologue, FPV039, Interacts with Activated Bax and a Discrete Subset of BH3-Only Proteins To Inhibit Apoptosis. Journal of Virology, 83(14), 7085–7098. https://doi.org/10.1128/jvi.00437-09

Banks, R. E., Dunn, M. J., Hochstrasser, D. F., Sanchez, J. C., Blackstock, W., Pappin, D. J., & Selby, P. J. (2000). Proteomics: New perspectives, new biomedical opportunities. Lancet, 356(9243), 1749–1756. https://doi.org/10.1016/S0140-6736(00)03214-1

Bao, D., Wang, R., Qiao, S., Wan, B., Wang, Y., Liu, M., Shi, X., Guo, J., & Zhang, G. (2013). Antibody-dependent enhancement of PRRSV infection down-modulates TNF-α and IFN-β transcription in macrophages. Veterinary Immunology and Immunopathology, 156(1–2), 128–134. https://doi.org/10.1016/j.vetimm.2013.09.006

Barranco, I., Gómez-Laguna, J., Rodríguez-Gómez, I. M., Salguero, F. J., Pallarés, F. J., Bernabé, A., & Carrasco, L. (2011). Immunohistochemical detection of extrinsic and intrinsic mediators of apoptosis in porcine paraffin-embedded tissues. Veterinary Immunology and Immunopathology, 139(2–4), 210–216. https://doi.org/10.1016/j.vetimm.2010.10.017

Baumann, A., Mateu, E., Murtaugh, M. P., & Summerfield, A. (2013). Impact of genotype 1 and 2 of porcine reproductive and respiratory syndrome viruses on interferon-α responses by plasmacytoid dendritic cells. Veterinary Research, 44(1), 33. https://doi.org/10.1186/1297-9716-44-33

Bautista, E. M., & Molitor, T. W. (1999). IFNγ inhibits porcine reproductive and respiratory syndrome virus replication in macrophages. Archives of Virology, 144(6), 1191–1200. https://doi.org/10.1007/s007050050578

Bautista, Elida M., & Molitor, T. W. (1997). Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunology, 10(2), 83–94. https://doi.org/10.1089/vim.1997.10.83

Belkaid, Y. (2007). Regulatory T cells and infection: A dangerous necessity. In Nature Reviews Immunology (Vol. 7, Issue 11, pp. 875–888). Nature Publishing Group. https://doi.org/10.1038/nri2189

Bowie, A. G., & Unterholzner, L. (2008). Viral evasion and subversion of pattern-recognition receptor signalling. In Nature Reviews Immunology (Vol. 8, Issue 12, pp. 911–922). https://doi.org/10.1038/nri2436

Boyle, J. J., Harrington, H. A., Piper, E., Elderfield, K., Stark, J., Landis, R. C., & Haskard, D. O. (2009). Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. American Journal of Pathology, 174(3), 1097–1108. https://doi.org/10.2353/ajpath.2009.080431

Brar, M. S., Shi, M., Ge, L., Carman, S., Murtaugh, M. P., & Leung, F. C. C. (2011). Porcine reproductive and respiratory syndrome virus in Ontario, Canada 1999 to 2010: Genetic diversity and restriction fragment length polymorphisms. Journal of General Virology, 92(6), 1391–1397.

https://doi.org/10.1099/vir.0.030155-0

Brockmeier, S. L., Loving, C. L., Nelson, E. A., Miller, L. C., Nicholson, T. L., Register, K. B., Grubman, M. J., Brough, D. E., & Kehrli, M. E. (2012). The presence of alpha interferon at the time of infection alters the innate and adaptive immune responses to porcine reproductive and respiratory syndrome virus. Clinical and Vaccine Immunology, 19(4), 508–514. https://doi.org/10.1128/CVI.05490-11

Brockmeier, S. L., Loving, C. L., Palmer, M. V., Spear, A., Nicholson, T. L., Faaberg, K. S., & Lager, K. M. (2017). Comparison of Asian porcine high fever disease isolates of porcine reproductive and respiratory syndrome virus to United States isolates for their ability to cause disease and secondary bacterial infection in swine. Veterinary Microbiology, 203, 6–17. https://doi.org/10.1016/j.vetmic.2017.02.003

Brockmeier, S. L., Palmer, M. V., Bolin, S. R., & Rimler, R. B. (2001). Effects of intranasal inoculation with Bordetella bronchiseptica, porcine reproductive and respiratory syndrome virus, or a combination of both organisms on subsequent infection with Pasteurella multocida in pigs. American Journal of Veterinary Research, 62(4), 521–525. https://doi.org/10.2460/ajvr.2001.62.521

Buddaert, W., Van Reeth, K., & Pensaert, M. (1998). In vivo and in vitro interferon (IFN) studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Advances in Experimental Medicine and Biology, 440, 461–467. https://doi.org/10.1007/978-1-4615-5331-1_59

Buechler, C., Eisinger, K., & Krautbauer, S. (2013). Diagnostic and prognostic potential of the macrophage specific receptor CD163 in inflammatory diseases. Inflammation and Allergy - Drug Targets, 12(6), 391–402. https://doi.org/10.2174/18715281113126660060

Buechler, C., Ritter, M., Orsó, E., Langmann, T., Klucken, J., & Schmitz, G. (2000). Regulation of scavenger receptor CD163 expression in human monocytes and macrophages by pro- and antiinflammatory stimuli. Journal of Leukocyte Biology, 67(1), 97–103. https://doi.org/10.1002/jlb.67.1.97

Burkard, C., Lillico, S. G., Reid, E., Jackson, B., Mileham, A. J., Ait-Ali, T., Whitelaw, C. B. A., & Archibald, A. L. (2017). Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLoS Pathogens, 13(2), e1006206. https://doi.org/10.1371/journal.ppat.1006206

Butler, J. E., Lager, K. M., Golde, W., Faaberg, K. S., Sinkora, M., Loving, C., & Zhang, Y. I. (2014). Porcine reproductive and respiratory syndrome (PRRS): An immune dysregulatory pandemic. Immunologic Research, 59(1–3), 81–108. https://doi.org/10.1007/s12026-014-8549-5

Cai, Q., Guo, Y., Xiao, B., Banerjee, S., Saha, A., Lu, J., Glisovic, T., & Robertson, E. S. (2011). Epstein-

barr virus nuclear antigen 3C stabilizes gemin3 to block p53-mediated apoptosis. PLoS Pathogens, 7(12), e1002418. https://doi.org/10.1371/journal.ppat.1002418

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S.-K. W. (2007). CD163 Expression Confers Susceptibility to Porcine Reproductive and Respiratory Syndrome Viruses. Journal of Virology, 81(14), 7371–7379. https://doi.org/10.1128/jvi.00513-07

Calzada-Nova, G., Schnitzlein, W. M., Husmann, R. J., & Zuckermann, F. A. (2011). North American Porcine Reproductive and Respiratory Syndrome Viruses Inhibit Type I Interferon Production by Plasmacytoid Dendritic Cells. Journal of Virology, 85(6), 2703–2713. https://doi.org/10.1128/jvi.01616-10

Calzada-Nova, Gabriela, Schnitzlein, W., Husmann, R., & Zuckermann, F. A. (2010). Characterization of the cytokine and maturation responses of pure populations of porcine plasmacytoid dendritic cells to porcine viruses and toll-like receptor agonists. Veterinary Immunology and Immunopathology, 135(1–2), 20–33. https://doi.org/10.1016/j.vetimm.2009.10.026

Canelli, E., Catella, A., Borghetti, P., Ferrari, L., Ogno, G., De Angelis, E., Corradi, A., Passeri, B., Bertani, V., Sandri, G., Bonilauri, P., Leung, F. C., Guazzetti, S., & Martelli, P. (2017). Phenotypic characterization of a highly pathogenic Italian porcine reproductive and respiratory syndrome virus (PRRSV) type 1 subtype 1 isolate in experimentally infected pigs. Veterinary Microbiology, 210(July), 124–133. https://doi.org/10.1016/j.vetmic.2017.09.002

Cao, J., Grauwet, K., Vermeulen, B., Devriendt, B., Jiang, P., Favoreel, H., & Nauwynck, H. (2013). Suppression of NK cell-mediated cytotoxicity against PRRSV-infected porcine alveolar macrophages in vitro. Veterinary Microbiology, 164(3–4), 261–269. https://doi.org/10.1016/j.vetmic.2013.03.001

Cao, Q. M., Subramaniam, S., Ni, Y.-Y., Cao, D., & Meng, X.-J. (2016). The non-structural protein Nsp2TF of porcine reproductive and respiratory syndrome virus down-regulates the expression of Swine Leukocyte Antigen class I. Virology, 491, 115–124. https://doi.org/10.1016/j.virol.2016.01.021

Cao, X. (2020). COVID-19: immunopathology and its implications for therapy. In Nature Reviews Immunology (Vol. 20, Issue 5, pp. 269–270). Nature Research. https://doi.org/10.1038/s41577-020-0308-3

Carrasco, L., Nú N ˜ Ez, A., Salguero, F. J., Díaz, F., Segundo, S., Sá Nchez-Cordó, P., Gó Mez-Villamandos, J. C., & Sierra, M. A. (2002). African Swine Fever: Expression of Interleukin-1 alpha and Tumour Necrosis Factor-alpha by Pulmonary Intravascular Macrophages. J. Comp. Path, 126, 194–201. https://doi.org/10.1053/jcpa.2001.0543

Carrasco, L., Núez, A., Salguero, F. J., Díaz San Segundo, F., Sánchez-Cordón, P., Gómez-Villamandos, J. C., & Sierra, M. A. (2002). African swine fever: Expression of interleukin-1 alpha and tumour necrosis factor-alpha by pulmonary intravascular macrophages. Journal of Comparative

Pathology, 126(2–3), 194–201. https://doi.org/10.1053/jcpa.2001.0543

Cavanagh, D. (1997). Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Archives of Virology, 142(3), 629–633.

Cecere, T. E., Todd, S. M., & LeRoith, T. (2012). Regulatory T cells in arterivirus and coronavirus infections: Do they protect against disease or enhance it? In Viruses (Vol. 4, Issue 5, pp. 833–846). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/v4050833

Chand, R. J., Trible, B. R., & Rowland, R. R. R. (2012). Pathogenesis of porcine reproductive and respiratory syndrome virus. In Current Opinion in Virology (Vol. 2, Issue 3, pp. 256–263). Elsevier. https://doi.org/10.1016/j.coviro.2012.02.002

Chang, H. C. H. luan, Peng, Y. T., Chang, H. C. H. luan, Chaung, H. C., & Chung, W. Bin. (2008). Phenotypic and functional modulation of bone marrow-derived dendritic cells by porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 129(3–4), 281–293. https://doi.org/10.1016/j.vetmic.2007.12.002

Charavaryamath, C., Janardhan, K. S., Caldwell, S., & Singh, B. (2006). Pulmonary intravascular monocytes/macrophages in a rat model of sepsis. Anatomical Record - Part A Discoveries in Molecular, Cellular, and Evolutionary Biology, 288(12), 1259–1271. https://doi.org/10.1002/ar.a.20401

Chaung, H. C., Chen, C. W., Hsieh, B. L., & Chung, W. Bin. (2010). Toll-like receptor expressions in porcine alveolar macrophages and dendritic cells in responding to poly IC stimulation and porcine reproductive and respiratory syndrome virus (PRRSV) infection. Comparative Immunology, Microbiology and Infectious Diseases, 33(3), 197–213. https://doi.org/10.1016/j.cimid.2008.10.001

Chen, Z., Lawson, S., Sun, Z., Zhou, X., Guan, X., Christopher-Hennings, J., Nelson, E. A., & Fang, Y. (2010). Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology, 398(1), 87–97. https://doi.org/10.1016/j.virol.2009.11.033

Chitko-McKown, C. G., Chapes, S. K., Brown, R. E., Phillips, R. M., McKown, R. D., & Blecha, F. (1991). Porcine alveolar and pulmonary intravascular macrophages: Comparison of immune functions. Journal of Leukocyte Biology, 50(4), 364–372. https://doi.org/10.1002/jlb.50.4.364

Cho, J. G., Dee, S. A., Deen, J., Guedes, A., Trincado, C., Fano, E., Jiang, Y., Faaberg, K., Collins, J. E., Murtaugh, M. P., & Soo Joo, H. (2006). Evaluation of the effects of animal age, concurrent bacterial infection, and pathogenicity of porcine reproductive and respiratory syndrome virus on virus concentration in pigs. American Journal of Veterinary Research, 67(3), 489–493. https://doi.org/10.2460/ajvr.67.3.489

Conzelmann, K. K., Visser, N., Van Woensel, P., & Thiel, H. J. (1993). Molecular characterization of porcine reproductive and respiratory syndrome virus, a member of the arterivirus group.

