SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

REPRODUCTIVE AND RESPIRATORY SYNDROME

INMUNOPATOGENIA DEL PRRS:

ESTUDIO COMPARADO EN PULMÓN Y ÓRGANOS LINFOIDES PRIMARIOS DE ANIMALES INFECTADOS CON CEPAS DE DISTINTA VIRULENCIA DEL PRRSV-1

IMMUNOPATHOGENESIS OF PRRS:

COMPARATIVE STUDY IN THE LUNG AND PRIMARY LYMPHOID ORGANS OF ANIMALS INFECTED WITH DIFFERENT VIRULENCE PRRSV-1 STRAIN

DPTO. ANATOMÍA Y ANATOMÍA PATOLÓGICA COMPARADA

FACULTAD DE VETERINARIA UNIVERSIDAD DE CÓRDOBA

CÓRDOBA - NOVIEMBRE 2015

TÍTULO: *Inmunopatogenia del PRRS: Estudio comparado en pulmón y órganos linfoides primarios de animales infectados con cepas de distintas virulencias del PRRSV-1.*

AUTOR: *Shyrley Paola Amarilla*

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

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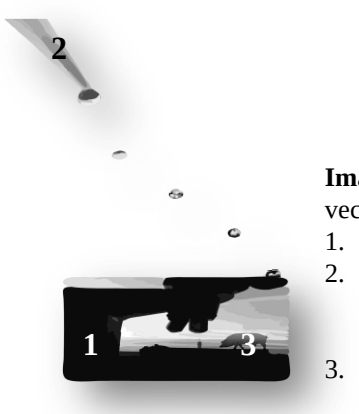

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Trabajo presentado por la Lincenciada en Veterinaria Dña ***Shyrley Paola Amarilla*** para optar al grado de Doctora por la Universidad de Córdoba.

Dpto. Anatomía y Anatomía Patológica Comparadas

Córdoba - Noviembre 2015

Al regalo más hermoso y valioso que
Dios me ha dado y va a donde yo voy,

MI FAMILIA

La lógica te llevará de A a B. La imaginación te llevará a todas partes.

(Albert Einstein)

TÍTULO DE LA TESIS: Inmunopatogénesis del PRRS: Estudio comparado en pulmón y órganos linfoides primarios de animales infectados con cepas de distinta virulencia del PRRSV-1

DOCTORANDO/A: Shyrley Paola Amarilla

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La doctoranda ha realizado bajo nuestra dirección y asesoramiento la tesis doctoral de título "Inmunopatogénesis del PRRS: Estudio comparado en pulmón y órganos linfoides primarios de animales infectados con cepas de distinta virulencia del PRRSV-1" , un trabajo que se ha estructurado como el estudio comparado del efecto de cuatro cepas de diferente virulencia del virus del genotipo europeo del virus del PRRS sobre el pulmón, como órgano diana, y los órganos linfoides primarios, timo y médula ósea. El trabajo se ha realizado principalmente en el departamento de Anatomía y Anatomía Patológica Comparadas de la Universidad de Córdoba, aunque una parte del estudio se realizó durante una estancia de la doctoranda en la Universidad de Surrey (Reino Unido) centro de trabajo de uno de los codirectores. Los resultados obtenidos han sido presentados a diferentes reuniones científicas y han dado lugar a tres artículos, uno de los cuales ya ha sido publicado en la revista *Veterinary Immunology and Immunopathology* y los otros dos han sido enviados y están en proceso de revisión en las revistas *Veterinary Microbiology* y *Fronteirs in Immunology*. Por todo ello consideramos que el trabajo realizado y la tesis presentada reúnen las condiciones y calidad científica necesaria para que la doctoranda opte al Grado de Doctor por la Universidad de Córdoba

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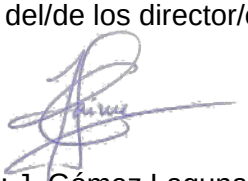

Fdo.: J. Gómez Laguna

Fdo.: F.J. Salguero Bodes

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

Facultad de Veterinaria
Universidad de Córdoba

Librado Carrasco Otero, Catedrático de Universidad adscrito al Departamento de Anatomía y Anatomía Patológica Comparadas de la Universidad de Córdoba

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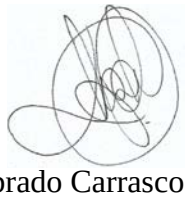

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Jaime Gómez Laguna, Doctor por la Universidad de Córdoba, responsable del área de producción primaria del Departamento de I+D+i del Centro de Investigación y Calidad Agroalimentaria del Valle de los Pedroches (CICAP), Pozoblanco (Córdoba)

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Apdo. Correos 105 · Ctra. de la Canaleja, s/n
14400 Pozoblanco (Córdoba) · T: +34 957 116 254
cicap@cicap.es · www.cicap.es

UNIVERSITY OF SURREY
Faculty of Health and Medical Sciences

School of Veterinary Medicine
Duke of Kent Building
Guildford, Surrey GU2 7TE UK

Dr. F. Javier Salguero Bodes
Reader in Comparative Pathology

T: +44 (0)1483 689872
F: +44 (0)1483 686711

f.salguerobodes@surrey.ac.uk
www.surrey.ac.uk

Dr. Francisco Javier Salguero Bodes,
DVM, PhD, DiplECPHM, FRCpath, MRCVS

Informa

Que la Licenciada en Veterinaria Dña **Shyrley Paola Amarilla** a realizado bajo mi dirección y asesoramiento el trabajo **"Inmunopatogenia del PRRS: Estudio comparado en pulmón y órganos linfoides primarios de animales infectados con cepas de distinta virulencia del PRRSV-1"**, que considero que reúne las condiciones y calidad científica necesaria para optar al Grado de Doctora por la Universidad de Córdoba

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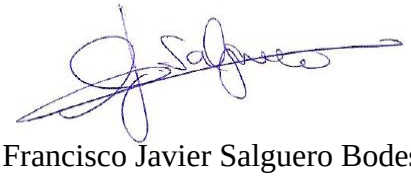

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LISTA DE ABREVIATURAS

LIST OF ABREVIATIONS

LISTA DE ABREVIATURAS
LIST OF ABBREVIATIONS

ABC	*Avidin-Biotin-peroxidase Complex*
ADE	*Antibody-Dependent Enhancement*
AN(s)	Anticuerpo(s) neutralizante(s)
APC(s)	*Antigen Presenting Cell(s);* Célula(s) Presentadora(s) de antígeno(s)
BALFs	*BronchoAlveolar Lavage Fluids*
BFU-E	*Burst Forming Unit–Erythroid;* Líneas de eritrocitos precursores
BMDC	*Bone Marrow-derived Dendritic Cells;* Células dendríticas derivadas de médula ósea
C:M ratio	*Cortex:Medulla ratio*
cCasp	*cleaved Caspase*
CD	*Cluster Differentiation*
Células NK	*Natural killer Cells*
CFU-GM	*Colony-Forming Unit–Granulocyte-Macrophage;* Unidades formadoras de colonias de granulocitos y monocitos
COX-2	Ciclooxigenasa-2
DC(s)	*Dendritic Cell(s);* Célula(s) dendrítica(s)
DMV	Vesículas de doble membrana peri-nuclear
dpi	*Days post-infection or inoculation;* Días post-infección o inoculación
gARN	Ácido de ribonucleico genómico
G-CSF	*Granulocyte Colony-Stimulating Factors*
GM-CSF	*Granulocyte-Macrophage Colony-Stimulating Factors*
GP	*GlicoProteine;* Glicoproteínas

h(s)	*hour(s);* hora(s)
hpi	Horas post-infección o inoculación
HP-PRRSV	*Highly Pathogenic PRRSV;* Altamente patógena PRRSV
HTAR	*High Temperature Antigen Retrievel*
IFN(s)	*InterFeroN(s);* InterferóN(es)
Ig	*Immunoglobulin;* Imnunoglobulina
IHQ	*ImmunoHistoChemistry;* Inmunohistoquimica
IL	*InterLeukin;* Interleucina
JAK/STAT	*The JAK-STAT system*: *JAnus Kinase* and *Signal Transducer and Activator of Transcription*
JNK	*c-Jun N-terminal Kinases*
kb	Kilobase
kDa	kiloDalton
LV	Lelystad virus
M:E ratio	*Myeloid:Erythroid ratio*
MA-104	Células de riñón de mono verde.
mAb	*Monoclonal Antibody;* Anticuerpo monoclonal
mARN	*messenger RiboNucleic Acid;* Ácido Ribonucleico mensajero
MHC	*Major Histocompatibility Complex;* Complejo mayor de histocompatibilidad
min	*minutes*, minutos
MLV	*Modified Live Virus;* vacuna viva modificada
MoDCs	Macrófagos derivados de monocitos
N	*Nucleocapside protein;* Proteína de la nucleocapside
NF-κB	*Nuclear Factor kappa-light-chain-enhancer of Activated B cells*

Nsp	*No structural protein;* Proteína no estructural
ORFs	*Open Reading Frames*
pAb	*polyclonal Antibody;* Anticuerpo policlonal
PAM(s)	*Porcine Alveolar Macrophages;* Macrófagos alveolares porcinos
PBS	Phosphate-Buffered Saline
PD	*Post-infection or inoculation;* Post-infección o inoculación
PIMs	*Porcine Interstitial and Intravascular Macrophages;* Macrófagos intersticiales e intravasculares (Macrófagos residentes del pulmón)
PRRs	Receptores de patrones de reconocimiento
PRRS	Síndrome reproductivo y respiratorio porcino
PRRSV	Virus del síndrome reproductivo y respiratorio porcino.
PRRSV-1	Genotipo Europeo o tipo 1 del PRRSV
PRRSV-2	Genotipo Norteamericano o tipo 2 del PRRSV
RdRp	Ácido ribonucleico dependiente de ácido ribonucleico polimerasa.
RFS	*Ribosomal FrameShift*
RNA	*RiboNucleic Acid;* Ácido Ribonucleico
SD	*Standard Deviation,*
sgRNA	Ácido ribonucleico sub-genómicos.
SIRS	*Swine Infertility and Respiratory Syndrome;* Síndrome de infertilidad y respiratorio porcino
$TCID_{50}$	*50% Tissue Culture Infectious Dose*
TGF-β	*Transforming Growth Factor beta;* Factor de crecimiento transformante beta

TNF-α	*Tumor Necrosis Factor Alpha;* Factor de necrosis tumoral alfa
Tregs	*Regulatory T cells;* Células T reguladoras
TUNEL	*Terminal deoxynucleotidyl transferase-mediated dUtp Nick-End Labelling*

INTRODUCCIÓN

INTRODUCTION

INTRODUCCIÓN

El síndrome reproductivo y respiratorio porcino (PRRS del inglés, *Porcine Reproductive and Respiratory Syndrome*) es una enfermedad vírica que ocasiona grandes pérdidas económicas al sector porcino, debido a que puede producir tanto fallos reproductivos en hembras adultas como procesos respiratorios en cerdos en crecimiento. A pesar de los esfuerzos realizados por todos los organismos competentes para controlar esta enfermedad y disminuir su impacto económico desde su aparición; en la actualidad, el PRRS sigue siendo considerado como uno de los problemas más graves en el sector porcino.

Una característica importante de esta enfermedad es que el virus del PRRS (PRRSV del inglés, *Porcine Reproductive and Respiratory Syndrome Virus*) modula la respuesta inmune del hospedador para favorecer su replicación, distribución y latencia en el organismo del animal infectado. Asimismo, el PRRSV es capaz de instaurar en el hospedador un estado de inmunosupresión en el que podrían estar implicados, una alteración en la expresión de diferentes citoquinas, tanto pro-inflamatorias como inmunomoduladoras y los fenómenos de apoptosis, que pueden estar o no asociados a la replicación del virus.

Nuestro grupo de investigación ha centrado sus estudios en conocer cómo se desencadena y establece la respuesta inmune en el hospedador frente a la infección por el PRRSV utilizando una cepa prototipo del virus del genotipo Europeo, el Lelystad (LV). Sin embargo, y debido a la aparición de las denominadas cepas altamente patógenas HP-PRRSV (HP, del inglés *highly pathogenic*), hemos considerado que

era el momento oportuno para realizar estudios que compararen los efectos producidos por cepas del PRRSV de diferente virulencia, incluidas las HP-PRRSV.

El principal objetivo de esta tesis ha sido "comparar la inmunopatogenia tras la infección con cepas del PRRSV-1 de distinta virulencia (la cepa estándar del PRRSV-1, LV; dos cepas de campo, la cepa altamente virulenta SU1-bel y la cepa 215-06, de moderada virulencia; y una cepa de vacuna atenuada, DV) en el pulmón y órganos linfoides primarios de cerdos infectados experimentalmente". La inoculación de los animales con las diferentes cepas del PRRSV-1 fueron realizados en el APHA *(Animal and Plant Health Agency*, Reino Unido) y fueron financiados tanto por los proyectos europeos PoRRSCon y NADIR (FP7 228394), el Departamento de Agricultura del Reino Unido (Defra Project SE0529) como por el Sector Porcino Británico (BPEX, del inglés *Bristish Pork Executive* ahora AHDB PORK). Los estudios de laboratorio fueron realizados en la Universidad de Córdoba dentro de las actividades del proyecto de investigación AGL2009-12438/GAN, financiado por el Ministerio de Educación y Ciencia. La doctoranda, Shyrley Paola Amarilla, ha desarrollado sus estudios de doctorado en la Universidad de Córdoba y las estancias tanto en esta Universidad como en la Universidad de Surrey en el Reino Unido gracias a la beca DET/GP/0024/2009 de ITAIPU BINACIONAL-PARAGUAY, del gobierno de Paraguay y la ayuda para la realización de estancias de la Universidad de Córdoba.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRSV) is a viral disease that causes major economic losses to pork producers. It can induce both reproductive failure in sows and respiratory tract disease in growing pigs. Currently, PRRS is still considered one of the most important problems in the swine industry worldwide, despite efforts by all partners involved in controlling the disease and reduce its economic impact.

An important characteristic of the disease is that PRRS virus (PRRSV) modulates the host immune response to facilitate its replication, distribution and latency in the organism of the infected animal. Additionally, PRRSV is able to produce a state of immunosuppression in the host with the probable involvement and alteration of the expression of several pro-inflammatory and immunomodulatory cytokines, together with the apoptosis phenomenon, which may or may be not associated with viral replication.

Previous studies conducted by our research group have been focused on understanding how PRRSV triggers and establishes an immune response in the host using the prototype PRRSV-1 strain Lelystad (LV). However, due to the emergence of highly pathogenic-PRRSV strains (HP-PRRSV), we have considered appropriate to conduct studies comparing several strains of different virulence, including HP-PRRSV.

The main aim of this thesis was "to compare the immunopathogenesis of PRRSV-1 infection with strains of differing virulence (the prototype PRRSV-1, Lelystad virus, LV; two field strains, the highly virulent SU1-bel strain and the strain 215-06 of moderate virulence; and a live-attenuated vaccine, DV) in the lung and primary lymphoid organs of experimentally infected pigs". The experimental animal infection with different PRRSV-1 strains was conducted in the Animal and Plant Health Agency, United Kingdom (APHA, UK). This work was supported by the European Union PoRRSCon Cost Action and NADIR (FP7 228394), United Kingdom Department of Agriculture (Defra Project SE0529) as the British Pork Executive (BPEX now AHDB PORK). Laboratory studies were performed at the University of Cordoba in the activities of the research project of the Spanish Ministry of Education and Science (Grant #AGL2009-12438/GAN). The PhD student, Shyrley Paola Amarilla, was based at the University of Córdoba and carried out an internship at the University of Surrey in the United Kingdom supported by a fellowship from the ITAIPU BINACIONAL-PARAGUAY under project number DET/GP/0024/2009 of the government of Paraguay and the support for the realization of stays at the University of Cordoba.

RESUMEN

SUMMARY

RESUMEN

El PRRSV es el causante del síndrome reproductivo y respiratorio porcino; una enfermedad que, a pesar de los esfuerzos realizados por controlarla y disminuir su impacto económico, sigue siendo considerada como una de las enfermedades más importantes que afectan al sector porcino.

Los dos genotipos del PRRSV, el genotipo Europeo o tipo 1 (PRRSV-1), representado por la cepa Lelystad (LV), y el genotipo Norteamericano o tipo 2 (PRRSV-2), cuyo prototipo es el aislado ATCC-VR-2332, presentan entre sí una elevada variabilidad genética y biológica. Inicialmente, se creía que todas las cepas del genotipo Europeo eran genéticamente homólogas. Sin embargo, el análisis de la secuencia de la ORF5 y OFR7 han justificado la división del genotipo Europeo en cuatro subtipos genéticos bien diferenciados: el subtipo 1 o Pan-europeo, y los subtipos 2, 3 y 4 de Europa del Este.

La amplia variabilidad biológica, genética e incluso antigénica de los diferentes aislados del PRRSV-1, así como las elevadas pérdidas económicas que esta enfermedad ocasiona en el sector porcino, justifican la realización de estudios que tengan como objetivo aclarar los mecanismos patogénicos desencadenados por las diferentes cepas de este virus. En esta tesis doctoral hemos evaluado la expresión del antígeno viral, el cuadro lesional y la expresión de diferentes citoquinas pro-inflamatorias e inmunomoduladoras en el pulmón y en los órganos linfoides primarios (timo y médula ósea) tras la infección con una cepa

estándar del PRRSV-1 (LV), dos cepas de campo (SU1-bel, de alta virulencia, y 215-06, de moderada virulencia) y una cepa atenuada, utilizada para la producción de vacunas (DV).

La expresión del antígeno del PRRSV en todos los grupos infectados, a excepción del grupo DV y el grupo control, fue principalmente en macrófagos alveolares del pulmón y macrófagos del timo, con una expresión máxima a los 7 días post-infección (dpi). En el pulmón y el timo se detectó la replicación más alta en el grupo infectado con la cepa de alta virulencia (SU1-bel) y tanto con esta cepa como con la cepa estándar LV existía una correlación positiva entre las lesiones macroscópicas del pulmón y la presencia del antígeno vírico.

En la médula ósea se demostró la expresión de RNA viral mediante RT-qPCR desde las primeras fases de la infección. La mayor expresión de RNA viral se observó en el grupo infectado con la cepa estándar LV a los 3 dpi, la cual se mantuvo hasta el final del estudio (35 dpi). Los grupos infectados con las cepas de alta (SU1-bel) y moderada (215-06) virulencia expresaron RNA del virus a los 3 y 7 dpi, con la mayor expresión a los 7 dpi. Estos resultados demuestran que tanto la expresión del antígeno como del RNA del PRRSV difiere de acuerdo al órgano y al grado de patogenicidad de las cepas.

En nuestro estudio el mayor grado de neumonía intersticial fue observado a los 7 dpi, siendo el grupo de animales infectados con la cepa de alta virulencia (SU1-bel) el que presentó el mayor grado de lesión. En

el timo, las principales alteraciones consistieron en una reducción del número de timocitos asociada a una disminución de la expresión de las células $CD3^+$, que se correlacionó negativamente con células que expresaron algún marcador de muerte celular (TUNEL o cCasp3) y se correlacionó positivamente con las células que expresaron el antígeno viral. Estos hallazgos fueron observados principalmente en los animales infectados con la cepa de alta virulencia SU1-bel, seguido por las cepas de moderada virulencia LV y 215-06 a los 7 dpi. Estos resultados refuerzan la hipótesis de que el PRRSV induce mecanismos de muerte celular de manera directa e indirecta en el timo y apoyan la hipótesis de que en el transcurso de las infecciones con el PRRSV-1 se produce una regulación negativa del sistema inmune de los lechones.

Además en nuestro estudio, hemos observado el desarrollo de una hipoplasia transitoria y moderada de la línea eritroide que se asoció con un cambio en la relación de las líneas mieloide y eritroide (M:E) al inicio de la infección (3 dpi) en todos los animales infectados, principalmente en los cerdos infectados con la cepa prototipo LV y a los 7 dpi en los infectados con la cepa de alta virulencia SU1-bel. Esto indica que la infección con el PRRSV-1 induce cambios en el parénquima de la médula ósea y afecta la producción cuantitativa y/o posiblemente la cualitativa de células progenitoras lo que estaría relacionado con la existencia de fallos en otros órganos y/o en la instauración de la respuesta inmune del huésped, lo que contribuiría a aumentar la susceptibilidad de los animales a sufrir infecciones secundarias.

En el transcurso de la infección por la cepa de alta virulencia (SU1-bel) se observó una correlación positiva entre la expresión del antígeno vírico y la expresión de la citoquina IL-1α, sugiriendo que esta cepa sería una buena inductora de esta citoquina en el pulmón que, a su vez, sería la responsable de la intensidad de la neumonía intersticial en los animales infectados con SU1-bel. En cambio no se observaron cambios en la expresión local de IL-1α en el timo de los animales infectados con las diferentes cepas utilizadas en nuestro estudio. Curiosamente, la muerte celular de los timocitos estuvo precedida por un incremento en la expresión local, de TNF-α y/o IL-10, principalmente en la médula tímica de todos los animales infectados, independientemente de la virulencia de la cepa con que estaban infectados. Estos resultados sugieren que, al igual que en otros órganos, el PRRSV-1 podría utilizar un mecanismo indirecto para inducir los fenómenos de muerte celular en el timo.

El aumento de la expresión local de IL-1α e IL-6, que son poderosos factores estimulantes de la proliferación y diferenciación de las unidades formadoras de colonias de granulocitos y monocitos (CFU-GM del inglés, *Colony-Forming Unit–Granulocyte-Macrophage*) en la médula ósea, en la primeras fases de la infección por el PRRSV es indicativo de que existe una respuesta del tejido ante la pérdida inicial de las células hematopoyéticas. Por otra parte, el incremento en la expresión local de TNF-α a los 3 dpi en los grupos infectados con las cepas de moderada (215-06) y alta (SU1-bel) virulencia, podría jugar un papel en la inducción de los fenómenos de muerte celular observados. Además, la expresión local de esta citoquina en la médula ósea puede inhibir la formación de la línea de eritrocitos precursores (BFU-E del inglés, *Burst*

Forming Unit–Erythroid) e inducir la síntesis de CFU-GM. Todos estos hallazgos sugieren que la presencia del PRRSV en médula ósea produce de manera directa e indirecta una alteración de las líneas precursoras eritroide y mieloide.

Finalmente, en nuestro estudio se demuestra que la mayor virulencia de la cepa SU1-bel está relacionada con una mayor capacidad de replicación local del virus en el pulmón y en el timo, así como una mayor expresión de IL-1α en el parénquima pulmonar y TNF-α e IL-10 en el timo, asociada a una mayor muerte celular en el timo y alteraciones en el parénquima de la médula ósea, que podrían comprometer el normal funcionamiento del sistema inmune.

SUMMARY

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of a disease of pigs, named porcine reproductive and respiratory syndrome (PRRS). Despite efforts in controlling it and reducing its economic impact, PRRS is still considered one of the most important diseases affecting the swine industry.

PRRSV is divided into two distinct genotypes, European PRRSV or Type 1 (PRRSV-1) with the prototype Lelystad strain (LV), and North American or Type 2 (PRRSV-2), whose prototype is the ATCC-VR-2332 strain, with high genetic and biological diversity among both genotypes. Initially, PRRSV-1 was thought to be genetically homogenous. However, deep analysis of PRRSV-1 ORF5 and OFR7 sequences defined four distinct genetic subtypes: subtype 1 or Pan-European, and subtypes 2, 3 and 4 in Eastern Europe.

The wide biological, genetic and antigenic diversity of the different PRRSV-1 strains, as well as the high economic losses to the pork industry associated with this disease justify studies that aim to clarify the pathogenic mechanisms triggered by different virus strains. In the present thesis we evaluated the expression of viral antigen, the morphologic lesion and the expression of several proinflammatory and immunomodulatory cytokines in lungs and primary lymphoid organs (thymus and bone marrow) after the infection with a prototype PRRSV-1 Lelystad (LV), two field strains (SU1-bel, of high virulence, and 215-06,

of moderate virulence) and an attenuated strain that is used as a vaccine (DV).

PRRSV antigen in all virus-infected groups, except for the DV and the control groups was mainly present within pulmonary alveolar macrophages and macrophages of the thymus, with a maximum expression at 7 days post-infection (dpi). The highest replication rate of PRRSV was detected in the lung and thymus of the group infected with the highly virulent SU1-bel strain, and a positive correlation was observed between the number of PRRSV-positive cells and macroscopic lesions in the lung of SU1-bel and the prototype LV strains.

PRRSV RNA was detected in the bone marrow by RT-qPCR in early stages of infection. The highest RNA viral load was detected in pigs experimentally infected with the prototype PRRSV-1 LV, remaining high until the end of the experiment (35 dpi). PRRSV viral load was also observed in the groups infected with the moderate virulent 215-06 strain and the highly virulent SU1-bel strain at 3 and 7 dpi, with a higher viral load at 7 dpi. These data suggest that the presence of virus antigen and the PRRSV-RNA viral load differs according to the organ and the degree of pathogenicity of the different strains.

In our study, the high score of the interstitial pneumonia was displayed at 7 dpi. Animals infected with the highly virulent SU1-bel strain showed the highest score. In the thymus, the main changes were characterised by a decrease in the number of thymocytes associated with

a decrease in the expression of CD3 positive cells. Moreover, there was a negative correlation in the expression of some markers for cell death (TUNEL and cCasp3) and a positive correlation with the expression of PRRSV antigen, mainly in animals infected with the highly virulent SU1-bel strain, followed by the moderate virulent LV and 215-06 strains at 7 dpi. These results reinforce the hypotheses that PRRSV induce cell death by direct or indirect mechanisms in the thymus and support the fact that PRRSV-1 infection suppresses the immune system in piglets.

In addition, we found a moderate transient hypoplasia of erythroid cells and an increased M:E ratio at the early stages of infection (3dpi) in all experimentally infected piglets, more remarkable in those animals infected with the prototype PRRSV-1 LV and at 7 dpi in the piglets infected with the highly virulent SU1-bel strains. These data may suggest that infection with PRRSV-1 induces changes in the parenchyma of the bone marrow and affects the quantitative and/or qualitative production of stem cells. This fact could be related to the existence of an immune system failure in other organs and may therefore contribute to an increase susceptibility to secondary infections of PRRSV infected animals.

During the infection by the highly virulent SU1-bel strain we observed a positive correlation between the expression of viral antigen and the expression of IL-1α. This finding suggests that SU1-bel strain is a good inducer of IL-1α in the lung, which would be associated to the severity of the interstitial pneumonia. However, no changes were observed in the local expression of IL-1α in the thymus of those piglets

infected with the other strains used in our study. Surprisingly, thymocyte cell death was preceded by a peak in the local expression of TNF-α and IL-10 within the thymic medulla of all infected animals, independently of the PRRSV-1 strain. These results suggest that, as in other organs, PRRSV-1 may induce cell death in the thymus mainly by indirect mechanism.

The increase in the local expression of IL-1α and IL-6, two powerful immune stimulating factors for the proliferation and differentiation of colony-forming unit–granulocyte-macrophage (CFU-GM) in the bone marrow, at early stages of PRRSV infection, may indicate a tissue response to the initial loss of hematopoietic cells. On the other hand, the increase of the expression of TNF-α in the piglets infected with the moderately virulent strain 215-06 and the highly virulent strain SU1-bel at 3dpi, could play a role in cell death. Moreover, the expression of TNF-α in the bone marrow can inhibit the formation of the burst forming unit–erythroid (BFU-E) and induce the synthesis of the CFU-GM. These results suggest that PRRSV could produce direct or indirect changes in erythroid and myeloid cells in bone marrow.

Finally, our results point out that the higher virulence reported for the SU1-bel strain could be associated with a) a higher local PRRSV replication rate in the lung and thymus and b) an increased expression of IL-1α in the lung parenchyma, as well as TNF-α and IL-10 in the thymus. In addition, it may be associated with an increased cell death in the thymus and changes in the bone marrow parenchyma, and therefore compromise the function of the normal immune system.

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A. SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

I. INTRODUCCIÓN

A finales del año 1987, comienzos de 1988, se señala en los estados de Carolina del Norte, Minnesota y Iowa la aparición de fallos reproductivos y respiratorios en diferentes explotaciones porcinas que fueron asociadas a una nueva enfermedad que se denominó como *"Enfermedad misteriosa porcina"*; en 1990, esta enfermedad había sido identificada en once estados de los Estados Unidos, dos provincias de Canadá y en Europa; así a finales del año 1990 fue diagnosticada en Münster (Alemania) extendiéndose también con posterioridad rápidamente por otros países europeos como Holanda, Francia, Bélgica, Dinamarca y Reino Unido *(Wensvoort et al., 1991; Plana et al., 1992; Wensvoort et al., 1992; Yoon et al., 1992; Conzelmann et al., 1993; Goyal, 1993).*

El primer aislamiento del agente etiológico fue realizado en los laboratorios de Lelystad (Holanda) por *Wensvoort et al. (1991)*, por lo que se denominó a este virus como virus Lelystad (LV, del inglés *Lelystad Virus*), siendo considerada esta cepa como el prototipo del genotipo Europeo del virus. Seis meses después, *Collins et al. (1992)* realizan el primer aislamiento del virus desde los casos que afectaban a cabaña de Estados Unidos, denominando a la enfermedad como síndrome de infertilidad y respiratorio porcino (SIRS, del inglés *Swine Infertility and Respiratory Syndrome*); la cepa aislada, denominada como VR-2332, es considerada como el prototipo del genotipo

Norteamericano del virus *(Benfield et al., 1992)*. Debido a la existencia de estos dos aislados víricos y a que la enfermedad presentaba unas características diferentes en Estados Unidos y Europa, esta enfermedad recibió diferentes nombres hasta que se celebró en St. Paul (Minnesota, EE.UU) el primer Simposio Internacional sobre SIRS, donde se acordó denominar a la enfermedad como *"síndrome reproductivo y respiratorio porcino"* (PRRS, del inglés *porcine reproductive and respiratory syndrome*) y a su agente causal *"virus del síndrome reproductivo y respiratorio porcino"* (PRRSV) *(Terpstra et al., 1991; Wensvoort et al., 1992; Goyal, 1993)*.

Ambos aislados del PRRSV, Europeos y Norteamericanos, a pesar de compartir similitud con los virus que conforman la Familia *Arteriviridae*, muestran entre sí una lejana relación filogenética y una clara diferencia en la gravedad de los signos clínicos *(Halbur et al., 1995; Han et al., 2013 ab; Snijder et al., 2013; Stadejek et al., 2013; Kappes y Faaberg, 2015)* pero, sorprendentemente, a pesar de estas diferencias los aislados del PRRS aparecieron casi al mismo tiempo en ambos continentes, con las mismas características clínicas y epidemiológicas *(Bautista et al., 1993; Goyal, 1993; Nelson et al., 1993; Magar et al., 1995; Rossow, 1998; Meulenberg, 2000; Han et al., 2013 a,b)*.

En la actualidad está demostrado que todos los aislados Europeos del PRRSV están estrechamente relacionados entre sí, al igual que los aislados Norteamericanos, pero que no existe relación entre ellos; por lo que se consideran como dos genotipos biológica y genéticamente diferentes, y denominados como genotipo Europeo o Tipo 1 (PRRSV-1)

y genotipo Norteamericano o Tipo 2 (PRRSV-2) *(Meulenberg, 2000; Murtaugh et al., 2010; Stadejek et al., 2013)*; siendo en la actualidad considerada como una enfermedad endémica de distribución mundial *(Meulenberg, 2000; Cho y Dee, 2006)*.

Después de, aproximadamente, 27 años de estudios sobre los diferentes aspectos del PRRSV, es frustrante, para la comunidad científica y los productores del sector, no haber sido capaces de controlar y erradicar esta enfermedad lo que posiblemente esté relacionado con la capacidad intrínseca que tiene este virus de adaptarse, persistir y evolucionar *(Kappes y Faaberg, 2015)*, unas capacidades que se han puesto de manifiesto en los últimos años debido a la aparición de diferentes subtipos a nivel intragenotipo y de cepas cada vez más divergentes y virulentas *(Stadejek et al., 2008; Murtaugh et al., 2010; Stadejek et al., 2013; Kappes y Faaberg, 2015)* como por ejemplo la responsable de la pandemia que se produjo en China en 2006 *(Tian et al., 2007)*.

La amplia variabilidad biológica, genética e incluso antigénica de los diferentes aislados del PRRSV, así como las elevadas pérdidas económicas que ocasiona en el sector porcino, justifican la realización de estudios que tengan como objetivo aclarar los mecanismos patogénicos que utilizan las diferentes cepas de este virus, a fin de controlar y, en lo posible, erradicar esta enfermedad, haciendo uso de todas las estrategias biológicas y no biológicas disponibles al alcance de los productores.

II. VIRUS DEL SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

1. Genoma

El PRRSV es un virus RNA, de cadena simple, de sentido positivo y envuelto, con un tamaño aproximado de entre los 50-65 nanómetros *(Meulenberg et al., 1997; Meulenberg, 2000)*; correspondiente al orden *Nidovirales*, familia *Arteriviridae*, la cual se compone de otros cuatro virus más que comparten características genéticas y biológicas similares, el virus de la arteritis equina, el virus elevador de la lactato-deshidrogenasa del ratón, el virus de la fiebre hemorrágica del simio y la reciente enfermedad reportada de la zarigüeya tambaleante (WPDV, del inglés *Wobbly Possum Disease Virus*) *(Meulenberg, 2000; Cho y Dee, 2006; Dokland, 2010; Dubovi, 2011; Mac Lachlan y Duboi, 2011; Chand et al., 2012; Dunowska et al., 2012; Snijder et al., 2013; Kappes y Faaberg, 2015; http://talk.ictvonline.org/files/proposals/animal_ssrna_viruses/m/animal_rna_plus_newly_submitted/5421.aspx)*.

El genoma del PRRSV presenta entre 14,9 kb a 15,5 kb de longitud, el cual expresa 8 proteínas estructurales y entre 14-16 proteínas no estructurales (nsp, del inglés *non structural proteins*), a través de 10 fragmentos de lectura abierta (ORFs, del inglés *Open Reading Frames*) y dos mecanismos de transcripción distintos (RFS, del inglés *ribosomal frameshift*) y RNAs sub-genómicos (sgRNA, del inglés *subgenomic RiboNucleic Acid)* (Figura 1) *(Dokland, 2010; Fang y Snijder, 2010; Chand et al., 2012; Badaoui et al., 2013a; Choi et al., 2014; Han y Yoo, 2014; Kappes y Faaberg, 2015; Zhang y Yoo, 2015)*.

El extremo proximal 5´(5´UTR del inglés, 5´-*untranslated region*) contiene los ORF1a y ORF1b, que son grandes y están superpuestos y codifican las proteínas no estructurales (replicasas) y, aunque comparten un solo sitio de traducción, al traducir en dos RFS, pueden codificar diferentes tipos de proteínas no estructurales (nsp): nsp1α, nsp1β, nsp2, nsp4 *(Meulenberg et al., 1993, 1998; Snijder y Meulenberg, 1998; Nelsen et al., 1999; Han et al., 2009; Music y Gagnon, 2010; Snijder et al., 2013; Kappes et al., 2015; Kappes y Faaberg, 2015; Li et al., 2015).*

En contraste, las proteínas estructurales del virus se codifican y expresan individualmente por un conjunto de sgRNA *(van Marle et al., 1999)* denominándose como glicoproteínas GP2, GP3, GP4, GP5, GP5a, y proteínas E, M y N. Las glicoproteínas GP2, GP3 y GP4 forman un complejo trimérico que facilita la entrada del virus en la célula *(Wissink et al., 2005; Das et al., 2010)*. La GP5 es considerada como la principal glicoproteína y tiene un número variable de residuos N-glicano, y, junto con la proteína M, forman un complejo mayor de glicoproteína *(Faaberg et al., 1995; Mardassi et al., 1996)* que es requerido para la infectividad de los arterivirus *(Snijder et al., 2013)*. Las proteínas N y M son necesarias para la formación de la partícula vírica *(Wissink et al., 2005)*. La GP5a es necesaria para la viabilidad del virus *(Meulenberg et al., 1995; Mardassi et al., 1996; Johnson et al., 2011; Robinson et al., 2013)*. La proteína E es esencial para facilitar la unión del virus a su célula diana *(Lee y Yoo, 2006)* y por último la proteína N que se dispone de forma asimétrica tapizando el virus *(Dokland, 2010; Music y Gagnon, 2010)* es altamente inmunógena, lo que la convierte en una proteína con un alto valor diagnóstico para la detección de anticuerpos frente al PRRSV *(Dea et al., 2000 a,b; Music y Gagnon, 2010)*. La gran variación genética y proteica de todas estas proteínas

estructurales y no estructurales, desde la menos conservada "nsp2" *(Han et al., 2006; Tian et al., 2007)* a la más conservada, la proteína M *(Veit et al., 2014)*, demuestra la complejidad y la plasticidad del genoma del PRRSV y por lo tanto la estructura del virión.

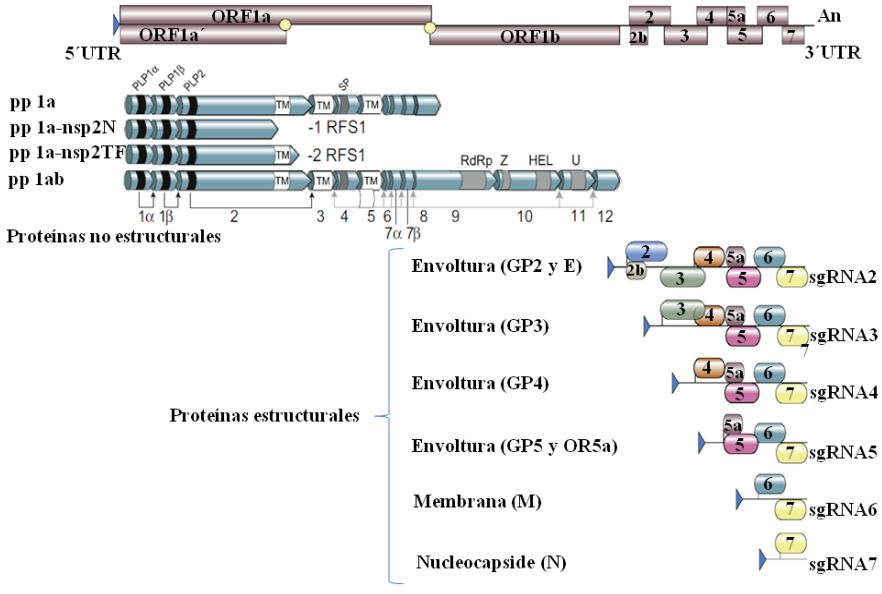

Figura 1: Representación esquemática del genoma del virus del síndrome reproductivo y respiratorio porcino *(Kappes y Faaberg, 2015).*

2. Replicación

Aunque el macrófago alveolar porcino (PAM, del inglés *Porcine Alveolar Macrophage*) es considerado la célula diana del PRRSV, el virus es capaz también de replicarse en otros macrófagos residentes del pulmón como los macrófagos intersticiales y los macrófagos intravaculares pulmonares (PIMs, del inglés *Pulmonary Intravascular Macrophage*), subpoblaciones de macrófagos de diferentes órganos

linfoides (tonsila, nódulos linfáticos y bazo), macrófagos intravasculares de la placenta, cordón umbilical y células dendríticas *(Halbur et al., 1996 a,b; Duan et al., 1997a; Lawson et al., 1997; Snijder y Meulenberg, 1998; Thanawongnuwech et al., 2000; Gómez-Laguna et al., 2010; Rodríguez-Gómez et al., 2013)*. Recientemente se ha descrito un cierto tropismo de cepas altamente virulentas (aislado asiático, JXwn06) a células epiteliales *(Li et al., 2007; Hu et al., 2013)*. Por otro lado, estudios *in vitro* demuestran que el virus es capaz de replicarse en monocitos, macrófagos derivados de monocitos, células dendríticas derivadas de monocitos (MoDCs, del inglés *Monocyte-derived Dendritic Cells*) *(Charerntantanakul et al., 2006; Loving et al., 2007; Wang et al., 2007; Flores-Mendoza et al., 2008; Park et al., 2008; Silva-Campa et al., 2009, 2010; Wongyanin et al., 2010)*, células dendríticas derivadas de médula ósea (BMDCs, del inglés *Bone Marrow-derived Dendritic Cells*) *(Gimeno et al., 2011)* y en líneas celulares derivadas de riñón de mono verde (MA-104, CL2621, MARC-145 y CRL11171) *(Kim et al., 1993; Mengeling et al., 1995; Zhang y Yoo, 2015)*.

La interacción virus/célula aún no está completamente definida a pesar de que se reconocen al menos seis moléculas diferentes como potenciales receptores de entrada del PRRSV, incluyendo el heparán sulfato (HpS), vimentina, CD151, CD163 (CD, del inglés *Cluster of Differentiation)*. Receptor del complejo hemoglobina-haptoglobina), sialoadhesina (Sn; Siglec-1 o también conocido como CD169) y DC-SIGN (también conocido como CD209) *(Jusa et al., 1997; Duan et al., 1998; Kim et al., 2006; Calvert et al., 2007; Shanmukhappa et al., 2007; Huang et al., 2009)*. La mayoría de los estudios coinciden en que el receptor de la membrana celular CD163 podría ser el receptor primario del PRRSV, jugando al mismo tiempo un importante papel en la susceptibilidad de las células al

virus *(Calvert et al., 2007; Van Gorp et al., 2008; Patton et al., 2009; Wang et al., 2013c; Welch y Calvert, 2010)*, mientras que la sialoadesina podría funcionar como una proteína accesoria para la entrada del virus a la célula *(Vanderheijden et al., 2003; Delputte y Nauwynck, 2004; De Baere et al., 2012)*.

Además, se postula que la internalización del virus se realiza mediante un proceso de endocitosis mediado por clatrinas, ya que la replicación del virus se realiza en vesículas de doble membrana peri-nuclear, que posiblemente derivan del retículo endoplásmico, y que es necesario que exista un microambiente ácido para la liberación del RNA genómico del virus (gRNA, del inglés *genomic RiboNucleic Acid*) en el citoplasma del macrófago *(Labarque et al., 2003; Costers et al., 2008; Chen et al., 2012; Sun et al., 2012; Yin et al., 2012; Huo et al., 2013; Wang et al., 2014).* El gRNA actuaría directamente como RNA mensajero (mRNA, del inglés *messenger RiboNucleic Acid*), comenzando el ciclo de replicación con la expresión de los genes de los ORFs 1a/1b *(Kroese et al., 2008)* y, posteriormente, la transcripción y traducción del resto del genoma vírico *(Music y Gagnon, 2010)*, lo que facilitaría la rápida expresión de las nsp a partir del gRNA y, posteriormente, la amplificación de transcriptos mediante el sgRNA *(Pasternak et al., 2004)*.

Parece que el principal motor de la maquinaria de replicación del PRRSV es la RNA-polimerasa RNA-dependiente (RdRp; nsp9) *(Ulferts y Ziebuhr, 2011; Kappes y Faaberg, 2015)*, que no posee la capacidad de corrección de pruebas *(Lauber et al., 2013)*, lo que supone la introducción en su genoma de mutaciones aleatorias a velocidades elevadas *(Forsberg et al., 2001, 2002, 2005)*, contribuyendo a una tasa de evolución

anormalmente alta *(Hanada et al., 2005)*. En este sentido, merece la pena mencionar que se ha descrito la recombinación entre cepas de campo del mismo genotipo del PRRSV, así como la existencia de casos de co-infección en cerdos *(Li et al., 2009; Liu et al., 2011; Shi et al., 2013; Martin-Valls et al., 2014; Kappes et al., 2015)*.

En resumen, en la replicación del PRRSV se relacionan estrechamente tres características clave: 1) el re-ordenamiento de las membranas del hospedador para estabilizar los complejos de replicación virales; 2) la síntesis y expresión del gRNA; y, 3) la transcripción de sgRNA para la expresión eficiente de las proteínas estructurales *(Yuan et al., 2000, 2004; Van Breedam et al., 2010; Kappes y Faaberg, 2015)*.

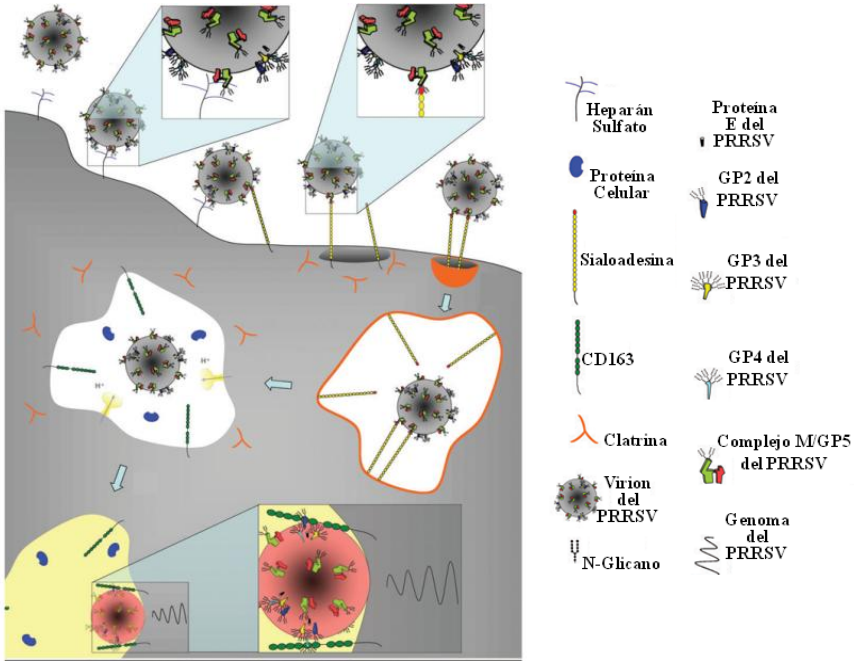

Figura 2: Modelo de entrada del PRRSV al macrófago porcino *(Van Breedam et al., 2010)*

3. Genotipo, subtipo y linaje del virus

Se reconocen dos principales genotipos del PRRSV con una alta variabilidad genética y biológica *(Nelsen et al., 1999; Forsberg, 2005)*: el genotipo Europeo o tipo 1 (PRRSV-1), representado por la cepa Lelystad, y el genotipo Norteamericano o tipo 2 (PRRSV-2), cuyo prototipo es el aislado ATCC-VR-2332 *(Wensvoort et al., 1991; Collins et al., 1992; Nelsen et al., 1999; Badaoui et al., 2013a)*. Ambos genotipos sólo comparten del 55% al 70% de los nucleótidos y del 50% al 80% de los amino-ácidos *(Nelsen et al., 1999; Forsberg, 2005; Fang et al., 2007; Dokland, 2010)*. Inicialmente, se creía que todas las cepas del genotipo Europeo eran genéticamente homólogas, pero en la actualidad los estudios genéticos basados en los análisis de la secuencia del ORF5 y ORF7 justifican la división del PRRSV-1 en cuatro subtipos genéticos bien diferenciados: el subtipo 1 o Pan-europeo, y los subtipos 2, 3 y 4 de Europa del Este *(Stadejek et al., 2006, 2008; Darwich et al., 2011; Stadejek et al., 2013)*. Con relación al genotipo Norteamericano, en base a análisis filogenéticos de la secuencia del ORF5, se describe la existencia de nueve linajes diferentes *(Shi et al., 2010b; Brar et al., 2011)*. Es importante destacar que esta construcción filogenética no representa la diversidad completa del genotipo Norteamericano, debido a la falta de información con relación a la secuencia de las cepas aisladas en Canadá *(Shi et al., 2010a)*.

La aparición de brotes atípicos agudos de la enfermedad son un reflejo de esta diversidad, no sólo genética sino también fenotípica (patogenicidad y virulencia) del PRRVS *(Meng et al., 1996; Mengeling et al.,*

1998). Una variabilidad que, además, es un problema tanto para el diagnóstico serológico como molecular del virus *(Truyen et al., 2006)*.

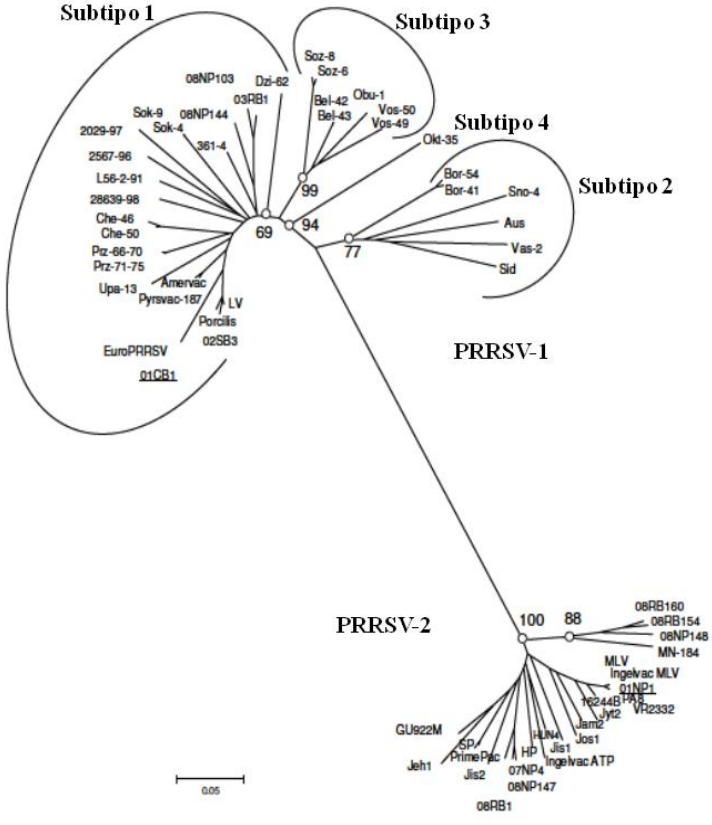

Figura 3: Modelo filogenético del PRRSV, basado en la secuencia del OFR5 *(Amonsin et al., 2009)*

III. SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

1. Epidemiología

Actualmente el PRRS es considerado como una enfermedad endémica de distribución mundial, a pesar de que aún no hay información de la enfermedad en varios países de Latinoamérica y África y no ha sido descrita en Finlandia, Bulgaria y Australia *(Meulenberg, 2000; Cho y Dee, 2006; http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap)*. Además, el PRRS presenta un comportamiento epidémico que se asocia con la forma reproductiva de la enfermedad *(Dewey et al., 1999)* y un comportamiento endémico que está relacionado con un fallo en la respuesta inmunológica y la presentación de la forma respiratoria *(Drew, 2000)*.

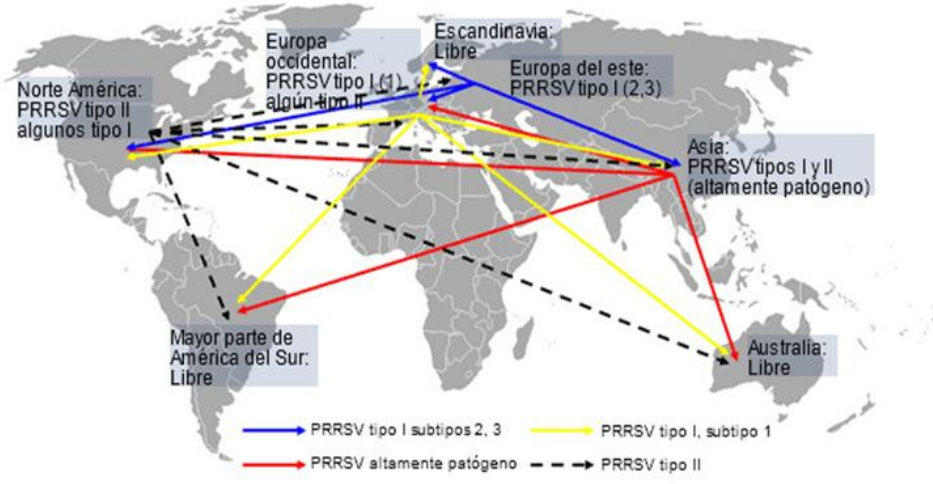

Figura 4: Distribución global del PRRSV e hipotética transmisión intercontinental *(Cano, 2012)*.

La transmisión vertical ha sido identificada prácticamente desde la aparición de la enfermedad *(Christianson et al., 1992)* y a pesar de que esta transmisión es más marcada al final de la gestación *(Christianson et al., 1992; Mengeling et al., 1994; Karniychuk et al., 2011, 2012)*, también es posible al comienzo de la misma *(Lager et al., 1994)*.

En la transmisión horizontal del virus juegan un papel importante tanto la vía aerógena *(Otake et al., 2002a; Dee et al., 2009; Pitkin et al., 2009; Otake et al., 2010)*, como la transmisión mediante vectores, ya sea a través de insectos como el *Aedes vexans* y *Musca domestica* *(Otake et al., 2002c, 2003, 2004; Schurrer et al., 2005)* como a través de fómites contaminados, como ropa de trabajo, agujas, vehículos de transporte, botas, guantes, equipos, visitantes, etc. *(Dee et al., 2002; Otake et al., 2002b, 2004; Dee et al., 2005a; Pitkin et al., 2009; Otake et al., 2010)*. En la transmisión horizontal cobra especial importancia el transporte de animales de una granja infectada a una granja susceptible; la utilización de vehículos comunes, como son las pajuelas de semen *(Christopher-Hennings et al., 1995)* y los mecanismos iatrogénicos, como la utilización de vacuna con virus vivo atenuado *(Otake et al., 2002b)*.

El principal mecanismo de transmisión del PRRSV está representado por el contacto directo de animales sanos con la sangre, aerosoles, excreciones (orina, semen y heces) y secreciones (saliva, leche o calostro y secreciones nasales) de animales infectados *(Wills et al., 1997; Bierk et al., 2001; Wagstrom et al., 2001)*. Aunque algunos estudios sugieren la existencia de una vía de transmisión alternativa, como la ingestión de tejido muscular infectado *(van der Linden et al., 2003; Molina et al., 2009)*.

En un brote de la enfermedad, la transmisión del virus se produce a amplias zonas geográficas *(Tian et al., 2009; Shi et al., 2010 a,b)* y el uso adecuado y controlado de las posibles fuentes del PRRSV, que actúan como origen de transmisión, junto con la implementación de estrategias de manejo, reducen el nivel de contaminación del virus y su diseminación mecánica *(Dee et al., 2005a; Schurrer et al., 2005; Spronk et al., 2010; Alonso et al., 2012; Dee et al., 2012; Alonso et al., 2013 a,b)*.

***Tabla 1**:* Aislamiento del PRRSV en diferentes tipos de muestras y tiempos post-infección (dpi).

Tipo de muestra	***dpi***	***Referencias***
Agua residual	7	*Pirtle y Beran, 1996; Dee et al., 2005b*
Agua limpia	11	*Pirtle y Beran, 1996; Dee et al., 2005b*
Orina	14	*Wills et al., 1997*
Heces	28 - 35	*Yoo et al., 1993*
Saliva	42	*Wills et al., 1997*
Semen	43 - 92	*Christopher-Hennings et al., 1995*
Tonsila	56 - 251	*Duan et al., 1997b; Wills et al., 2000, 2003*
Orofaringe	84	*Duan et al., 1997b*
Suero	90	*Christopher-Hennings et al., 1995*

El PRRSV no es un virus resistente a condiciones desfavorables de temperatura, pH y exposición a ciertos detergentes; aunque soporta

bajas temperaturas durante largos periodos de tiempo *(Benfield et al., 1992)* y es estable a un pH de entre 6.5 – 7.5 *(Bloemraad et al., 1994)*. En la *tabla 1* se especifica las fuentes posibles del PRRSV y el tiempo de identificación o aislamiento del virus después de la contaminación.

2. Patogenia

En el transcurso de la infección por el PRRSV se pueden establecer dos fases, (1) una infección aguda, caracterizada por una viremia que puede durar un promedio de entre 9 a 15 dpi y alcanzar el mes en animales adultos (verracos y reproductoras), mientras que en cerdos jóvenes el promedio es de entre 28 y 35 dpi, aunque puede llegar hasta los 3 meses, con un pico de replicación vírica más o menos conservado entre cepas, a nivel pulmonar, durante la primera semana de la infección *(Mengeling et al., 1994; Rossow et al., 1995; Prieto et al., 1997; Duan et al., 1997a; Labarque et al., 2000, 2003; Martínez-Lobo, 2010; Rodríguez-Gómez, 2012)*; y (2) una infección crónica, caracterizada por la ausencia de viremia, aunque el antígeno vírico puede estar persistente y ser aislado de los órganos linfoides secundarios incluso hasta los 300 dpi (Figura 5) *(Wills et al., 2000, 2003)*.

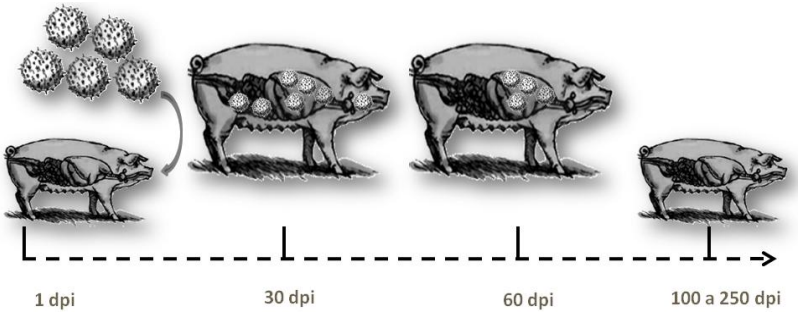

Figura 5: Representación esquemática de la patogenia del PRRSV en el cerdo (modificado de *Prieto et al., 2005*).

Normalmente el PRRSV ingresa al organismo por la vía oro-nasal desde donde el virus alcanza los pulmones e infecta los PAMs, considerados como las células diana del PRRSV, y, en menor medida, los macrófagos septales (macrófagos intersticiales y PIMs) *(Plagemann y Moennig, 1992; Lawson et al., 1997; Rossow, 1998; Snijder y Meulenberg, 1998; Gómez-Laguna et al., 2010; Snijder et al., 2013)*. Células en las que la replicación tiene una velocidad sorprendentemente alta, en tan sólo 6 a 12 horas post-infección (hpi) es posible detectar el antígeno vírico en el citoplasma de las células infectadas *(Pol et al., 1997b)*.

Varios estudios experimentales coinciden que los picos de replicación del virus se producen entre los 7 y 14 dpi *(van Reeth y Nauwynck, 2000; Costers et al., 2008)* y postulan que, durante la replicación, las células son conducidas hacia un estadio de anti-apoptosis, de forma que cuando la replicación ha culminado la célula entra en apoptosis. De esta manera la liberación del PRRSV se produce mediante la lisis de la célula infectada *(Sirinarumitr et al., 1998)*. Este proceso de lisis, junto con los fenómenos directos e indirectos de apoptosis y la liberación de diferentes tipos de citoquinas, pro-inflamatorias (IL-1α y β, TNF-α, IL-6, IL-12) y anti-inflamatorias (IL-10), por parte de los macrófagos infectados y no infectados, linfocitos y neutrófilos, tiene un papel relevante en la patogenia y el establecimiento de las lesiones en la enfermedad *(Sirinarumitr et al., 1998; Van Reeth et al., 1999; Labarque et al., 2003; Thanawongnuwech et al., 2003 a,b; Thanawongnuwech y Thacker, 2003; Thanawongnuwech et al., 2004b; Costers et al., 2008; Barranco et al., 2011, 2012a; Gómez-Laguna et al., 2013; Rodríguez-Gómez et al., 2014)*.

Además, como consecuencia de los fenómenos anteriormente descritos, en el transcurso de las infecciones con el PRRSV se incrementa la susceptibilidad de los animales a una amplio rango de patógenos durante un período aproximado de hasta 4 semanas *(Drew, 2000; Cho et al., 2006a)*, dando como resultado un incremento en la gravedad de los signos clínicos y daños tisulares *(van Reeth y Nauwynck, 2000; Gómez-Laguna et al., 2013)*.

Una vez que el virus es liberado por los PAMs se disemina al resto del organismo vía hematógena en su forma libre (12 hpi) y asociada a monocitos y linfocitos (1 dpi), aunque también se ha señalado una diseminación vía linfática *(Rossow et al., 1995; Martínez-Lobo, 2010)*, a la tonsila, nódulos linfáticos y bazo *(Wills et al., 1997; Allende et al., 2000)*.

Los resultados de varios estudios sugieren que el pulmón es el principal sitio de persistencia del virus y, por ende, fuente de la viremia, sobre todo en animales jóvenes *(van Reeth y Nauwynck, 2000)*. Sin embargo, otros autores han señalado que los nódulos linfáticos también serían un sitio preferente de replicación y liberación del PRRSV al torrente circulatorio, lo que estaría relacionado con la viremia prolongada que se produce en esta enfermedad *(Rossow et al., 1995; Wills et al., 1997; Allende et al., 2000; van Reeth y Nauwynck, 2000; Chand et al., 2012)*. El virus se puede recuperar de varios órganos durante las primeras 2 semanas post-infección, siendo positivos a partir de la segunda semana solamente algunos órganos (pulmones, suero, tonsilas y nódulos linfáticos). En la *tabla 2* se detalla el órgano, célula y tiempo de identificación del antígeno viral.

Tabla 2: Antígeno viral del PRRSV en diferentes órganos y tiempos post-infección (dpi).

Órganos	***Células***	***dpi***	***Referencias***
Mucosa Nasal	Macrófagos	0,5-7	*Halbur et al., 1996a*
Tráquea-Bronquio	Epitelio	*ND	*Halbur et al., 1996a*
Pulmón	PAMs, PIMs y células epiteliales alveolares	1-28	*Halbur et al., 1996a; Gómez-Laguna et al., 2010; Hu et al., 2013*
Nódulo linfáticos	Macrófagos	2 -35	*Halbur et al., 1996a; Morgan et al., 2014*
Tonsila	Macrófagos	1-251	*Halbur et al., 1996a; Wills et al., 2003*
Timo	Macrófagos y Células dendríticas	2-35	*Halbur et al., 1996a; He et al., 2012; Morgan et al., 2014*
Bazo	Macrófagos	1-35	*Morgan et al., 2014*
Ileón	Macrófagos	1-21	*Halbur et al., 1996a*
Corazón	Macrófagos	3-21	*Halbur et al., 1996a*
Hígado	Células de Kupffer	7	*Halbur et al., 1996a*
Riñón	Macrófagos	3-7	*Halbur et al., 1996a*
Adrenales	*ND	3-10	*Halbur et al., 1996a*
SNC	*ND	7	*Rossow et al., 1996*
Testículos **Glándula-Vesicular** **Pene**	Epitelio del túbulo seminífero Macrófagos Linfocitos	7	*Christopher-Hennings et al., 1998*

***ND:** no determinado

2.1 Forma respiratoria

La infección por el PRRSV está asociada con la destrucción del sistema mucociliar de la mucosa nasal *(Done y Paton, 1995; Halbur et al., 1995)*, con una destrucción masiva, aunque de forma transitoria en la primera semana post-infección, de los PAMs *(Martínez-Lobo, 2010)* y con la apoptosis y/o necrosis de linfocitos *(Thanawongnuwech et al., 2001; Labarque et al., 2003; Gómez-Laguna et al., 2012b, 2013)*, lo que se traduce en una predisposición a la invasión por parte de agentes secundarios al estar comprometidas las barreras de defensa del sistema respiratorio *(Done y Paton, 1995; Halbur et al., 1995)*. Varios estudios han demostrado que las citoquinas juegan un papel relevante en la forma respiratoria de la enfermedad *(Van Reeth et al., 1999; van Reeth y Nauwynck, 2000; Gómez-Laguna et al., 2013)* y que, aunque el PRRSV no se comporta como un virus respiratorio "clásico", es capaz de inducir la producción de citoquinas pro-inflamatorias y anti-inflamatorias principalmente por los macrófagos septales del pulmón que mediarán los fenómenos inflamatorios locales dando lugar a la neumonía intersticial típica de esta enfermedad *(Van Reeth et al., 1999; van Reeth y Nauwynck, 2000; Van Reeth et al., 2002; Labarque et al., 2003; Gómez-Laguna et al., 2010, 2013; Frydas et al., 2013; Weesendorp et al., 2014)*.