Virology, 193(1), 329–339. https://doi.org/10.1006/viro.1993.1129

Cortey, M., Díaz, I., Martín-Valls, G. E., & Mateu, E. (2017). Next-generation sequencing as a tool for the study of Porcine reproductive and respiratory syndrome virus (PRRSV) macro- and micro- molecular epidemiology. In Veterinary Microbiology (Vol. 209, pp. 5–12). Elsevier B.V. https://doi.org/10.1016/j.vetmic.2017.02.002

Cortey, Martí, Arocena, G., Pileri, E., Martín-Valls, G., & Mateu, E. (2018). Bottlenecks in the transmission of porcine reproductive and respiratory syndrome virus (PRRSV1) to naïve pigs and the quasi-species variation of the virus during infection in vaccinated pigs.Veterinary Research, 49(1), 107. https://doi.org/10.1186/s13567-018-0603-1

Costa-Hurtado, M., Olvera, A., Martinez-Moliner, V., Galofré-Milà, N., Martínez, P., Dominguez, J., & Aragon, V. (2013). Changes in macrophage phenotype after infection of pigs with haemophilus parasuis strains with different levels of virulence. Infection and Immunity, 81(7), 2327–2333. https://doi.org/10.1128/IAI.00056-13

Costers, S., Lefebvre, D. J., Delputte, P. L., & Nauwynck, H. J. (2008). Porcine reproductive and respiratory syndrome virus modulates apoptosis during replication in alveolar macrophages. Archives of Virology, 153(8), 1453–1465. https://doi.org/10.1007/s00705-008-0135-5

Craven, R. A., & Banks, R. E. (2001). Laser capture microdissection and proteomics: Possibilities and limitation. PROTEOMICS, 1(10), 1200–1204. https://doi.org/10.1002/1615-9861(200110)1:10<1200::AID-PROT1200>3.0.CO;2-Q

D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. In Cell Biology International (Vol. 43, Issue 6, pp. 582–592). Wiley-Blackwell Publishing Ltd. https://doi.org/10.1002/cbin.11137

Darwich, L., Díaz, I., & Mateu, E. (2010). Certainties, doubts and hypotheses in porcine reproductive and respiratory syndrome virus immunobiology. In Virus Research (Vol. 154, Issues 1–2, pp. 123–132). Elsevier. https://doi.org/10.1016/j.virusres.2010.07.017

Darwich, L., Gimeno, M., Sibila, M., Diaz, I., de la Torre, E., Dotti, S., Kuzemtseva, L., Martin, M., Pujols, J., & Mateu, E. (2011). Genetic and immunobiological diversities of porcine reproductive and respiratory syndrome genotype 1 strains. Veterinary Microbiology, 150(1–2), 49–62. https://doi.org/10.1016/j.vetmic.2011.01.008

Das, P. B., Dinh, P. X., Ansari, I. H., De Lima, M., Osorio, F. A., & Pattnaik, A. K. (2010). The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor CD163. Journal of Virology, 84(4), 1731–1740. https://doi.org/10.1128/JVI.01774-09

Davidovich, P., Kearney, C. J., & Martin, S. J. (2014). Inflammatory outcomes of apoptosis, necrosis and necroptosis. Biological Chemistry, 395(10), 1163–1171. https://doi.org/10.1515/hsz-2014-0164

De Baere, M. I., Van Gorp, H., Delputte, P. L., & Nauwynck, H. J. (2012). Interaction of the European

genotype porcine reproductive and respiratory syndrome virus (PRRSV) with sialoadhesin (CD169/Siglec-1) inhibits alveolar macrophage phagocytosis. Veterinary Research, 43(1), 47. https://doi.org/10.1186/1297-9716-43-47

de Lima, M., Pattnaik, A. K., Flores, E. F., & Osorio, F. A. (2006). Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus. Virology, 353(2), 410–421. https://doi.org/10.1016/j.virol.2006.05.036

Delputte, P. L., Costers, S., & Nauwynck, H. J. (2005). Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: Distinctive roles for heparan sulphate and sialoadhesin. Journal of General Virology, 86(5), 1441–1445. https://doi.org/10.1099/vir.0.80675-0

Delputte, P. L., Vanderheijden, N., Nauwynck, H. J., & Pensaert, M. B. (2002). Involvement of the Matrix Protein in Attachment of Porcine Reproductive and Respiratory Syndrome Virus to a Heparinlike Receptor on Porcine Alveolar Macrophages. Journal of Virology, 76(9), 4312–4320. https://doi.org/10.1128/jvi.76.9.4312-4320.2002

di Mauro, G., Cristina, S., Concetta, R., Francesco, R., & Annalisa, C. (2020). SARS-Cov-2 infection: Response of human immune system and possible implications for the rapid test and treatment. International Immunopharmacology, 84. https://doi.org/10.1016/j.intimp.2020.106519

Díaz, I., Darwich, L., Pappaterra, G., Pujols, J., & Mateu, E. (2006). Different European-type vaccines against porcine reproductive and respiratory syndrome virus have different immunological properties and confer different protection to pigs. Virology, 351(2), 249–259. https://doi.org/10.1016/j.virol.2006.03.046

Díaz, Ivan, Darwich, L., Pappaterra, G., Pujols, J., & Mateu, E. (2005). Immune responses of pigs after experimental infection with a European strain of Porcine reproductive and respiratory syndrome virus. Journal of General Virology, 86(7), 1943–1951. https://doi.org/10.1099/vir.0.80959-0

Díaz, Ivan, Gimeno, M., Darwich, L., Navarro, N., Kuzemtseva, L., López, S., Galindo, I., Segalés, J., Martín, M., Pujols, J., & Mateu, E. (2012). Characterization of homologous and heterologous adaptive immune responses in porcine reproductive and respiratory syndrome virus infection. Veterinary Research, 43(1), 30. https://doi.org/10.1186/1297-9716-43-30

Domingo, E., & Holland, J. J. (1997). RNA VIRUS MUTATIONS AND FITNESS FOR SURVIVAL. Annual Review of Microbiology, 51(1), 151–178. https://doi.org/10.1146/annurev.micro.51.1.151

Du, J., Ge, X., Liu, Y., Jiang, P., Wang, Z., Zhang, R., Zhou, L., Guo, X., Han, J., & Yang, H. (2015). Targeting swine SLA-I molecules for proteasomal degradation by the nsp1alpha replicase protein of the Chinese highly pathogenic PRRSV strain JXwn06. Journal of Virology. https://

doi.org/10.1128/JVI.02307-15

Duan, X., Nauwynck, H. J., Favoreel, H. W., & Pensaert, M. B. (1998). Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. Journal of Virology, 72(5), 4520–4523. http://www.ncbi.nlm.nih.gov/pubmed/9557752

Duan, X., Nauwynck, H. J., & Pensaert, M. B. (1997). Virus quantification and identification of cellular targets in the lungs and lymphoid tissues of pigs at different time intervals after inoculation with porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary Microbiology, 56(1–2), 9–19. https://doi.org/10.1016/S0378-1135(96)01347-8

Dwivedi, V., Manickam, C., Binjawadagi, B., Linhares, D., Murtaugh, M. P., & Renukaradhya, G. J. (2012). Evaluation of immune responses to porcine reproductive and respiratory syndrome virus in pigs during early stage of infection under farm conditions. Virology Journal, 9(1), 45. https://doi.org/10.1186/1743-422X-9-45

Dwivedi, V., Manickam, C., Patterson, R., Dodson, K., Murtaugh, M., Torrelles, J. B., Schlesinger, L. S., & Renukaradhya, G. J. (2011). Cross-protective immunity to porcine reproductive and respiratory syndrome virus by intranasal delivery of a live virus vaccine with a potent adjuvant. Vaccine, 29(23), 4058–4066. https://doi.org/10.1016/j.vaccine.2011.03.006

Dwivedi, V., Manickam, C., Patterson, R., Dodson, K., Weeman, M., & Renukaradhya, G. J. (2011). Intranasal delivery of whole cell lysate of Mycobacterium tuberculosis induces protective immune responses to a modified live porcine reproductive and respiratory syndrome virus vaccine in pigs. Vaccine, 29(23), 4067–4076. https://doi.org/10.1016/j.vaccine.2011.03.005

Elhelu, M. A. (1983). The role of macrophages in immunology. In Journal of the National Medical Association (Vol. 75, Issue 3, pp. 314–317). National Medical Association.

Elmore, M. R. P., Burton, M. D., Conrad, M. S., Rytych, J. L., Van Alstine, W. G., & Johnson, R. W. (2014). Respiratory viral infection in neonatal piglets causes marked microglia activation in the hippocampus and deficits in spatial learning. Journal of Neuroscience, 34(6), 2120–2129. https://doi.org/10.1523/JNEUROSCI.2180-13.2014

Etzerodt, A., Berg, R. M. G., Plovsing, R. R., Andersen, M. N., Bebien, M., Habbeddine, M., Lawrence, T., Møller, H. J., & Moestrup, S. K. (2017). Soluble ectodomain CD163 and extracellular vesicle-associated CD163 are two differently regulated forms of â €soluble CD163' in plasma. Scientific Reports, 7(1), 1–10. https://doi.org/10.1038/srep40286

Etzerodt, A., Maniecki, M. B., Møller, K., Møller, H. J., & Moestrup, S. K. (2010). Tumor necrosis factor □-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. Journal of Leukocyte Biology, 88(6), 1201–1205. https://doi.org/10.1189/jlb.0410235

Etzerodt, A., & Moestrup, S. K. (2013). CD163 and inflammation: Biological, diagnostic, and therapeutic aspects. In Antioxidants and Redox Signaling (Vol. 18, Issue 17, pp. 2352–2363).

Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA . https://doi.org/10.1089/ars.2012.4834

Etzerodt, A., Rasmussen, M. R., Svendsen, P., Chalaris, A., Schwarz, J., Galea, I., Møller, H. J., & Moestrup, S. K. (2014). Structural basis for inflammation-driven shedding of CD163 ectodomain and tumor necrosis factor-□ in macrophages. Journal of Biological Chemistry, 289(2), 778–788. https://doi.org/10.1074/jbc.M113.520213

Fabriek, B. O., Bruggen, R. Van, Deng, D. M., Ligtenberg, A. J. M., Nazmi, K., Schornagel, K., Vloet, R. P. M., Dijkstra, C. D., & Van Den Berg, T. K. (2009). The macrophage scavenger receptor CD163 functions as an innate immune sensor for bacteria. Blood, 113(4), 887–892. https://doi.org/10.1182/blood-2008-07-167064

Fan, B., Liu, X., Bai, J., Li, Y., Zhang, Q., & Jiang, P. (2015). The 15N and 46R Residues of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus Nucleocapsid Protein Enhance Regulatory T Lymphocytes Proliferation. PLOS ONE, 10(9), e0138772. https://doi.org/10.1371/journal.pone.0138772

Feng, W. H., Tompkins, M. B., Xu, J. S., Brown, T. T., Laster, S. M., Zhang, H. X., & McCaw, M. B. (2002). Thymocyte and peripheral blood T lymphocyte subpopulation changes in piglets following in utero infection with porcine reproductive and respiratory syndrome virus. Virology, 302(2), 363–372. https://doi.org/10.1006/viro.2002.1650

Ferrari, L., Canelli, E., De Angelis, E., Catella, A., Ferrarini, G., Ogno, G., Bonati, L., Nardini, R., Borghetti, P., & Martelli, P. (2018). A highly pathogenic porcine reproductive and respiratory syndrome virus type 1 (PRRSV-1) strongly modulates cellular innate and adaptive immune subsets upon experimental infection. Veterinary Microbiology, 216, 85–92. https://doi.org/10.1016/j.vetmic.2018.02.001

Firth, A. E., Zevenhoven-Dobbe, J. C., Wills, N. M., Go, Y. Y., Balasuriya, U. B. R., Atkins, J. F., Snijder, E. J., & Posthuma, C. C. (2011). Discovery of a small arterivirus gene that overlaps the gp5 coding sequence and is important for virus production. Journal of General Virology, 92(5), 1097–1106. https://doi.org/10.1099/vir.0.029264-0

Fritsch, M., Günther, S. D., Schwarzer, R., Albert, M. C., Schorn, F., Werthenbach, J. P., Schiffmann, L. M., Stair, N., Stocks, H., Seeger, J. M., Lamkanfi, M., Krönke, M., Pasparakis, M., & Kashkar, H. (2019). Caspase-8 is the molecular switch for apoptosis, necroptosis and pyroptosis. Nature, 575(7784), 683–687. https://doi.org/10.1038/s41586-019-1770-6

Frydas, I. S., Verbeeck, M., Cao, J., & Nauwynck, H. J. (2013). Replication characteristics of porcine reproductive and respiratory syndrome virus (PRRSV) European subtype 1 (Lelystad) and subtype 3 (Lena) strains in nasal mucosa and cells of the monocytic lineage: Indications for the use of new receptors of PRRSV (Lena). Veterinary Research, 44(1), 73. https://doi.org/10.1186/1297-9716-44-73

Galliher-Beckley, A., Li, X., Bates, J. T., Madera, R., Waters, A., Nietfeld, J., Henningson, J., He, D.,

Feng, W., Chen, R., & Shi, J. (2015). Pigs immunized with Chinese highly pathogenic PRRS virus modified live vaccine are protected from challenge with North American PRRSV strain NADC-20. Vaccine, 33(30), 3518–3525. https://doi.org/10.1016/j.vaccine.2015.05.058

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., … Kroemer, G. (2018). Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. In Cell Death and Differentiation (Vol. 25, Issue 3, pp. 486–541). Nature Publishing Group. https://doi.org/10.1038/s41418-017-0012-4

García-Nicolás, O., Baumann, A., Vielle, N. J., Gómez-Laguna, J., Quereda, J. J., Pallarés, F. J., Ramis, G., Carrasco, L., & Summerfield, A. (2014). Virulence and genotype-associated infectivity of interferon-treated macrophages by porcine reproductive and respiratory syndrome viruses. Virus Research, 179(1), 204–211. https://doi.org/10.1016/j.virusres.2013.08.009

García-Nicolás, O., Rosales, R. S., Pallarés, F. J., Risco, D., Quereda, J. J., Graham, S. P., Frossard, J. P., Morgan, S. B., Steinbach, F., Drew, T. W., Strickland, T. S., & Salguero, F. J. (2015). Comparative analysis of cytokine transcript profiles within mediastinal lymph node compartments of pigs after infection with porcine reproductive and respiratory syndrome genotype 1 strains differing in pathogenicity. Veterinary Research, 46(1). https://doi.org/10.1186/s13567-015-0161-8