2.2 Forma reproductiva

Durante la diseminación orgánica el PRRSV llega al aparato reproductor y, como consecuencia, en el verraco se produce la eliminación intermitente, y en muy bajas cantidades, del virus a través del semen, por lo que en condiciones de campo parece que sería difícil alcanzar las dosis infectivas necesarias para la transmisión *(Prieto et al.,*

1996b, 2003). Por el contrario, en las reproductoras, las consecuencias de la infección dependerán en gran medida de la fase de la gestación en la que se produzca dicha infección *(Martínez-Lobo,, 2010; Karniychuk et al., 2011, 2012)*. Así, la importancia de la infección al comienzo de la gestación (entre los días 1 y 21 de gestación) es relativamente baja comparada con las afectadas al final de la misma (a partir de los 85 días de gestación) debido a que es necesario que se produzca la placentación para que el virus pueda infectar a los embriones y a los fetos *(Mengeling et al., 1994; Lager y Halbur, 1996; Prieto et al., 1996b, 1997; Karniychuk et al., 2011, 2012)*.

3. Signos clínicos

Los signos clínicos de la enfermedad pueden presentar una gran variabilidad, ya que la intensidad de estos va a depender de la virulencia de la cepa, del estado inmune y susceptibilidad del hospedador, de las infecciones concurrentes, de la exposición a lipopolisacáridos, y de diferentes factores relacionados con el manejo, como ser: el flujo de los animales, el diseño de los edificios, la regulación de la temperatura, la ventilación, etc. *(Halbur et al., 1995; Mengeling et al., 1998; Blaha, 2000; Karniychuk et al., 2010; Chand et al., 2012; Charerntantanakul, 2012)*.

Clínicamente, la infección aguda tiene una fase inicial de, aproximadamente, unas 2 semanas caracterizada por la presencia anorexia y letargo, como consecuencia de la viremia, en animales de todas las edades (entre el 5 al 75%). Los signos clínicos aparecen en una o más etapas de la producción y, en tan sólo de 3 a 7 días, se propaga en toda la explotación. Clínicamente los animales afectados pueden

presentar pelo hirsuto, linfopenia, hipertermia (con unas temperaturas rectales de entre 39 y 41°C), hiperpnea, dispnea, hiperemia cutánea, que suele ser transitoria y localizada, apareciendo en forma de "manchas" y cianosis en las extremidades y una reducción de la ganancia media diaria, lo que genera lotes irregulares *(Rossow et al., 1994a; Van Reeth et al., 1996; Labarque et al., 2002; Zimmerman et al., 2012)*.

Los lechones infectados en el útero o aquellos infectados a las pocas horas, o días, después de su nacimiento son los animales que presentan una sintomatología más grave, caracterizada por una marcada disnea y taquipnea, edema periocular y palpebral, conjuntivitis, diarrea y signos nerviosos *(Lager y Halbur, 1996; Zimmerman et al., 2012)*. La mortalidad, en estos casos, es de aproximadamente el 100%, mientras que en las etapas de pre-destete puede alcanzar el 60% *(Hopper et al., 1992; Rossow et al., 1994b, 1995; Rossow, 1998; Zimmerman et al., 2012)*.

La segunda fase de esta infección aguda, puede prolongarse de 1 hasta 4 meses y se caracteriza por la aparición de un fallo reproductivo que afecta, principalmente, a las reproductoras que son virémicas en su tercer trimestre de gestación y se asocia con una alta mortalidad pre-destete *(Zimmerman et al., 2012)*. Aunque no todas las reproductoras afectadas presentan signos clínicos, suelen perder entre el 1 al 3% de los lechones cuando la infección se produce entre los 21 a 109 días de gestación y, posteriormente, presentar estros irregulares y bajas tasas de concepción. La mortalidad en reproductoras suele ser muy baja, entre un 1 y un 4%, y puede observarse, de manera irregular, agalaxia, falta de coordinación, y/o una exacerbación dramática de enfermedades

endémicas de la granja como la sarna sarcóptica, rinitis atrófica, o cistitis/pielonefritis *(Hopper et al., 1992; Zimmerman et al., 2012)*. Normalmente, del 5 al 80% de las reproductoras que se infectan entre los 100 y 118 días de gestación tienen camadas compuestas tanto por lechones normales como débiles, así como nacidos muertos, que pueden estar parcialmente momificados o autolíticos. Los cerdos nacidos muertos pueden representar desde el 0 hasta el 100% de cada camada afectada y desde el 7 al 35% del total de los cerdos nacidos en cada lote de partos *(Christianson et al., 1992; Zimmerman et al., 2012)*.

Durante la infección aguda los verracos pueden ser asintomáticos o presentar una disminución de la líbido y/o una reducción, variable, en la calidad del semen *(Martínez-Lobo, 2010)*. Entre las alteraciones descritas en los espermatozoides procedentes de verrracos, a las 2-10 semanas después de la infección, se encuentran la disminución de la motilidad, el aumento de las morfoanomalías (gotas citoplasmáticas proximales y distales) y las alteraciones del acrosoma *(Prieto et al., 1996 a,b; Prieto y Castro, 2005)*.

En condiciones de campo las infecciones por el PRRSV suelen estar acompañadas de infecciones secundarias, lo que se traduce en una mayor incidencia de las enfermedades endémicas de la explotación, y de un incremento, de entre el 12 al 20%, de la tasa de mortalidad. Entre las enfermedades que más frecuentemente se asocian con el PRRS se encuentran la meningitis estreptocócica, la salmonelosis septicémica, la enfermedad de Glässer, la dermatitis exudativa, la sarna sarcóptica, la bronconeumonía bacteriana, la gripe porcina, la enfermedad de Aujeszky

y las infecciones por circovirus porcino tipo 2 y coronavirus respiratorio porcino *(Galina et al., 1994; Done y Paton, 1995; Van Reeth et al., 1996; Pol et al., 1997a; Thanawongnuwech et al., 2000; Wills et al., 2000; Feng et al., 2001; van Gucht et al., 2003; Cho et al., 2006b)*.

La forma crónica o endémica del PRRS se instaura en las explotaciones tras superar la forma aguda de la enfermedad y se mantiene durante largos períodos de tiempo, dependiendo del tamaño y el manejo de la granja *(Stevenson et al., 1993)*. Los principales signos observados son de tipo respiratorio, en cerdos en etapas de crecimiento, acompañado de una disminución de los índices productivos y un aumento de infecciones secundarias *(Christopher-Hennings et al., 1995; Allende et al., 2000; Zimmerman et al., 2012)*.

4. Lesiones

Las lesiones macroscópicas y microscópicas se observan, de forma constante, en los pulmones y los nódulos linfáticos, principales órganos de replicación del virus, entre los 4 a 28 dpi. Sin embargo, en otros órganos donde hay menor replicación del virus, como son los riñones, el cerebro y el corazón, las lesiones microscópicas suelen presentarse tan sólo entre los 7 a 14 dpi. De manera esporádica pueden observarse lesiones microscópicas en el útero de cerdas reproductoras con fallo reproductivo y en los testículos de verracos infectados. La intensidad y distribución de las lesiones van a depender de diferentes factores como la virulencia de la cepa, las condiciones del hospedador (edad, estado inmunológico y factores genéticos) y de diferentes condiciones ambientales y de manejo *(Halbur et al., 1995, 1996 a,b; Rossow et*

al., 1995, 1996; Wills et al., 1997; Chand et al., 2012; Gómez-Laguna et al., 2013; Kappes y Faaberg, 2015).

4.1 Aparato respiratorio

La gravedad y distribución de las lesiones en el aparato respiratorio pueden variar considerablemente, por lo que nos podemos encontrar desde pulmones aparentemente normales, hasta casos en los que las lesiones por el PRRSV aparecen enmascaradas por la existencia de infecciones secundarias o concomitantes por otros virus y/o bacterias, y que estarían englobadas dentro del complejo respiratorio porcino, pasando por aquellos animales que muestran un cuadro de neumonía intersticial, entre leve a grave, y con una distribución que puede ser multifocal o difusa *(Done y Paton, 1995; Halbur et al., 1995, 1996 a,b; Gómez-Laguna et al., 2009, 2010; Morgan et al., 2013; Weesendorp et al., 2014)*.

La neumonía intersticial que se produce en el PRRS, y que suele estar asociada con un aumento de tamaño de los nódulos linfáticos traqueobronquiales y mediastínicos, suele presentarse entre los 3 y 28 dpi, siendo las lesiones más intensas entre los 10 y 14 dpi. Macroscópicamente, los pulmones pueden presentarse desde ligeramente aumentados de consistencia, no colapsados y con un aspecto moteado de color gris-marrón, hasta un pulmón afectado de forma difusa, firme, muy húmedo y rojizo en los casos más graves (Figura 6A) *(Halbur et al., 1995, 1996 a,b; Caswell y Williams, 2012; Gómez-Laguna et al., 2010; Zimmerman et al., 2012; Morgan et al., 2013)*.

Microscópicamente, es característica la existencia de un engrosamiento de los septos alveolares, por la infiltración de macrófagos y, en menor medida, de linfocitos, que se acompaña de la hiperplasia e hipertrofia de los neumocitos tipo II, así como de la presencia de restos celulares, material proteináceo, macrófagos descamados y, ocasionalmente, células sincitiales en los alveolos. Además, también puede existir un infiltrado mononuclear linfoplasmocitario alrededor del árbol bronquial y vasos sanguíneos y, en ocasiones, se ha descrito una hipertrofia del tejido linfoide asociado a los bronquios (Figura 6B) *(Collins et al., 1992; Done y Paton, 1995; Halbur et al., 1996 a,b; Gómez-Laguna et al., 2010; Balka et al., 2013)*.

La mucosa nasal puede presentar, a partir de los 7 dpi, lesiones tanto a nivel de la lámina epitelial de la mucosa, y consistentes en la agrupación o ausencia de cilios, la presencia de fenómenos de tumefacción de las células del epitelio o la pérdida de las mismas y la aparición de una metaplasia escamosa, como a nivel de la submucosa, donde puede observarse un infiltrado inflamatorio mononuclear constituido, principalmente, por linfocitos y macrófagos *(Collins et al., 1992; Rossow et al., 1995; Halbur et al., 1996 a,b)*. Adicionalmente, en el epitelio bronquial se ha señalado la presencia del antígeno vírico en células que mostraban fenómenos de tumefacción y pérdida de los cilios *(Rossow et al., 1994a; Done y Paton, 1995; Halbur et al., 1995)*

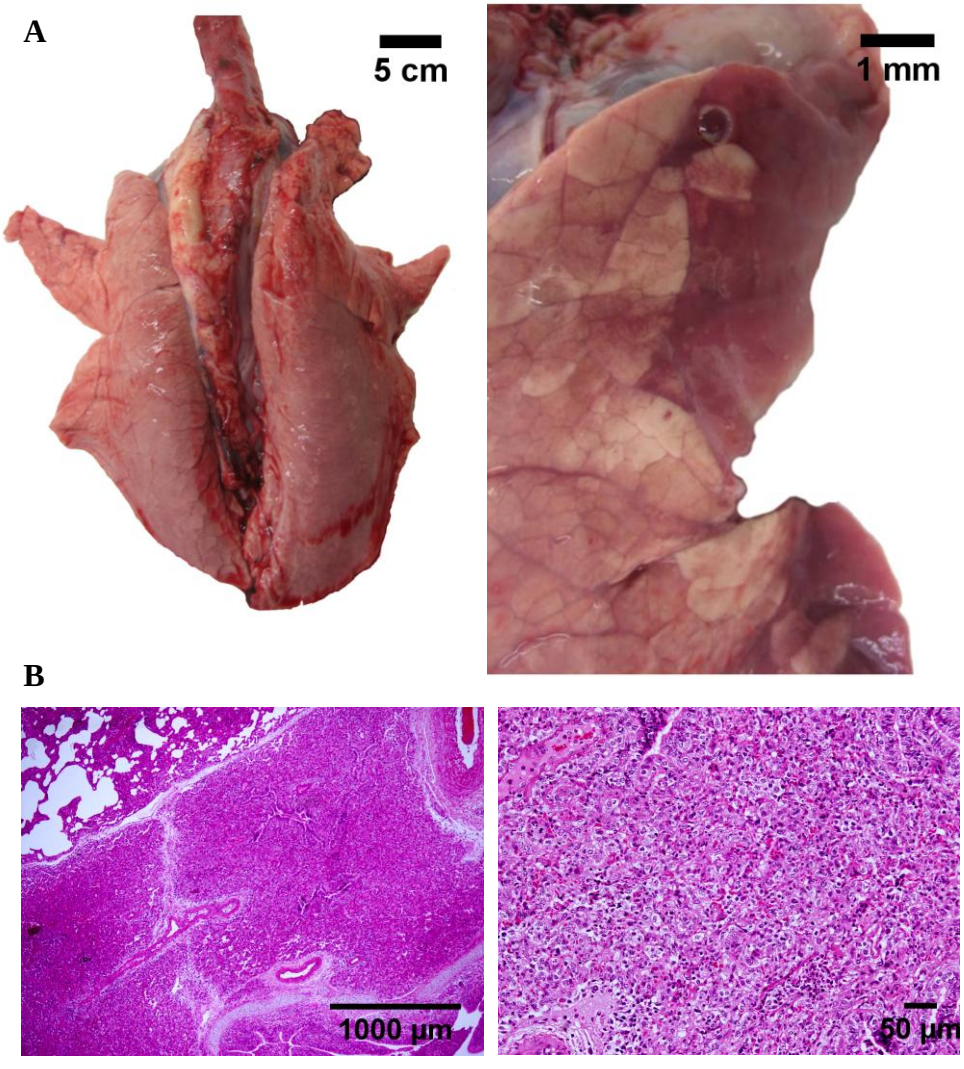

***Figura 6*:** Lesiones pulmonares de un cerdo infectado experimentalmente con la cepa SU1-bel a los 7 dpi. **A)** Lóbulos pulmonares no colapsados, de aspecto moteado con edema interlobulillar. **B)** Neumonía intersticial difusa severa caracterizada por el engrosamiento de los septos alveolares, infiltración de macrófagos, linfocitos y neutrófilos y la hiperplasia e hipertrofia de los neumocitos tipo II.

4.2 Órganos linfoides

Con relación a los órganos linfoides en animales infectados por el PRRSV es conveniente diferenciar entre órganos linfoides primarios, (el timo y la médula ósea), y los órganos linfoides secundario (nódulos linfáticos, tonsilas, placa de Peyer y bazo).

En el timo de lechones infectados de forma intrauterina (a los 98 días de gestación) se ha descrito una reducción de la relación peso corporal/peso del timo al nacimiento de un 50-52% y una reducción de más de dos tercios del peso del timo a los 7 y 14 días de edad *(Feng et al., 2001, 2002)*. En el caso de las cepas altamente virulentas se ha señalado, en lechones de 28 días de edad infectados con la cepa HuN4 (HP-PRRSV-2), una reducción del tamaño del timo de entre un 50 a un 90% *(Wang et al., 2011).* Sin embargo, en la medula ósea no se han descrito lesiones macroscópicas significativas.

En la mayoría de los cerdos infectados por el PRRSV los nódulos linfáticos son el órgano linfoide secundario que presenta cambios macroscópicos más significativos. Así, suelen presentar un marcado aumento de tamaño, entre 2 a 10 veces el tamaño normal, aspecto edematoso, color oscurecido y consistencia moderadamente firme. Conforme transcurre la infección los nódulos linfáticos se vuelven más firmes de consistencia, presentan un color blanco o marrón claro, con un patrón nodular o difuso, y, en ocasiones, puede observase en la corteza la existencia de múltiples quites, de unos 2 a 5 mm de diámetro *(Collins et al., 1992; Rossow et al., 1994b; Halbur et al., 1995, 1996 a,b; Lawson et al., 1997; Gómez-Laguna et al., 2009; Morgan et al., 2014)*.

Las lesiones microscópicas descritas en el timo de lechones infectados a los 98 días de gestación y entre los 28 y 35 días de edad con PRRSV, varían desde una leve depleción de timocitos hasta una marcada atrofia, como consecuencia de la pérdida de linfocitos T. Una pérdida de linfocitos que puede presentar un patrón multifocal o difuso, y que origina una disminución de la relación corteza:médula, y la desaparición de los límites que demarca la región cortico-medular, acompañándose de un aumento de los componentes estromales y de la presencia de restos celulares, cuerpos apoptóticos y macrófagos de cuerpo tangibles (Figura 7A) *(Feng et al., 2001, 2002; Wang et al., 2011; He et al., 2012; Li et al., 2014).*

La médula ósea de lechones de 7 y 12 días de vida tras una infección intrauterina presenta fenómenos de hipoplasia que puede variar desde leve a grave, y que se caracteriza por la falta de precursores celulares de las líneas mieloide y eritroide. Además, se describe la aparición de forma difusa o focal de células mononucleares, de pequeño o mediano tamaño y de núcleo redondo o vesicular, un aumento del número de megacariocitos y la existencia de ocasionales focos de precursores eritroides nucleados (Figura 7B) *(Feng et al., 2001).* Sin embargo, también se ha descrito un aumento significativo de la relación mieloidea:eritroidea desde los 3 hasta los 10 dpi en cerdos de 4 semanas de edad *(Halbur et al., 2002).*

Microscópicamente, los órganos linfoides secundarios, pueden presentar desde fenómenos de atrofia y depleción hasta de hiperplasia e hipertrofia de los folículos linfoides que, en ocasiones, pueden acompañarse de la existencia de fenómenos de necrosis de los centros

germinales y de la aparición de sincitios celulares (Figura 7C y 7D) *(Collins et al., 1992; Rossow et al., 1994b; Halbur et al., 1995, 1996 a,b; Lawson et al., 1997; Gómez-Laguna et al., 2009; Wang et al., 2011; Barranco et al., 2012b; He et al., 2012; Li et al., 2014; Morgan et al., 2014)*.

4.3 Aparato reproductor

Las lesiones microscópicas descritas en el aparato reproductor son escasas, y están representadas, en las reproductoras, por una endometritis, caracterizada por la existencia de edema e infiltrado perivascular linfohistiocitario y, menos frecuentemente, por la existencia de micro separaciones entre el epitelio del trofoblasto y el endometrio placentario, que están ocupadas por un fluido proteico eosinofílico y restos celulares *(Christianson et al., 1992; Lager y Halbur, 1996; Karniychuk et al., 2011, 2012)*. En los verracos se ha señalado la existencia de atrofia de los túbulos seminíferos, caracterizada por la presencia de células gigantes, fenómenos de apoptosis y agotamiento de las células germinales, entre los 7 y 25 dpi *(Prieto et al., 1996 a,b; Martínez-Lobo, 2010)*.

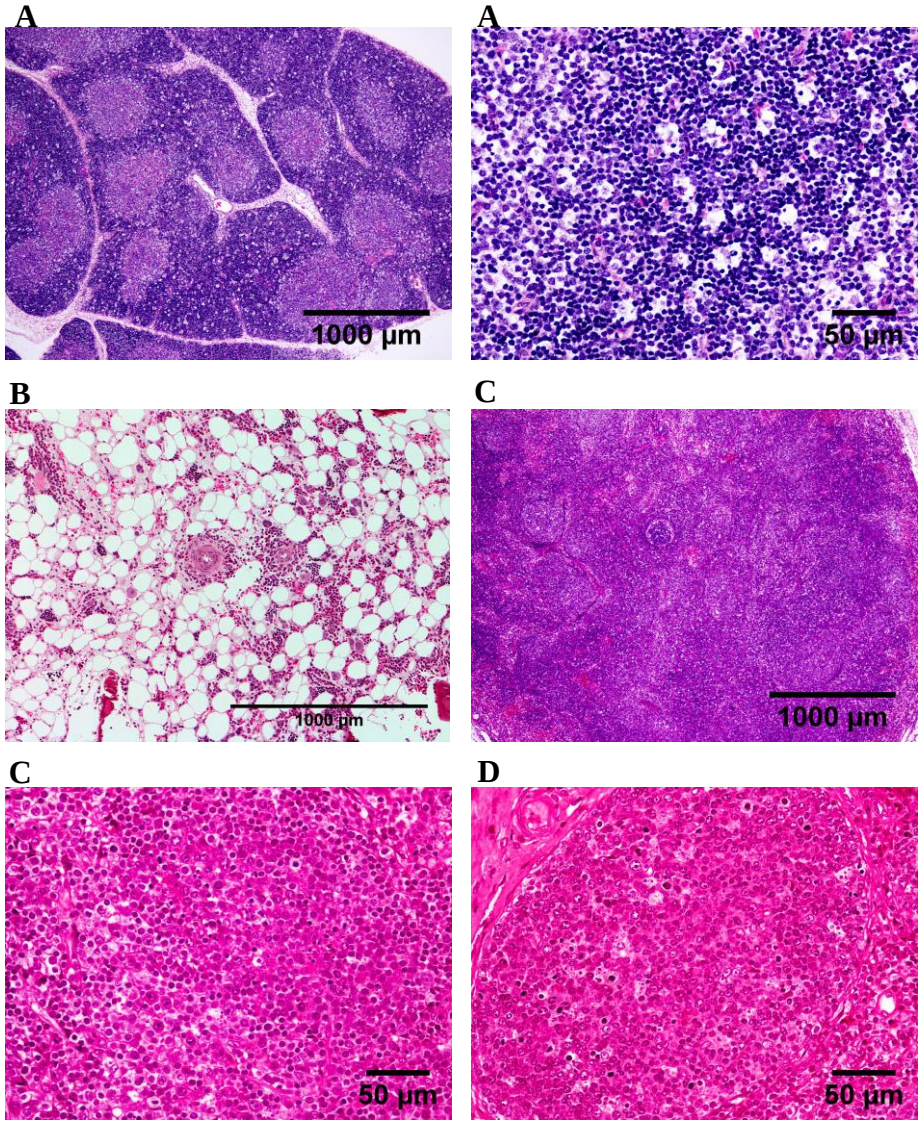

Figura 7: Cerdo infectado experimentalmente con la cepa SU1-bel a los 7 dpi. **A)** Timo, con disminución de la relación corteza:médula y aumento de los componentes estromales. La corteza presenta un aspecto de "cielo estrellado" por la presencia de gran numero de macrófagos de cuerpo tangible. **B)** Médula ósea, con hipoplasia del tejido hematopoyético y aumento del estroma. **C)** Nódulo linfático mediastínico, con depleción moderada de los folículos linfoides, presencia de cuerpos apoptóticos y macrófagos de cuerpo tangibles. **D)** Tonsila, existencia de cuerpos apoptóticos y macrófagos de cuerpo tangible en los folículos linfoides.

4.4 Fetos y lechones

Las lesiones en fetos y lechones nacidos muertos son infrecuentes y rara vez contribuyen a un diagnóstico definitivo de la enfermedad; sin embargo, la ausencia de lesiones no descarta la infección por el PRRSV. Las lesiones macroscópicas descritas en los fetos incluyen la existencia de ascitis e hidrotórax, edema perirrenal, en el ligamento esplénico y mesentérico. Los lechones muertos están, comúnmente, recubiertos con una mezcla de meconio y líquido amniótico, de color marrón, y que sugiere la existencia de fenómenos de estrés y/o hipoxia fetal *(Lager y Halbur, 1996; Zimmerman et al., 2012)*.

En infecciones experimentales de lechones de 13 días de edad, se ha descrito la existencia de trastornos en el drenaje, representado por la aparición de edemas subcutáneo, periocular y escrotal, a partir del segundo día post infección *(Lager y Halbur, 1996; Rossow et al., 1996, 1999; Martínez-Lobo., 2010; Zimmerman et al., 2012)*. Las principales lesiones microscópicas descritas en fetos abortados y lechones se localizan en pulmón y consisten en un cuadro de neumonía intersticial similar a la descrita anteriormente; en el resto de órganos y tejidos las lesiones están representadas por la existencia de vasculitis, caracterizada por la presencia de un infiltrado inflamatorio linfoplasmocitario en la túnica media, que puede llegar a presentar una extensión transmural y, en raras ocasiones, se asocia a una necrosis fibrinoide *(Christianson et al., 1992; Halbur et al., 1996 a,b; Lager y Halbur, 1996; Martínez-Lobo, 2010; Zimmerman et al., 2012).* En el tejido nervioso se ha descrito una leucoencefalitis linfohistiocitaria leve que en ocasiones puede estar acompañada por la existencia de un infiltrado histiocitario en los plexos coroideos *(Collins et al., 1992; Rossow et al., 1995; Lager y Halbur, 1996; Rossow et al., 1999)*.

5. Respuesta inmune

El PRRSV se caracteriza por modular la respuesta inmune del hospedador para favorecer tanto su replicación, como su distribución y latencia en el organismo. Aunque se sabe que el virus es capaz de desarrollar diferentes mecanismos para evadir dicha respuesta inmune, a pesar de todos los esfuerzos desarrollados no se conocen aún con exactitud los mecanismos concretos utilizados por el virus.

La liberación de citoquinas y la existencia de células T reguladoras (Tregs, del inglés *regulatory T cells*) tienen una gran importancia en la homeostasis de la respuesta inmune del hospedador *(Sinkora y Butler, 2009; Badaoui et al., 2013b)*. Por otra parte, y con el fin de sobrevivir y propagarse, los virus han desarrollado diferentes mecanismos para alterar el sistema de defensa del hospedador mediante la codificación de proteínas que se dirigen a componentes clave de las vías de señalización del sistema inmune *(Huang et al., 2015)*. Recientemente se ha señalado que el PRRSV podría afectar a varias de estas etapas, como la producción y señalización de los interferones (IFNs), la modulación de la expresión de citoquinas, la manipulación de las rutas apoptóticas y la regulación de la inmunidad adaptativa *(Huang et al., 2015)*.

5.1 Inmunidad innata versus PRRSV

Estudios *in vivo* e *in vitro* confirman que tanto los IFN tipo 1 (IFNα/β) o IFN tipo 2 (IFNγ) como los IFNs porcinos recombinante o RNA de doble cadena sintéticos, pueden inhibir la replicación del

PRRSV y proteger a los animales de la infección *(Albina et al., 1998; Buddaert et al., 1998; Huang et al., 2015)*. Pero, al mismo tiempo, se ha señalado que el PRRSV utiliza varias estrategias que antagonizarían con esta respuesta del hospedador, entre las que se encuentran:

a) Un retraso de la detección del virus por parte de los receptores de patrones de reconocimiento (PRRs, del inglés *Pattern Recognition Receptors*) *(Chen et al., 2012; Yin et al., 2012; Huo et al., 2013; Wang et al., 2014; Huang et al., 2015)*.

b) La interferencia con la detección y señalización de las vías de inducción de IFNs *(Chen et al., 2010; Sun et al., 2010; Sagong y Lee, 2011; Sun et al., 2012; Butler et al., 2014; Chen et al., 2014; Sun et al., 2014).*

c) La inhibición de las vías de señalización mediadas por IFNs *(Patel et al., 2010; Barranco et al., 2012a; Sun et al., 2012; Wang et al., 2013 a,b)*.

Además, existen diferentes estudios que han demostrado que la expresión y producción de determinadas citoquinas en el transcurso de la infección con determinadas cepas del PRRSV es baja *(Van Reeth et al., 1999; Thanawongnuwech et al., 2001; Chung y Chae, 2003; Labarque et al., 2003; Gómez-Laguna et al., 2009, 2010; Renukaradhya et al., 2010; Gimeno et al., 2011; Barranco et al., 2012 a,b; García-Nicolás et al., 2015)*. Así, se ha señalado que en el transcurso de la infección por algunas cepas del PRRSV, se produciría una inhibición de la producción del TNF-α *(López-Fuertes et al., 2000; Labarque et al., 2003; Gómez-Laguna et al., 2010; Gimeno et al., 2011; Gómez-Laguna et al., 2013; Huang et al., 2015).* Una citoquina que, entre sus funciones, se encuentra la citolisis selectiva de células infectadas por

virus, la modulación de la apoptosis celular y la impermeabilización de células no infectadas *(Toews, 2001; Goetz et al., 2004)*.

Igualmente, se ha descrito la inhibición en la síntesis y liberación de citoquinas pro-inflamatorias, coincidiendo en el transcurso de la infección por el PRRSV, con la inducción de citoquinas anti-inflamatorias, como la IL-10 *(Suradhat et al., 2003; Flores-Mendoza et al., 2008; Wang et al., 2011)* que, a su vez, inhibe y modula la producción de otras citoquinas, contrarrestando la inmunidad adaptativa *(Moore et al., 2001; Díaz et al., 2005; Sabat et al., 2010)*. Adicionalmente, en la infección por el PRRSV se ha demostrado que las cepas consideradas más patógenas y virulentas inducen una mayor expresión de citoquinas pro-inflamatorias, como la IL-1β, tanto a nivel local como sistémico, lo que se asocia con la mayor gravedad de los signos clínicos y lesiones *(Weesendorp et al., 2013a)*.

La apoptosis se considera como un mecanismo de defensa innata crucial, ya que inhibe la replicación vírica y elimina a las células infectadas por diferentes virus. Sin embargo, algunos virus han desarrollado estrategias para bloquear o retrasar los fenómenos de apoptosis durante su replicación, de tal manera que se garantiza la producción de nuevas partículas víricas *(Thomson, 2001; Yordy y Iwasaki, 2011; Huang et al., 2015)*. En este sentido, se ha sugerido que el PRRSV podría inhibir la apoptosis durante las fases tempranas de la infección para favorecer la replicación del virus, induciendo la apoptosis en fases posteriores favoreciendo, en este caso, la liberación y dispersión de las partículas víricas *(Costers et al., 2008)*. Así, la GP5 o la nsp4 del virus se ha

asociado con la inducción de fenómenos de apoptosis, en los que la vía de señalización de JNK (del inglés, *c-Jun N-terminal Kinas*) podría ejercer un papel crucial, mientras que, contradictoriamente, la proteína GP2a inhibe la apoptosis *(Suárez et al., 1996; Yin et al., 2012; Huo et al., 2013; Ma et al., 2013; Huang et al., 2015)*.

Estos mecanismos de regulación de los fenómenos de apoptosis tanto en las células infectadas como no infectadas por el PRRSV se considera que juegan un papel muy importante en la patogénesis de esta enfermedad *(Sirinarumitr et al., 1998; Summerfield et al., 2000; Labarque et al., 2003; Karniychuk et al., 2011; Li et al., 2014; Wang et al., 2014; Morgan et al., 2014)*. Como se ha señalado anteriormente el PRRSV es capaz de inducir apoptosis directamente en las células infectadas, tanto a través de la activación de la vía de las caspasas como por otras vías independientes de la caspasa 3, pero también es capaz de desarrollar mecanismos indirectos para la inducción de la apoptosis de las células no infectadas a través de la liberación de diferentes mediadores, como podrían ser algunas citoquinas apoptogénicas *(Costers et al., 2008; Barranco et al., 2011; Rodríguez-Gómez et al., 2014)*.

5.1 Inmunidad adaptativa versus PRRSV

5.1.1 Respuesta humoral

En el transcurso de la infección por el PRRSV se produce una producción temprana de anticuerpos. Así, los anticuerpos IgM específicos son detectables en el suero entre los 5 a 7 dpi, alcanzando los mayores niveles a los 14 dpi *(Mulupuri et al., 2008)*. Unos anticuerpos que,

en esta etapa temprana de la infección, están dirigidos frente a las proteínas N, M, GP5 y nsp2 *(de Lima et al., 2006)*. Por otro lado, las concentraciones de anticuerpos IgG alcanzan su pico entre los 21 - 49 dpi *(Loemba et al., 1996; Vezina et al., 1996; Mulupuri et al., 2008),* no alcanzando los títulos máximos, que generalmente son modestos, hasta las 10-18 semanas post-infección *(Nelson et al., 1994; Yoo et al., 2010)*.

Desafortunadamente esta rápida respuesta de anticuerpos no se corresponde con la producción de anticuerpos neutralizantes (ANs) que no son detectados antes de las 4 semanas post-infección *(Benfield et al., 1992; Díaz et al., 2005, 2006; Yoo et al., 2010; Butler et al., 2014, Huang et al., 2015)*, con títulos muy bajos, de entre 1/32 a 1/64 o incluso inferiores *(Loemba et al., 1996; Díaz et al., 2005),* y que se dirigen principalmente frente a la GP5 del virus *(Benfield et al., 1992; Díaz et al., 2005, 2006; Yoo et al., 2010; Butler et al., 2014)*. Aunque la GP5 del PRRSV se considera el epítopo más importante para la inducción de anticuerpos neutralizantes (ANs) *(Plagemann et al., 2002; Wissink et al., 2003, 2005)*, también se ha observado la producción de ANs asociados a otras proteínas del virus como GP3, GP4 y M *(Meulenberg et al., 1997; Yang et al., 2000; Cancel-Tirado et al., 2004; Kim y Yoon, 2008; Costers et al., 2010; Vanhee et al., 2011; Li y Murtaugh, 2012)*. Este hecho cuestiona la dependencia de la proteína GP5 para la inducción de ANs como han sugerido algunos autores *(Butler et al., 2014)*. Por otro lado, no se han observado diferencias entre lechones y animales adultos con relación a la fecha de aparición de estos anticuerpos *(Klinge et al., 2009)*.

Aunque algunos estudios señalan que los ANs son importantes para la protección del hospedador frente a la infección del PRRSV

(Vezina et al., 1996; López et al., 2007; Mateu y Díaz, 2008), existen resultados contradictorios al respecto. Así, se ha demostrado que la viremia y la replicación vírica pueden persistir incluso en presencia de ANs *(Rossow, 1998; López et al., 2007)*, y que la viremia se resuelva antes de que aparezcan los ANs *(Nelson et al., 1994; Vezina et al., 1996; Mateu y Díaz, 2008)*. Sin embargo, en otro estudio se ha observado una correlación entre la aparición de ANs y la eliminación del virus *(Butler et al., 2014)*. Por otro lado, se ha señalado que las glicoproteínas frente a las cuales están dirigidos los ANs están retenidas en la membrana del retículo endoplásmico rugoso de la célula hospedadora, por lo que sólo en el caso en el que se produzca la lisis celular estos anticuerpos podrán interactuar con ellas *(Nauwynck et al., 2012)*.

Además de los ANs, se ha demostrado la existencia de otro mecanismo basado en anticuerpos antivirales, la estimulación dependiente de anticuerpo (ADE, del inglés *Antibody-Dependent Enhancement*), por el que se facilita su entrada a la célula a través del receptor Fc que media la endocitosis *(Mateu y Díaz, 2008; Qiao et al., 2011)*. También se ha señalado que la ADE altera la respuesta antiviral innata mediante la modulación de la expresión de factores antivirales clave *(Bao et al., 2013; Zhang et al., 2013; Zhang y Yoo, 2015.)*

Por último señalar que la respuesta humoral en fetos y lechones recién nacidos infectados con el PRRSV tienen una serie de características similares como el desarrollo de una hipergammaglobulinemia, por activación policlonal de células B, y la aparición de auto-anticuerpos, lo que sugiere que se produce una

desregulación de la diferenciación de células B durante el periodo inmunológico crítico *(Lemke et al., 2004; Butler et al., 2007, 2008, 2014; Sinkora et al., 2014)*.

Todos estos acontecimientos podrían explicar, al menos en parte, la ineficiente respuesta inmune humoral que se observa frecuentemente en las infecciones por el PRRSV.

5.1.2 Respuesta celular

La respuesta celular en las infecciones por el PRRSV es considerada como débil, retardada y variable, apareciendo entre las 2 y 8 semanas post-infección *(Bautista y Molitor, 1997, 1999; Xiao et al., 2004)* y no estando correlacionada con los niveles de replicación vírica en los órganos linfoides *(Xiao et al., 2004)*. En las infecciones por el PRRSV se puede observar la existencia de una leucopenia temprana aguda *(Christianson et al., 1993; Nielsen y Botner, 1997; Feng et al., 2002; Halbur et al., 2002; Gómez-Laguna et al., 2009)*, una atrofia del timo, por la pérdida de células T inmaduras *(Halbur et al., 1995; He et al., 2012; Guo et al., 2013a; Li et al., 2014)*, y una linfadenopatía asociada a la presencia de antígeno vírico *(Rossow et al., 1994a, 1995; Halbur et al., 1996 a,b; Lemke et al., 2004)*, por lo que el desarrollo de una respuesta inmune adaptativa eficaz puede verse comprometida, al no producirse el desarrollo y circulación de poblaciones de linfocitos T *(Butler et al., 2014)*.

El PRRSV es capaz, como mecanismos de defensa, de inducir tanto la disminución de la expresión de las moléculas de MHC clase I y

II por las células presentadoras de antígeno (APCs, del inglés *Antigen Presenting Cells*) *(Wang et al., 2007; Rodríguez-Gómez , 2012, 2013; Weesendorp et al., 2013b; Huang et al., 2015)* como de inducir la muerte celular, tanto mediante fenómenos de apoptosis como de necrosis, de estas células *(Rodríguez-Gómez et al., 2013, 2014)*. Estos mecanismos podrían estar implicados en que la respuesta celular no aparezca hasta las 4-8 semanas post-infección, y que las poblaciones de células T $CD4^+$ y $CD8^+$ permanezcan a unos niveles bajos y constantes después de la infección, lo que es indicativo de que la inmunidad mediada por células (CMI, del inglés *Cellular Mediated Immunity*) es transitoria y está retardada en las infecciones por el PRRSV *(Bautista y Molitor, 1997, 1999; López Fuertes et al., 1999, 2000; Feng et al., 2002; Xiao et al., 2004)*. Aunque algunos estudios han demostrado un aumento de la subpoblación de linfocitos T $CD8^{high}$, estos linfocitos parecen ser no funcionales *(Lamontagne et al., 2003; Costers et al., 2009; Gómez-Laguna et al., 2009)*.

Las células Tregs son responsables del equilibrio de la respuesta inmune y del mantenimiento de la homeostasis inmune, mediante el control o inhibición de las funciones de las células efectoras inmunocompetentes. En los últimos años, varios estudios han señalado que el PRRSV podría activar a las células Tregs tanto *in vitro* como *in vivo* *(Wongyanin et al., 2010; LeRoith et al., 2011; Manickam et al., 2013)* como una estrategia que favorecería la persistencia del virus en el hospedador *(Huang et al., 2015)*. Los estudios inciales demostraron la generación de células Tregs del fenotipo $CD4^+CD8^-CD25^+Foxp3^{high}$ a partir de células dendríticas infectadas con el PRRSV del genotipo Norteamericano *(Silva-Campa et al., 2009, 2012)* pero no con cepas del genotipo Europeo *(Silva-Campa et al., 2010)*. Curiosamente, un estudio reciente en el que se ha

llevado a cabo una caracterización detallada de MoDCs infectadas con cepas de ambos genotipos del PRRSV demuestra la ausencia de proliferación de células Tregs, y propone la necesidad de desarrollar una misma estrategia que permita una caracterización más detallada de las MoDC infectadas con el PRRSV para poder obtener resultados más consistentes a este respecto *(Rodríguez-Gómez et al., 2015)*.

Los linfocitos citotóxicos naturales (NK, del inglés *Natural killer cells*) son células del sistema inmune innato que tienen la capacidad de reconocer y matar algunas células infectadas por patógenos intracelulares como son los virus. Por otra parte el sistema inmune adaptativo tiene a los linfocitos T citotóxicos (CTL, del inglés *MHC-restricted cytotoxic T lymphocytes*) también, capaces de inducir la muerte celular de células infectadas por virus; deteniendo de esta forma, mediante estas poblaciones de linfocitos NK y CTL, la generación de nuevas partículas virales *(Murphy, 2012)*. Estudios *in vitro* han demostrado la capacidad del PRRSV para suprimir las células NK a partir de las 6 hpi *(Cao et al., 2013)*. Además en el transcurso del PRRS, los linfocitos CTL son muy débiles y de lento desarrollo *(Costers et al., 2009)*. Otra población de linfocitos T que se ve afectada de igual manera que las células NK y CTL en las infecciones con el PRRSV son los linfocitos T γ/δ *(Olin et al., 2005; Martelli et al., 2009; Käser et al., 2011; Sinkora et al., 2014.)*. Estos linfocitos además de servir como puente entre la respuesta innata y la adaptativa, producen la secreción de múltiples citoquinas y ejercen una actividad citotóxica y colaboradora en la producción de inmunoglobulinas *(Murphy, 2012)*. Sin embargo, hasta el momento no se ha podido concretar ninguna hipótesis sobre el papel que jugarían los

posibles cambios en la población de células T γ/δ en las infecciones por el PRRSV *(Butler et al., 2014)*.

Por último señalar que, aunque la respuesta celular se ha detectado frente a todas las proteínas estructurales del PRRSV *(Bautista y Molitor, 1997, 1999; López Fuertes et al., 1999; Díaz et al., 2009)*, los estudios más recientes se centran en la búsqueda de epítopos conservados entre diferentes cepas del PRRSV, tanto en proteínas estructurales como no estructurales *(Díaz et al., 2009; Rodríguez-Gómez, 2012)*.

B. DIVERSIDAD Y VARIABILIDAD DEL VIRUS DEL SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO: CEPAS ALTAMENTE PATÓGENAS

Como se ha señalado anteriormente una de las principales características del PRRS es la gran variabilidad genética y antigénica del virus *(Wensvoort et al., 1992; Nelson et al., 1993; Forsberg et al., 2002; Fang et al., 2007; Wang et al., 2011; Gómez-Laguna et al., 2013; Stadejek et al., 2013; Weesendorp et al., 2013b; Butler et al., 2014; Brar et al., 2015; Kappes y Faaberg, 2015)* que estaría relacionada tanto con el curso de la enfermedad como con la intensidad de las formas respiratoria *(Halbur et al., 1995, 1996 a,b; Gómez-Laguna et al., 2013)* o reproductiva *(Christianson et al., 1992, 1993; Mengeling et al., 1998; Karniychuk et al., 2012)*. En este sentido, mientras que las cepas de baja o moderada virulencia están relacionadas con cuadros sub-clínicos o infecciones endémicas, las cepas altamente virulentas causan cuadros graves, en los que la intensidad de la clínica y de las lesiones dependen del estado inmunológico de los animales de la explotación *(Zimmerman et al., 2012)*. Además, según demuestran diversos estudios, las cepas del PRRSV-2 parecen ser más neumovirulentas que las cepas del PRRSV-1 *(Halbur et al., 1995, 1996b; Johnson et al., 2004; Martínez-Lobo et al., 2011)*.

Desde la identificación del PRRSV en 1991 como agente etiológico del PRRS en Europa y Norteamérica, existen numerosos trabajos que señalan la amplia variabilidad genética y fenotípica entre las cepas aisladas en estos dos continentes, así como entre sus diferentes subtipos y linajes *(Wensvoort et al., 1991; Benfield et al., 1992; Wensvoort et al., 1992; Goyal, 1993; Halbur et al., 1996 a,b; Kapur et al., 1996)*. En la actualidad

sabemos que el PRRSV-1 (Figura 8) tiene, a su vez tres subtipos bien diferenciados y un cuarto subtipo tentativo. El subtipo 1 circula, principalmente, en Europa Occidental y Central (Figura 9), y engloba los aislados responsables de los nuevos brotes de la enfermedad *(Indik et al., 2000; Pesente et al., 2006; Balka et al., 2008; Greiser-Wilke et al., 2010; Shi et al., 2010a; Stadejek et al., 2013)*. El subtipo 2 engloba a las cepas procedentes de Bielorrusia, Lituania y Rusia (Figura 9). El subtipo 3 del PRRSV-1, en el que estaría la cepa aislada en Belorrusia, y el recientemente propuesto subtipo 4 tentativo aislado de muestras proveniente de Bielorrusia y Rusia *(Shi et al., 2010 a,b; Stadejek et al., 2013)*. Además, se ha señalado la presencia del PRRSV-1 en países no Europeos, como EE.UU. *(Ropp et al., 2004; Fang et al., 2007)*, Canadá *(Dewey et al., 2000)*, Corea del Sur *(Lee et al., 2010)*, China y Tailandia *(Thanawongnuwech et al., 2004a)*.

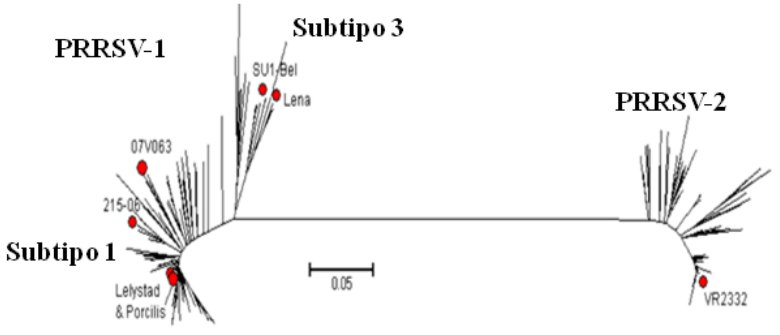

***Figura 8*:** Modelo filogenético del PRRSV, basado en la secuencia del ORF7, resaltando las cepas HP-PRRSV y las estudiadas en la tesis *(Morgan et al.,2014)*

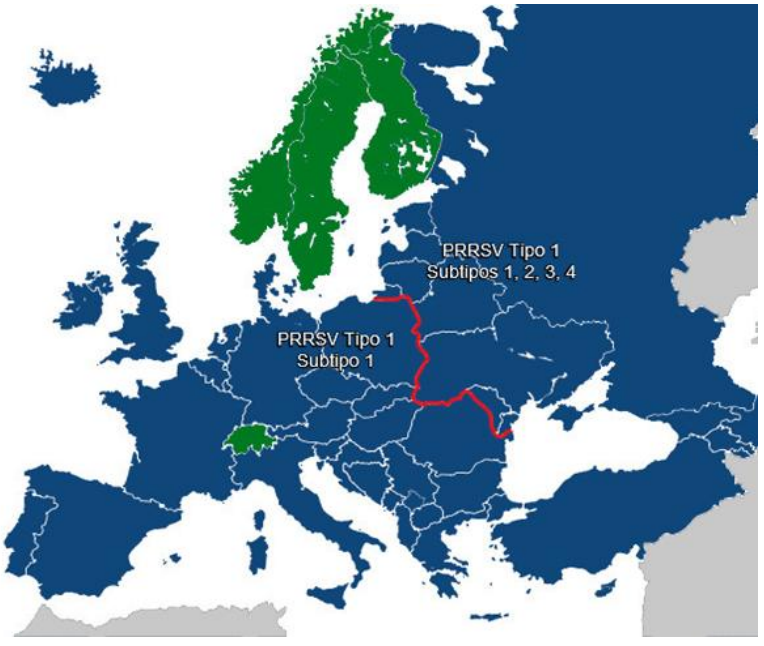

Figura 9: Distribución de PRRSV-1 *(Stadejek, 2015)*

El PRRSV-2 presenta nueve linajes diferentes, de los cuales siete están ampliamente distribuidos por Norteamérica y sólo dos se encuentran en países asiáticos *(Shi et al., 2010 a,b; Brar et al., 2015)*. Se ha propuesto que el brote original, observado a finales de 1980, fue producido por la cepa VR-2332 del PRRSV-2 *(Shi et al., 2010a)*. *Meng et al.*, en *1996*, indican la existencia de 8 cepas que, posteriormente *Halbur et al. (1996 b)* dividen en 2 grupos de acuerdo a su virulencia, señalando a la cepa VR-2385 como la de mayor virulencia. En la actualidad se sabe que una parte significativa de los aislamientos Norteamericanos realizados entre los años 1989 y 1990 estaban estrechamente relacionados y se correspondían a cepas encuadradas en el quinto linaje del PRRSV-2 *(Shi et al., 2010 a,b)*.

A finales del año 1996, en los estados de Iowa y Carolina del Norte, se registraron brotes caracterizados por graves fallos reproductivos en explotaciones infectadas de forma endémica por el PRRSV, afectando tanto a animales vacunados como no vacunados, y que fueron descritos como *"formas agudas del PRRS"*, ya que presentaban una alta mortalidad en reproductoras y verracos, de entre un 5% a un 10%, en un período de tiempo entre 1 a 5 semanas y con una tasa de abortos de entre el 10% y el 50%, independientemente de la fase de gestación *(Mengeling et al., 1998; Key et al., 2001; Shi et al., 2010a)*.

El aparente fracaso de las vacunas vivas atenuadas (Ingelvac-PRRS MLV y PrimePac-PRRS) utilizadas para controlas estos brotes pudo deberse al hecho de que los aislados del PRRSV en esta epidemia eran muy heterogéneos y encuadrados en los linajes 8 y 9 *(Key et al., 2001; Shi et al., 2010 a,b)* y, por tanto, genéticamente distintos a los linajes utilizados para la producción de vacunas en ese momento (linajes 5 y 7). A finales del año 2001 se produjo, en el estado de Minnesota, otro brote de la enfermedad, con una cepa de alta virulencia a la que se le ha denominado MN184 *(Han et al., 2006)*, la cual podría estar relacionada con cepas Canadienses *(Shi et al., 2010a)*.

En el verano del año 2006 surgió en China una cepa altamente patógena que, en pocos meses, se extendió a más de 20 provincias de este país asiático, causando enormes pérdidas económicas. A partir de este brote se empezó a usar el término enfermedad de la fiebre alta (del inglés, *High fever disease*), hasta diagnosticarla como un PRRSV altamente patógeno HP-PRRSV *(Tian et al., 2007; Zhou et al., 2008; An et al.,*

2010; Zhou y Yang, 2010). Las reconstrucciones filogenéticas realizadas han demostrado que esta cepa altamente patógena del PRRSV es una evolución de las cepas presentes en China y que se remontan a una sola cepa del PRRSV-2 que se introdujo en este país asiático. La diferencia con las demás cepas Chinas es una deleción en la región Nsp2 codificante que resulta en la pérdida de 90 nucleótidos que traducen 30 amino-ácidos *(Zhou et al., 2008; An et al., 2010; Shi et al., 2010 a,b; Zhou y Yang, 2010)*. Esta deleción de la región Nsp2 en la cepa China HP-PRRSV no puede ser utilizada como un marcador definitivo de virulencia, aunque puede servir como un marcador para distinguir cepas Chinas altamente patógenas a partir de cepas del PRRSV-2. Se especula que la virulencia del virus chino altamente patógeno puede estar asociada con la región que codifica el ORF1b *(Zhou y Yang 2010)*.

Por otra parte, este brote se caracterizó en comparación con otros brotes de PRRS típico por:

- Su rápida expansión en las granjas productoras de cerdos, en casi de todo el país. Entre los primeros 3 a 5 días de infección todo el hato de cerdos estaba infectado y en las siguientes 1 a 2 semanas la enfermedad se extendía a toda la granja y se transmitía a las áreas cercanas;

- La temperatura corporal de los cerdos afectados era alta entre 41 a 42 ◦C, y el curso de la enfermedad era por lo general entre 1 a 3 semanas;

- La enfermedad afectaba a cerdos de todas las edades, con una alta tasa de morbilidad, de entre el 50 al 100%, y una tasa de mortalidad que podía oscilar de entre el 10% al 100%, dependiendo de las características de los animales afectados. Así, lechones lactantes y cerdos en crecimiento presentaron las tasas más alta (normalmente de entre el 70 al 100%), mientras que en los cerdos de engorde y las cerdas gestante presentaron unas tasas muchos más bajas de mortalidad (de entre un 10 y un 20%). Las cerdas en gestación podían verse afectadas, con tasas de aborto de más del 40%.

En la mayoría de las granjas, la enfermedad surgió inicialmente en animales adultos (cerdas gestantes o cerdos de cebo) que, clínicamente presentaban depresión, anorexia, letargia y coloración rojiza en la piel y en las orejas. Los cerdos enfermos mostraron síntomas respiratorios evidentes, como estornudos, tos y disnea, que se acompañaba de un aumento de la secreción ocular, conjuntivitis, estreñimiento o diarrea y, en algunos casos, signos neurológicos. Los animales afectados que sobrevivieron al brote presentaron pelo hirsuto y estaban pálidos y emaciados. Las principales lesiones observadas fueron la existencia de hemorragias en la dermis, edema y consolidación pulmonar grave y nódulos linfáticos edematosos *(Tian et al., 2007; Zhou et al., 2008; An et al., 2010; Zhou y Yang, 2010)*.

La cepa HP-PRRSV, JXwn06 retrasaría la activación de PRRs, lo que favorecería la replicación del virus durante las primeras etapas de la infección. Además, parece que la proteína nsp4 de esta cepa HP-

PRRSV-2 tendría un mayor efecto inhibidor sobre la producción de IFNs en comparación con la cepa de menor virulencia la CH-1a; lo que sugiere que la propiedad inhibidora del nsp4 puede estar correlacionada con la virulencia del PRRSV *(Huang et al., 2015)*.

También se han señalado brotes atípicos y agudos de la enfermedad asociados a cepas del PRRSV-1 (HP-PRRSV-1). Así, en enero del año 2007, se registraron en el Este de Bielorrusia un brote de la forma reproductiva del PRRS que se acompañaba de trastornos respiratorios y una tasa de mortalidad que alcanzó el 70% en cerdos en crecimiento. Esta cepa, denominada Lena, y que está clasificada como perteneciente al subtipo 3 del PRRSV-1, es también considerada una cepa HP-PRRSV (Figura 8) *(Karniychuk et al., 2010; Weesendorp et al., 2013b, 2014)*, que comparada con otras cepas de Bielorrusia del subtipo 3, presenta una proteína N de menor tamaño y una proteína GP4 más grande *(Karniychuk et al., 2010)*. Además, se observó que los virus de la cepa Lena replican en una población de células monocíticas $CD163^{+}$ $sialodesina^{-}$ (Sn^{-}), indicando el desarrollo de nuevas estrategias por parte de las cepas HP-PRRSV para conseguir una mayor replicación vírica *(Frydas et al., 2013)*. La cepa Lena presenta una tasa de mortalidad de aproximadamente un 40%, y una rápida evolución de la enfermedad que se caracteriza por fiebre alta, de entre unos 40 a 42,1 °C, anorexia, depresión, edema periocular y, en algunos casos, tos *(Karniychuk et al., 2010)*.

Las infecciones experimentales han demostrado que la respuesta inflamatoria en los cerdos infectados con la cepa Lena es fuerte y

temprana, con altos títulos del virus en el suero y altos niveles de expresión de diferentes citoquinas, como IL-12, IFN-α/γ, IL-1β, IL-10 y, principalmente, TNF-α *(Weesendorp et al., 2013a, 2014)*. Los signos clínicos están claramente relacionados tanto con el grado de lesión pulmonar como con la expresión de IL-1β y TNF-α en el pulmón *(Weesendorp et al., 2014)*. También se ha descrito una regulación de la respuesta inmune asociada a la disminución de los linfocitos T colaboradores, linfocitos T γδ y de los linfocitos B en sangre *(Weesendorp et al., 2013a)*. Por otro lado, la infección *in vitro* con virus de la cepa Lena induce un mayor porcentaje de células en apoptosis y una menor expresión de las moléculas de superficie celular SLA-I, SLA-II y CD80/86, lo que podría explicar el retraso y la disminución de la respuesta inmune adaptativa en animales infectados con esta cepa altamente patógena *(Weesendorp et al., 2013b)*.

Otra cepa del subtipo 3 del PRRSV-1 es la denominada SU1-bel, aislada en el año 2010 a partir de tejido pulmonar de lechones de 30 días de edad de una granja en Bielorrusia *(Morgan et al., 2013; Lu et al., 2015)*. Esta cepa presenta una similitud del 88% con respecto a la cepa Lena, aunque con un genoma más corto y una alta variabilidad en ciertos grupos de genes (Figura 8) *(Lu et al., 2015)*. Los animales infectados experimentalmente con la cepa SU1-bel presentaron una temperatura rectal superior a los 40 °C, a partir del 2 dpi, y signos clínicos más graves que los animales infectados con la cepa Lena. Las lesiones neumónicas en estos animales llegan a extenderse hasta un 30% de la superficie pulmonar y se caracterizan por la presencia de lesiones multifocales de color más intenso y consistencia gomosa (Figura 6A). Por otra parte, la viremia y la carga viral en el fluido del lavado bronco-

alveolar (BALF, del inglés *broncho-alveolar lavage fluid*) en los animales infectados con la cepa SU1-bel fueron más bajas que la de los animales infectados con las cepas del subtipo 1 PRRSV-1 (LV y 215-06), lo que sugiere que la gravedad de la enfermedad en estos animales no está relacionada con la capacidad de la replicación de la cepa *(Morgan et al., 2013)*.

Los animales infectados con la cepa SU1-bel presentaron, en el suero, la existencia de anticuerpos no neutralizantes frente al PRRSV en etapas más tempranas (7 dpi) que los animales infectados con la cepa Lena (10 dpi). Anticuerpos no neutralizantes también fueron detectados en el BALF de los animales infectados con la cepa SU1-bel desde los 7 dpi hasta el final del experimento (35 dpi). Además, ambas cepas altamente patógenas demuestran una eliminación del virus en suero más rápida en comparación con otras cepas del subtipo 1 del PRRSV-1 (LV, 215-06 y Belgium A) *(Morgan et al., 2013; Weesendorp et al., 2013a)*. Describiéndose la existencia en circulación de un aumento de las poblaciones de linfocitos T naïve, linfocitos T de memoria, CD8 y NK; y una disminución de monocitos y linfocitos B en los animales infectados con la cepa SU1-bel *(Morgan et al., 2013)*.

En resumen, podríamos destacar que se ha descrito una marcada variabilidad en la respuesta de IFNs (*in vitro* e *in vivo*) y de citoquinas pro-inflamatorias para las cepas HP-PRRSV del PRRSV-1, las cuales suelen presentar una mayor expresión de estas citoquinas e inducir una temprana y mejorada respuesta inmune que serían las responsables de los signos clínicos y de las lesiones pulmonares, pero que también ayudan a

una eliminación más rápida del virus, a diferencia de la reducida y retrasada respuesta inmune que inducen las cepas de baja virulencia del subtipo 1 del PRRSV-1 (LV, 215-06 y Belgium A) *(Murtaugh et al., 2002; Morgan et al., 2013; Wang et al., 2013a; Butler et al., 2014; Weesendorp et al., 2014)*.

A raíz de la gran variabilidad presentada por las distintas cepas ha surgido la necesidad de caracterizar las cepas del PRRSV no sólo en función de sus características genotípicas sino también atendiendo a ciertas características fenotípicas. En este sentido, *Gimeno et al. (2011)* propusieron una clasificación de las cepas del PRRSV en función de su capacidad, o no, de inducir TNF-α y/o IL-10. Así, la inducción del TNF-α podría estar relacionada con la virulencia de las cepas *(Gómez-Laguna et al., 2013; Huang et al., 2015)*

C. ÓRGANOS LINFOIDES PRIMARIOS: FUNCIÓN BIOLÓGICA Y PAPEL EN EL PRRS

I. FUNCIÓN BIOLÓGICA DE LOS ÓRGANOS LINFOIDES PRIMARIOS

La estructura y funciones biológicas de los órganos linfoides primarios y secundarios son muy diferentes. Mientras que los órganos linfoides primarios, médula ósea y timo, son los encargados de la producción y diferenciación de los linfocitos B y T respectivamente, los órganos linfoides secundarios son los órganos especializados en la presentación de los antígenos a los linfocitos maduros ahí presentes, para su posterior activación, proliferación clonal y la generación de células de memoria *(Carrasco et al., 2004; Murphy, 2012).*

En la vida fetal los órganos hematopoyéticos son el saco embrionario y posteriormente el hígado y bazo *(Murphy, 2012)*. El esbozo del timo se observa a partir de los 21 días de gestación; a los 30 días puede caracterizarse fenotípicamente los linfocitos $CD3^+$ en el timo y alrededor de los 44 días se detecta en este órgano la existencia de sub-poblaciones de linfocitos $CD4^+$ y $CD8^+$. Los linfocitos T γ/δ están presentes tanto dentro como fuera del timo, pero el órgano prenatal inicial de origen de estos linfocitos no se conoce. Aproximadamente a los 40 días se observan las primeras células B (μ-cadena+) en el hígado. y las células B IgM^+ se encuentran en el bazo a los 50 días y en médula ósea a los 60 días. Las células B del bazo e hígado son capaces de

producir una pequeña cantidad de inmunoglobulinas a partir de los 50 días de la gestación. Como no hay transferencia de inmunoglobulinas de la madre al embrión y/o feto porcino, las células B fetales en estos animales son una población naïve, sin influencia de idiotipo y/o anti-idiotipo *(Rothkötter, 2009)*. Al nacimiento, la médula ósea se convierte en el principal centro hematopoyético y el timo es el primero de los órganos linfoides en completar su desarrollo, creciendo considerablemente, en respuesta a los estímulos antigénicos postnatal y a la gran demanda de linfocitos T maduros *(Pearse., 2006b; Murphy, 2012)*.

En animales jóvenes, la medula ósea se encuentra dentro de las cavidades centrales de los huesos axial y largos, y está compuesta por (i) un parénquima conformado por islas de tejido hematopoyético que consiste básicamente en una variedad de diferentes tipos celulares como las células primordiales hematopoyéticas pluripotenciales, y las células primordiales progenitoras de las líneas linfoide y mieloide y (ii) un estroma compuestos por senos vasculares, tejido adiposo y espículas óseas. Además, este estroma contiene células progenitoras multipotenciales no hematopoyéticas capaces de diferenciarse en diversos tejidos de origen mesenquimal, incluyendo los osteoblastos, células endoteliales, células reticulares, fibroblastos y adipocitos *(Travlos., 2006b; Zhao., et al 2012; Murphy, 2012; Vatti T, 2012)*.

El timo está considerado como un órgano linfo-epitelial, localizado principalmente en el mediastino anterior y rodeado por una cápsula que lo divide parcialmente en lóbulos, en los cuales se distinguen claramente: (i) una corteza con una alta densidad de linfocitos

T inmaduros (timocitos), una población minoritaria de linfocitos B y células plasmáticas; y (ii) una médula con una menor densidad celular y constituida por linfocitos T maduros, células epiteliales grandes, corpúsculos de Hassalls, macrófagos, células dendríticas, linfocitos B y raramente mastocitos y eosinófilos. Todos los linfocitos de la corteza están sostenidos por una red de células epiteliales que contribuyen a establecer un microambiente favorable para el desarrollo y maduración de los timocitos. Además, en la corteza del timo está presente una población de macrófagos que son los encargados de la fagocitosis de los linfocitos muertos, y que varía de número de acuerdo a varios factores. Ambas áreas, corteza y médula, están separadas por una zona, conocida como región cortico-medular, que contiene abundantes vasos sanguíneos, un escaso tejido conectivo perivascular y linfocitos T maduros e inmaduros *(Pearse., 2006b; Murphy, 2012)*.

En los cerdos adultos la médula ósea funcional se concentra en la columna vertebral, la pelvis, el esternón, las costillas, la bóveda craneal y en los extremos proximales de los huesos de las extremidades. En cambio el timo alcanza su máximo desarrollo alrededor de la pubertad para entrar en un proceso de involución durante la vida adulta *(Vatti T., 2012)*

En los procesos de crecimiento y diferenciación de las células progenitoras de la médula ósea participan una variedad de factores de crecimiento como son los factores de crecimiento de colonias de granulocitos y monocitos (GM-CSF, G-CSF); factores estimuladores, como la IL-1, IL-3 (acción multi-lineal), IL-6, IL-7 (línea linfoide), IL-

11 (generación de plaquetas); y factores inhibidores como el TNF-α *(Travlos., 2006b; Murphy, 2012; Zhao., et al 2012; Vatti T., 2012)*. Durante la diferenciación de los linfocitos B participan activamente las células del estroma mediante la liberación de citoquinas y factores de crecimiento; en esta etapa, los linfocitos B que muestran autorreactividad son eliminadas mediante fenómenos de apoptosis, lo que sucede en aproximadamente el 50% de los casos. Finalmente, el linfocito B maduro emerge de la médula y, a través de la circulación, se dirige a los órganos linfoides secundarios para ejercer su función efectora *(Murphy, 2012)*.