Geldhof, M. F., Vanhee, M., Van Breedam, W., Van Doorsselaere, J., Karniychuk, U. U., & Nauwynck, H. J. (2012). Comparison of the efficacy of autogenous inactivated Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. BMC Veterinary Research, 8(1), 182. https://doi.org/10.1186/1746-6148-8-182

Gerner, W., Käser, T., & Saalmüller, A. (2009). Porcine T lymphocytes and NK cells - An update. Developmental and Comparative Immunology, 33(3), 310–320. https://doi.org/10.1016/j.dci.2008.06.003

Gimeno, M., Darwich, L., Diaz, I., De La Torre, E., Pujols, J., Martín, M., Inumaru, S., Cano, E., Domingo, M., Montoya, M., & Mateu, E. (2011). Cytokine profiles and phenotype regulation of antigen presenting cells by genotype-I porcine reproductive and respiratory syndrome virus isolates. Veterinary Research, 42(1), 9. https://doi.org/10.1186/1297-9716-42-9

Gleissner, C. A., Shaked, I., Erbel, C., Böckler, D., Katus, H. A., & Ley, K. (2010). CXCL4 Downregulates the Atheroprotective Hemoglobin Receptor CD163 in Human Macrophages. Circulation Research, 106(1), 203–211. https://doi.org/10.1161/CIRCRESAHA.109.199505

Gómez-Laguna, J., Salguero, F. J., Barranco, I., Pallarés, F. J., Rodríguez-Gómez, I. M., Bernabé, A., & Carrasco, L. (2010). Cytokine Expression by Macrophages in the Lung of Pigs Infected with the Porcine Reproductive and Respiratory Syndrome Virus. Journal of Comparative Pathology, 142(1), 51–60. https://doi.org/10.1016/j.jcpa.2009.07.004

Gómez-Laguna, J., Salguero, F. J., Fernández de Marco, M., Barranco, I., Rodríguez-Gómez, I. M., Quezada, M., & Carrasco, L. (2013). Type 2 Porcine Reproductive and Respiratory Syndrome Virus Infection Mediated Apoptosis in B- and T-Cell Areas in Lymphoid Organs of Experimentally Infected Pigs. Transboundary and Emerging Diseases, 60(3), 273–278. https://doi.org/10.1111/j.1865-1682.2012.01338.x

Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., & Carrasco, L. (2013). Immunopathogenesis of porcine reproductive and respiratory syndrome in the respiratory tract of pigs. The Veterinary Journal, 195(2), 148–155. https://doi.org/10.1016/j.tvjl.2012.11.012

Gómez-Laguna, J., Salguero, F. J., Pallarés, F. J., Fernández de Marco, M., Barranco, I., Cerón, J. J., Martínez-Subiela, S., Van Reeth, K., & Carrasco, L. (2010). Acute phase response in porcine reproductive and respiratory syndrome virus infection. Comparative Immunology, Microbiology and Infectious Diseases, 33(6), e51–e58. https://doi.org/10.1016/j.cimid.2009.11.003

Gorbalenya AE, Krupovic M, Siddell S, Varsani A, K. J. (2018). International Committee on Taxonomy of Viruses (ICTV). Retrieved 6 April 2019, from International Committee on Taxonomy of Viruses (ICTV) Website: Https://Talk.Ictvonline.Org/Taxonomy/p/Taxonomy-History?Taxnode_id=20186087. https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20186087

Gordon, S., & Martinez, F. O. (2010). Alternative activation of macrophages: Mechanism and functions. In Immunity (Vol. 32, Issue 5, pp. 593–604). https://doi.org/10.1016/j.immuni.2010.05.007

Gordon, S., & Plüddemann, A. (2017). Tissue macrophages: Heterogeneity and functions. In BMC Biology (Vol. 15, Issue 1, pp. 1–18). BioMed Central Ltd. https://doi.org/10.1186/s12915-017-0392-4

Grau-Roma, L., & Segalés, J. (2007). Detection of porcine reproductive and respiratory syndrome virus, porcine circovirus type 2, swine influenza virus and Aujeszky's disease virus in cases of porcine proliferative and necrotizing pneumonia (PNP) in Spain. Veterinary Microbiology, 119(2–4), 144–151. https://doi.org/10.1016/j.vetmic.2006.09.009

Guo, B., Lager, K. M., Henningson, J. N., Miller, L. C., Schlink, S. N., Kappes, M. A., Kehrli, M. E., Brockmeier, S. L., Nicholson, T. L., Yang, H. C., & Faaberg, K. S. (2013). Experimental infection of United States swine with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. Virology, 435(2), 372–384. https://doi.org/10.1016/j.virol.2012.09.013

Gustafsson, J. O. R., Oehler, M. K., Ruszkiewicz, A., McColl, S. R., & Hoffmann, P. (2011). MALDI Imaging Mass Spectrometry (MALDI-IMS)□Application of Spatial Proteomics for Ovarian Cancer Classification and Diagnosis. International Journal of Molecular Sciences, 12(1), 773–794. https://doi.org/10.3390/ijms12010773

Guth, A. M., Janssen, W. J., Bosio, C. M., Crouch, E. C., Henson, P. M., & Dow, S. W. (2009). Lung environment determines unique phenotype of alveolar macrophages. American Journal of Physiology - Lung Cellular and Molecular Physiology, 296(6). https://doi.org/10.1152/ajplung.90625.2008

Gutstein, H. B., & Morris, J. S. (2007). Laser capture sampling and analytical issues in proteomics. In Expert Review of Proteomics (Vol. 4, Issue 5, pp. 627–637). Taylor & Francis. https://doi.org/10.1586/14789450.4.5.627

Halbur, P. G., Miller, L. D., Paul, P. S., Meng, X. J., Huffman, E. L., & Andrews, J. J. (1995). Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Veterinary Pathology, 32(2), 200–204. https://doi.org/10.1177/030098589503200218

Halbur, Patrick G., Paul, P. S., Meng, X. J., Lum, M. A., Andrews, J. J., & Rathje, J. A. (1996). Comparative pathogenicity of nine US porcine reproductive and respiratory syndrome virus (PRRSV) isolates in a five-week-old cesarean-derived, colostrum-deprived pig model. Journal of Veterinary Diagnostic Investigation, 8(1), 11–20. https://doi.org/10.1177/104063879600800103

Han, D., Hu, Y., Li, L., Tian, H., Chen, Z., Wang, L., Ma, H., Yang, H., & Teng, K. (2014). Highly pathogenic porcine reproductive and respiratory syndrome virus infection results in acute lung injury of the infected pigs. Veterinary Microbiology, 169(3–4), 135–146. https://doi.org/10.1016/j.vetmic.2013.12.022

Han, J., Wang, Y., & Faaberg, K. S. (2006). Complete genome analysis of RFLP 184 isolates of porcine reproductive and respiratory syndrome virus. Virus Research, 122(1–2), 175–182. https://doi.org/10.1016/j.virusres.2006.06.003

Han, J., Zhou, L., Ge, X., Guo, X., & Yang, H. (2017). Pathogenesis and control of the Chinese highly pathogenic porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 209, 30–47. https://doi.org/10.1016/j.vetmic.2017.02.020

Han, Z., Liu, Y., Wang, G., He, Y., Hu, S., Li, Y., Shi, W., Wu, J., Wang, S., Liu, H., & Cai, X. (2015). Comparative Analysis of Immune Responses in Pigs to High and Low Pathogenic Porcine Reproductive and Respiratory Syndrome Viruses Isolated in China. Transboundary and Emerging Diseases, 62(5), e1–e10. https://doi.org/10.1111/tbed.12190

Hanada, K., Suzuki, Y., Evolution, T. N.-… biology and, & 2005, U. (2005). The origin and evolution of porcine reproductive and respiratory syndrome viruses. Academic.Oup.Com. https://academic.oup.com/mbe/article-abstract/22/4/1024/1083231

He, L., Long, L. R., Antani, S., & Thoma, G. R. (2012). Histology image analysis for carcinoma detection and grading. Computer Methods and Programs in Biomedicine, 107(3), 538–556. https://doi.org/10.1016/j.cmpb.2011.12.007

He, Y., Wang, G., Liu, Y., Shi, W., Han, Z., Wu, J., Jiang, C., Wang, S., Hu, S., Wen, H., Dong, J., Liu, H., &

Cai, X. (2012). Characterization of thymus atrophy in piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 160(3–4), 455–462. https://doi.org/10.1016/j.vetmic.2012.05.040

Hintz, K.A., Rassias, A. J., Wardwell, K., Moss, M. L., Morganelli, P. M., Pioli, P.A., Givan, A. L., Wallace, P. K., Yeager, M. P., & Guyre, P. M. (2002). Endotoxin induces rapid metalloproteinase-mediated shedding followed by up-regulation of the monocyte hemoglobin scavenger receptor CD163. Journal of Leukocyte Biology, 72(4), 711–717. https://doi.org/10.1189/jlb.72.4.711

Högger, P., & Sorg, C. (2001). Soluble CD163 inhibits phorbol ester-induced lymphocyte proliferation. Biochemical and Biophysical Research Communications, 288(4), 841–843. https://doi.org/10.1006/bbrc.2001.5845

Hu, S. P., Zhang, Z., Liu, Y. G., Tian, Z. J., Wu, D. L., Cai, X. H., & He, X. J. (2013). Pathogenicity and Distribution of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus in Pigs. Transboundary and Emerging Diseases, 60(4), 351–359. https://doi.org/10.1111/j.1865-1682.2012.01354.x

Huang, C., Zhang, Q., Guo, X. -k., Yu, Z. -b., Xu, A.-T., Tang, J., & Feng, W. -h. (2014). Porcine Reproductive and Respiratory Syndrome Virus Nonstructural Protein 4 Antagonizes Beta Interferon Expression by Targeting the NF- B Essential Modulator. Journal of Virology, 88(18), 10934–10945. https://doi.org/10.1128/jvi.01396-14

Huang, Y. W., Dryman, B. A., Li, W., & Meng, X. J. (2009). Porcine DC-SIGN: Molecular cloning, gene structure, tissue distribution and binding characteristics. Developmental and Comparative Immunology, 33(4), 464–480. https://doi.org/10.1016/j.dci.2008.09.010

Huo, Y., Fan, L., Yin, S., Dong, Y., Guo, X., Yang, H., & Hu, H. (2013). Involvement of unfolded protein response, p53 and Akt in modulation of porcine reproductive and respiratory syndrome virus-mediated JNK activation. Virology, 444(1–2), 233–240. https://doi.org/10.1016/j.virol.2013.06.015

Hussell, T., & Bell, T. J. (2014). Alveolar macrophages: Plasticity in a tissue-specific context. In Nature Reviews Immunology (Vol. 14, Issue 2, pp. 81–93). Nature Publishing Group. https://doi.org/10.1038/nri3600

Jan, J.-T., Chen, B.-H., Ma, S.-H., Liu, C.-I., Tsai, H.-P., Wu, H.-C., Jiang, S.-Y., Yang, K.-D., & Shaio, M.-F. (2000). Potential Dengue Virus-Triggered Apoptotic Pathway in Human Neuroblastoma Cells: Arachidonic Acid, Superoxide Anion, and NF-kappa B Are Sequentially Involved. Journal of Virology, 74(18), 8680–8691. https://doi.org/10.1128/jvi.74.18.8680-8691.2000

Johnsen, C. K., Bøtner, A., Kamstrup, S., Lind, P., & Nielsen, J. (2002). Cytokine mRNA profiles in bronchoalveolar cells of piglets experimentally infected in utero with porcine reproductive and respiratory syndrome virus: Association of sustained expression of IFN-γ and IL-10 after viral clearance. Viral Immunology, 15(4), 549–556. https://doi.org/10.1089/088282402320914494

Johnson, C. R., Griggs, T. F., Gnanandarajah, J., & Murtaugh, M. P. (2011). Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative orf5 present in all arteriviruses. Journal of General Virology, 92(5), 1107–1116. https://doi.org/10.1099/vir.0.030213-0

Jorgensen, I., Rayamajhi, M., & Miao, E. A. (2017). Programmed cell death as a defence against infection. In Nature Reviews Immunology (Vol. 17, Issue 3, pp. 151–164). Nature Publishing Group. https://doi.org/10.1038/nri.2016.147

Jusa, E. R., Inaba, Y., Kouno, M., & Hirose, O. (1997). Effect of heparin on infection of cells by porcine reproductive and respiratory syndrome virus. American Journal of Veterinary Research, 58(5), 488–491. http://www.ncbi.nlm.nih.gov/pubmed/9140556

Kaempfer, T., Duerst, E., Gehrig, P., Roschitzki, B., Rutishauser, D., Grossmann, J., Schoedon, G., Vallelian, F., & Schaer, D. J. (2011). Extracellular hemoglobin polarizes the macrophage proteome toward Hb-clearance, enhanced antioxidant capacity and suppressed HLA class 2 expression. Journal of Proteome Research, 10(5), 2397–2408. https://doi.org/10.1021/pr101230y

Kappes, M. A., & Faaberg, K. S. (2015). PRRSV structure, replication and recombination: Origin of phenotype and genotype diversity. Virology, 479–480, 475–486. https://doi.org/10.1016/j.virol.2015.02.012

Karniychuk, U. U., De Spiegelaere, W., & Nauwynck, H. J. (2013). Porcine reproductive and respiratory syndrome virus infection is associated with an increased number of Sn-positive and CD8-positive cells in the maternal-fetal interface. Virus Research, 176(1–2), 285–291. https://doi.org/10.1016/j.virusres.2013.05.005

Karniychuk, U. U., Geldhof, M., Vanhee, M., Van Doorsselaere, J., Saveleva, T. A., & Nauwynck, H. J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Veterinary Research, 6(1), 30. https://doi.org/10.1186/1746-6148-6-30

Karst, S. M. (2016). The influence of commensal bacteria on infection with enteric viruses. Nature Reviews Microbiology, 14(4), 197–204. https://doi.org/10.1038/nrmicro.2015.25

Käser, T., Gerner, W., Hammer, S. E., Patzl, M., & Saalmüller, A. (2008). Detection of Foxp3 protein expression in porcine T lymphocytes. Veterinary Immunology and Immunopathology, 125(1–2), 92–101. https://doi.org/10.1016/j.vetimm.2008.05.007

Kavanová, L., Prodělalová, J., Nedbalcová, K., Matiašovic, J., Volf, J., Faldyna, M., & Salát, J. (2015). Immune response of porcine alveolar macrophages to a concurrent infection with porcine reproductive and respiratory syndrome virus and Haemophilus parasuis in vitro. Veterinary Microbiology, 180(1–2), 28–35. https://doi.org/10.1016/j.vetmic.2015.08.026

Keffaber, K., 1989. Reproductive failure of unknown etiology. Am Assoc Swine Pract Newsl, 1: 1-9.