El linfocito T inmaduro (pro-timocito), que también se origina en la médula ósea, llega al timo guiado por señales quimiotácticas generadas por quimiocinas, ingresando al timo a través de la vasculatura de la región cortico-medular. Durante su migración intratímica, los pro-timocitos proliferan y se diferencian a timocitos, lo que se acompaña de la expresión del antígeno de diferenciación y de receptores de la membrana celular de estas células T (TCR, del inglés *T cell receptor*). Se produce así, la selección positiva, propiciando el desarrollo de los linfocitos capacitados para reaccionar frente a moléculas extrañas y se induce la apoptosis de los que muestran afinidad por moléculas propias. Finalmente, los linfocitos T maduros con sus marcadores CD4 (Th, linfocito cooperador) o CD8 (Tc, linfocito citotóxico) salen del timo a través de las vénulas y entran al torrente sanguíneo en donde, aproximadamente, el 75% permanece como linfocitos T circulantes y el resto se dirige a los órganos linfoides secundarios donde se producirá el reconocimiento de los antígenos *(Pearse., 2006b; Murphy, 2012)*.

II. PAPEL DE LOS ÓRGANOS LINFOIDES PRIMARIOS EN EL PRRS

Como se ha señalado anteriormente el principal sitio de persistencia del PRRSV y fuente de la viremia son los pulmones *(van Reeth y Nauwynck, 2000)*. Sin embargo, son varios los trabajos que han demostrado que los órganos linfoides, tanto primarios como secundarios. son también un sitio preferente de replicación del virus *(Rossow et al., 1995; Wills et al., 1997; Allende et al., 2000; van Reeth y Nauwynck, 2000; Feng et al., 2002; Halbur et al., 2002; Wang et al., 2011; He et al., 2012; Li et al., 2014;)*.

La expresión de citoquinas pro-inflamatorias y/o inmunomoduladoras inducidas por las diferentes cepas del PRRSV en órganos linfoides, principalmente en los nódulos linfáticos sugiere que este órgano tiene un papel importante en la patogenia del PRRSV *(Gómez-Laguna et al., 2009, 2012a; Barranco et al., 2012 a,b; Weesendorp et al., 2014; García-Nicolás et al., 2015)*.

Sin embargo, hasta el momento son escasos los estudios que se han centrado en el papel que desempeñan los órganos linfoides primarios en la patogenia del PRRSV. En este sentido hay que tener en cuenta que tanto la hematopoyesis como la timopoyesis son procesos complejos en el que participan múltiples factores que propician un microambiente adecuado para la supervivencia, proliferación, diferenciación y maduración de las diferentes células del sistema inmune; por este motivo, un desequilibrio de este microambiente, en respuesta a una infección vírica puede conducir a una desregulación y/o destrucción de

los progenitores celulares y de las células maduras, contribuyendo de esta forma a la persistencia del virus en el organismo y a la instauración de fallos inmunológicos que facilitarían la aparición de infecciones secundarias *(Feng et al., 2002; Halbur et al., 2002; Kolb-Maürer y Goebel, 2003; Pearse, 2006a; Travlos, 2006a; Butler et al., 2014)*.

Debemos recordar que la médula ósea es una fuente importante de las células del sistema inmune innato y adaptativo *(Murphy, 2012)*, y que los cambios producidos en la misma por una infección vírica puede originar cuadros de anemia, leucopenia y/o trombocitopenia por hipoplasia o atrofia de los precursores de las líneas celulares *(Rebar, 1993; Gómez-Villamandos et al 2003; Ezquerra et al 2009)*. En este sentido se ha señalado que la infección con el PRRSV de cerdas en el tercio final de la gestación se relaciona con el nacimiento de lechones con una mayor susceptibilidad a infecciones cuando al exponerlos a *Streptococcus suis*.

La presencia del antígeno del PRRSV en el timo *(Halbur et al., 1996a; Feng et al., 2002; He et al., 2012; Li et al., 2014; Morgan et al., 2014)* y el impacto negativo de la infección en la celularidad de este órgano *(Feng et al., 2002; Wang et al., 2011; Guo et al., 2013b; Wang et al., 2015)* se ha relacionado, principalmente, con la pérdida de linfocitos CD4/CD8 doblemente positivas *(Feng et al., 2001)* y los cambios en la producción y naturaleza de las células T *(Butler et al., 2014)*. Aunque la infección de las células tímicas no estaría relacionada con la el desarrollo de una linfopenía sino con el efecto del virus en las células T periféricas *(Butler et al., 2014)*.

Finalmente, es importante señalar que la intensidad de las lesiones en médula ósea y en el timo en animales infectados con el PRRSV varían considerablemente de acuerdo al grado de patogenicidad de la cepa y al modelo animal que se utilice. Así, se ha señalado cambios más graves tanto en la infección con cepas HP-PRRSV como cuando la infección se produce en el último tercio de la gestación *(Halbur et al., 1996 a,b; Feng et al., 2001, 2002; Halbur et al., 2002; He et al., 2012; Li et al., 2014)*. Además, la presencia de antígenos del PRRSV en el timo podría ser responsable de la inducción de una tolerancia inmunológica frente a este virus *(Silva-Campa et al., 2012)*. Los mecanismos precisos de esta inmunosupresión aún son desconocidos, pero las evidencias sugieren una disminución y/o pérdida de la función fagocítica de los macrófagos *(Feng et al., 2001)*. Además la infección de las APCs presentes en el timo tendría un efecto perjudicial sobre el desarrollo de las células T naïve y, por tanto, un efecto negativo en el desarrollo de una respuesta inmune capaz de eliminar al virus del hospedador *(Butler et al., 2014)*.

CAPÍTULO 2 / CHAPTER 2

OBJETIVOS DE LA TESIS

AIMS OF THE THESIS

OBJETIVO GENERAL

Comparar la inmunopatogenia tras la infección con cepas del PRRSV-1 de distinta virulencia (la cepa estándar del PRRSV-1 el Lelystad, LV; dos cepas de campo, la cepa altamente virulenta SU1-bel y la cepa 215-06 de moderada virulencia; y una cepa de vacuna atenuada, DV) en el pulmón y órganos linfoides primarios de cerdos infectados experimentalmente.

OBJETIVOS ESPECÍFICOS

1. Estudiar la expresión local, a nivel pulmonar, de las citoquinas pro-inflamatorias IL-1α, TNF-α e IL-6, e inmunomoduladoras IFN-γ e IL-10, en el transcurso de la infección por una cepa estándar del PRRSV-1 (LV), una cepa altamente virulenta del PRRSV (SU1-bel), una cepa de moderada virulencia (215-06) y una cepa vacunal (DV).

Este objectivo será abordado en el primer estudio experimental de título "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"

2. Evaluar los cambios histomorfométricos, la expresión local de citoquinas pro-inflamatorias (IL-1α y TNF-α) e inmunomoduladoras (IL-10), y su correlación con los fenómenos de apoptosis, en el timo de cerdos infectados experimentalmente con una cepa estándar del PRRSV-1, LV; una cepa altamente virulencia, SU1-bel y una cepa de moderada virulencia, 215-06.

Este objectivo será abordado en el segundo estudio experimental de título "Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains"

3. Evaluar los cambios que ocasiona cepas del PRRSV-1 de diferente virulencia (LV, 215-06 y SU1-bel) en la médula ósea de cerdos infectados experimentalmente.

Este objectivo será abordado en el tercer estudio experimental de título, "Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the viral load and virulence"

GENERAL AIM

To compare the immunopathogenesis of PRRSV-1 infection with strains of differing virulence (the prototype PRRSV-1 Lelystad virus, LV; two field strains, the highly virulent SU1-bel strain and the strain 215-06, of moderate virulence; and a live-attenuated vaccines, DV) in the lung and primary lymphoid organs of experimentally infected pigs.

SPECIFICS AIMS

1. To characterize the production of different proinflammatory (IL-1α, TNF-α and IL-6) and immunomodulatory (IFN-γ and IL-10) cytokines by subpopulations of pulmonary macrophages in pigs experimentally infected with different PRRSV-1 strains (LV, 215-06, SU1-bel and DV).

This aim is developed in the first experimental study, called "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"

2. To evaluate the impact of different virulent PRRSV-1 strains (LV, 215-06 and SU1-bel) in the thymus of piglets by analysing the histomorphometry, the presence of apoptotic phenomena and the local expression of cytokines (IL-1α, TNF-α and IL-10).

This aim is developed in the second experimental study, called "Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains"

3. To evaluate the effect of different virulence of PRRSV-1 strains (LV, 215-06 and SU1-bel) in the bone marrow of experimentally infected piglets.

This aim is developed in the third experimental study, called "Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the viral load and virulence"

CAPÍTULO 3 / CHAPTER 3

EXPERIMENTAL STUDIES

EXPERIMENTAL STUDIES

I. Common experimental design.

II. 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.

III. Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains.

IV. Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the viral load and virulence.

I. COMMON EXPERIMENTAL DESIGN

To assess all the aims of this PhD Thesis a common experiment was carried out, using several techniques for the different approaches. In this section, the common experimental design has been included.

Animal selection and housing

The experimental design is described in detail by *Morgan et al. (2013)*. In short, sixty-five 5-weeks old male piglets, negative for PRRSV were obtained from an isolated, specific-pathogen-free pig farm in The Netherlands. All pigs were confirmed negative against PRRSV, porcine circovirus type 2 (PCV-2) and *Mycoplasma hyopneumoniae* by ELISA and PCR. Besides *M. hyopneumoniae* culture from all animals at the end of the study yielded negative results. The animals were randomly allocated into five groups and housed in separate pens of a containment facility at the Animal and Plant Health Agency (APHA). In this study we analysed five animals within the infected groups and two animals within the control group per dpi. This experiment was approved by the APHA ethical review committee, and all procedures were carried out under the Animals (Scientific Procedures) Act, 1986, UK.

Viruses

Four PRRSV-1 strains were used in this study, as previously reported *(Morgan et al., 2013)*: Lelystad virus-Ter Huurne (LV), the prototype PRRSV-1 strain; the moderate virulent 215-06 strain, isolated from the serum of a post-weaning piglet showing signs of wasting and poor condition in 2006; the highly virulent SU1-bel strain, isolated from

lung tissue homogenate from a 30-day old piglet from a farm in Belarus in 2010; and, the attenuated strain DV (Porcilis® PRRS, Intervet BV, Boxmeer, The Netherlands). LV, 215-06 and SU1-bel strains were propagated in PAMs and cultured in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (Autogen Bioclear, Calne, UK) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies) (cRPMI) for three days in a humidified incubator at 37 ºC with 5% CO_2. Both SU1-bel and 215-06 strains were used at the 4^{th} passage and the LV strain was used at the 8^{th} passage *(Morgan et al., 2013)*.

Experimental design and animal monitoring

Pigs were randomly allocated and statistically blocked by weight as follows: the control group consisted of six pigs while each virus-infected group contained 14 to 15 animals. At seven weeks of age, after acclimatising for 14 days, the piglets were intranasally inoculated with 10^5 $TCID_{50}$ of the respective virus (either LV, 215-06 or SU1-bel) in 1.5 ml of cRPMI. The group infected with strain DV was inoculated with 10^4 $TCID_{50}$ *(Amarilla et al., 2015)*. Controls were inoculated with 1.5 ml of PAMs cryolysate diluted in cRPMI. Every day, from 3 days before experimental inoculation until the end of the experiment (35 dpi) rectal temperatures and clinical signs were recorded. Clinical signs associated with PRRSV infection were scored between 0-3, where 0 was normal and a score between 1-3 represented increasing severity of each observation as previously described *(Weesendorp et al., 2013a)*. No equipment was shared and staff changed in between rooms to prevent virus transmission between the groups. At days 3, 7 and 35 dpi, two pigs from the control group and four to five pigs from each infected group

were euthanized by administration of an intravenous lethal dose of pentobarbitone, followed by exsanguinations.

Immunohistochemistry

The Chemmate Dako Envision detection kit (Dako, Burlingame, CA, USA) was used for the detection of PRRSV nucleocapsid protein (lung and thymus), in formalin-fixed tissues *(Morgan et al., 2014)*. The Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA) was used for the detection of CD3, MAC387 (thymus) (formaldehyde-fixed tissues) and cytokines as IL-1α, TNF-α, IL-6, IL-10 (lung, thymus and bone marrow) and IFN-γ (lung) (Bouin-fixed tissues) *(Gómez-Laguna et al., 2010; Barranco et al., 2011; Amarilla et al., 2015)*, with the following modifications: the Labelled Streptavidin Biotin (LSAB) method was used specifically for the detection of IL-6. Cleaved Caspase 3 (cCasp3) immunolabelling and Terminal dUTP Nick End-Labeling (TUNEL) (thymus and bone marrow) were carried out in Bouin-fixed tissues by commercial kits (SignalStain® Apoptosis Cleaved Caspase-3 IHC Detection Kit, Cell Signaling, USA; and, In Situ Cell Death Detection Kit, POD, Roche, Germany, respectively) following the manufacturer's instructions *(Barranco et al., 2011)*.

Briefly, 4-µm sections were dewaxed and rehydrated, followed by endogenous peroxidase inhibition with 3% H_2O_2 solution in methanol for 45 minutes (min). The slides were exposed to different antigen retrieval methods or left untreated as summarized in *Table 1*. Sections were washed with PBS (pH 7.4, 0.01 M) and Tris-buffered saline (pH 7.6, 0.005 M) (Sigma–Aldrich, USA) with 0.01% Tween20 (TBST) and

incubated for 30 min at room temperature with 100 µl of blocking solution in a humid chamber. All primary antibodies against cytokines and cCasp3 were incubated overnight at 4 ºC in a humid chamber. IL-6 was incubated 1 hour (h) at 37 ºC. Primary anti-PRRSV antibody was incubated for 2 hs at room temperature. Anti-CD3 and anti-MAC387 antibodies and TUNEL were incubated for 1 h at room temperature.

In each case, the corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, USA) was incubated for 30 min at room temperature. Chemmate Dako Envision detection kit was applied for 30 min at room temperature with the addition of 5% normal swine serum (Life Technologies) (PRRSV) and avidin-biotin-peroxidase complex was applied for 30 min (CD3 and MAC387) and for 1 h (cytokines) at room temperature. Immunostaining was 'visualized' by application of the NovaRED^{TM} substrate kit (Vector Laboratories) and 3,30-diaminobenzidine tetrahydrochloride (DAB). Dako LSAB+System-HRP and liquid DAB+Substrate Chromogen System were used for the antibody polyclonal anti-pig IL-6 visualization. Sections were counterstained with Harris's haematoxylin, dehydrated and mounted. For negative controls, the primary antibody was replaced by an isotype control.

Antibody specificity was verified by substituting the primary antibody by isotype matched reagents of irrelevant specificity.

Table 1. Summary of immunohistochemical methodology.

Specificity	Type of antibody	Commercial Origin	Fixative	Blocking solution	Dilution	Antigen retrieval
Anti-PRRSV (clone SDOW17)	mAb	Rural Technologies, Brookings, SD,USA	Formalin	BSA	1 in 70	Protease
Anti-human CD3 (clone F7.2.38)	mAb	Dako	Formalin	10% NGS	1 in 1000	Protease
Anti-human MAC-387 (clone MAC387)	mAb	Serotec	Formalin	10% NGS	1 in 100	Protease
Anti-pig IL-1α	pAb	Endogen, Woburn, USA	Bouin	BSA	1 in 100	Tween20 0.01%
Anti-human TNF-α (clone 68B6A3 L1)	mAb	Invitrogen, Camarillo, USA	Bouin	BSA	1 in 75	Tween20 0.01%
Anti-pig IL-10	pAb	R&D Systems, Abingdon, UK	Bouin	10% NGS	1 in 20	Tween20 0.01%
Anti-pig IFN-γ	pAb	R&D Systems	Bouin	BSA	1 in 20	Tween20 0.01%
Anti-pig IL-6	pAb	Thermo Scientific, Rockford, USA	Bouin	1,5% NRS	1 in 100	Tween20 0.01%

mAb, monoclonal antibody; pAb, polyclonal antibody; Tween 20 diluted 0.01% in phosphate-buffered saline for 10 min; BSA, Bovine Serum Albumin; NGS, Normal Goat Serum; NRS, Normal Rabbit Serum; Protease, enzymatic digestion with trypsin (0.5%) / chemotrypsin (0.5%) at 37°C for 10 min; PRRSV, Porcine reproductive and respiratory syndrome virus.

Statistical analysis

Several univariate models were estimated for different response variables, and also a multivariate analysis of variance, for the measuring of the macroscopic lung lesions, histopathological lung *(Amarilla et al., 2015)* and thymus lesions, histomorphometry of the thymus and bone marrow, cortex/medulla ratio of the thymus, the number of tingible-body macrophages, the number of myeloid and erythroid cells, myeloid:erythroid ratio and the expression of the PRRSV antigen, MAC387, CD3, cCasp3, TUNEL and cytokines (IL-1α, TNF-α, IL-6, IFN-γ and IL-10), depending on the factors that may be cause of variation: time-point, lung lobes and different strains.

When analysing the lung, the control group was only included in the study as a reference of the basal level and was not included in the statistical analysis because it had no sufficient number of animals at each time-point for lung. Since vaccinated animals (DV group) presented a similar evolution than control animals along the study, all infected groups were compared among them and with the DV group and the differences were assessed by Dunnet, t-student and Mann Whitney tests, according to the parameters under study *(Amarilla et al., 2015)*.

All infected groups were compared to each other and with the control group and the differences were assessed by Dunnet and Mann Whitney tests, according to the parameters under study. A bidimensional contingency table and a log-linear model for the analysis of the interaction between different strains and histopathology score of the thymus were performed.

Pearson and Spearman tests were performed to assess the correlation (1) between gross and histopathological lung lesions and the local expression of virus and cytokines (IL-1α, TNF-α, IL-6, IFN-γ and IL-10) in lung *(Amarilla et al., 2015)*; (2) between PRRSV antigen labelled cells with the thymus histomorphometry measurements, the score of the histopathology and the expression of cCasp 3, TUNEL and cytokines (IL-1α, TNF-α and IL-10) in thymus; and, (3) between PRRSV viral load, the histomorphometric measurements of the bone marrow and the local expression of cCasp3, TUNEL and cytokines (IL-1α, IL-6, TNF-α and IL-10) in bone marrow. Differences with p-values ≤ 0.05 were considered to be statistically significant (SPSS 22.0, IBM SPSS Statistics, Chicago, USA).

II. A COMPARATIVE STUDY OF THE LOCAL CYTOKINE RESPONSE IN THE LUNGS OF PIGS EXPERIMENTALLY INFECTED WITH DIFFERENT PRRSV-1 STRAINS: UPREGULATION OF IL-1A IN HIGHLY PATHOGENIC STRAIN INDUCED LESIONS

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Introduction

Porcine reproductive and respiratory syndrome virus is a single stranded RNA virus belonging to the order Nidovirales, family Arteriviridae, genus Arterivirus *(Meulenberg, 2000; Snijder et al., 2013)*. PRRSV strains are classified into two distinct genotypes, type 1 or European PRRSV (PRRSV-1) and type 2 or North American PRRSV (PRRSV-2), with significant genetic differences among them. The European type strains can be further divided into at least three subtypes: Pan-European subtype 1 and Eastern European subtypes 2 and 3 *(Stadejek et al., 2008)*. Several authors have described differences in virulence among European PRRSV subtypes, finding that subtype 3 strains, such as Lena, are significantly more pathogenic than subtype 1 strains *(Karniychuk et al., 2010; Morgan et al., 2013, 2014; Weesendorp et al., 2013a, 2014; García-Nicolás et al., 2014a)*. Numerous studies have demonstrated that PRRSV-2 strains induce more severe respiratory disease comparing to PRRSV-1 strains *(Martinez-Lobo et al., 2011; Han et al., 2013a, 2013b, 2014)*. Moreover, it has been proved that several isolates of PRRSV-2 possess different virulence and pathogenic characteristics *(Halbur et al., 1996b; Shang et al., 2013)*.

The increased clinical and pathological effect of the SU1-bel strain, an Eastern European subtype 3 strain, has recently been associated to an enhanced inflammatory immune response rather than higher levels of virus in blood or broncho-alveolar lavage fluid (BALF) *(Morgan et al., 2013)*. The mechanisms by which a PRRSV strain exerts its virulence are not fully understood, but it has been suggested to be related to the in vivo replication capacity, tissue distribution, or immunomodulatory properties *(Haynes et al., 1997; Johnson et al., 2004; Loving et al., 2008; Rodríguez-Gómez et al., 2012; Rodríguez-Gómez et al., 2013)*. In addition, the immune response varies in pigs infected with different European PRRSV strains. Whereas, *Morgan et al. (2013)* evidenced significantly higher numbers of PRRSV-specific IFN-γ producing cells in SU1-bel infected animals when compared with subtype 1 strains, *Weesendorp et al. (2013a)* detected a lower level of IFN-γ secreting cells in Lena infected-pigs. In this latter study higher cytokine mRNA levels in blood, mainly TNF-α, were observed in pigs infected with the Lena strain than in animals infected with Belgium A and LV strains during the first week post-infection. Besides, $CD8^-$ γδ T cells displayed a significant decreased in Lena-infected animals along the first month post-infection as long as Natural Killer (NK) and cytotoxic T cell subsets were enhanced in the animals infected with other PRRSV-1 strains *(Weesendorp et al., 2013b)*.

Cytokines play an important role in the induction and regulation of immune responses, and their production by macrophages and other immune or non-immune cells plays a key role in the induction of pathology or protective immunity *(van Reeth and Nauwynck, 2000; Barranco et al., 2012a)*. PRRSV replicates primarily in PAMs, and the expression of proinflammatory cytokines has been observed mainly in septal

macrophages, suggesting that they are activated indirectly by the replication of PRRSV in bystander cells *(Gómez-Laguna et al., 2010, 2013)*. Under most conditions the degree of pathological damage in respiratory viral infections does not correlate with the number of virus particles in the infected tissues, rather aberrant induction and imbalance of cytokines may induce a severe systemic inflammatory response syndrome *(Kimura et al., 2013)*. Cytokines, such as IL-1α, IL-6, and TNF-α, secreted by macrophages and other immune cells have various biological effects on the organism that may protect against infection. However, these cytokines may also induce inflammation and tissue damage when they are over-expressed *(Liu et al., 2010; Kimura et al., 2013)*.

Gimeno et al. (2011) found that different PRRSV-1 strains are able to induce in vitro different patterns of expression of IL-10 and TNF-α and classified four possible phenotypes based on their ability to induce IL-10 and/or TNF-α. Moreover, the expression of IL-1α, IL-6 and TNF-α in the lungs of pigs infected with PRRSV-1 has been correlated with the development of the interstitial pneumonia typical of this disease *(Gómez-Laguna et al., 2010)*. The variability in cytokine profiles induced by different PRRSV strains, as well as the emergence of HP-PRRSV in Asia and Eastern Europe, highlight the need to evaluate differences in the immunobiology among PRRSV strains of differing virulence *(Gómez-Laguna et al., 2013)*. This study aimed to compare and characterize the production of different cytokines by subpopulations of pulmonary macrophages in pigs experimentally infected with different PRRSV-1 strains.

Materials and Methods

Animal selection and housing, viruses, experimental design and animal monitoring used in this experiment has been described above in the section "Common experimental design".

Table 1. The organization in this study of all pigs experimentally infected with four different PRRSV-1 strains: LV, 215-06, SU1-bel and DV. Animals were euthanized at 3, 7 and 35 dpi.

DPI	Control	LV[a]	215-06[b]	SU1-bel[c]	DV[d]
3	2	4	5	5	5
7	2	5	5	5	5
35	2	5	5	5*	5

a LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain; **b** 215-06: British field strain; **c** SU1-bel: Highly virulent strain; **d** DV: Attenuated vaccine strain; * Two animals in the SU1-bel group displayed a prolonged fever along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi.

Gross and histopathological lesions

At necropsy, gross pathology scores of the lungs were performed based on the method developed by *Halbur et al. (1995)*. After gross pathology examination, samples of the right lung (including apical, cardiac and diaphragmatic lobes) were collected and fixed by immersion in 10% neutral buffered formalin (Fisher Scientific Ltd., Leicestershire, UK) and in Bouin´s solution (Fisher Scientific Ltd.) and routinely processed, for the histopathological and immunohistochemical studies. Four µm sections were stained with haematoxylin and eosin (HE) for microscopical examination. Lung sections were blindly examined by two

pathologists and scored for the estimated severity and distribution of the interstitial pneumonia as previously reported *(Halbur et al., 1995)*.

Immunohistochemistry

The number of labelled cells was determined as previously described *(Salguero et al., 2005)*. Briefly, the labelled cells were counted in 50 non-overlapping and consecutively selected high magnification fields of 0.20 mm^2. Results were expressed as the number of cells per mm^2. Immunolabelled cells were identified and counted morphologically as macrophages, lymphocytes or neutrophils. Pulmonary intravascular macrophages and interstitial macrophages were grouped together and described as 'septal macrophages'. The results are expressed as the median of the immunolabelled cells in the three lung lobes of each animal at each time-point.

Results

Clinical signs and gross pulmonary lesions

The clinical signs and gross pulmonary lesions have previously been described elsewhere *(Morgan et al., 2013, 2014)*. Briefly, SU1-bel infected animals had mean temperatures above 40 ºC and were significantly higher than controls at 3 and between 6 and 10 dpi. These temperatures were higher than controls at 3 and between 6 and 10 dpi. Mean temperatures of the LV, 215-06 and DV groups were not different from controls. Clinical observations showed that the SU1-bel group had higher mean clinical scores between 5 and 17 dpi compared to the other groups. Two animals in the SU1-bel group displayed a prolonged

hyperthermia along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi. The mean scores of the other groups did not increase above 3 for the duration of the study.

At post-mortem, a score dependent on the percentage of the lung surface affected by gross lesions was given, with a maximum of 100 points. At 3 dpi gross lesions, consistent with mottled tan and rubbery pulmonary parenchyma which failed to collapse, were only detected in three and two animals of the 215-06 and SU1-bel groups, respectively, with the latter displaying the highest average score. Statistically significant differences were detected in the score for gross lesions at different time-points ($p = 0.001$) and between different strains ($p < 0.001$), with a significant interaction between time-point and strain ($p < 0.001$). SU1-bel ($p = 0.027$) and 215-06 groups ($p < 0.001$) displayed the highest score for gross lesions when compared with DV group. Moreover, at 7 dpi SU1-bel group displayed statistically significant higher score for gross lesions than LV and 215-06 groups ($p \leq 0.004$ in each group) (Fig. 1A). The SU1-bel animals euthanized at 12 and 13 dpi presented high gross pathology scores of 66 and 39, respectively. No gross pathology was seen in animals from the DV group along the study *(Morgan et al., 2013)*.

Microscopic lung lesions

The microscopic lesions were characterised by thickened alveolar septa with increased numbers of interstitial mononuclear cells (macrophages and lymphocytes), hyperplasia and hypertrophy of type II pneumocytes and alveoli filled with necrotic/apoptotic cell debris and

macrophages. These lesions when present were multifocal to diffuse, mild to severe. Mild to moderate perivascular lymphohistiocytic infiltrate was observed in some animals, mostly in apical, cardiac and diaphragmatic lung lobes, from 3 dpi onwards. Microscopic lung lesion scores are summarized in Fig. 1B. All infected groups displayed a similar kinetics with a peak of microscopical lesions at 7 dpi. No remarkable microscopic lung lesions were observed in control animals throughout the study. Statistically significant differences were observed at different time-points and between strains ($p < 0.001$), but not among different lung lobes. In addition, a significant interaction between time-point and strain was detected ($p < 0.001$). Statistically significant differences were observed between LV, SU1-bel and 215-06 groups with respect to DV group ($p < 0.001$). SU1-bel and 215-06 strains showed statistically significant higher score for microscopic lesions than LV group at 3 dpi ($p < 0.001$ in each group), with SU1-bel group showing the highest score at 7 dpi ($p < 0.001$ with respect to LV; and, $p = 0.010$ with respect to 215-06) (Fig. 1B). At 35 dpi 215-06 infected animals displayed the highest score for microscopic lung lesion when compared with SU1-bel ($p = 0.033$) and LV ($p = 0.018$) groups (Fig. 1B). Animals from DV group displayed minimal lesions throughout the study. The SU1-bel animals euthanized at 12 and 13 dpi had the highest microscopic lung lesion score (3.3 and 3.5, respectively).

Labelling of PRRSV antigen

PRRSV antigen was detected from 3 dpi until the end of the study in all virus-infected animals except for the animals infected with DV strain and control group. Statistical analysis of expression of PRRSV antigen demonstrated significant differences between different time-

points and different strains ($p < 0.001$), with a significant interaction between both variables ($p < 0.001$). Differences in the expression of PRRSV antigen were detected for each strain at different time-points. Statistically significant differences were detected between LV, 215-06 and SU1-bel groups with respect to DV group ($p < 0.010$). In addition, SU1-bel and 215-06 groups showed a statistically significant higher expression of viral antigen than LV group ($p < 0.001$ and $p = 0.005$, respectively), with SU1-bel group displaying the highest expression of PRRSV antigen at 7 dpi ($p = 0.002$ with respect to LV group; and, $p = 0.004$ with respect to 215-06 group) (Fig. 1C).

Tissue expression of proinflammatory cytokines

The expression of proinflammatory cytokines was mainly observed in the cytoplasm of septal macrophages and PAMs, and for IL-1α also in the cytoplasm of neutrophils. All proinflammatory cytokines were mostly detected in the cytoplasm of septal macrophages compared to PAMs and neutrophils (Fig. 2E, 2F). Differences in the expression of IL-1α were statically significant for different time-points, lung lobes and PRRSV strains ($p < 0.001$ in each case), with a significant interaction between all of them. SU1-bel and LV groups showed statistical significant differences in the expression of IL-1α with respect to DV group ($p < 0.006$ and $p < 0.001$, respectively). Furthermore, the animals infected with SU1-bel showed statistically significant higher levels of IL1-α than LV and 215-06 groups at 3 dpi ($p < 0.001$ and $p < 0.001$, respectively), and both SU1-bel and LV groups displayed a significantly enhanced expression of IL1-α with respect to 215-06 group at 7dpi ($p < 0.001$ and $p < 0.001$, respectively) (Fig. 1D).

The expression of TNF-α yielded significant differences for the evaluated variables: time-point ($p \leq 0.001$), lung lobe ($p = 0.057$) and PRRSV strain ($p \leq 0.001$), but no significant interaction was detected among any of them. SU1-bel and LV groups showed a significantly higher expression of TNF-α than DV group ($p < 0.001$), but no significant differences were observed for 215-06 group. At 35 dpi LV group showed higher levels of TNF-α when compared with SU1-bel ($p = 0.018$) and 215-06 group ($p = 0.018$) (Fig. 1E). The expression of IL-6 was only significantly enhanced for the variable time-point ($p = 0.005$). In animals infected with SU1-bel strain an increase in the expression of IL-6 was detected at 7 dpi ($p = 0.068$) (Fig. 1F).

The immunolabelling of proinflammatory cytokines was observed mainly in areas of lung with moderate to severe, diffuse, interstitial pneumonia (Fig. 2F). Fewer immunolabelled cells were observed in areas with mild to moderate, multifocal, interstitial pneumonia or in areas without lesions. Inoculated animals showed always higher counts of cells expressing proinflammatory cytokines than DV group at 3 and 7 dpi. The apical lung lobe displayed statistically significant higher levels for the expression of IL1-α than cardiac and diaphragmatic lobes ($p < 0.001$ and $p < 0.005$, respectively); however, these lobes showed higher levels of expression of TNF-α than the apical lobe ($p = 0.024$ and $p = 0.003$, respectively).

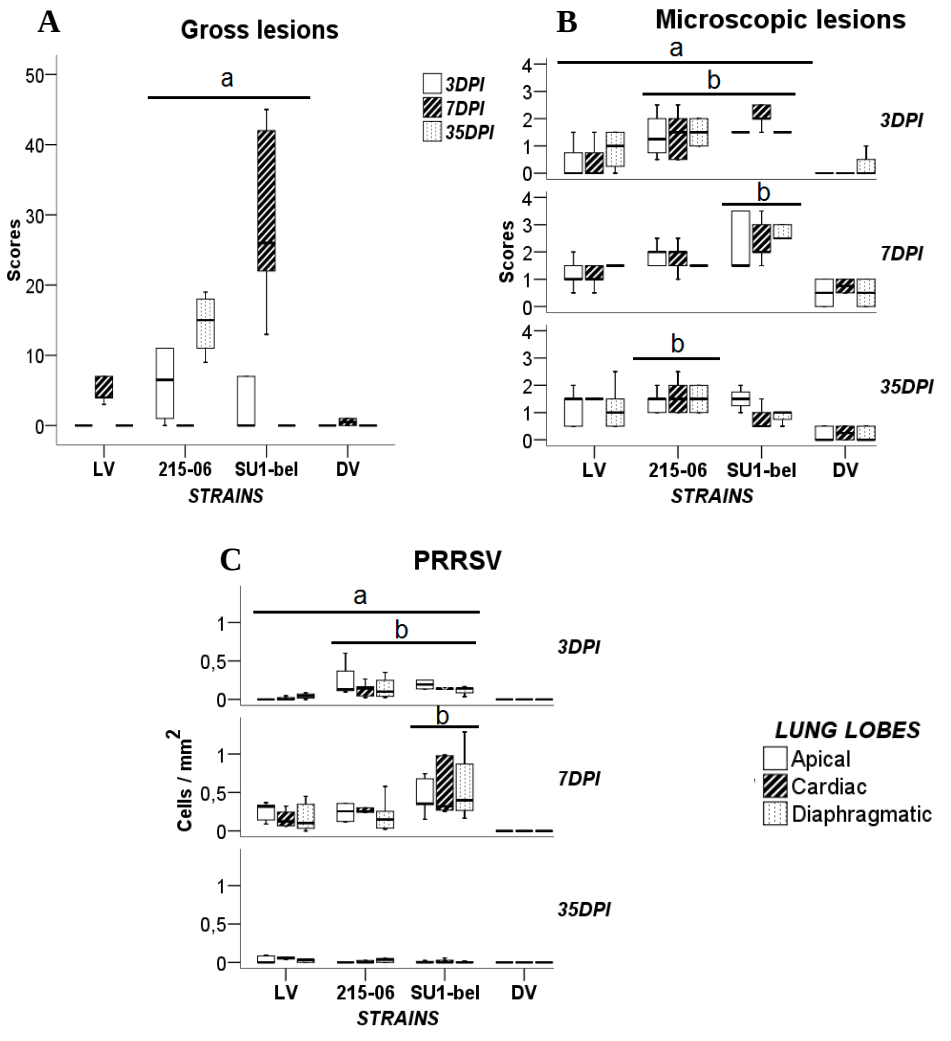

Fig. 1 Gross pathology **(A)** and microscopic lesions **(B)** scores, and counts for PRRSV positive cells **(C)** in lungs from pigs experimentally infected with four different PRRSV-1 strains: LV, 215-06, SU1-bel and DV and compared to animals from the control group. Animals were euthanized at 3, 7 and 35 dpi. Two animals from SU1-bel group were euthanized at 12 and 13 dpi due to welfare considerations. The median is marked with a line, the box shows the 25th– 75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between the inoculated group(s) and DV group at one time-point, whereas the letter "b" indicates significant differences between a specific inoculated group and the remaining groups (both inoculated and DV groups) at one time-point ($p < 0.05$).

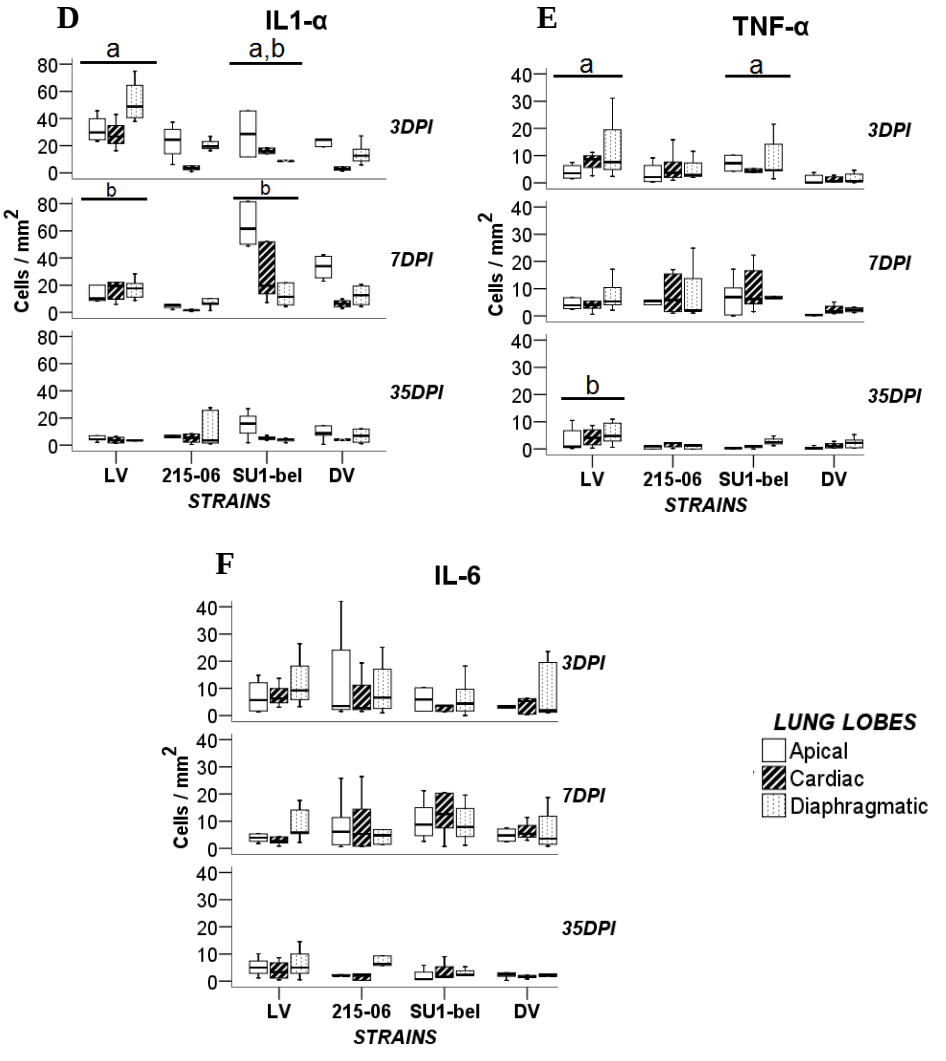

Fig. 1 **(D - F)** counts for IL-1α, TNF-α and IL-6 positive cells in lungs from pigs experimentally infected with four different PRRSV-1 strains: LV, 215-06, SU1-bel and DV and compared to animals from the control group. Animals were euthanized at 3, 7 and 35 dpi. Two animals from SU1-bel group were euthanized at 12 and 13 dpi due to welfare considerations. The median is marked with a line, the box shows the 25th– 75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between the inoculated group(s) and DV group at one time-point, whereas the letter "b" indicates significant differences between a specific inoculated group and the remaining groups (both inoculated and DV groups) at one time-point ($p < 0.05$).

Tissue expression of IFN-γ and IL-10

IFN-γ was expressed mainly in the cytoplasm of septal macrophages and lymphocytes. The kinetics of expression of IFN-γ was similar in LV, 215-06 and SU1-bel infected animals. Statistically significant differences in the expression of IFN-γ were only detected for the variant time-point ($p = 0.001$), without significant differences among any of the PRRSV strains evaluated in this study (Fig. 3A).

IL-10 was expressed in the cytoplasm of PAMs and septal macrophages (Fig. 3B). Statistical analysis of the expression of IL-10 demonstrated significant differences between different time-points ($p = 0.001$), lung lobes ($p < 0.001$) and different strains ($p = 0.035$), but no interaction was detected among them. A significantly enhanced expression of IL-10 was observed in 215-06 infected animals when compared with DV group ($p = 0.027$). In addition, a higher number of IL-10 immunolabelled cells in 215-06 group than in LV group was evidenced at 7 dpi ($p = 0.034$) (Fig. 3C, 3D).

Immunolabelling for IFN-γ and IL-10 was mostly observed in areas of mild to moderate interstitial pneumonia. The apical lung lobe displayed a statistically significant higher expression of IL-10 than cardiac and diaphragmatic lobes ($p < 0.001$ and $p = 0.002$, respectively) and in turn the expression of this cytokine trended to increase in the diaphragmatic lung lobe when compared with the cardiac lobe ($p = 0.055$).

Correlation study

LV and SU1-bel-infected pigs showed a statistically significant positive correlation between the expression of PRRSV antigen and gross lesions (Table 2). In addition, SU1-bel-infected pigs displayed a statistically significant positive correlation between the expression of PRRSV antigen and both microscopic lesions ($r = 0.645$, $p < 0.001$) and the expression of IL1-α ($r = 0.517$, $p < 0.001$) and also between gross lesions and both the expression of IL1-α and IL10 ($r = 0.731$, $p = 0.005$; and, $r = 0.575$, $p = 0.040$, respectively). A significant positive correlation was detected in all groups between the expression of IL-6 and IFN-γ, and also between the expression of IL-6 and IL-10 for 215-06 and DV groups (Table 3). In all groups, but LV-infected pigs, the expression of IFN-γ was positively correlated with the expression of IL-10. In LV and SU1-bel groups the expression of TNF-α displayed a significant positive correlation with the expression of IL-6 and IFN-γ (Table 2).

Table 2. Correlation detected between the expression of PRRSV antigens and gross lesions, microscopic lesions and cytokines in animals infected with each one of PRRSV-1 strains (LV, 215-06 and SU1-bel) throughout the study. NS: Not statistically significant.

	PRRSV/Gross		**PRRSV/Microscopic**		**PRRSV/IL-1α**		**PRRSV/TNF-α**		**PRRSV/IL-6**		**PRRSV/IL-10**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
LV	0.838	< 0.001	0.414	0.008	NS		NS		NS		NS	
215-06	NS		0.322	0.035	NS		NS		NS		0.375	0.013
SU1-bel	0.694	0.008	0.645	< 0.001	0.517	0.001	0.369	0.045	0.336	0.045	NS	

Table 3. Correlation detected between the expression of different cytokines and gross lesions and microscopic lesions in animals infected with each one of PRRSV-1 strains (LV, 215-06, SU1-bel and DV) throughout the study. NS: Not statistically significant.

	IL-1α/ Gross		**IL-1α/ Microscopic**		**TNF-α/IL-6**		**TNF-α/IFN-γ**		**TNF-α/IL-10**		**IL-6/IFN-γ**		**IL-6/IL-10**		**IL-10/ Gross**		**IL-10/IFN-γ**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***	***r***	***p***
LV	0.838	< 0.001	NS		0.766	< 0.001	0.637	< 0.001	NS		0.761	< 0.001	0.437	0.005	NS		0.312	0.050
215-06	NS		NS		NS		0.464	0.002	0.305	0.047	0.715	< 0.001	0.518	< 0.001	NS		0.594	< 0.001
SU1-bel	0.694	0.008	0.517	0.001	0.652	< 0.001	0.713	< 0.001	NS		0.741	< 0.001	0.445	0.007	0.575	0.040	0.593	< 0.001
DV	NS		NS		0.408	0.005	0.404	0.006	NS		0.576	< 0.001	0.510	< 0.001	NS		0.503	< 0.001

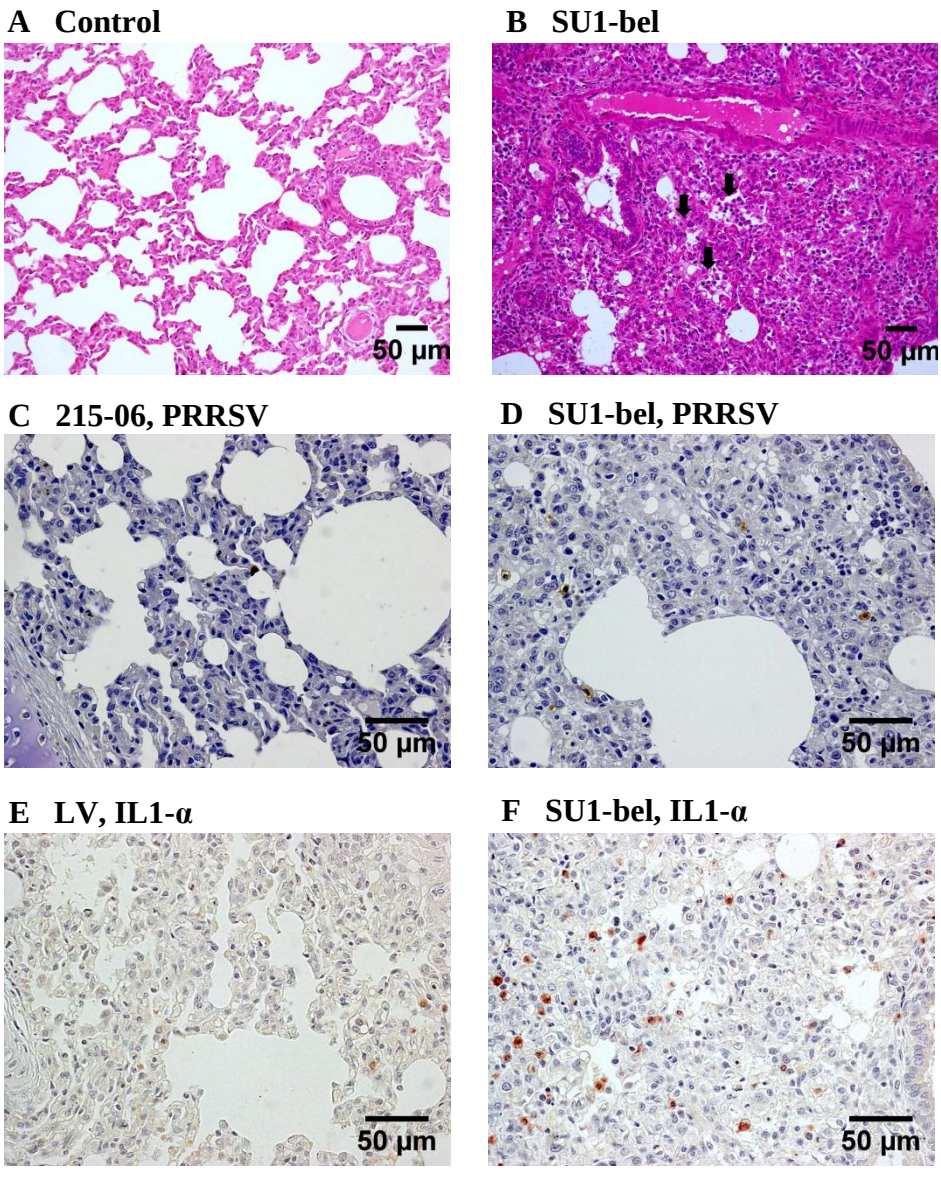

Fig. 2. Representative photomicrographs of the apical lobe of the right lung from a pig from control group **(A)** and from a pig inoculated with SU1-bel strain and euthanized at 7dpi **(B)**. Interstitial pneumonia was characterised by thickened alveolar septa with increased numbers of interstitial mononuclear cells, hyperplasia and hypertrophy of type II pneumocytes and alveoli filled necrotic/apoptotic cell debris and macrophages (black arrow). HE. Bar, 50 µm. Alveolar and septal macrophages expressing PRRSV antigen in pigs from 215-06 and SU1-bel strains, respectively, euthanized at 7dpi. IHC. Bar, 50µm **(C-D)**. Few septal macrophages and neutrophils expressing IL-1α in the lung of a pig from LV group euthanized at 7 dpi. IHC. Bar, 50µm **(E)**. Marked infiltration of macrophages and neutrophils expressing IL-1α in the lung parenchyma of a pig from SU1-bel euthanized at 7 dpi **(F)**. IHC. Bar, 50µm.

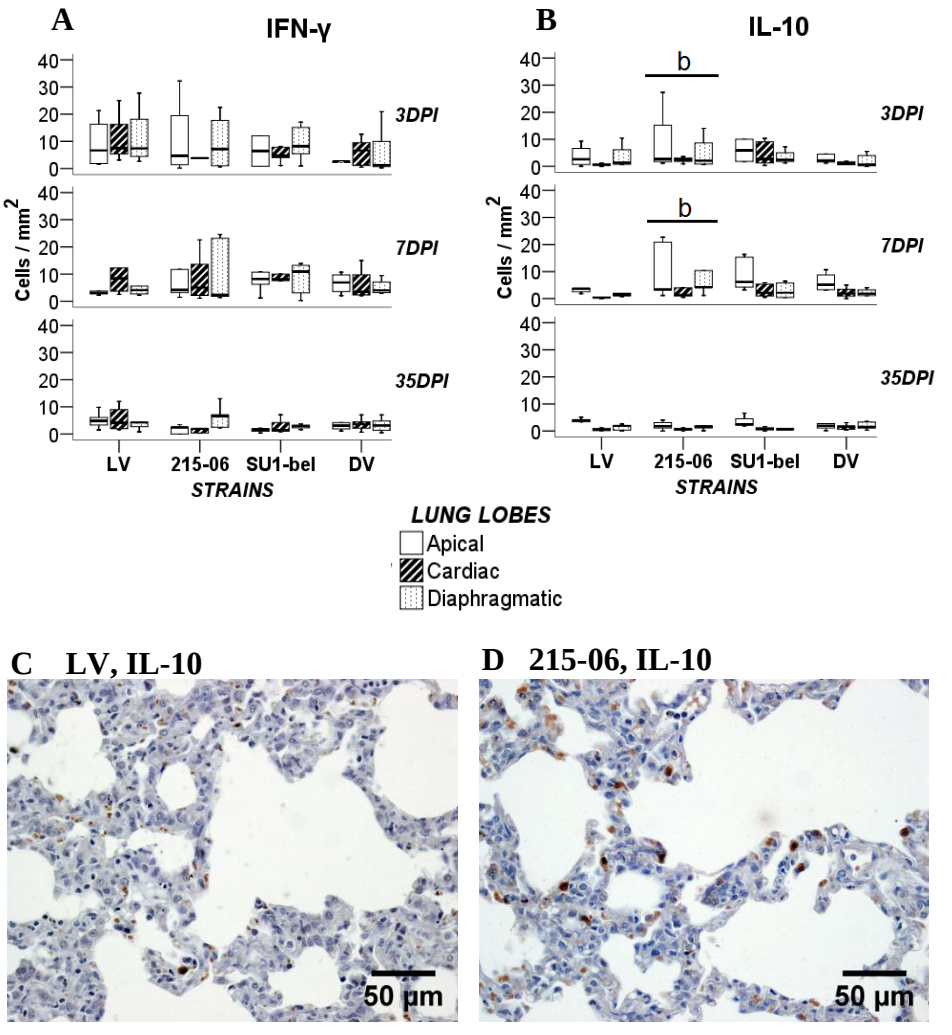

Fig. 3. Counts for IFN-γ and IL-10 in the animals infected with four different PRRSV-1 strains: **(A - B)** Lelystad virus, 215-06 a British field strain, SU1-bel from Belarus and strain DV (Porcilis® PRRS) and in animals from the Control group. Animals were sacrificed at 3, 7 and 35 dpi. Two animals from SU1-bel group were euthanized at 12 and 13 dpi due to welfare considerations. The median is marked with a line, the box shows the 25th– 75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between the inoculated group(s) and DV group at one time-point, whereas the letter "b" indicates significant differences between a specific inoculated group and the remaining groups (both inoculated and DV groups) at one time-point ($p < 0.05$). Photomicrograph of the apical lobe of the lung from pigs from LV and 215-06 strains euthanized at 7dpi showing few alveolar and septal macrophages expressing IL-10 **(C)** and numerous cells expressing IL-10 in the pulmonary parenchyma **(D)**. IHC. Bar, 50µm.

Discussion

An up regulation of proinflammatory cytokines has been correlated with lung pathology and disease during different porcine viral respiratory infections *(van Reeth and Nauwynck, 2000)*. The measurement of the expression of cytokines in the lung of infected animals may contribute to elucidate the mechanisms or pathways used by different PRRSV strains for developing respiratory disease. The immune response against PRRSV infection has previously been analysed by determining the changes observed in different immune mediators, such as cytokines in blood *(Loving et al., 2008; Wang et al., 2011; Guo et al., 2013a; Guo et al., 2013b; Morgan et al., 2013; Weesendorp et al., 2013a)*, whereas other studies characterise the expression of cytokines in situ in target organs, such as lung or lymphoid organs, in PRRSV-infected pigs *(Choi et al., 2002; Chung and Chae, 2003; Thanawongnuwech et al., 2003; Chung et al., 2004; Gomez-Laguna et al., 2010; Barranco et al., 2012 a,b)*.

Differences in pathogenicity have been reported between strains belonging to subtypes 1, 2 and 3 of the European genotype *(Karniychuk et al., 2010; Han et al., 2013a; Morgan et al., 2013, 2014; Weesendorp et al., 2013a, 2014)*. Thus the present manuscript compared the expression of cytokines in the lung of pigs infected with a highly pathogenic subtype 3 PRRSV-1 strain (SU1-bel strain) with low virulent strains of the pan-European subtype 1 (LV, 215-06 and DV strains).

In our study, the animals infected with the SU1-bel strain developed the highest clinical score and more severe gross and microscopic lesions compared to the LV, 215-06 and DV groups.

Besides, in this group two animals were euthanized because they had prolonged fever and high clinical scores.

All animals infected with subtype 1 and subtype 3 PRRSV-1 strains expressed PRRSV antigen in the lung throughout the study with a maximum expression at 7 dpi. The highest replication of PRRSV was detected in the SU1-bel group and a positive correlation was observed between the number of PRRSV-positive cells and gross pathology in the lung of SU1-bel and LV infected animals. Moreover, a positive correlation was detected between the expression of PRRSV and the expression of IL-1α in SU1-bel group, which suggests that the virus induces the expression of IL-1α in the lung of PRRSV-infected animals inducing the typical interstitial pneumonia of this disease. Our results are in agreement with those recently published by *Weesendorp et al. (2014)* who reported a higher number of PRRSV positive cells and an increase in the IL-1β mRNA expression in the lung of Lena-infected animals than in animals infected with other PRRSV-1 strains (LV, Belgium A).

PRRSV replicated mainly in PAM and all cytokines were mostly detected in the cytoplasm of septal macrophages and for IL-1α also in the cytoplasm of neutrophils. Similar results have previously been published by our research group *(Gómez-Laguna et al., 2010)* and support the hypothesis that septal macrophages play a key role in the synthesis of cytokines in pigs infected with PRRSV *(Gómez-Laguna et al., 2013)*. In the present study the profile of lung expression of TNF-α and IL-6 was similar for each infected-group, nonetheless the expression of IL-1α differed significantly among groups. Animals infected with the SU1-bel strain trended to display the highest expression of all three

proinflammatory cytokines at 7 dpi compared to the other PRRSV-infected groups. Our results evidence a main role of IL-1α in the onset of the local inflammatory response during PRRS in the lung, which may synergistically act together with other proinflammatory mediators in the development of the lesions in the lung and the clinical signs in infected pigs *(Van Reeth and Nauwynck, 2000; Kimura et al., 2013)*. This idea is supported by the significant positive correlation between the levels of TNF-α and IL-6 in LV and SU1-bel groups, which is suggestive of the synergistic action among proinflammatory cytokines *(Van Reeth et al., 2002)*.

The data presented in this manuscript show that the highly virulent subtype 3 SU1-bel PRRSV strain replicates more efficiently and induce more severe lesions in the lung of infected animals, mainly associated to a higher expression of IL-1α, than other moderately virulent subtype 1 PRRSV strains, such as LV and 215-06 strains, or the attenuated vaccine strain DV.

In a parallel study carried out by *Morgan et al. (2013)* a significantly higher viral load in serum and BALF in LV-infected animals when compared to SU1-bel and 215-06 infected animals at 3 and 7 dpi was reported. These authors suggested that the virulence of the SU1-bel strain could be associated to an enhanced inflammatory immune response and not to a higher viral replication capability. Our results highlight that the higher virulence reported for the SU1-bel strain could be associated both to a higher local PRRSV replication rate and to an increased expression of IL-1α in the lung parenchyma.

In our study, the kinetics of IFN-γ was similar in all PRRSV infected groups showing only a mild to moderate enhancement at 3 dpi. In a previous study carried out by our group an enhancement in the expression of IFN-γ in pigs infected with the strain 2982, which presents a 93% of homology with LV, was already detected at 3 dpi, coinciding with the results observed in the present study *(García-Nicolás et al., 2014b)*. Furthermore, the results of the present study are partially in agreement with the PRRSV-specific IFN-γ response in the blood of SU1-bel-infected animals previously reported by *Morgan et al. (2013)*, who found an enhancement only between 14 and 28 dpi with a peak at 21 dpi. This increase in the expression of IFN-γ has also previously been reported in infections with a HP-PRRSV-2 strain, from 10 to 28 dpi *(Thanawongnuwech et al., 2003)*, whereas other studies with the subtype 3 PRRSV strain Lena could not evidence changes in the expression of this cytokine *(Weesendorp et al., 2013a)*.

IFN-γ participates in regulating the immune response and its production by pulmonary macrophages is induced by the expression of other cytokines including TNF-α and IFN-α *(Gómez-Laguna et al., 2010)*. Interestingly, a significant positive correlation between the expression of IFN-γ with respect to the expression of TNF-α and IL-6 was detected in all groups infected with PRRSV in our study, which suggests that proinflammatory cytokines may play a role in the early induction of IFN-γ in PRRS.

PRRSV strains may induce the production of IL-10 *(Suradhat et al., 2003; Díaz et al., 2006; Gimeno et al., 2011; Wang et al., 2011)*, which has been proposed as a mechanism which may prolong viraemia and inhibition of

cell mediated immunity at an early stage of infection *(Díaz et al., 2006; Wang et al., 2011)*. According to *Gimeno et al. (2011)*, PRRSV-1 strains may be classified as IL-10, TNF-α, both IL-10 and TNF-α inducers or none of them. In the present study the expression of IL-10 was significantly higher in the lungs of 215-06 infected pigs and in a lesser extent in SU1-bel-infected pigs. In the 215-06 group the expression of IL-10 was positively correlated with the expression of PRRSV antigen and the expression of TNF-α, pointing out that this strain may be a strain inducer of IL10 and TNF-α. The 3262 PRRSV-1 strain, classified also as IL-10^{+}TNF-α^{+} by *Gimeno et al. (2011)*, was characterized by a down-regulation of the co-stimulatory molecules CD80/86 which may be a mechanism of PRRSV strains with this cytokine profile to avoid an efficient host immune response. The higher expression of IL-10 detected in the animals infected with the PRRSV strain 215-06 than LV and DV strains was associated with a lower expression of IL-1α which may be the cause of the moderate virulent of this strain. In addition all infected groups had a significant positive correlation between the expression of IL-10 with the expression of IFN-γ and IL-6, confirming the role of this cytokine as an immunomodulatory and anti-inflammatory cytokine in PRRSV infection.

The absence of clinical signs, gross lesions and PRRSV replication in all animals inoculated with DV strain suggests that there was no infection in these animals, but a mild interstitial pneumonia associated to a mild expression of IL-1α and IL-10 at 7 dpi was found. Modified-live DV vaccines are known to induce a relatively weak cell mediated immunity *(Charerntantanakul, 2012)*, which is in agreement with the lack of significant changes in the expression of IFN-γ in the lung.

In conclusion, the results of the present study indicates that subtype 3 European SU1-bel strain replicated more efficiently in the lung of infected animals, as well as induced a higher expression of IL-1α than what LV, 215-06 and DV strains which may have had a key role in the increased pathogenicity and the development of the onset of the clinical signs.

III. THYMIC DEPLETION OF LYMPHOCYTES IS ASSOCIATED WITH THE VIRULENCE OF PRRSV-1 STRAINS.

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Introduction

Porcine reproductive and respiratory syndrome virus is a single stranded positive sense RNA virus belonging to the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* *(Meulenberg, 2000; Snijder et al., 2013)*. PRRSV is divided into two distinct viruses, type 1 (formerly European; PRRSV-1) and type 2 (formerly North American; PRRSV-2), with significant genetic differences between both genotypes *(Salguero et al., 2015)*. Sequence analysis of different PRRSV-1 strains led to the definition of three distinct subtypes, namely pan-European subtype 1 and East European subtypes 2 and 3, and recently a fourth subtype has been proposed *(Stadejek et al., 2013)*. Highly pathogenic PRRSV strains firstly emerged in China and were classified within the PRRSV-2 genotype; these strains caused high morbidity and mortality, high fever and multifocal skin haemorrhages in pigs of all ages *(Tong et al., 2007)*. Later HP-PRRSV strains were also identified in Eastern Europe *(Karniychuk et al., 2010)*. The emergence of HP-PRRSV in Asia and Eastern Europe have revived the interest in understanding the immunobiology of PRRSV strains of differing virulence *(Gómez-Laguna et al., 2013; Salguero et al., 2015)*.

Differences in the virulence among PRRSV-1 strains have been associated with either an enhanced inflammatory immune response, mainly associated to an increased pulmonary expression of IL-1α/β, or to

higher levels of virus replication *(Gómez-Laguna et al., 2010; Karniychuk et al., 2010; Morgan et al., 2013, 2014; Weesendorp et al., 2013a; Amarilla et al., 2015; Salguero et al., 2015)*. As of yet the exact mechanisms by which PRRSV-1 exert its virulence are unknown.

The thymus is the lymphoid organ responsible for T lymphocyte differentiation and maturation and it is essential for the normal development and function of the immune system *(Pearse, 2006b)*. A decrease in the relative thymus weight, associated to a decreased cellularity in the cortex and less commonly in the medulla, is a sensible indicator for immunosuppresion *(Elmore, 2006; Pearse, 2006 a,b)*. Cortical involution of the thymus with a poor demarcation of cortico-medullary boundary and apoptosis of cortical thymocytes have been reported in PRRSV-2 infected piglets but data for PRRSV-1 strains are very scarce *(Feng et al., 2002; Wang et al., 2011; He et al., 2012; Li et al., 2014)*.

Apoptosis of immune cells can be involved in the immunopathogenesis of viral diseases and has been suggested to play a significant role in PRRSV infection, with increased apoptotic cells widely distributed within both PRRSV-1 and PRRSV-2 infected tissues *(Feng et al., 2002; Labarque et al., 2003; Wang et al., 2011; Gómez-Laguna et al., 2012; He et al., 2012; Li et al., 2014; Morgan et al., 2014; Rodríguez-Gómez et al., 2014)*. PRRSV replicates in thymic macrophages and dendritic cells *(Halbur et al., 1996 a,b)*, which through their interaction with thymocytes can deliver pro-apoptotic signals *(He et al., 2012; Li et al., 2014)*. Several cytokines including TNF-α and IL-1α/β, promote apoptosis of T cell lines *(Feng et al., 2002; Salguero et al., 2005)*. In addition, during the course of PRRSV infection macrophages and to a lesser extent neutrophils and

lymphocytes have been shown to upregulate the expression of IL-1α, IL-6 and TNF-α *(Labarque et al., 2003; Gómez-Laguna et al., 2010; Barranco et al., 2012a)*.