Kerr, J. F. R., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer, 26(4), 239–257. https://doi.org/10.1038/bjc.1972.33

Khatri, M., Dwivedi, V., Krakowka, S., Manickam, C., Ali, A., Wang, L., Qin, Z., Renukaradhya, G. J., & Lee, C.-W. (2010). Swine Influenza H1N1 Virus Induces Acute Inflammatory Immune Responses in Pig Lungs: a Potential Animal Model for Human H1N1 Influenza Virus. Journal of Virology, 84(21), 11210–11218. https://doi.org/10.1128/jvi.01211-10

Kim, J.-K., Fahad, A.-M., Shanmukhappa, K., & Kapil, S. (2006). Defining the Cellular Target(s) of Porcine Reproductive and Respiratory Syndrome Virus Blocking Monoclonal Antibody 7G10. Journal of Virology, 80(2), 689–696. https://doi.org/10.1128/jvi.80.2.689-696.2006

Kinter, A. L., Hennessey, M., Bell, A., Kern, S., Lin, Y., Daucher, M., Planta, M., McGlaughlin, M., Jackson, R., Ziegler, S. F., & Fauci, A. S. (2004). $CD25^{+}CD4^{+}$ regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate $CD4^{+}$ and $CD8^{+}$ HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. Journal of Experimental Medicine, 200(3), 331–343. https://doi.org/10.1084/jem.20032069

Kneidl, J., Löffler, B., Erat, M. C., Kalinka, J., Peters, G., Roth, J., & Barczyk, K. (2012). Soluble CD163 promotes recognition, phagocytosis and killing of Staphylococcus aureus via binding of specific fibronectin peptides. Cellular Microbiology, 14(6), 914–936. https://doi.org/10.1111/j.1462-5822.2012.01766.x

Kowal, K., Silver, R., Sławińska, E., Bielecki, M., Chyczewski, L., & Kowal-Bielecka, O. (2011). CD163 and its role in inflammation. In Folia Histochemica et Cytobiologica (Vol. 49, Issue 3, pp. 365–374). Via Medica. https://doi.org/10.5603/FHC.2011.0052

Kreeger, P., Carcinogenesis, D. L.-, & 2010, U. (2010). Cancer systems biology: a network modeling perspective. Academic.Oup.Com. https://academic.oup.com/carcin/article-abstract/31/1/2/2391695

Kuhn, J. H., Lauck, M., Bailey, A. L., Shchetinin, A. M., Vishnevskaya, T. V., Bào, Y., Ng, T. F. F., LeBreton, M., Schneider, B. S., Gillis, A., Tamoufe, U., Diffo, J. L. D., Takuo, J. M., Kondov, N. O., Coffey, L. L., Wolfe, N. D., Delwart, E., Clawson, A. N., Postnikova, E., … Goldberg, T. L. (2016). Reorganization and expansion of the nidoviral family Arteriviridae. Archives of Virology, 161(3), 755–768. https://doi.org/10.1007/s00705-015-2672-z

Kui, S. V., Peng, G., Guo, Z., Fu, T., Li, Y., Frappier, L., & Wang, R. F. (2005). Functional characterization of EBV-encoded nuclear antigen 1-specific $CD4^{+}$ helper and regulatory T cells elicited by in vitro peptide stimulation. Cancer Research, 65(4), 1577–1586. https://doi.org/10.1158/0008-5472.CAN-04-2552

Labarque, G., Van Gucht, S., Nauwynck, H., Van Reeth, K., & Pensaert, M. (2003). Apoptosis in the lungs of pigs infected with porcine reproductive and respiratory syndrome virus and

associations with the production of apoptogenic cytokines. Veterinary Research, 34(3), 249–260. https://doi.org/10.1051/vetres:2003001

Labarque, G., Van Reeth, K., Van Gucht, S., Nauwynck, H., & Pensaert, M. (2002). Porcine reproductive-respiratory syndrome virus infection predisposes pigs for respiratory signs upon exposure to bacterial lipopolysaccharide. Veterinary Microbiology, 88(1), 1–12. https://doi.org/10.1016/S0378-1135(02)00104-9

Lamontagne, L., Pagé, C., Larochelle, R., & Magar, R. (2003). Porcine Reproductive and Respiratory Syndrome Virus Persistence in Blood, Spleen, Lymph Nodes, and Tonsils of Experimentally Infected Pigs Depends on the Level of CD8 high T Cells. Viral Immunology, 16(3), 395–406. https://doi.org/10.1089/08828240332239618 1

Law, S. K. A., Micklem, K. J., Shaw, J. M., Zhang, X. □P, Dong, Y., Willis, A. C., & Mason, D. Y. (1993). A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. European Journal of Immunology, 23(9), 2320–2325. https://doi.org/10.1002/eji.1830230940

Lawson, S. R., Rossow, K. D., Collins, J. E., Benfield, D. A., & Rowland, R. R. (1997). Porcine reproductive and respiratory syndrome virus infection of gnotobiotic pigs: sites of virus replication and co-localization with MAC-387 staining at 21 days post-infection. Virus Research, 51(2), 105–113. https://doi.org/10.1016/s0168-1702(97)00086-5

Lazova, R., Seeley, E. H., Kutzner, H., Scolyer, R. A., Scott, G., Cerroni, L., Fried, I., Kozovska, M. E., Rosenberg, A. S., Prieto, V. G., Shehata, B. M., Durham, M. M., Henry, G., Rodriguez-Peralto, J. L., Riveiro-Falkenbach, E., Schaefer, J. T., Danialan, R., Fraitag, S., Vollenweider-Roten, S., … Caprioli, R. M. (2016). Imaging mass spectrometry assists in the classification of diagnostically challenging atypical Spitzoid neoplasms. Journal of the American Academy of Dermatology, 75(6), 1176-1186.e4. https://doi.org/10.1016/j.jaad.2016.07.007

LeRoith, T., Hammond, S., Todd, S. M., Ni, Y., Cecere, T., & Pelzer, K. D. (2011). A modified live PRRSV vaccine and the pathogenic parent strain induce regulatory T cells in pigs naturally infected with Mycoplasma hyopneumoniae. Veterinary Immunology and Immunopathology, 140(3–4), 312–316. https://doi.org/10.1016/j.vetimm.2010.12.011

Li, B., Fang, L., Xu, Z., Liu, S., Gao, J., Jiang, Y., Chen, H., & Xiao, S. (2009). Recombination in vaccine and circulating strains of porcine reproductive and respiratory syndrome viruses. Emerging Infectious Diseases, 15(12), 2032–2035. https://doi.org/10.3201/eid1512.090390

Li, C., Zhuang, J., Wang, J., Han, L., Sun, Z., Xiao, Y., Ji, G., Li, Y., Tan, F., Li, X., & Tian, K. (2016). Outbreak Investigation of NADC30-Like PRRSV in South-East China. Transboundary and Emerging Diseases, 63(5), 474–479. https://doi.org/10.1111/tbed.12530

Li, Chengzhi, Li, Z., Tuo, Y., Ma, D., Shi, Y., Zhang, Q., Zhuo, X., Deng, K., Chen, Y., Wang, Z., & Huang, P. (2017). MALDI-TOF MS as a Novel Tool for the Estimation of Postmortem Interval in Liver Tissue Samples. Scientific Reports, 7(1). https://doi.org/10.1038/s41598-017-05216-0

Li, L., Zhao, Q., Ge, X., Teng, K., Kuang, Y., Chen, Y., Guo, X., & Yang, H. (2012). Chinese highly pathogenic porcine reproductive and respiratory syndrome virus exhibits more extensive tissue tropism for pigs. Virology Journal, 9, 203. https://doi.org/10.1186/1743-422X-9-203

Li, Y., Wang, G., Liu, Y., Tu, Y., He, Y., Wang, Z., Han, Z., Li, L., Li, A., Tao, Y., & Cai, X. (2014). Identification of apoptotic cells in the thymus of piglets infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Virus Research, 189, 29–33. https://doi.org/10.1016/j.virusres.2014.04.011

Liu, C. H., Chaung, H. C., Chang, H. luan, Peng, Y. T., & Chung, W. Bin. (2009). Expression of Toll-like receptor mRNA and cytokines in pigs infected with porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 136(3–4), 266–276. https://doi.org/10.1016/j.vetmic.2008.11.016

Liu, D., Zhou, R., Zhang, J., Zhou, L., Jiang, Q., Guo, X., Ge, X., & Yang, H. (2011). Recombination analyses between two strains of porcine reproductive and respiratory syndrome virus in vivo. Virus Research, 155(2), 473–486. https://doi.org/10.1016/j.virusres.2010.12.003

Liu, Y., Shi, W., Zhou, E., Wang, S., Hu, S., Cai, X., Rong, F., Wu, J., Xu, M., Xu, M., & Li, Q. (2010). Dynamic changes in inflammatory cytokines in pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. Clinical and Vaccine Immunology, 17(9), 1439–1445. https://doi.org/10.1128/CVI.00517-09

Lodoen, M. B., & Lanier, L. L. (2006). Natural killer cells as an initial defense against pathogens. In Current Opinion in Immunology (Vol. 18, Issue 4, pp. 391–398). https://doi.org/10.1016/j.coi.2006.05.002

Loemba, H. D., Mounir, S., Mardassi, H., Archambault, D., & Dea, S. (1996). Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Archives of Virology, 141(3–4), 751–761. https://doi.org/10.1007/BF01718333

Logue, S. E., & Martin, S. J. (2008). Caspase activation cascades in apoptosis. Biochemical Society Transactions, 36(1), 1–9. https://doi.org/10.1042/BST0360001

Longworth, K. E. (1997). THE COMPARATIVE BIOLOGY OF PULMONARY INTRAVASCULAR MACROPHAGES. In Frontiers in Bioscience (Vol. 2).

López-Fuertes, L., Campos, E., Doménech, N., Ezquerra, A., Castro, J. M., Domínguez, J., & Alonso, F. (2000). Porcine reproductive and respiratory syndrome (PRRS) virus down- modulates TNF-□ production in infected macrophages. Virus Research, 69(1), 41–46. https://doi.org/10.1016/S0168-1702(00)00172-6

López Fuertes, L., Doménech, N., Alvarez, B., Ezquerra, A., Domínguez, J., Castro, J. M., & Alonso, F. (1999). Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Research, 64(1), 33–42. https://doi.org/10.1016/s0168-1702(99)00073-8

Lopez, O. J., Oliveira, M. F., Alvarez Garcia, E., Kwon, B. J., Doster, A., & Osorio, F. A. (2007). Protection against Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. Clinical and Vaccine Immunology, 14(3), 269–275. https://doi.org/10.1128/CVI.00304-06

Lopez, O. J., & Osorio, F. A. (2004). Role of neutralizing antibodies in PRRSV protective immunity. In Veterinary Immunology and Immunopathology (Vol. 102, Issue 3, pp. 155–163). Elsevier. https://doi.org/10.1016/j.vetimm.2004.09.005

Loving, C. L., Osorio, F. A., Murtaugh, M. P., & Zuckermann, F. A. (2015). Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. In Veterinary Immunology and Immunopathology (Vol. 167, Issues 1–2, pp. 1–14). Elsevier. https://doi.org/10.1016/j.vetimm.2015.07.003

Loula, T., 1991. Mystery pig disease. Agri-Practice, 12: 23-34.