Due to the limited available data on the effects of PPRVS-1 infection on the thymus, the present study aimed to evaluate the impact of PRRSV-1 strains of different virulence in the thymus of piglets by analysing the histomorphometry, the presence of apoptotic phenomena and the local expression of cytokines.

Materials and Methods

Animal selection and housing, viruses, experimental design and animal monitoring used in this experiment has been described above in the section "Common experimental design".

Table 1. Distribution in this study of all pigs experimentally infected within each group, namely control and three different PRRSV-1 strains: LV, 215-06 and SU1-bel. Animals were euthanized at 3, 7 and 35 dpi.

DPI	Control	LV[a]	215-06[b]	SU1-bel[c]
3	3	4	5	5
7	4	5	5	5
35	3	5	5	5*

a LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain; **b** 215-06: British field strain; **c** SU1-bel: High virulence strain; * Two animals in the SU1-bel group displayed a prolonged fever along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi.

Histopathology and histomorphometry analysis of thymus

Four micrometre tissue sections were stained with haematoxylin and eosin (H&E). The severity of the lesions in thymus was scored as follows *(adapted and modified from Contreiras et al., 2004)*:

Grade 0: The cortex:medulla ratio (C/M) is about 2:1 with typical histological characteristics of the thymus.

Grade I: Diffuse cortical reduction with focal cortical disappearance, 5-9 tingible-body macrophages/mm^2 within the thymic cortex, typical medulla and extraparenchymal compartment, which includes capsule, interlobular connective tissue septa and perivascular spaces (PVS).

Grade II: Focal or multifocal decrease of C/M (< 2:1), decrease of cortical layer with slight proportional increase of the extraparenchymal compartment and 10-15 tingible-body macrophages/mm^2 within the thymic cortex.

Grade III: Focal to multifocal disappearance of cortico-medullary boundary distinction, increase of the extraparenchymal compartment, mainly PVS, occasional increase in the number of lymphocytes, mast and plasma cells and ≥16 tingible-body macrophages/ mm^2, which give the tissue a "starry sky" appearance.

Histomorphometry was conducted using the image analysis software ImageJ 1.45 and NIS Elements BR (4.20.00 Build 967, 64 bits, University of Surrey, Guildford, UK). H&E -stained sections of the thymus were photographed to produce digital images. The percentage of

the parenchyma (thymic cortex and thymic medulla) and stroma (capsule, interlobular connective tissue septa, PVS and adipose tissues) were assessed in a field of 400 mm^2. Differentiation of the parenchyma and stroma were performed according to the typical histological features of the thymus *(Pearse, 2006b)*. Automatic and manual quantification of thymocytes and tingible-body macrophages in thymic cortex were assessed in 25 non-overlapping, consecutively selected high magnification fields of 0.2 mm^2. Results were expressed in number of cells per mm^2.

Immunohistochemistry

Labelled cells were analysed in 25 non-overlapping and consecutively selected high magnification fields of 0.2 mm^2. The assessment if the expression of CD3 was calibrated according to the intensity of immunollabeling with threshold colour adjustment and expressed in percentage. While the expression of MAC387, cytokines and apoptosis cells (cCasp3 and TUNEL) were manual counted and expressed as the number of cells per mm^2. Immunolabelled cells were identified and counted, and morphologically differentiated as macrophages, thymocytes or neutrophils.

Results

Histopathology score and histomorphometry analysis of thymus

Control animals did not show significant microscopic changes in the thymus throughout the study. In the infected animals the microscopic lesions were characterised by diffuse cortical reduction with focal

cortical disappearance, slight increase of the extraparenchymal compartment and focal to multifocal cortico-medullary inversion and, occasionally variable number of macrophages, mast cells and eosinophils were observed (Figs. 1A, C, E and G). *Table 3* summarises the microscopic scores for the thymus for each group and time-point.

Two animals infected with the SU1-bel strain and one animal infected with the LV strain displayed focal to multifocal disappearance of cortico-medullary boundary with an increase of the extraparenchymal compartment, mainly PVS, at 7 dpi. A significant increase in the number of tingible-body macrophages in the thymic cortex was detected between different strains ($p < 0.001$), with 215-06 and SU1-bel groups differing significantly from the control group ($p \leq 0.003$) (Figs. 1B, D, F and H). Furthermore, at 3 and 7 dpi, SU1-bel group displayed more significant differences when compared with LV and 215-06 groups ($p \leq 0.03$) and 215-06 group displayed more significant differences with respect to LV group ($p < 0.009$) (Fig. 2A).

Statistically significant association was detected between different strains and the histopathology score ($p < 0.001$), mainly at 7dpi; besides, SU1-bel group displayed more significant differences of the histopathology score when compared with 215-06 group ($p < 0.03$).

The histomorphometry analysis of the thymus displayed significant differences in the percentage of parenchyma and stroma of the thymus between different strains and the control group ($p < 0.001$) (Fig. 2B). In addition, statistically significant differences were observed

in the percentage of thymic stroma at different time-points ($p < 0.005$). The percentage of the thymic cortex showed significant differences between different strains and control group ($p \leq 0.002$) (Fig. 2C). Moreover C/M ratio in LV and SU1-bel infected animals showed the lowest ratio at 7 dpi ($p < 0.05$) (Table 2).

The number of thymocytes in the thymic cortex showed statistically significant differences at different time-points ($p = 0.001$) and between different strains and control group ($p \leq 0.011$). SU1-bel group displayed more statistically significant differences when compared with the other PRRSV-1 strains at 7 and 35 dpi ($p < 0.0001$) (Fig. 2D).

Table 2. Cortex:Medulla ratio and histopathology score in the thymus of piglets from each group at 3, 7 and 35 dpi in relation to the total number of animals per group, classified according to the ratio and scores obtained.

DPI	Ratio	1:1	2:1	Histophatology Score	Control	LV	215-06	SU1-bel
	Control		3/3	**Grade 0**	3/3	2/4	2/5	1/5
	LV		4/4	**Grade I**		2/4	2/5	2/5
3	**215-06**	1/5	4/5	**Grade II**			1/5	2/5
	SU1-bel		5/5	**Grade III**				
DPI	**Ratio**	**1:1**	**2:1**	**Histophatology Score**	**Control**	**LV** [a]	**215-06** [a]	**SU1-bel** [a,c]
	Control		4/4	**Grade 0**	4/4			
	LV [a]	2/5	3/5	**Grade I**		3/5	2/5	
7	**215-06**		5/5	**Grade II**		1/5	3/5	3/5
	SU1-bel [a]	2/5	3/5	**Grade III**		1/5		2/5
DPI	**Ratio**	**1:1**	**2:1**	**Histophatology Score**	**Control**	**LV**	**215-06**	**SU1-bel**
	Control		3/3	**Grade 0**	3/3	3/5	2/5	
	LV		5/5	**Grade I**		1/5	2/5	2/3
35	**215-06**		5/5	**Grade II**		1/5	1/5	
	SU1-bel		3/3	**Grade III**				1/3

Normal Cortex:Medulla ratio: 2:1. Grade 0: normal histology. **Grade I:** Diffuse cortical reduction with focal cortical disappearance, about 5 to 9 tingible-body macrophages by mm^2 within the thymic cortex. **Grade II:** Focal or multifocal decrease of C/M (< 2:1), decrease of cortical layer with slight proportional increase of the extraparenchymal compartment and about 10 to 15 of tingible-body macrophages by mm2 within the thymic cortex. **Grade III:** Focal to multifocal disappearance of cortico-medullary boundary distinction, increase of the extraparenchymal compartment and higher number of tingible-body macrophages (≥ 16 / mm^2), which give the tissue a "starry sky" appearance. Different letters indicate statistically significant differences ($p \leq 0.05$): "a" Statistically significant differences between different strains and control group; "b" Statistically significant differences between different time-points; "c" Statistically significant differences with low virulent strains (LV and/or 215-06 strains).

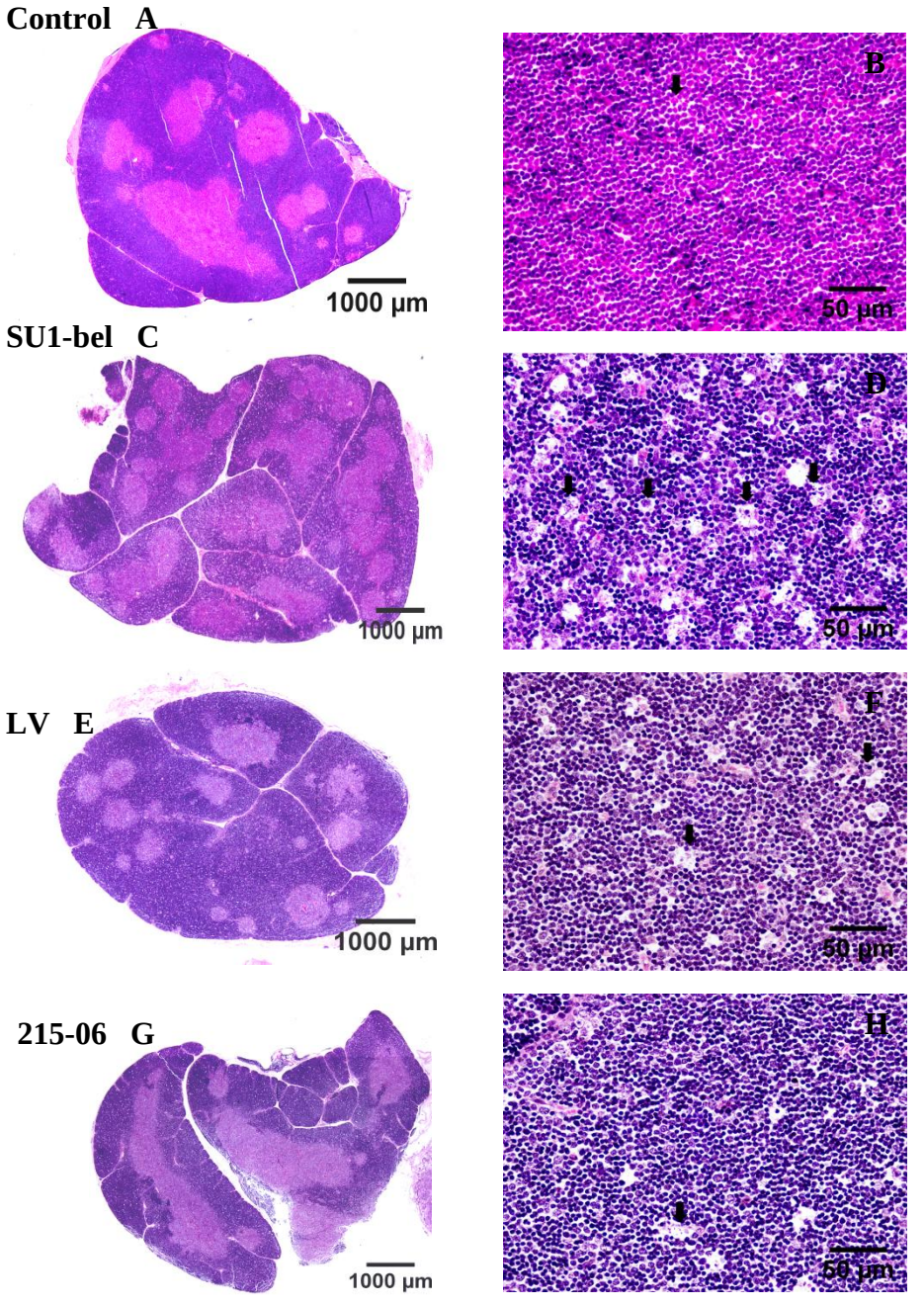

Fig. 1. Representative photomicrographs of the thymus from a pig from control group and from pigs inoculated with different PRRSV-1 strains and euthanized at 7 days post-infection. H&E stained thymus sections a pig from **(A-B)** control group and from pigs infected with **(C-D)** SU1-bel, **(E-F)** LV and **(G-H)** 215-06 strains are presented. Bar, 1000 µm **(A, C, E and G)** and 50 µm **(B, D, F and H)**. The SU1-bel infected animal thymus presented a grade III histopathology score, whereas the LV and 215-06 infected animals presented a grade II. Focal to multifocal disappearance of cortico-medullary boundary distinctions are shown by white arrows, tingible-body macrophages by black arrows.

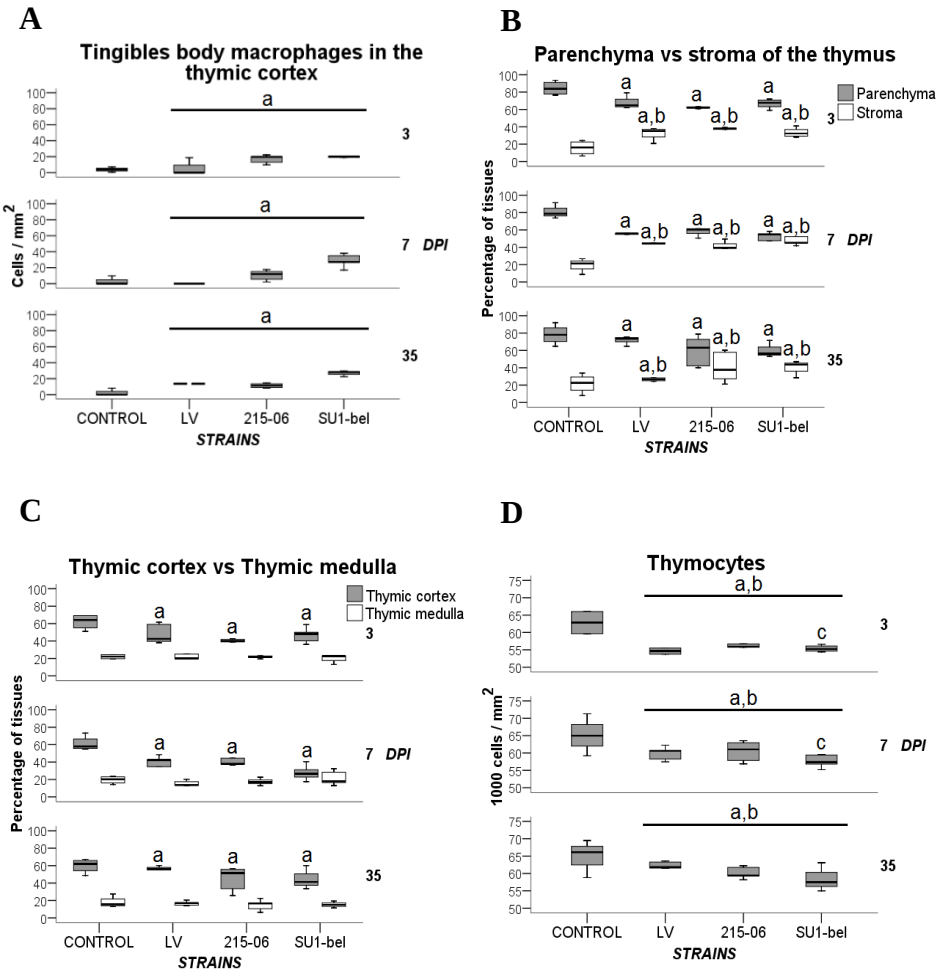

Fig. 2. Graphs representing the percentage of the tissue in the thymus and number of tingible-body macrophages and thymocytes in the cortex of the thymus at all time-points in all virus-infected animals and control group. **(A)** number of the tingible-body macrophages, **(B)** percentage of parenchyma *vs* stroma, **(C)** percentage of the thymic cortex *vs* thymic medulla, and **(D)** thymocytes per mm^2 in the thymus in control animals and in the animals infected with PRRSV-1 strains differing in virulence. Animals were euthanized at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25^{th}–75^{th} percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains and the control group, whereas the letter "b" indicates significant differences between different time-points and "c" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. The asterisks indicate statistical significant differences between thymic cortex and thymic medulla. Statistical significant differences were considered at p-values ≤ 0.05.

Evidence of PRRSV replication in the thymus

PRRSV antigen was detected at all time-points in all virus-infected animals except in control group. The highest number of PRRSV antigen expressing cells was observed in SU1-bel group and secondly in LV group, whereas 215-06 group displayed the lowest viral expression. Statistical analysis of the expression of PRRSV antigen evidenced significant differences between different strains ($p = 0.003$) (Fig. 3A).

Tissue distribution of macrophages and T cells in the thymus

The expression of MAC387 as a marker of macrophages was higher in the thymic medulla than in the cortex ($p \leq 0.01$) being mainly observed in the cytoplasm of macrophages and monocytes around the Hassall´s corpuscles, PVS in the thymic medulla and in a lesser extent in PVS, subcapsular region and areas of decreased cellular density in the cortex (Figs. 4A, C, E and G). Tingible-body macrophages did not stain for MAC387. A statistically significant interaction between different strains and time-points was observed for the expression of MAC387 in the medulla of the thymus ($p = 0.005$), whereas statistically significant differences were only observed between different strains in the cortex ($p < 0.035$). Specifically, SU1-bel group displayed a significant increase in the number of MAC387 positive cells in both cortex and medulla of the thymus when compared with the control group ($p = 0.018$) (Fig. 3B).

CD3 as a marker for T cells was observed mainly in the thymic cortex and only in some scattered cells in the medulla (Figs. 4B, D, F and H). The SU1-bel group displayed the lowest percentage of the expression of CD3 in the thymus cortex, presenting statistically

significant differences with respect to LV and 215-06 groups ($p < 0.001$) at 7 and 35 dpi. Besides, the expression of CD3 in the thymic cortex was lower in all PRRSV-1 strains compared to control animals at the different time-points ($p < 0.0001$) (Fig. 3C).

Apoptosis in the thymus of PRRSV-1 infected pigs

cCasp3 staining was mainly found in the cortex of the thymus ($p \leq 0.02$), within the nuclei of cells and secondly in free apoptotic bodies (Figs. 5B, D, F, H, and insets). The expression of cCasp3 yielded statistically significant differences both in the thymic cortex and medulla between different time-points ($p < 0.001$) and between the infected and control group ($p \leq 0.051$). The expression of cCasp3 displayed a significant increase in the thymic medulla of SU1-bel group at 3 dpi when compared to 215-06 group ($p < 0.03$) (Fig. 3D).

TUNEL labelling, was mostly observed in the cortex and to a lesser extent in the medulla of the thymus of all piglets including the control group (Figs. 5A, C, E and G). TUNEL labelling was mainly observed in apoptotic bodies inside macrophages and in only occasionally in free apoptotic bodies (Fig. 5C inset). Statistically significant differences in the expression of TUNEL in thymic cortex were observed between different time-points and between the infected and control group ($p < 0.001$ respectively). In the medulla of the thymus, statistically significant differences were only detected at different time-points ($p = 0.025$). SU1-bel group displayed a significant increase in apoptosis cells in both the thymic cortex and medulla at 3 and 7 dpi when compared with 215-06 group ($p < 0.03$) and at 7 dpi in the thymic cortex when compared with LV group ($p < 0.01$) (Fig. 3E).

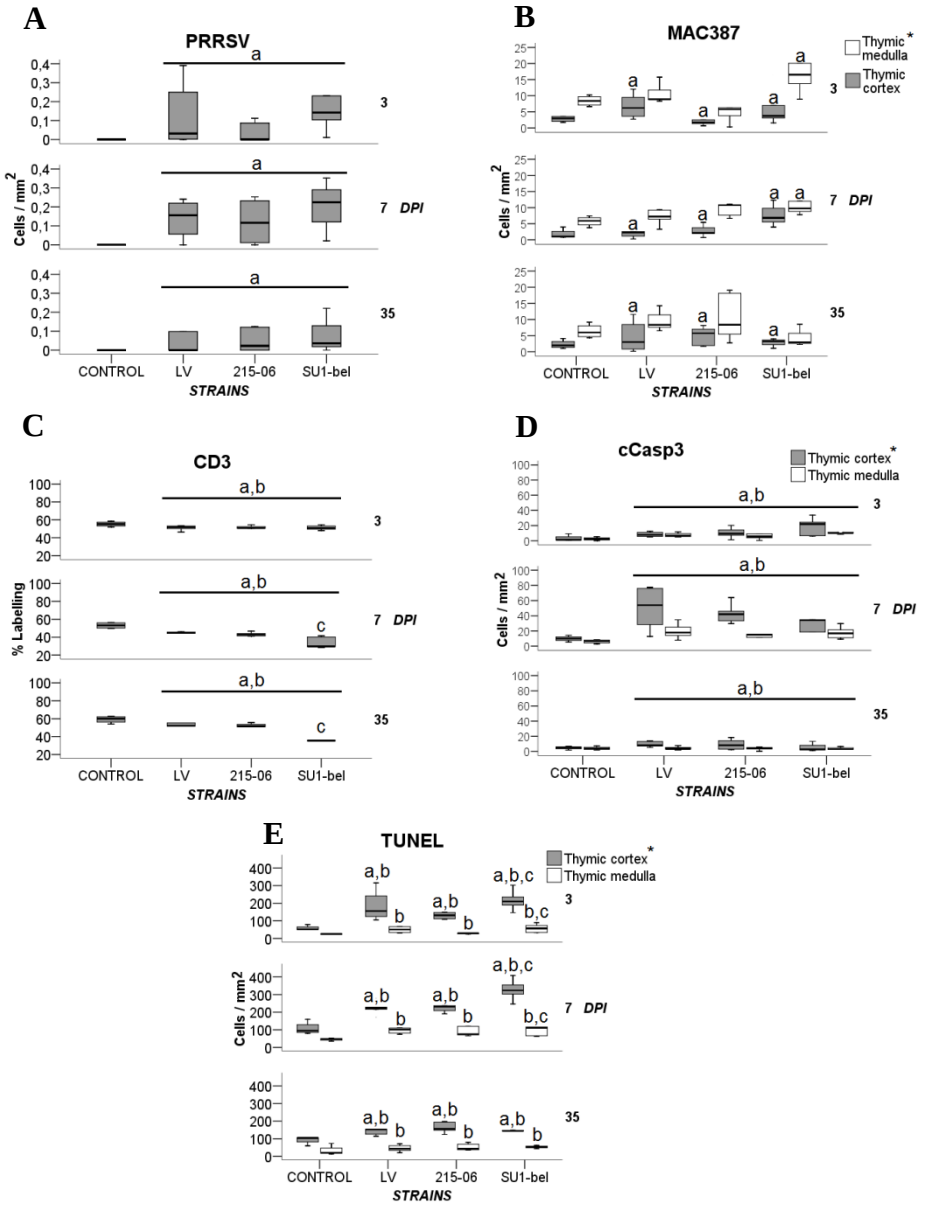

Fig. 3. Graphs representing the percentage of the expression of CD3 and the number of immunostained PRRSV-antigen, MAC387 and apoptosis-positive cells per mm^2 in the thymus from all the groups at all time-points. **(A)** counts for PRRSV-antigen, **(B)** percentage of the expression of CD3 in the thymic cortex, **(C)** counts for MAC387-positive cells, **(D)** counts for TUNEL and **(E)** cCasp3-positive cells in the thymus of control animals and animals infected with the three different PRRSV-1 strains included in the study. Animals were euthanized at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25^{th}–75^{th} percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains and control group, whereas the letter "b" indicates significant differences between different time-points and "c" indicates significant differences with low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. The asterisks indicate statistical significant differences between thymic cortex and thymic medulla. Statistical significant differences were considered at p-values ≤ 0.05.

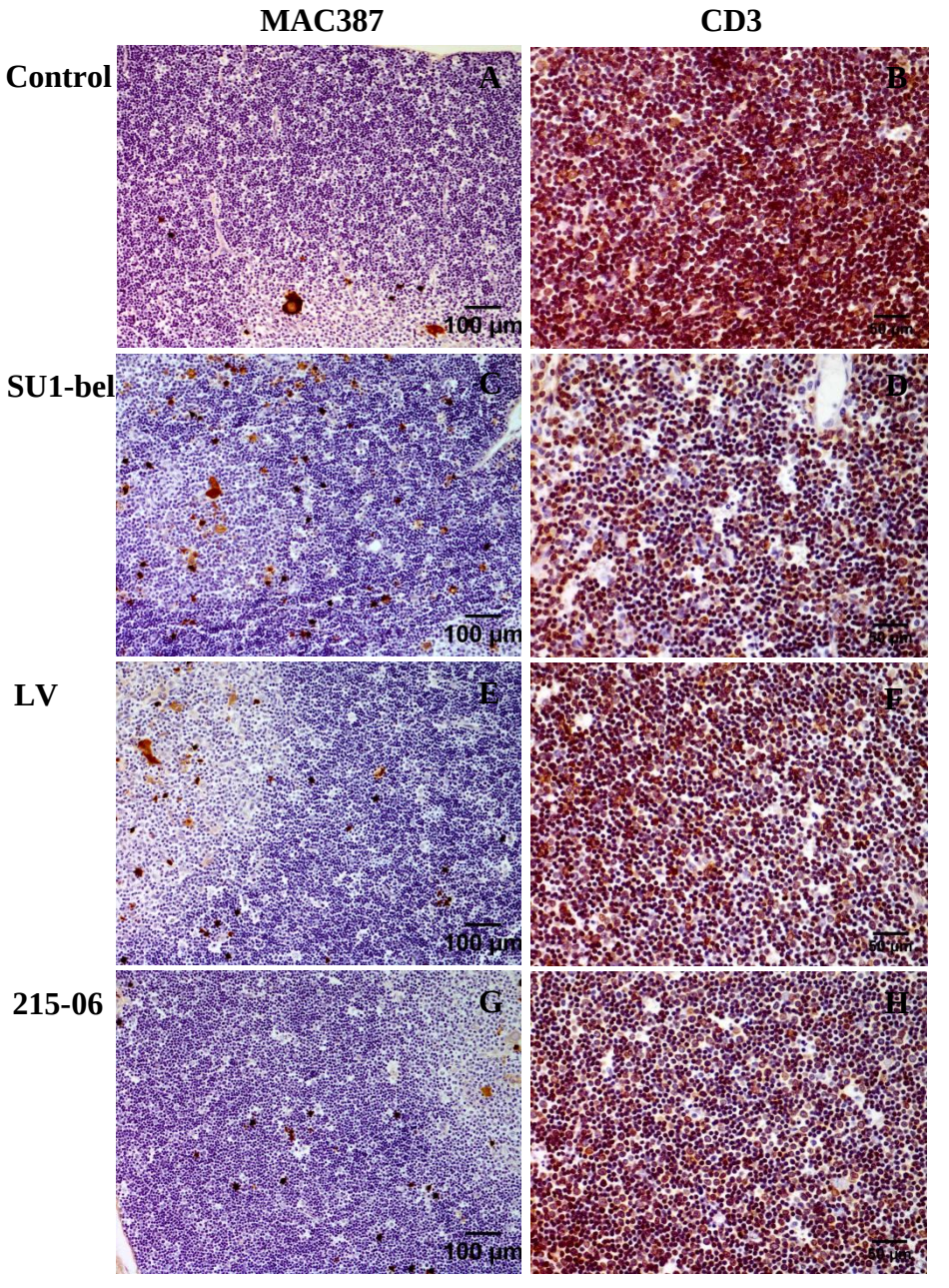

Fig. 4. Representative photomicrographs of MAC387 and CD3 immunollabeling in the thymus from a pig from control group and from pigs inoculated with different PRRSV-1 strains and euthanized at 7 dpi. IHC stained thymus sections from **(A-B)** a control pig and from pigs infected with **(C-D)** SU1-bel, **(E-F)** LV and **(G-H)** 215-06 strains are presented. Bar, 100 µm and 50 µm. The SU1-bel group showed an increase in the number of MAC387 positive cells in both cortex and medulla of the thymus and the lowest labelled of CD3 in the thymic cortex when compared with the control group and other PRRVS-1 strains.

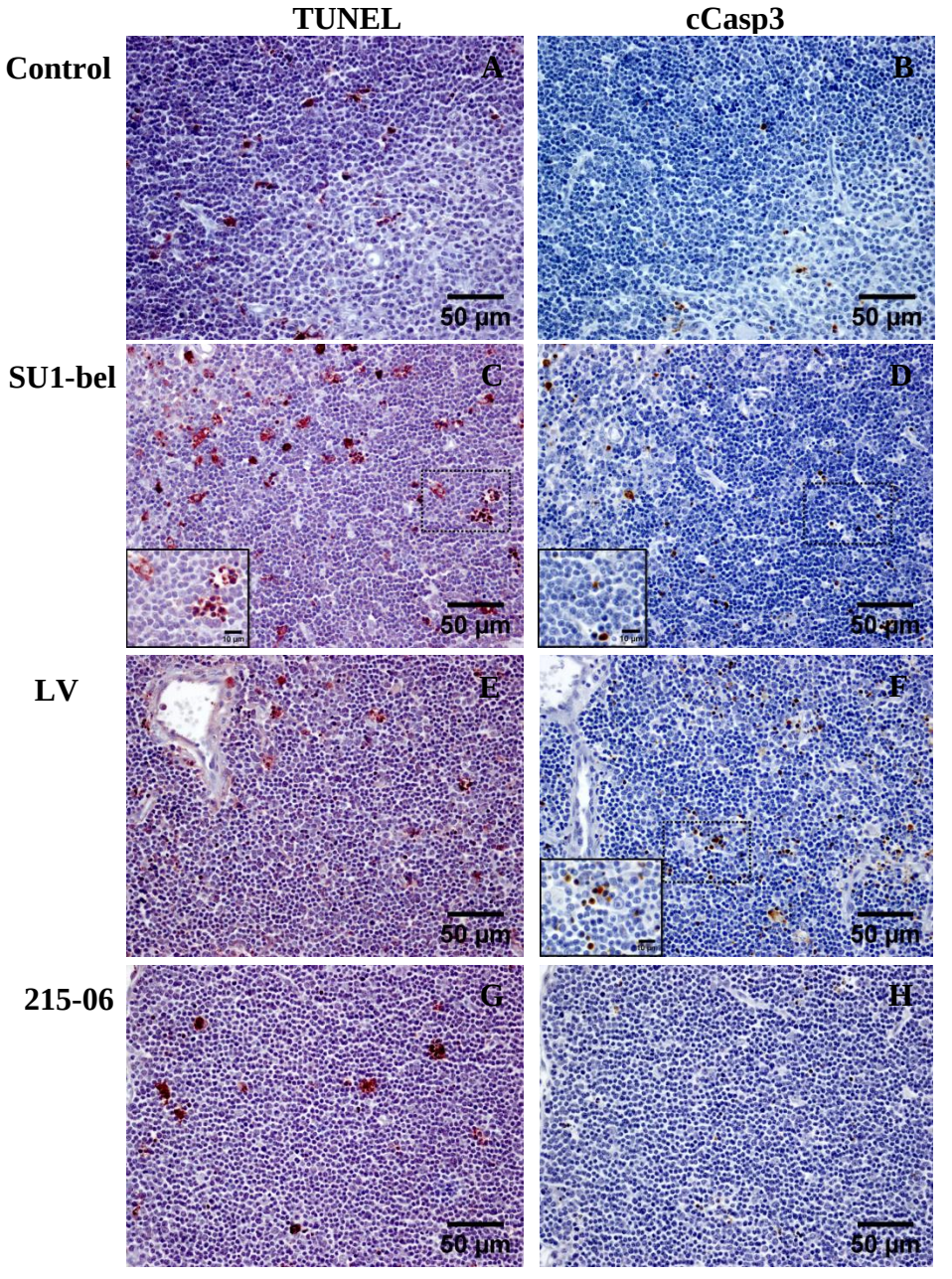

Fig. 5. Representative photomicrographs of TUNEL and cCasp3 labelling in the thymus from a pig from control group and from pigs inoculated with different PRRSV-1 strains and euthanized at 7 dpi. TUNEL labelling of tingible-body macrophages and apoptotic bodies in the thymic cortex of **(A)** a control animal, **(C)** SU1-bel, **(E)** LV and **(G)** 215-06 infected animal. TUNEL. Bar, 50 µm. **C**, *inset*: detail of tingible-body macrophages labelled with TUNEL method. TUNEL. Bar, 10 µm. cCasp3 labelling of lymphocytes and macrophages in the thymus of **(B)** a control animal, **(D)** SU1-bel, **(F)** LV and **(H)** 215-06 infected animal. IHC. Bar, 50 µm. **D and F**, *insets*: detail of a lymphocyte **(D)** and a macrophage **(F)** immunolabelled against cCasp3. IHC. Bar, 10 µm.