Lu, Z. H., Wang, X., Wilson, A. D., Dorey-Robinson, D. L. W., Archibald, A. L., Ait-Ali, T., & Frossard, J.-P. (2017). Quasispecies evolution of the prototypical genotype 1 porcine reproductive and respiratory syndrome virus early during in vivo infection is rapid and tissue specific. Archives of Virology, 162(8), 2203–2210. https://doi.org/10.1007/s00705-017-3342-0

Lunney, J. K., Fang, Y., Ladinig, A., Chen, N., Li, Y., Rowland, B., & Renukaradhya, G. J. (2016). Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. Annual Review of Animal Biosciences, 4(1), 129–154. https://doi.org/10.1146/annurev-animal-022114-111025

Lunney, J. K., Fritz, E. R., Reecy, J. M., Kuhar, D., Prucnal, E., Molina, R., Christopher-Hennings, J., Zimmerman, J., & Rowland, R. R. R. (2010). Interleukin-8, interleukin-1beta, and interferon-gamma levels are linked to PRRS virus clearance. Viral Immunology, 23(2), 127–134. https://doi.org/10.1089/vim.2009.0087

Ma, S. M., Mao, Q., Yi, L., Zhao, M. Q., & Chen, J. D. (2019). Apoptosis, autophagy, and pyroptosis: Immune escape strategies for persistent infection and pathogenesis of classical swine fever virus. In Pathogens (Vol. 8, Issue 4). MDPI AG. https://doi.org/10.3390/pathogens8040239

Maier, S. K., Hahne, H., Gholami, A. M., Balluff, B., Meding, S., Schoene, C., Walch, A. K., & Kuster, B. (2013). Comprehensive identification of proteins from MALDI imaging. Molecular and Cellular Proteomics, 12(10), 2901–2910. https://doi.org/10.1074/mcp.M113.027599

Mair, K. H., Sedlak, C., Käser, T., Pasternak, A., Levast, B., Gerner, W., Saalmüller, A., Summerfield, A., Gerdts, V., Wilson, H. L., & Meurens, F. (2014). The porcine innate immune system: An update. In Developmental and Comparative Immunology (Vol. 45, Issue 2, pp. 321–343). Elsevier Ltd. https://doi.org/10.1016/j.dci.2014.03.022

Mair, Kerstin H., Essler, S. E., Patzl, M., Storset, A. K., Saalmüller, A., & Gerner, W. (2012). NKp46 expression discriminates porcine NK cells with different functional properties. European Journal of Immunology, 42(5), 1261–1271. https://doi.org/10.1002/eji.201141989

Mair, Kerstin H., Stadler, M., Talker, S. C., Forberg, H., Storset, A. K., Müllebner, A., Catharina Duvigneau, J., Hammer, S. E., Saalmüller, A., & Gerner, W. (2016). Porcine $CD3^{+}NKp46^{+}$ lymphocytes have NK-cell characteristics and are present in increased frequencies in the lungs of influenza-infected animals. Frontiers in Immunology, 7(JUL). https://doi.org/10.3389/fimmu.2016.00263

Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases, 277 Immunological Reviews 61 (2017). https://doi.org/10.1111/imr.12534

Manickam, C., Dwivedi, V., Patterson, R., Papenfuss, T., & Renukaradhya, G. J. (2013). Porcine reproductive and respiratory syndrome virus induces pronounced immune modulatory responses at mucosal tissues in the parental vaccine strain VR2332 infected pigs. Veterinary Microbiology, 162(1), 68–77. https://doi.org/10.1016/j.vetmic.2012.08.021

Martin-Valls, G. E., Kvisgaard, L. K., Tello, M., Darwich, L., Cortey, M., Burgara-Estrella, A. J., Hernandez, J., Larsen, L. E., & Mateu, E. (2014). Analysis of ORF5 and Full-Length Genome Sequences of Porcine Reproductive and Respiratory Syndrome Virus Isolates of Genotypes 1 and 2 Retrieved Worldwide Provides Evidence that Recombination Is a Common Phenomenon and May Produce Mosaic Isolates. Journal of Virology, 88(6), 3170–3181. https://doi.org/10.1128/jvi.02858-13

Martínez-Lobo, F. J., Díez-Fuertes, F., Simarro, I., Castro, J. M., & Prieto, C. (2011). Porcine Reproductive and Respiratory Syndrome Virus isolates differ in their susceptibility to neutralization. Vaccine, 29(40), 6928–6940. https://doi.org/10.1016/j.vaccine.2011.07.076

Mateu, E., & Diaz, I. (2008). The challenge of PRRS immunology. The Veterinary Journal, 177(3), 345–351. https://doi.org/10.1016/j.tvjl.2007.05.022

Medigeshi, G. R., Lancaster, A. M., Hirsch, A. J., Briese, T., Lipkin, W. I., DeFilippis, V., Fruh, K., Mason, P. W., Nikolich-Zugich, J., & Nelson, J. A. (2007). West Nile Virus Infection Activates the Unfolded Protein Response, Leading to CHOP Induction and Apoptosis. Journal of Virology, 81(20), 10849–10860. https://doi.org/10.1128/jvi.01151-07

Meier, W. A., Galeota, J., Osorio, F. A., Husmann, R. J., Schnitzlein, W. M., & Zuckermann, F. A. (2003). Gradual development of the interferon-γ response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology, 309(1), 18–31. https://doi.org/10.1016/S0042-6822(03)00009-6

Mengeling, W., Vorwald, A., Lager, K., Clouser, D., & Wesley, R. (1999). Identification and clinical assessment of suspected vaccine-related field strains of porcine reproductive and respiratory syndrome virus. American Journal of Veterinary Research, 60(3), 334–340.

Metz, G. E., Serena, M. S., Abeyá, M. M., Dulbecco, A. B., Massone, A., Díaz, S., & Echeverría, M. G. (2014). Equine arteritis virus gP5 protein induces apoptosis in cultured insect cells. Virus Research, 183, 81–84. https://doi.org/10.1016/j.virusres.2014.01.026

Meulenberg, J. J. M. (2000). PRRSV, the virus. Veterinary Research, 31(1), 11–21. https://doi.org/10.1051/vetres:2000103

Møller, Holger J. (2012). Soluble CD163. Scandinavian Journal of Clinical and Laboratory Investigation, 163(1), 1–13. https://doi.org/10.3109/00365513.2011.626868

Møller, Holger Jon, Peterslund, N. A., Graversen, J. H., & Moestrup, S. K. (2002). Identification of the hemoglobin scavenger receptor/CD163 as a natural soluble protein in plasma. Blood, 99(1), 378–380. https://doi.org/10.1182/blood.V99.1.378

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., & O'Garra, A. (2001). I NTERLEUKIN -10 AND THE I NTERLEUKIN -10 R ECEPTOR . Annual Review of Immunology, 19(1), 683–765. https://doi.org/10.1146/annurev.immunol.19.1.683

Morgan, S. B., Frossard, J. P., Pallares, F. J., Gough, J., Stadejek, T., Graham, S. P., Steinbach, F., Drew, T. W., & Salguero, F. J. (2016). Pathology and Virus Distribution in the Lung and Lymphoid Tissues of Pigs Experimentally Inoculated with Three Distinct Type 1 PRRS Virus Isolates of Varying Pathogenicity. Transboundary and Emerging Diseases, 63(3), 285–295. https://doi.org/10.1111/tbed.12272

Morgan, S. B., Graham, S. P., Salguero, F. J., Sánchez Cordón, P. J., Mokhtar, H., Rebel, J. M. J., Weesendorp, E., Bodman-Smith, K. B., Steinbach, F., & Frossard, J. P. (2013). Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. Veterinary Microbiology, 163(1–2), 13–22. https://doi.org/10.1016/j.vetmic.2012.11.024

Morganelli, P. M., & Guyre, P. M. (1988). IFN-gamma plus glucocorticoids stimulate the expression of a newly identified human mononuclear phagocyte-specific antigen. The Journal of Immunology, 140(7), 2296–2304.

Mosser, D. M. (2003). The many faces of macrophage activation. Journal of Leukocyte Biology, 73(2), 209–212. https://doi.org/10.1189/jlb.0602325

Mosser, D. M., & Edwards, J. P. (2008). Exploring the full spectrum of macrophage activation. In Nature Reviews Immunology (Vol. 8, Issue 12, pp. 958–969). https://doi.org/10.1038/nri2448

Murphy, T. L., Grajales-Reyes, G. E., Wu, X., Tussiwand, R., Briseño, C. G., Iwata, A., Kretzer, N. M., Durai, V., & Murphy, K. M. (2016). Transcriptional Control of Dendritic Cell Development. Annual Review of Immunology, 34(1), 93–119. https://doi.org/10.1146/annurev-immunol-032713-120204

Murtaugh, M. P., Stadejek, T., Abrahante, J. E., Lam, T. T. Y., & Leung, F. C. C. (2010). The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. In Virus Research (Vol. 154, Issues 1–2, pp. 18–30). Elsevier B.V. https://doi.org/10.1016/j.virusres.2010.08.015

Murtaugh, M. P., Yuan, S., & Faaberg, K. S. (2001). Appearance of novel PRRSV isolates by recombination in the natural environment. Advances in Experimental Medicine and Biology, 494, 31–36. https://doi.org/10.1007/978-1-4615-1325-4_4

Music, N., & Gagnon, C. A. (2010). The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. In Animal health research reviews / Conference of Research Workers in Animal Diseases (Vol. 11, Issue 2, pp. 135–163). https://doi.org/10.1017/S1466252310000034

Nailwal, H., & Chan, F. K. M. (2019). Necroptosis in anti-viral inflammation. In Cell Death and Differentiation (Vol. 26, Issue 1, pp. 4–13). Nature Publishing Group. https://doi.org/10.1038/s41418-018-0172-x

Nauwynck, H. J., Duan, X., Favoreel, H. W., Van Oostveldt, P., & Pensaert, M. B. (1999). Entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages via receptor-mediated endocytosis. Journal of General Virology, 80(2), 297–305. https://doi.org/10.1099/0022-1317-80-2-297

Neagu, A. N. (2019). Proteome Imaging: From Classic to Modern Mass Spectrometry-Based Molecular Histology. In Advances in experimental medicine and biology (Vol. 1140, pp. 55–98). NLM (Medline). https://doi.org/10.1007/978-3-030-15950-4_4

Nelson, E. A., Christopher-Hennings, J., & Benfield, D. A. (1994). Serum Immune Responses to the Proteins of Porcine Reproductive and Respiratory Syndrome (PRRS) Virus. Journal of Veterinary Diagnostic Investigation, 6(4), 410–415. https://doi.org/10.1177/104063879400600402

Norris, J. L., & Caprioli, R. M. (2013). Imaging mass spectrometry: A new tool for pathology in a molecular age. Proteomics - Clinical Applications, 7(11–12), 733–738. https://doi.org/10.1002/prca.201300055

Oleksiewicz, M. B., Botner, A., Toft, P., Normann, P., & Storgaard, T. (2001). Epitope Mapping Porcine Reproductive and Respiratory Syndrome Virus by Phage Display: the nsp2 Fragment of the Replicase Polyprotein Contains a Cluster of B-Cell Epitopes. Journal of Virology, 75(7), 3277–3290. https://doi.org/10.1128/jvi.75.7.3277-3290.2001

Olin, M. R., Batista, L., Xiao, Z., Dee, S. A., Murtaugh, M. P., Pijoan, C. C., & Molitor, T. W. (2005). γδ Lymphocyte Response to Porcine Reproductive and Respiratory Syndrome Virus. Viral Immunology, 18(3), 490–499. https://doi.org/10.1089/vim.2005.18.490

Orzalli, M. H., & Kagan, J. C. (2017). Apoptosis and Necroptosis as Host Defense Strategies to Prevent Viral Infection. Trends in Cell Biology, 27(11), 800–809. https://doi.org/10.1016/j.tcb.2017.05.007

Osorio, F. A., Galeota, J. A., Nelson, E., Brodersen, B., Doster, A., Wills, R., Zuckermann, F., & Laegreid, W. W. (2002). Passive transfer of virus-specific antibodies confers protection against reproductive failure induced by a virulent strain of porcine reproductive and respiratory syndrome virus and establishes sterilizing immunity. Virology, 302(1), 9–20. https://doi.org/10.1006/viro.2002.1612

Overend, C., Mitchell, R., He, D., Rompato, G., Grubman, M. J., & Garmendia, A. E. (2007). Recombinant swine beta interferon protects swine alveolar macrophages and MARC-145

cells from infection with Porcine reproductive and respiratory syndrome virus. Journal of General Virology, 88(3), 925–931. https://doi.org/10.1099/vir.0.82585-0

Pallarés, F. J., Halbur, P. G., Opriessnig, T., Sorden, S. D., Villar, D., Janke, B. H., Yaeger, M. J., Larson, D. J., Schwartz, K. J., Yoon, K. J., & Hoffman, L. J. (2002). Porcine Circovirus Type 2 (PCV-2) Coinfections in US Field Cases of Postweaning Multisystemic Wasting Syndrome (PMWS). Journal of Veterinary Diagnostic Investigation, 14(6), 515–519. https://doi.org/10.1177/104063870201400614

Pasternak, J. A., MacPhee, D. J., & Harding, J. C. S. (2019). Development and application of a porcine specific ELISA for the quantification of soluble CD163. Veterinary Immunology and Immunopathology, 210, 60–67. https://doi.org/10.1016/j.vetimm.2019.03.011

Pathogenesis and antigenic characterization of a new East European Subtype 3 PRRSV isolate. (2010). 1–10.

Peng, Y. T., Chaung, H. C., Chang, H. C. H. luan, Chang, H. C. H. luan, & Chung, W. Bin. (2009). Modulations of phenotype and cytokine expression of porcine bone marrow-derived dendritic cells by porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 136(3–4), 359–365. https://doi.org/10.1016/j.vetmic.2008.11.013

Pereira, L. M. S., Gomes, S. T. M., Ishak, R., & Vallinoto, A. C. R. (2017). Regulatory T Cell and Forkhead Box Protein 3 as Modulators of Immune Homeostasis. Frontiers in Immunology, 8(May), 1–24. https://doi.org/10.3389/fimmu.2017.00605

Pérez, C., Ortuño, E., Gómez, N., García-Briones, M., Álvarez, B., Martínez De La Riva, P., Alonso, F., Revilla, C., Domínguez, J., & Ezquerra, A. (2008). Cloning and expression of porcine CD163: its use for characterization of monoclonal antibodies to porcine CD163 and development of an ELISA to measure soluble CD163 in biological fluids Introduction 1. In INIA) Spanish Journal of Agricultural Research (Issue 6). www.inia.es/sjar

Pileri, E., & Mateu, E. (2016). Review on the transmission porcine reproductive and respiratory syndrome virus between pigs and farms and impact on vaccination. Veterinary Research, 47(1), 1–13. https://doi.org/10.1186/s13567-016-0391-4

Plagemann, P. G. W., Rowland, R. R. R., & Faaberg, K. S. (2002). The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Archives of Virology, 147(12), 2327–2347. https://doi.org/10.1007/s00705-002-0887-2

Poderoso, T., Martínez de la Riva, P., Uenishi, H., Alvarez, B., Toki, D., Nieto-Pelegrín, E., Alonso, F., Domínguez, J., Ezquerra, A., & Revilla, C. (2019). Analysis of the expression of porcine CD200R1 and CD200R1L by using newly developed monoclonal antibodies. Developmental & Comparative Immunology, 100(June), 103417. https://doi.org/10.1016/j.dci.2019.103417

Pujhari, S., Baig, T. T., & Zakhartchouk, A. N. (2014). Potential role of porcine reproductive and respiratory syndrome virus structural protein GP2 in apoptosis inhibition. BioMed Research

International, 2014, 160505. https://doi.org/10.1155/2014/160505

Qi, P., Liu, K., Wei, J., Li, Y., Li, B., Shao, D., Wu, Z., Shi, Y., Tong, G., Qiu, Y., & Ma, Z. (2017). Nonstructural Protein 4 of Porcine Reproductive and Respiratory Syndrome Virus Modulates Cell Surface Swine Leukocyte Antigen Class I Expression by Downregulating □2-Microglobulin Transcription. Journal of Virology, 91(5). https://doi.org/10.1128/JVI.01755-16

Qiao, S., Feng, L., Bao, D., Guo, J., Wan, B., Xiao, Z., Yang, S., & Zhang, G. (2011). Porcine reproductive and respiratory syndrome virus and bacterial endotoxin act in synergy to amplify the inflammatory response of infected macrophages. Veterinary Microbiology, 149(1–2), 213–220. https://doi.org/10.1016/j.vetmic.2010.11.006

Qiao, S., Jiang, Z., Tian, X., Wang, R., Xing, G., Wan, B., Bao, D., Liu, Y., Hao, H., Guo, J., & Zhang, G. (2011). Porcine Fc□RIIb Mediates Enhancement of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Infection. PLoS ONE, 6(12), e28721. https://doi.org/10.1371/journal.pone.0028721

Rajkhowa, T. K., Jagan Mohanarao, G., Gogoi, A., Hauhnar, L., & Isaac, L. (2015). Porcine reproductive and respiratory syndrome virus (PRRSV) from the first outbreak of India shows close relationship with the highly pathogenic variant of China. Veterinary Quarterly, 35(4), 186–193. https://doi.org/10.1080/01652176.2015.1066043

Renson, P., Rose, N., Le Dimna, M., Mahé, S., Keranflec'h, A., Paboeuf, F., Belloc, C., Le Potier, M. F., & Bourry, O. (2017). Dynamic changes in bronchoalveolar macrophages and cytokines during infection of pigs with a highly or low pathogenic genotype 1 PRRSV strain. Veterinary Research, 48(1), 15. https://doi.org/10.1186/s13567-017-0420-y

Renukaradhya, G. J., Alekseev, K., Jung, K., Fang, Y., & Saif, L. J. (2010). Porcine reproductive and respiratory syndrome virus-Induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunology, 23(5), 457–466. https://doi.org/10.1089/vim.2010.0051

Rinaldo, C. R. (1994). Modulation of major histocompatibility complex antigen expression by viral infection. In American Journal of Pathology (Vol. 144, Issue 4, pp. 637–650). American Society for Investigative Pathology.

Ritter, M., Buechler, C., Langmann, T., & Schmitz, G. (1999). Genomic organization and chromosomal localization of the human CD163 (M130) gene: A member of the scavenger receptor cysteine-rich superfamily. Biochemical and Biophysical Research Communications, 260(2), 466–474. https://doi.org/10.1006/bbrc.1999.0866

Robinson, S. R., Li, J., Nelson, E. A., & Murtaugh, M. P. (2015). Broadly neutralizing antibodies against the rapidly evolving porcine reproductive and respiratory syndrome virus. Virus Research, 203, 56–65. https://doi.org/10.1016/j.virusres.2015.03.016

Rodríguez-Gómez, I. M., Gómez-Laguna, J., Barranco, I., Pallarés, F. J., Ramis, G., Salguero, F. J., & Carrasco, L. (2013). Downregulation of antigen-presenting cells in tonsil and lymph nodes

of porcine reproductive and respiratory syndrome virus-infected pigs. Transboundary and Emerging Diseases, 60(5), 425–437. https://doi.org/10.1111/j.1865-1682.2012.01363.x

Rodríguez-Gómez, Irene M., Barranco, I., Amarilla, S. P., García-Nicolás, O., Salguero, F. J., Carrasco, L., & Gómez-Laguna, J. (2014). Activation of extrinsic- and Daxx-mediated pathways in lymphoid tissue of PRRSV-infected pigs. Veterinary Microbiology, 172(1–2), 186–194. https://doi.org/10.1016/j.vetmic.2014.05.025

Rodríguez-Gómez, Irene M., Käser, T., Gómez-Laguna, J., Lamp, B., Sinn, L., Rümenapf, T., Carrasco, L., Saalmüller, A., & Gerner, W. (2015). PRRSV-infected monocyte-derived dendritic cells express high levels of SLA-DR and CD80/86 but do not stimulate PRRSV-naïve regulatory T cells to proliferate. Veterinary Research, 46(1), 54. https://doi.org/10.1186/s13567-015-0186-z

Rodríguez-Gómez, Irene M., Sánchez-Carvajal, J. M., Pallarés, F. J., Mateu, E., Carrasco, L., & Gómez-Laguna, J. (2019). Virulent Lena strain induced an earlier and stronger downregulation of CD163 in bronchoalveolar lavage cells. Veterinary Microbiology, 235, 101–109. https://doi.org/10.1016/j.vetmic.2019.06.011

Rodríguez-Gómez, Irene M, Gómez-Laguna, J., & Carrasco, L. (2013). Impact of PRRSV on activation and viability of antigen presenting cells. World Journal of Virology, 2(4), 146. https://doi.org/10.5501/wjv.v2.i4.146

Rossow, K. D., Collins, J. E., Goyal, S. M., Nelson, E. A., Christopher-Hennings, J., & Benfield, D. A. (1995). Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Veterinary Pathology, 32(4), 361–373. https://doi.org/10.1177/030098589503200404

Ruedas-Torres, I., Rodríguez-Gómez, I. M., Sánchez-Carvajal, J. M., Pallares, F. J., Barranco, I., Carrasco, L., & Gómez-Laguna, J. (2020). Activation of the extrinsic apoptotic pathway in the thymus of piglets infected with PRRSV-1 strains of different virulence. Veterinary Microbiology, 243, 108639. https://doi.org/10.1016/j.vetmic.2020.108639

Sabat, R., Grütz, G., Warszawska, K., Kirsch, S., Witte, E., Wolk, K., & Geginat, J. (2010). Biology of interleukin-10. Cytokine and Growth Factor Reviews, 21(5), 331–344. https://doi.org/10.1016/j.cytogfr.2010.09.002

Sánchez-Torres, C., Gómez-Puertas, P., Gómez-Del-Moral, M., Alonso, F., Escribano, J. M., Ezquerra, A., & Domínguez, J. (2003). Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. Archives of Virology, 148(12), 2307–2323. https://doi.org/10.1007/s00705-003-0188-4

Sánchez, C., Doménech, N., Alonso, F., Ezquerra, A., Domínguez, J., & Vázquez, J. (1999). The porcine 2A10 antigen is homologous to human CD163 and related to macrophage differentiation. Journal of Immunology, 162(9), 5230–5237.

Sang, Y., Ross, C. R., Rowland, R. R. R., & Blecha, F. (2008). Toll-like receptor 3 activation decreases

porcine arterivirus infection. Viral Immunology, 21(3), 303–313. https://doi.org/10.1089/vim.2008.0042

Sang, Y., Rowland, R. R. R., & Blecha, F. (2011). Interaction between innate immunity and porcine reproductive and respiratory syndrome virus. Animal Health Research Reviews, 12(2), 149–167. https://doi.org/10.1017/S1466252311000144

Schaer, D. J., Schaer, C. A., Buehler, P. W., Boykins, R. A., Schoedon, G., Alayash, A. I., & Schaffner, A. (2006). CD163 is the macrophage scavenger receptor for native and chemically modified hemoglobins in the absence of haptoglobin. Blood, 107(1), 373–380. https://doi.org/10.1182/blood-2005-03-1014

Schöne, C., Höfler, H., & Walch, A. (2013). MALDI imaging mass spectrometry in cancer research: Combining proteomic profiling and histological evaluation. In Clinical Biochemistry (Vol. 46, Issue 6, pp. 539–545). Elsevier. https://doi.org/10.1016/j.clinbiochem.2013.01.018

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. Journal of Leukocyte Biology, 75(2), 163–189. https://doi.org/10.1189/jlb.0603252

Seuberlich, T., Tratschin, J. D., Thür, B., & Hofmann, M. A. (2002). Nucleocapsid protein-based enzyme-linked immunosorbent assay for detection and differentiation of antibodies against European and North American porcine reproductive and respiratory syndrome virus. Clinical and Diagnostic Laboratory Immunology, 9(6), 1183–1191. https://doi.org/10.1128/CDLI.9.6.1183-1191.2002

Shanmukhappa, K., Kim, J. K., & Kapil, S. (2007). Role of CD151, a tetraspanin, in porcine reproductive and respiratory syndrome virus infection. Virology Journal, 4(1), 62. https://doi.org/10.1186/1743-422X-4-62

Shen, L., Zhang, X., Hu, D., Feng, T., Li, H., Lu, Y., & Huang, J. (2013). Hepatitis B virus X (HBx) play an anti-apoptosis role in hepatic progenitor cells by activating Wnt/β-catenin pathway. Molecular and Cellular Biochemistry, 383(1–2), 213–222. https://doi.org/10.1007/s11010-013-1769-5

Shi, M., Holmes, E. C., Brar, M. S., & Leung, F. C.-C. (2013). Recombination Is Associated with an Outbreak of Novel Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Viruses in China. Journal of Virology, 87(19), 10904–10907. https://doi.org/10.1128/jvi.01270-13

Shi, M., Lam, T. T.-Y., Hon, C.-C., Murtaugh, M. P., Davies, P. R., Hui, R. K.-H., Li, J., Wong, L. T.-W., Yip, C.-W., Jiang, J.-W., & Leung, F. C.-C. (2010). Phylogeny-Based Evolutionary, Demographical, and Geographical Dissection of North American Type 2 Porcine Reproductive and Respiratory Syndrome Viruses. Journal of Virology, 84(17), 8700–8711. https://doi.org/10.1128/jvi.02551-09

Shi, Mang, Lam, T. T. Y., Hon, C. C., Hui, R. K. H., Faaberg, K. S., Wennblom, T., Murtaugh, M. P.,

Stadejek, T., & Leung, F. C. C. (2010). Molecular epidemiology of PRRSV: A phylogenetic perspective. Virus Research, 154(1–2), 7–17. https://doi.org/10.1016/j.virusres.2010.08.014

Sierra, M. A., Carrasco, L., Gómez-Villamandos, J. C., Martin de las Mulas, J., Méndez, A., & Jover, A. (1990). Pulmonary intravascular macrophages in lungs of pigs inoculated with african swine fever virus of differing virulence. Journal of Comparative Pathology, 102(3), 323–334. https://doi.org/10.1016/S0021-9975(08)80021-7

Silva-Campa, E., Cordoba, L., Fraile, L., Flores-mendoza, L., Montoya, M., & Hernández, J. (2010). European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology, 396(2), 264–271. https://doi.org/10.1016/j.virol.2009.10.024

Silva-Campa, E., Flores-Mendoza, L., Reséndiz, M., Pinelli-Saavedra, A., Mata-Haro, V., Mwangi, W., & Hernández, J. (2009). Induction of T helper 3 regulatory cells by dendritic cells infected with porcine reproductive and respiratory syndrome virus. Virology, 387(2), 373–379. https://doi.org/10.1016/j.virol.2009.02.033

Silva-Campa, E., Mata-Haro, V., Mateu, E., & Hernández, J. (2012). Porcine reproductive and respiratory syndrome virus induces CD4 $^{+}$CD8 $^{+}$CD25 $^{+}$Foxp3^{+} regulatory T cells (Tregs). Virology, 430(1), 73–80. https://doi.org/10.1016/j.virol.2012.04.009

Singh, R., Letai, A., & Sarosiek, K. (2019). Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. Nature Reviews Molecular Cell Biology, 20(3), 175–193. https://doi.org/10.1038/s41580-018-0089-8

Sinkora, M., Butler, J. E., Lager, K. M., Potockova, H., & Sinkorova, J. (2014). The comparative profile of lymphoid cells and the T and B cell spectratype of germ-free piglets infected with viruses SIV, PRRSV or PCV2. Veterinary Research, 45(1), 91. https://doi.org/10.1186/s13567-014-0091-x

Sinn, L. J., Klingler, E., Lamp, B., Brunthaler, R., Weissenböck, H., Rümenapf, T., & Ladinig, A. (2016). Emergence of a virulent porcine reproductive and respiratory syndrome virus (PRRSV) 1 strain in Lower Austria. Porcine Health Management, 2, 1–10. https://doi.org/10.1186/s40813-016-0044-z

Sinn, L. J., Zieglowski, L., Koinig, H., Lamp, B., Jansko, B., Mößlacher, G., Riedel, C., Hennig-Pauka, I., & Rümenapf, T. (2016). Characterization of two Austrian porcine reproductive and respiratory syndrome virus (PRRSV) field isolates reveals relationship to East Asian strains. Veterinary Research, 47(1), 1–13. https://doi.org/10.1186/s13567-015-0293-x

Smyk-Pearson, S., Golden-Mason, L., Klarquist, J., Burton, Jr., J. R., Tester, I. A., Wang, C. C., Culbertson, N., Vandenbark, A. A., & Rosen, H. R. (2008). Functional Suppression by FoxP3^{+} CD4^{+} CD25 high Regulatory T Cells during Acute Hepatitis C Virus Infection . The Journal of Infectious Diseases, 197(1), 46–57. https://doi.org/10.1086/523651

Snijder, E. J., Kikkert, M., & Fang, Y. (2013). Arterivirus molecular biology and pathogenesis. In

Journal of General Virology (Vol. 94, Issue PART10, pp. 2141–2163). https://doi.org/10.1099/vir.0.056341-0

Snijder, E. J., & Meulenberg, J. J. M. (1998). The molecular biology of arteriviruses. In Journal of General Virology (Vol. 79, Issue 5, pp. 961–979). Microbiology Society. https://doi.org/10.1099/0022-1317-79-5-961

Song, S., Bi, J., Wang, D., Fang, L., Zhang, L., Li, F., Chen, H., & Xiao, S. (2013). Porcine reproductive and respiratory syndrome virus infection activates IL-10 production through NF-κB and p38 MAPK pathways in porcine alveolar macrophages. Developmental and Comparative Immunology, 39(3), 265–272. https://doi.org/10.1016/j.dci.2012.10.001

Sørensen, K. J., Strandbygaard, B., Bøtner, A., Madsen, E. S., Nielsen, J., & Have, P. (1998). Blocking ELISA's for the distinction between antibodies against European and American strains of porcine reproductive and respiratory syndrome virus. Veterinary Microbiology, 60(2–4), 169–177. https://doi.org/10.1016/s0378-1135(98)00159-x

Spight, D., Zhao, B., Haas, M., Wert, S., Denenberg, A., & Shanley, T. P. (2005). Immunoregulatory effects of regulated, lung-targeted expression of IL-10 in vivo. American Journal of Physiology - Lung Cellular and Molecular Physiology, 288(2 32-2), L251-65. https://doi.org/10.1152/ajplung.00122.2004

Spilman, M. S., Welbon, C., Nelson, E., & Dokland, T. (2009). Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: Organization of the nucleocapsid. Journal of General Virology, 90(3), 527–535. https://doi.org/10.1099/vir.0.007674-0

Stadejek, T., Larsen, L. E., Podgórska, K., Bøtner, A., Botti, S., Dolka, I., Fabisiak, M., Heegaard, P. M. H., Hjulsager, C. K., Huć, T., Kvisgaard, L. K., Sapierzyński, R., & Nielsen, J. (2017). Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs. Veterinary Microbiology, 209(April), 13–19. https://doi.org/10.1016/j.vetmic.2017.05.011

Stadejek, T., Stankevicius, A., Murtaugh, M. P., & Oleksiewicz, M. B. (2013). Molecular evolution of PRRSV in Europe: Current state of play. Veterinary Microbiology, 165(1–2), 21–28. https://doi.org/10.1016/j.vetmic.2013.02.029

Suárez, P., Díaz-Guerra, M., Prieto, C., Esteban, M., Castro, J. M., Nieto, A., & Ortín, J. (1996). Open reading frame 5 of porcine reproductive and respiratory syndrome virus as a cause of virus-induced apoptosis. Journal of Virology, 70(5), 2876–2882. https://doi.org/10.1128/jvi.70.5.2876-2882.1996

Subramaniam, S., Sur, J. H., Kwon, B., Pattnaik, A. K., & Osorio, F. A. (2011). A virulent strain of porcine reproductive and respiratory syndrome virus does not up-regulate interleukin-10 levels in vitro or in vivo. Virus Research, 155(2), 415–422. https://doi.org/10.1016/j.virusres.2010.11.010

Sulahian, T. H., Högger, P., Wahner, A. E., Wardwell, K., Goulding, N. J., Sorg, C., Droste, A., Stehling, M., Wallace, P. K., Morganelli, P. M., & Guyre, P. M. (2000). Human monocytes express CD163,

which is upregulated by IL-10 and identical to p155. Cytokine, 12(9), 1312–1321. https://doi.org/10.1006/cyto.2000.0720

Sun, N., Ly, A., Meding, S., Witting, M., Hauck, S. M., Ueffing, M., Schmitt-Kopplin, P., Aichler, M., & Walch, A. (2014). High-resolution metabolite imaging of light and dark treated retina using MALDI-FTICR mass spectrometry. Proteomics, 14(7–8), 913–923. https://doi.org/10.1002/pmic.201300407

Sun, Y., Ke, H., Han, M., Chen, N., Fang, W., & Yoo, D. (2016). Nonstructural Protein 11 of Porcine Reproductive and Respiratory Syndrome Virus Suppresses Both MAVS and RIG-I Expression as One of the Mechanisms to Antagonize Type I Interferon Production. PLOS ONE, 11(12), e0168314. https://doi.org/10.1371/journal.pone.0168314

Sur, J. H., Doster, A. R., Christian, J. S., Galeota, J. A., Wills, R. W., Zimmerman, J. J., & Osorio, F. A. (1997). Porcine reproductive and respiratory syndrome virus replicates in testicular germ cells, alters spermatogenesis, and induces germ cell death by apoptosis. Journal of Virology, 71(12), 9170–9179. https://doi.org/10.1128/jvi.71.12.9170-9179.1997

Suradhat, S., Thanawongnuwech, R., & Poovorawan, Y. (2003). Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus. In Journal of General Virology (Vol. 84, Issue 2, pp. 453–459). https://doi.org/10.1099/vir.0.18698-0

Tait, S. W. G., & Green, D. R. (2013). Mitochondrial regulation of cell death. Cold Spring Harbor Perspectives in Biology, 5(9). https://doi.org/10.1101/cshperspect.a008706

Takamatsu, H. H., Denyer, M. S., Stirling, C., Cox, S., Aggarwal, N., Dash, P., Wileman, T. E., & Barnett, P. V. (2006). Porcine γδ T cells: Possible roles on the innate and adaptive immune responses following virus infection. Veterinary Immunology and Immunopathology, 112(1–2), 49–61. https://doi.org/10.1016/j.vetimm.2006.03.011

Takeuchi, O., & Akira, S. (2010). Pattern Recognition Receptors and Inflammation. In Cell (Vol. 140, Issue 6, pp. 805–820). https://doi.org/10.1016/j.cell.2010.01.022

Thacker, E. L., Halbur, P. G., Ross, R. F., Thanawongnuwech, R., & Thacker, B. J. (1999). Mycoplasma hyopneumoniae potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia. Journal of Clinical Microbiology, 37(3), 620–627. https://doi.org/10.1128/jcm.37.3.620-627.1999

Thanawongnuwech, R., Brown, G. B., Halbur, P. G., Roth, J. A., Royer, R. L., & Thacker, B. J. (2000). Pathogenesis of Porcine Reproductive and Respiratory Syndrome Virus-induced Increase in Susceptibility to Streptococcus suis Infection. Veterinary Pathology, 37(2), 143–152. https://doi.org/10.1354/vp.37-2-143

PRRS Virus-Induced Damage to Intravascular Macrophages, 43 (testimony of R. Thanawongnuwech, Patrick G Halbur, R. Royer, & J. Bruna). https://lib.dr.iastate.edu/swinereports_1997/43

Thanawongnuwech, R., Thacker, B., Halbur, P., & Thacker, E. L. (2004). Increased production of

proinflammatory cytokines following infection with porcine reproductive and respiratory syndrome virus and Mycoplasma hyopneumoniae. Clinical and Diagnostic Laboratory Immunology, 11(5), 901–908. https://doi.org/10.1128/CDLI.11.5.901-908.2004

Thanawongnuwech, R., Thacker, E. L., & Halbur, P. G. (1998). Influence of pig age on virus titer and bactericidal activity of porcine reproductive and respiratory syndrome virus (PRRSV)-infected pulmonary intravascular macrophages (PIMs). Veterinary Microbiology, 63(2–4), 177–187. https://doi.org/10.1016/S0378-1135(98)00245-4

Thanawongnuwech, R., Young, T. F., Thacker, B. J., & Thacker, E. L. (2001). Differential production of proinflammatory cytokines: in vitro PRRSV and Mycoplasma hyopneumoniae co-infection model. Veterinary Immunology and Immunopathology, 79(1–2), 115–127. https://doi.org/10.1016/s0165-2427(01)00243-4

Theofilopoulos, A. N., Baccala, R., Beutler, B., & Kono, D. H. (2005). TYPE I INTERFERONS (α/β) IN IMMUNITY AND AUTOIMMUNITY. Annual Review of Immunology, 23(1), 307–335. https://doi.org/10.1146/annurev.immunol.23.021704.115843

Thompson, M. R., Kaminski, J. J., Kurt-Jones, E. A., & Fitzgerald, K. A. (2011). Pattern recognition receptors and the innate immune response to viral infection. In Viruses (Vol. 3, Issue 6, pp. 920–940). https://doi.org/10.3390/v3060920

Tian, D., Wei, Z., Zevenhoven-Dobbe, J. C., Liu, R., Tong, G., Snijder, E. J., & Yuan, S. (2012). Arterivirus Minor Envelope Proteins Are a Major Determinant of Viral Tropism in Cell Culture. Am Soc Microbiol. https://doi.org/10.1128/JVI.06836-11

Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., … Gao, G. F. (2007). Emergence of Fatal PRRSV Variants: Unparalleled Outbreaks of Atypical PRRS in China and Molecular Dissection of the Unique Hallmark. PLoS ONE, 2(6), e526. https://doi.org/10.1371/journal.pone.0000526

Tian, Z. J., An, T. Q., Zhou, Y. J., Peng, J. M., Hu, S. P., Wei, T. C., Jiang, Y. F., Xiao, Y., & Tong, G. Z. (2009). An attenuated live vaccine based on highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) protects piglets against HP-PRRS. Veterinary Microbiology, 138(1–2), 34–40. https://doi.org/10.1016/j.vetmic.2009.03.003

Tong, G. Z., Zhou, Y. J., Hao, X. F., Tian, Z. J., An, T. Q., & Qiu, H. J. (2007). Highly pathogenic porcine reproductive and respiratory syndrome, China [8]. In Emerging Infectious Diseases (Vol. 13, Issue 9, pp. 1434–1436). Centers for Disease Control and Prevention (CDC). https://doi.org/10.3201/eid1309.070399

Vahlenkamp, T. W., Tompkins, M. B., & Tompkins, W. A. F. (2005). The role of $CD4^+CD25^+$ regulatory T cells in viral infections. Veterinary Immunology and Immunopathology, 108(1–2), 219–225. https://doi.org/10.1016/j.vetimm.2005.07.011

Vaine, C. A., & Soberman, R. J. (2014). The CD200-CD200R1 Inhibitory Signaling Pathway.

Immune Regulation and Host-Pathogen Interactions. In Advances in Immunology (Vol. 121, pp. 191–211). Academic Press Inc. https://doi.org/10.1016/B978-0-12-800100-4.00005-2

Van Breedam, W., Delputte, P. L., Van Gorp, H., Misinzo, G., Vanderheijden, N., Duan, X., & Nauwynck, H. J. (2010). Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. Journal of General Virology, 91(7), 1659–1667. https://doi.org/10.1099/vir.0.020503-0

Van Den Heuvel, M. M., Tensen, C. P., Van As, J. H., Van Den Berg, T. K., Fluitsma, D. M., Dijkstra, C. D., Döpp, E. A., Droste, A., Van Gaalen, F. A., Sorg, C., Högger, P., & Beelen, R. H. J. (1999). Regulation of CD163 on human macrophages: Cross-linking of CD163 induces signaling and activation. Journal of Leukocyte Biology, 66(5), 858–866. https://doi.org/10.1002/jlb.66.5.858

van der Linden, I. F. A., Voermans, J. J. M., van der Linde-Bril, E. M., Bianchi, A. T. J., & Steverink, P. J. G. M. (2003). Virological kinetics and immunological responses to a porcine reproductive and respiratory syndrome virus infection of pigs at different ages. Vaccine, 21(17–18), 1952–1957. http://www.ncbi.nlm.nih.gov/pubmed/12706683

Van Gorp, H., Van Breedam, W., Delputte, P. L., & Nauwynck, H. J. (2008). Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. Journal of General Virology, 89(12), 2943–2953. https://doi.org/10.1099/vir.0.2008/005009-0

Van Gucht, S., Labarque, G., & Van Reeth, K. (2004). The combination of PRRS virus and bacterial endotoxin as a model for multifactorial respiratory disease in pigs. Veterinary Immunology and Immunopathology, 102(3), 165–178. https://doi.org/10.1016/j.vetimm.2004.09.006

Van Gucht, S., Reeth, K. Van, Nauwynck, H., & Pensaert, M. (2005). Porcine Reproductive and Respiratory Syndrome Virus Infection Increases CD14 Expression and Lipopolysaccharide-Binding Protein in the Lungs of Pigs. Viral Immunology, 18(1), 116–126. https://doi.org/10.1089/vim.2005.18.116

Van Reeth, K., Labarque, G., Nauwynck, H., & Pensaert, M. (1999). Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: Correlations with pathogenicity. Research in Veterinary Science, 67(1), 47–52. https://doi.org/10.1053/rvsc.1998.0277

Van Reeth, Kristien, Nauwynck, H., & Pensaert, M. (1996). Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: A clinical and virological study. Veterinary Microbiology, 48(3–4), 325–335. https://doi.org/10.1016/0378-1135(95)00145-X

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A., & Nauwynck, H. J. (2003). Involvement of Sialoadhesin in Entry of Porcine Reproductive and Respiratory Syndrome Virus into Porcine Alveolar Macrophages. Journal of Virology, 77(15), 8207–8215. https://doi.org/10.1128/jvi.77.15.8207-8215.2003

Vanderheijden, N., Delputte, P., Nauwynck, H., & Pensaert, M. (2001). Effects of heparin on the

entry of porcine reproductive and respiratory syndrome virus into alveolar macrophages. Advances in Experimental Medicine and Biology, 494, 683–689. https://doi.org/10.1007/978-1-4615-1325-4_101

Vézina, S. A., Loemba, H., Fournier, M., Dea, S., & Archambault, D. (1996). Antibody production and blastogenic response in pigs experimentally infected with porcine reproductive and respiratory syndrome virus. Canadian Journal of Veterinary Research = Revue Canadienne de Recherche Veterinaire, 60(2), 94–99. http://www.ncbi.nlm.nih.gov/pubmed/8785726

Wang, A., Chen, Q., Wang, L., Madson, D., Harmon, K., Gauger, P., Zhang, J., & Li, G. (2019). Recombination between vaccine and field strains of porcine reproductive and respiratory syndrome virus. Emerging Infectious Diseases, 25(12), 2335–2337. https://doi.org/10.3201/eid2512.191111

Wang, G., He, Y., Tu, Y., Liu, Y., Zhou, E. M., Han, Z., Jiang, C., Wang, S., Shi, W., & Cai, X. (2014). Comparative analysis of apoptotic changes in peripheral immune organs and lungs following experimental infection of piglets with highly pathogenic and classical porcine reproductive and respiratory syndrome virus. Virology Journal, 11(1), 2. https://doi.org/10.1186/1743-422X-11-2

Wang, G., Yu, Y., Tu, Y., Tong, J., Liu, Y., Zhang, C., Chang, Y., Wang, S., Jiang, C., Zhou, E. M., & Cai, X. (2015). Highly pathogenic porcine reproductive and respiratory syndrome virus infection induced apoptosis and autophagy in thymi of infected piglets. PLoS ONE, 10(6), 1–12. https://doi.org/10.1371/journal.pone.0128292

Wang, L., Hu, S., Liu, Q., Li, Y., Xu, L., Zhang, Z., Cai, X., & He, X. (2017). Porcine alveolar macrophage polarization is involved in inhibition of porcine reproductive and respiratory syndrome virus (PRRSV) replication. Journal of Veterinary Medical Science, 79(11), 1906–1915. https://doi.org/10.1292/jvms.17-0258

Wang, X., Eaton, M., Mayer, M., Li, H., He, D., Nelson, E., & Christopher-Hennings, J. (2007). Porcine reproductive and respiratory syndrome virus productively infects monocyte-derived dendritic cells and compromises their antigen-presenting ability. Archives of Virology, 152(2), 289–303. https://doi.org/10.1007/s00705-006-0857-1

Weesendorp, E., Morgan, S., Stockhofe-Zurwieden, N., Graaf, D. J. P. De, Graham, S. P., & Rebel, J. M. J. (2013). Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Veterinary Microbiology, 163(1–2), 1–12. https://doi.org/10.1016/j.vetmic.2012.09.013

Weesendorp, E., Rebel, J. M. J., Popma-De Graaf, D. J., Fijten, H. P. D., & Stockhofe-Zurwieden, N. (2014). Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Veterinary Microbiology, 174(1–2), 127–138. https://doi.org/10.1016/j.vetmic.2014.09.010

Weesendorp, E., Stockhofe-Zurwieden, N., Popma-De Graaf, D. J., Fijten, H., & Rebel, J. M. J. (2013). Phenotypic modulation and cytokine profiles of antigen presenting cells by European subtype 1 and 3 porcine reproductive and respiratory syndrome virus strains in vitro and in vivo. Veterinary Microbiology, 167(3–4), 638–650. https://doi.org/10.1016/j.vetmic.2013.09.021

Wells, K. D., Bardot, R., Whitworth, K. M., Trible, B. R., Fang, Y., Mileham, A., Kerrigan, M. A., Samuel, M. S., Prather, R. S., & Rowland, R. R. R. (2017). Replacement of Porcine CD163 Scavenger Receptor Cysteine-Rich Domain 5 with a CD163-Like Homolog Confers Resistance of Pigs to Genotype 1 but Not Genotype 2 Porcine Reproductive and Respiratory Syndrome Virus. Journal of Virology, 91(2), 1–11. https://doi.org/10.1128/jvi.01521-16

Wensvoort, G., Terpstra, C., Pol, J. M., ter Laak, E. A., Bloemraad, M., de Kluyver, E. P., Kragten, C., van Buiten, L., den Besten, A., & Wagenaar, F. (1991). Mystery swine disease in The Netherlands: the isolation of Lelystad virus. The Veterinary Quarterly, 13(3), 121–130. https://doi.org/10.1080/01652176.1991.9694296

Wensvoort, Gert, de Kluyver, E. P., Pol, J. M. A., Wagenaar, F., Moormann, R. J. M., Hulst, M. M., Bloemraad, R., Besten, A. den, Zetstra, T., & Terpstra, C. (1992). Lelystad virus, the cause of porcine epidemic abortion and respiratory syndrome: a review of mystery swine disease research at Lelystad. Veterinary Microbiology, 33(1–4), 185–193. https://doi.org/10.1016/0378-1135(92)90046-V

Whitworth, K. M., Rowland, R. R. R., Ewen, C. L., Trible, B. R., Kerrigan, M. A., Cino-Ozuna, A. G., Samuel, M. S., Lightner, J. E., McLaren, D. G., Mileham, A. J., Wells, K. D., & Prather, R. S. (2016). Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nature Biotechnology, 34(1), 20–22. https://doi.org/10.1038/nbt.3434

Wills, R W, Gray, J. T., Fedorka-Cray, P. J., Yoon, K. J., Ladely, S., & Zimmerman, J. J. (2000). Synergism between porcine reproductive and respiratory syndrome virus (PRRSV) and Salmonella choleraesuis in swine. Veterinary Microbiology, 71(3–4), 177–192. https://doi.org/10.1016/s0378-1135(99)00175-3

Wills, Robert W., Doster, A. R., Galeota, J. A., Sur, J. H., & Osorio, F. A. (2003). Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. Journal of Clinical Microbiology, 41(1), 58–62. https://doi.org/10.1128/JCM.41.1.58-62.2003

Wink, D. A., Hines, H. B., Cheng, R. Y. S., Switzer, C. H., Flores-Santana, W., Vitek, M. P., Ridnour, L. A., & Colton, C. A. (2011). Nitric oxide and redox mechanisms in the immune response. Journal of Leukocyte Biology, 89(6), 873–891. https://doi.org/10.1189/jlb.1010550

Wissink, E. H. J., Kroese, M. V., van Wijk, H. A. R., Rijsewijk, F. A. M., Meulenberg, J. J. M., & Rottier, P. J. M. (2005). Envelope Protein Requirements for the Assembly of Infectious Virions of Porcine Reproductive and Respiratory Syndrome Virus. Journal of Virology, 79(19), 12495–12506. https://doi.org/10.1128/jvi.79.19.12495-12506.2005

Wissink, Esther H.J., van Wijk, H. A. R., Kroese, M. V., Weiland, E., Meulenberg, J. J. M., Rottier, P. J. M., & van Rijn, P. A. (2003). The major envelope protein, GP5, of a European porcine reproductive and respiratory syndrome virus contains a neutralization epitope in its N-terminal ectodomain. Journal of General Virology, 84(6), 1535–1543. https://doi.org/10.1099/vir.0.18957-0

Wongyanin, P., Buranapraditkun, S., Chokeshai-usaha, K., Thanawonguwech, R., & Suradhat, S. (2010). Induction of inducible $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). Veterinary Immunology and Immunopathology, 133(2–4), 170–182. https://doi.org/10.1016/j.vetimm.2009.07.012

Wongyanin, Piya, Buranapraditkul, S., Yoo, D., Thanawongnuwech, R., Roth, J. A., & Suradhat, S. (2012). Role of porcine reproductive and respiratory syndrome virus nucleocapsid protein in induction of interleukin-10 and regulatory T-lymphocytes (Treg). Journal of General Virology, 93(6), 1236–1246. https://doi.org/10.1099/vir.0.040287-0

Xiao, Z., Batista, L., Dee, S., Halbur, P., & Murtaugh, M. P. (2004). The Level of Virus-Specific T-Cell and Macrophage Recruitment in Porcine Reproductive and Respiratory Syndrome Virus Infection in Pigs Is Independent of Virus Load. Journal of Virology, 78(11), 5923–5933. https://doi.org/10.1128/jvi.78.11.5923-5933.2004

Yin, S., Huo, Y., Dong, Y., Fan, L., Yang, H., Wang, L., Ning, Y., & Hu, H. (2012). Activation of c-Jun NH(2)-terminal kinase is required for porcine reproductive and respiratory syndrome virus-induced apoptosis but not for virus replication. Virus Research, 166(1–2), 103–108. https://doi.org/10.1016/j.virusres.2012.03.010

Yoo, D., Song, C., Sun, Y., Du, Y., Kim, O., & Liu, H. C. (2010). Modulation of host cell responses and evasion strategies for porcine reproductive and respiratory syndrome virus. In Virus Research (Vol. 154, Issues 1–2, pp. 48–60). https://doi.org/10.1016/j.virusres.2010.07.019

Yoon, J., Joo, H. S., Christianson, W. T., Kim, H. S., Collins, J. E., Morrison, R. B., & Dial, G. D. (1992). An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. Journal of Veterinary Diagnostic Investigation, 4(2), 144–147. https://doi.org/10.1177/104063879200400205

Yuan, S., Zhang, N., Xu, L., Zhou, L., Ge, X., Guo, X., & Yang, H. (2016). Induction of Apoptosis by the Nonstructural Protein 4 and 10 of Porcine Reproductive and Respiratory Syndrome Virus. PLOS ONE, 11(6), e0156518. https://doi.org/10.1371/journal.pone.0156518

Zanoni, I., & Granucci, F. (2013). Role of CD14 in host protection against infections and in metabolism regulation. In Frontiers in Cellular and Infection Microbiology (Vol. 4, Issue JUL). Frontiers Media SA. https://doi.org/10.3389/fcimb.2013.00032

Zhang, K., Hou, Q., Zhong, Z., Li, X., Chen, H., Li, W., Wen, J., Wang, L., Liu, W., & Zhong, F. (2013). Porcine reproductive and respiratory syndrome virus activates inflammasomes of porcine alveolar macrophages via its small envelope protein E. Virology, 442(2), 156–162. https://doi.

org/10.1016/j.virol.2013.04.007

Zhang, L., Liu, J., Bai, J., Wang, X., Li, Y., & Jiang, P. (2013). Comparative expression of Toll-like receptors and inflammatory cytokines in pigs infected with different virulent porcine reproductive and respiratory syndrome virus isolates. Virology Journal, 10, 135. https://doi.org/10.1186/1743-422X-10-135

Zhang, Q., & Yoo, D. (2015). PRRS virus receptors and their role for pathogenesis. In Veterinary Microbiology (Vol. 177, Issues 3–4, pp. 229–241). Elsevier. https://doi.org/10.1016/j.vetmic.2015.04.002

Zhou, L., Wang, Z., Ding, Y., Ge, X., Guo, X., & Yang, H. (2015). NADC30-like strain of porcine reproductive and respiratory syndrome virus, China. In Emerging Infectious Diseases (Vol. 21, Issue 12, pp. 2256–2257). Centers for Disease Control and Prevention (CDC). https://doi.org/10.3201/eid2112.150360

Zhou, L., & Yang, H. (2010). Porcine reproductive and respiratory syndrome in China. In Virus Research (Vol. 154, Issues 1–2, pp. 31–37). Elsevier. https://doi.org/10.1016/j.virusres.2010.07.016

Zhou, Y. J., Hao, X. F., Tian, Z. J., Tong, G. Z., Yoo, D., An, T. Q., Zhou, T., Li, G. X., Qiu, H. J., Wei, T. C., & Yuan, X. F. (2008). Highly virulent porcine reproductive and respiratory syndrome virus emerged in China. Transboundary and Emerging Diseases, 55(3–4), 152–164. https://doi.org/10.1111/j.1865-1682.2008.01020.x

Zimmerman, J. J., Dee, S. A., Holtkamp, D. J., Murtaugh, M. P., Stadejek, T., Stevenson, G. W., Torremorell, M., Yang, H., & Zhang, J. (2019). Porcine Reproductive and Respiratory Syndrome Viruses (Porcine Arteriviruses). In Diseases of Swine (pp. 685–708). Wiley. https://doi.org/10.1002/9781119350927.ch41

Zwadlo, G., Voegeíi, R., Osthoff, K. S., & Sorg, C. (1987). A Monoclonal Antibody to a Novel Differentiation Antigen on Human Macrophages Associated with the Down-Regulatory Phase of the Inflammatory Process. Pathobiology, 55(6), 295–304. https://doi.org/10.1159/000163432