Expression of cytokines in the thymus

The expression of IL-1α, TNF-α and IL-10 was mainly detected in the cytoplasm of macrophages and to a lesser extent in the cytoplasm of neutrophils and lymphocytes in the thymic medulla ($p \leq 0.01$) (Figs. 6A, C and E). The expression of IL-1α did not display statistically significant changes between different strains and time-points (Figs. 6B). At 3 dpi, the LV group displayed a significant increase in the expression of TNF-α when compared to control group ($p = 0.016$) and 215-06 group ($p = 0.032$) (Fig. 6D). A significant increase in the expression of IL-10 was observed in LV and SU1-bel groups at 3 dpi when compared with the control group ($p = 0.006$ and $p = 0.004$, respectively) (Fig. 6F).

Correlation of apoptosis and cytokine expression with PRRSV-1 infection

In the thymic cortex a significant negative correlation was found between the expression of cCasp3 and the percentage of parenchyma in LV and SU1-bel groups at different time-points ($r \geq - 0.70$; $p \leq 0.007$); whereas a significant negative correlation was observed between the expression of cCasp3 in the cortex and the expression of CD3 in the 215-06 group ($r = - 0.74$; $p < 0.001$). In the SU1-bel group at 3 dpi a significant positive correlation between the expression of TUNEL and PRRSV antigen in the thymic medulla ($r = 0.90$; $p = 0.037$), and in the thymic cortex between cCasp3 and PRRSV antigen ($r \geq 0.90$; $p \leq 0.037$); and between TUNEL and PRRSV antigen in the thymic cortex in the LV group ($r = 0.979$; $p = 0.021$). In addition, the expression of TUNEL was negatively correlated with the number of thymocytes in the cortex of the thymus of SU1-bel infected animals at 7 dpi ($r = -1.00$; $p < 0.001$).

A significant positive correlation was detected between the expression of TNF-α and IL-1α in LV and 215-06 strains ($r \geq 0.70$; $p \leq 0.004$) and between the expression of TNF-α and IL-10 in LV strain ($r = 0.83$; $p < 0.001$) in the thymic cortex at different time-points. A significant positive correlation between the three cytokines was only detected in the thymic medulla of the SU1-bel group at 7 dpi ($r = 0.90$; $p = 0.037$). Finally, the expression of IL-1α displayed a significant positive correlation with the number of MAC387 positive cells in the thymic cortex in LV group at different time-points ($r = 0.71$; $p < 0.004$) and in 215-06 and SU1-bel infected pigs at 3 dpi ($r \geq 0.90$; $p \leq 0.037$).

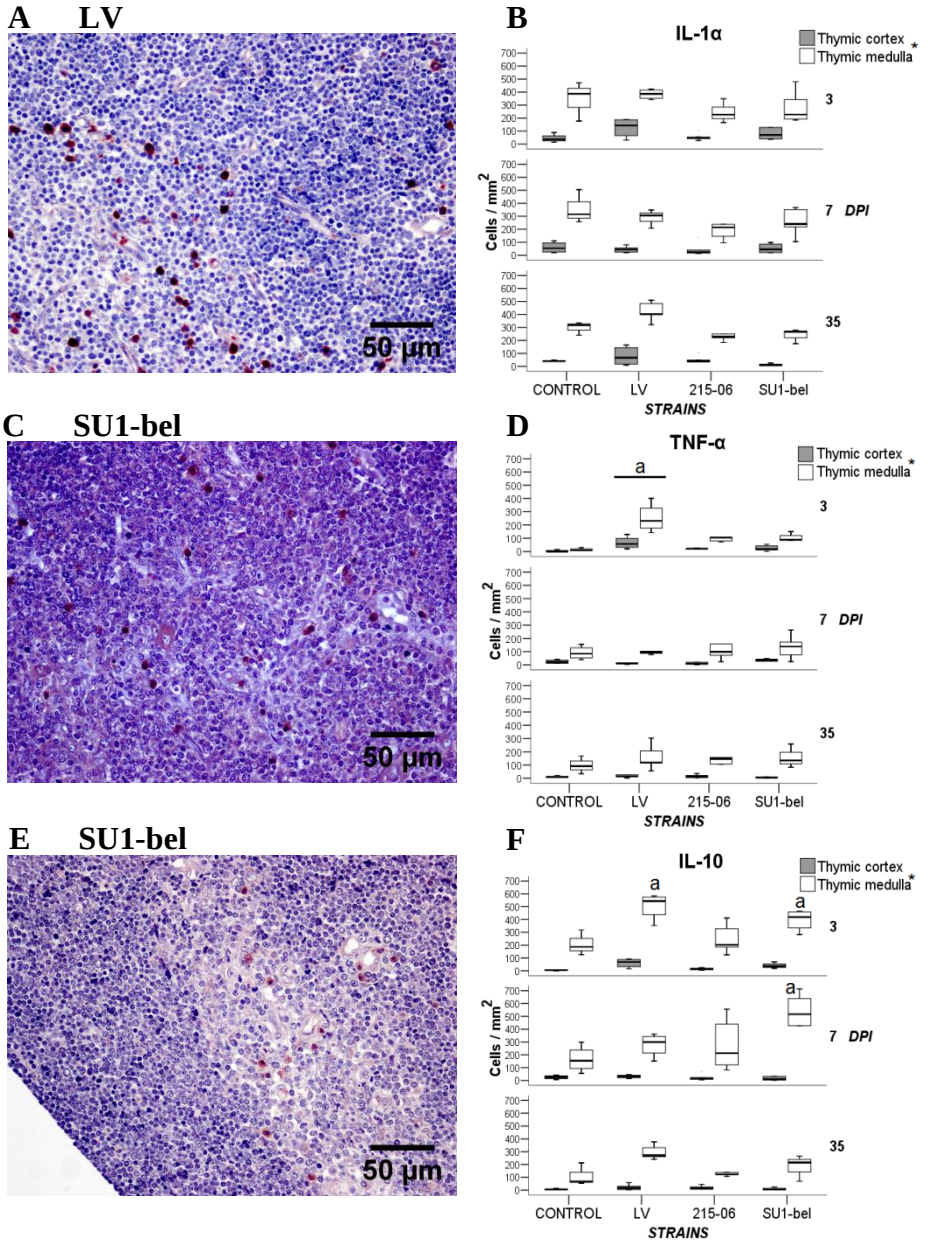

Fig. 6. Representative photomicrographs of the thymus from pigs infected with **(A)** LV and **(C, E)** SU1-bel strains at 3 dpi. Macrophages in the thymus, mainly in the thymic medulla expressing IL-1α of a pig from LV group and TNF-α and IL-10 of a pig from SU1-bel groups euthanized at 3 dpi. IHQ. Bar, 50µm. **(B, D, F)**. Counts for IL-1α, TNF-α, and IL-10 positive cells in the thymus from control animals and pigs infected with PRRSV-1 strains varying in virulence. Animals were euthanized at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains and control group, whereas the letter "b" indicates significant differences between the different time-points and "c" indicates significant differences with low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. The asterisks indicate statistical significant differences between thymic cortex and thymic medulla. Statistical significant differences were considered at p-values ≤ 0.05.

Discussion

PRRSV is characterized by a marked genetic variability and the impairment of the host immune response *(Stadejek et al., 2013)*. Atrophy and depletion of the thymus in piglets infected with PRRSV-2 strains of different pathogenicity have been associated with a loss of T cells in the cortex of the thymus and direct or indirect induction of apoptotic phenomena *(Feng et al., 2002; He et al., 2012; Li et al., 2014)*. In our study all infected animals, independently of the PRRSV-1 strain used, displayed changes in the histomorphometry of the thymus and significant amounts of apoptosis could be measured. Piglets infected PRRSV-1 strain (SU1-bel, the high virulence) presented the most severe changes when compared with two moderate virulent strains belonging to the pan European subtype 1 (LV and 215-06 strains).

All infected groups displayed a similar kinetics of the histopathology score with a peak at 7 dpi. The average score throughout the study was grade II for all the strains, although microscopic lesions observed in one animal from LV group and two animals from SU1-bel group were of grade III. A decrease in the percentage of parenchyma and thymic cortex was observed in all infected animals from 3 dpi onwards when compared to control group leading to a reduction of the C/M ratio. These changes were associated with a decrease in the cellularity of the cortex, specifically in the number of thymocytes and the expression of CD3 in the thymuses, which were significantly more severe for the animals infected with the SU1-bel strain. Some of these parameters were negatively correlated with the expression of cCasp3 in each of the PRRSV-1 strains. Decreased cellularity of the thymus is frequently associated with cell death and an increased number of tingible-body

macrophages *(Elmore, 2006)*. Accordingly, all PRRSV-1 strains showed an increase in the number of tingible-body macrophages compared to the control group, with SU1-bel infected animals presenting the highest counts, giving the tissue a "starry sky" appearance. These findings support the notion that PRRSV-1-infection suppresses the immune system in piglets and provides additional explanation regarding the pathogenesis of PRRSV-1 strains.

Several authors have previously measured PRRSV induced apoptosis in the thymus of infected animals using TUNEL method which was associated with severe thymus atrophy of thymocytes *(Feng et al., 2002; He et al., 2012)*. Interestingly, despite the differences between the different experimental designs, the results in all studies were similar and were characterized by moderate to severe atrophy of thymus which was accompanied by significant increase of apoptotic cells in the cortex of thymus. More so, caution should be given when evaluating apoptosis solely by TUNEL labelling since this technique may not differentiate between apoptosis and necrosis ***(****Charriaut-Marlangue and Ben-Ari, 1995****)***, and thus may rather be considered as marker for cell death. Therefore, in order to confirm apoptosis the use of a second assay based on a different principle is recommended ***(****Watanabe et al., 2002****)*****.** Our assessment of thymocyte death by means of TUNEL and cCasp3 labelling displayed a similar distribution, but the expression of TUNEL was higher than the expression of cCasp3. This finding could be attributed to several factors: (1) TUNEL assay is a sensible technique to detect cell death but may yield false positive results, from necrotic cells and cells in the process of DNA repair and gene transcription, *(Charriaut-Marlangue and Ben-Ari, 1995; Watanabe et al., 2002; Elmore, 2007)*; (2) TUNEL labels a later stage of the

apoptotic pathway, whereas cCasp3 labelling shows an earlier event in the apoptotic cascade *(Watanabe et al., 2002; Resendes et al., 2004)*; (3) Apoptosis may be also triggered by Casp3-independent pathways *(Rodríguez-Gómez et al., 2014)*, which may justify the lower expression of cCasp3 than TUNEL.

The results of the present study propose that different PRRSV-1 strains induced changes in the histopathology and histomorphometry in the thymus, mainly in the cortex, associated to apoptosis of thymocytes with the highest change observed in the thymus of piglets infected with the highly virulent SU1-bel strain. Indeed, in the animals infected with SU1-bel strain the expression of PRRSV antigen was positively correlated with both TUNEL and cCasp3 labelling. In addition, a significant positive correlation was also detected between TUNEL and the expression of PRRSV antigen in LV-infected animals.

Macrophages play a pivotal role in the induction of the host immune response through the secretion of mediators such as cytokines, the removal of dead cells and cellular debris as result of apoptosis, and the presentation of antigen to T -cells for the induction of an effective adaptive immune response *(Elmore, 2006; Pearse, 2006a; Rodríguez-Gómez et al., 2013)*. Macrophages may be classified attending to their polarization into classically (M1) or alternatively (M2) activated macrophages. M2 macrophages have been reported to be more susceptible than M1 macrophages to PRRSV infection and to induce higher levels of anti-inflammatory cytokines (e.g. IL-10), enhanced phagocytic activity but reduced antigen presenting functions *(Mosser, 2003; García-Nicolás et al.,*

2014). In the present study, SU1-bel infected piglets showed the highest increase in the number of macrophages both in the cortex and in the medulla of the thymus. Interestingly, the expression of IL-10 was also higher in SU1-bel infected animals on 7dpi than for the other PRRSV-1 strains coinciding also with a higher count of tingible body macrophages and an increase of apoptotic phenomena, as detected by the highest expression of both TUNEL and cCasp3. These results indicate the presence of alternatively activated M2 macrophages and reflect the requirement for enhanced phagocytosis after the increased cell death (tissue remodelling). Through the reduced generation of T cells it could result in an impaired T cell response, as previously suggested *(Rodríguez-Gómez et al., 2013; Salguero et al., 2015)*.

Cytokines are mediators of the immune response, some of which play a role in apoptosis and IL-1α and TNF-α are considered as pro-apoptotic cytokines, while the IL-10 plays a moderating role in pro-apoptotic effects *(Choi et al., 2002, Goetz et al., 2004; Salguero et al., 2004)*. In our previous study, animals infected with SU1-bel strain displayed a higher expression of IL-1α in the lung than piglets infected with LV or 215-06 strains *(Amarilla et al., 2015)*. Surprisingly, the expression of IL-1α in the thymus of infected animals was variable whereby a peak in the expression of TNF-α and IL-10 was observed in LV and SU1-bel groups, only. Previous studies have reported an association between apoptosis in the lungs, lymphoid tissues and the expression of several cytokines, such as IL-1, IL-10, IL-6, TNF-α and TGF-β, suggesting a mechanism for cytokine induced apoptosis in PRRSV infection *(Choi et al., 2002; Labarque et al, 2003; Rodríguez-Gómez et al., 2014)*. The results here with the higher counts for apoptotic cells rather than PRRSV-positive

cells point towards a strain-dependent behaviour in the induction of apoptosis and reinforce the hypotheses of both PRRSV-induced apoptosis and indirect mechanisms involved in the induction of apoptosis during PRRSV infection *(Li et al., 2014)*.

Conclusion

Our results demonstrate that different PRRSV-1 strains induced changes mainly in the cortex of the thymus due to apoptosis of thymocytes. High virulence PRRSV-1 (SU1-bel) showed the most severe depletion of the thymus with the highest expression of PRRSV antigen compared to the two moderate virulent strains of the pan European subtype 1 (LV and 215-06 strains). In addition, apoptosis of thymocytes was preceded in our study by a peak in the local expression of TNF-α and/or IL-10 chiefly in medulla of all infected animals.

IV. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUSES INDUCE HYPOPLASIA OF ERYTHROID CELLS AND MYELOID CELL HYPERPLASIA IN THE BONE MARROW OF EXPERIMENTALLY INFECTED PIGLETS INDEPENDENTLY OF THE VIRAL LOAD AND VIRULENCE.

Frontiers in immunology. Submitted

Introduction

Since the identification of the porcine reproductive and respiratory syndrome virus in 1991 as the causative agent of PRRS in Europe, several studies have demonstrated the remarkable phenotypic and genetic diversity between strains and within subtypes *(Wensvoort et al., 1991; Meulenberg et al., 1993; Han et al., 2013 a,b,c; Morgan et al., 2013; Stadejek et al., 2013; Weesendorp et al., 2014; Amarilla et al., 2015; Salguero et al., 2015)*.

PRRSV-induced gross and microscopic lesions in the lungs, secondary lymphoid organs and thymus are well characterized according to the different viral strains and their virulence *(Rossow et al., 1994; Halbur et al., 1995, 1996 a,b; Gómez-Laguna et al., 2009, 2010; Frydas et al., 2013; Hu et al., 2013; Butler et al., 2014; Li et al., 2014; Amarilla et al., 2015; García-Nicolás et al., 2015)*. Piglets infected *in utero* show mild to severe bone marrow hypoplasia characterized by an absence of normal myeloid and erythroid precursors *(Feng et al., 2001)*. In addition, non-regenerative anemia and marked increases in the myeloid:erythroid ratio (M:E) has been observed in five-weeks-old pigs inoculated with virulent genotype 2 PRRSV

(PRRSV-2) strains which likely reflects the effect of the virus on erythroid precursor cells in the bone marrow *(Halbur et al., 2002)*.

The proliferation of hematopoietic cells is regulated by hematopoietic growth factors and cytokines secreted in the bone marrow *(Majka et al., 2001)*. Disturbance of this complex network and inhibition of haematopoiesis by pathogens may occur by infection of stem cells, progenitor cells, and the stromal support *(Kolb-Maurer and Goebel, 2003)*. In the bone marrow, both IL-1 and IL-6 can synergistically stimulate the proliferation and differentiation of stem cells. Besides, IL-1 stimulates hematopoiesis through the induction of growth factors such as granulocyte-macrophage colony-stimulating factors (GM-CSF) and granulocyte colony-stimulating factors (G-CSF) by different cell types as progenitor cell proliferation, myeloid cells or of stromal cells *(Gronda and Bianchi de Di Risio, 1993; Majka et al., 2001; Kolb-Maurer and Goebel, 2003; Mak et al., 2005)*.

The expression of TNF-α in bone marrow can inhibit the formation of BFU-E and together with IL-3 and GM-CSF induces the synthesis of CFU-GM *(Férnandez-Delgado, 1992; Gronda and Bianchi de Di Risio, 1993)*. In contrast, IL-10 promotes the differentiation of lymphocytes and reduces the expression of GM-CSF *(Williams et al., 2004; Rodak, 2005)*.

Numerous studies have focussed on understanding how PRRSV interferes with antiviral host responses to establish persistent infection in pigs. Due to the limited available data on the effects of PRRSV-1

infection on the cellular components of the bone marrow and how this may contribute to compromising host immunity, the present study aimed to evaluate the effect of PRRSV-1 strains of different virulence in the bone marrow of experimentally infected piglets.

Materials and Methods

Animal selection and housing, viruses, experimental design and animal monitoring used in this experiment has been described above in the section "Common experimental design".

Table 1. Distribution in this study of all pigs experimentally infected within each group, namely control and three different PRRSV-1 strains: LV, 215-06 and SU1-bel. Animals were euthanized at 3, 7 and 35 dpi.

DPI	Control	LV[a]	215-06[b]	SU1-bel[c]
3	3	4	5	5
7	4	5	5	5
35	3	5	5	5*

a LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain; **b** 215-06: British field strain; **c** SU1-bel: Highly virulent strain; * Two animals in the SU1-bel group displayed a prolonged fever along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi.

At necropsy, femoral bone marrow samples were collected. For the quantitative real time reverse transcription PCR (RT-qPCR) analysis, samples of femoral bone marrow were stored at -70 °C.

Histomorphometric analysis and bone marrow myeloid to erythroid cells ratio

Four micrometre bone marrow sections were stained with haematoxylin and eosin and Giemsa. Stained sections were photographed to produce digital images and histomorphometry was conducted using the image analysis software ImageJ 1.45 (Bethesda, Maryland, United States). Differentiation of parenchyma (haematopoietic tissue) and stroma (plexus of venous sinuses, bone spicules and adipose cells) were performed according to the typical histological features of bone marrow described by *Travlos (2006a,b)*. The percentage of haematopoietic tissue and adipose cells were assessed in 25 non-overlapping and consecutively selected, high magnification fields of 0.2 mm^2. In addition, manual random quantification of nucleated cells in 5 non-overlapping and consecutively selected, high magnification fields of 0.01 mm^2 was performed. Results were expressed in number of cells per mm2. The ratio M:E was estimated by counting a total of 500 cells.

Immunohistochemistry

Labelled bone marrow cells were photographed to produce digital images and analysed in 15 non-overlapping and consecutively selected high magnification fields of 0.2 mm^2. The expression of all cytokines (IL-1α, TNF-α, IL-6 and IL-10) and dead cells (cCasp3 and TUNEL) were manually counted and expressed as number of cells per mm^2.

PRRSV RNA extraction and reverse transcription quantitative polymerase chain reaction

Total RNA was extracted from the femoral bone marrow using the MagNA Pure LC DNA Isolation kit II for tissues (Roche, Penzberg, Germany). For each 25 µl reaction, 2 ml of RNA was added to 12.5 µl of RT-PCR mastermix with 6.75 µl nuclease free water, 0.25 µl RT enzyme, and 0.5 µl of each probe (0.2 mM final concentration each) and primer (0.3 mM final concentration each). Thermocycling was performed on a Stratagene MX3000P instrument, with the following touchdown cycling parameters: 30 min at 50 °C and 15 min at 95 °C, followed by 10 cycles of denaturation (20 s at 94 °C), and annealing and extension (45 s at 72 °C), with the annealing temperature in these cycles reduced by 1 °C during each cycle. An additional 38 cycles of denaturation (20 s at 94 °C), annealing and extension (45 s at 55 °C) were then performed *(Frossard et al., 2012)*. Data was analysed by changes in the cycle threshold (Ct), and results were calculated as 38 – Ct, which represented the difference between the last cycle of the PRRSV RT-qPCR and the Ct for each sample *(García-Nicolás et al., 2015)*.

Results

Bone marrow histomorphometric analysis and M:E ratio

The histomorphometric analysis of the bone marrow in control animals at 3 dpi revealed that the proportions of parenchyma versus stoma in the bone marrow were comparable, but at 7 and 35 dpi there was a progressive decrease in parenchyma compared to stromal tissue. In contrast, at 3 dpi, bone marrow from all animals infected presented with significantly reduced percentages of hematopoietic tissue. At 7 dpi,

animals infected with the highly virulent SU1-bel strain and at 35 dpi piglets infected with the moderate virulent 215-06 strain additionally showed a significant decrease in parenchyma (Fig.1A). Significant differences in the percentage of hematopoietic tissue and stroma were observed in LV, 215-06 and SU1-bel groups when compared to control group at 3 dpi ($p \leq 0.02$). At 35 dpi, only the 215-06 group showed significant differences compared to control, LV and SU1-bel groups ($p \leq 0.03$). Moreover, significant differences were observed in the percentage of hematopoietic tissue between the different strains and, in the percentage of stroma at different time-points ($p \leq 0.05$, respectively) (Fig.1C).

The number of the erythroid cells in control group decreased moderately during the study; however, the number of the myeloid cells remained constant at the different time-points (Table 2). There was a significant decrease in the number of erythroid cells and a significant increase in the number of myeloid cells in all infected groups at 3 dpi when compared with the control group ($p \leq 0.03$) (Fig. 1B). The LV group was significantly different compared to SU1-bel group at 3 dpi ($p \leq 0.03$). The number of erythroid cells in 215-06 and SU1-bel groups showed a significant difference when compared to control group ($p \leq 0.03$), and SU1-bel group was significantly different compared to LV group ($p \leq 0.007$) at 7 dpi. At 35 dpi, LV and SU1-bel groups displayed an increase in the number of myeloid cells (Table 2).

The highest M:E ratio at 3 dpi was observed in the piglets infected with the moderate virulent LV strain and the highly virulent SU1-bel strain compared to control group ($p < 0.02$), and LV group was

significantly higher than SU1-bel ($p \leq 0.03$). Moreover, at 7 dpi SU1-bel group showed a higher M:E ratio compared to control group ($p \leq 0.01$) (Table 2).

Table 2. Median number of myeloid and erythroid cells and M:E ratio in 500 bone marrow cells. Animals were euthanized at 3, 7 and 35 days post-infected (DPI).

DPI	Different groups	Myeloid cells		Erythroid cells		M:E ratio	
		Mean	±SD	Mean	±SD	Mean	±SD
3	Control	258	47	242	47	1.06	1.00
	LV*	369[c,d]	17	131[c,d]	17	2.82[c,d]	1.00
	215-06†	313[c]	48	187[c]	48	1.67	1.00
	SU1-bel‡	329[c]	18	171[c]	18	1.92[c]	1.00
7	Control	259	44	241	44	1.08	1.00
	LV*	307	16	193	16	1.60	1.00
	215-06†	304	29	162[c]	65	1.88	0.45
	SU1-bel‡	312	90	131[c,d]	21	2.37[c]	4.22
35	Control	277	68	153	83	1.81	0.82
	LV*	301	117	123	64	2.45	1.81
	215-06†	238	123	135	79	1.77	1.57
	SU1-bel‡	342	58	158	58	2.17	1.00

*LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain; †215-06: British field strain; ‡SU1-bel: Highly virulent strain. Different letters indicate statistically significant differences ($p \leq 0.05$): The letter "a" indicates significant differences between different strains; the letter "b" indicates significant differences between different time-points, whereas the letter "c" indicates significant differences between different strains and the control group and "d" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. Statistical significant differences were considered at *p*-values ≤ 0.05.

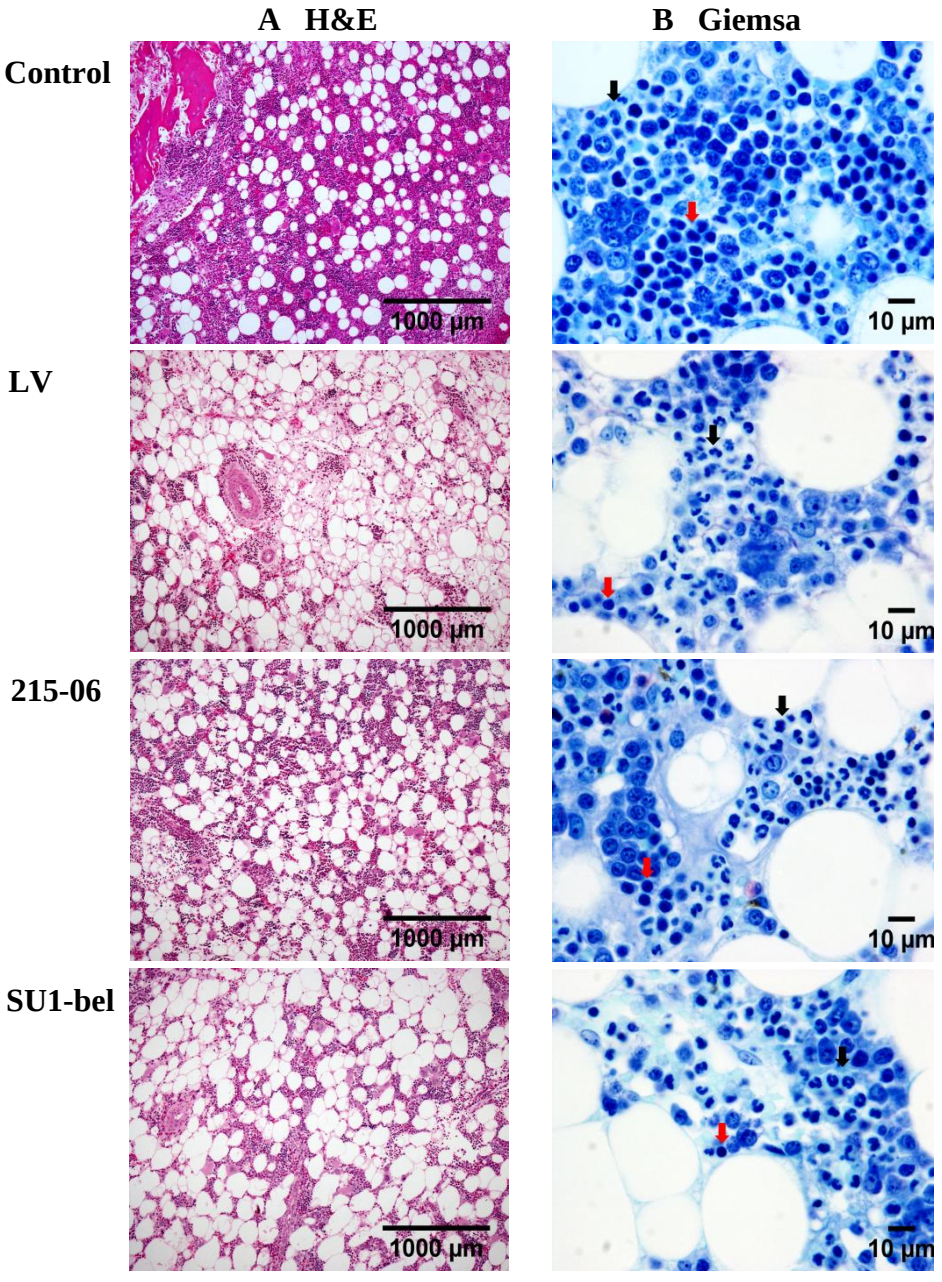

Fig. 1. Moderate transient hypoplasia of erythroid cells and an increased M:E ratio in piglets experimentally infected with the prototype PRRSV-1 Lelystad (LV), the moderate virulent 215-06 and the highly virulent SU1-bel strains. **(A)** Representative H&E stained photomicrographs of the bone marrow from pigs, from control group and from pigs inoculated with LV and 215-06 strains and euthanized at 3 days post-infection (dpi); and from pig inoculated with SU1-bel strains and euthanized at 7 dpi are presented. Bar, 1000 µm. **(B)** Representative photomicrographs of the bone marrow from pigs, from control group and from pigs inoculated with LV and 215-06 strains and euthanized at 3 days post-infection (dpi); and from pig inoculated with SU1-bel strains and euthanized at 7 dpi. Giemsa stained bone marrow sections a pig from control group and from pigs infected with different strains are presented. Bar, 10 µm. Examples of erythroid and myeloid cells are shown by red arrow and black arrows, respectively.

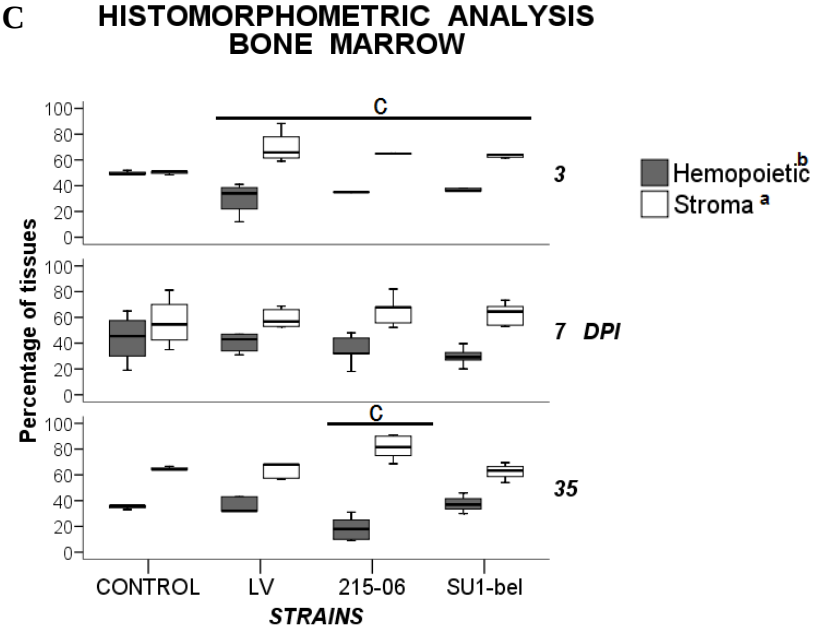

Fig. 1. **(C)** Graphs representing the percentage of the tissues in the bone marrow at all time-points in all virus-infected animals and control group. The histomorphometric analysis in bone marrow, in control animals and in the animals infected with PRRSV-1 strains differing in virulence. Animals were euthanized at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains; the letter "b" indicates significant differences between different time-points, whereas the letter "c" indicates significant differences between different strains and the control group and "d" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. Statistical significant differences were considered at p-values ≤ 0.05.

Cell-death in bone marrow of PRRSV-1 infected pigs

The number of TUNEL labelled cells was higher compared to that of cCasp3. cCasp3 staining was mostly observed within the cytoplasm of labelled cells. TUNEL labelling, however, was observed in apoptotic bodies inside macrophages, within the cytoplasm and nuclei of cells and only occasionally in free apoptotic bodies. Therefore, TUNEL labelling quantification was performed by identifying apoptotic bodies inside macrophages and within the cytoplasm of different cells (Fig.2A and insets). Throughout the study, control animals showed a mild increase in TUNEL expression and a decrease of cCasp3. LV group displayed higher TUNEL expression at 7 dpi, while 215-06 and SU1-bel groups showed a higher expression at 3 dpi. cCasp3 in LV group showed the highest expression at 3 dpi and in the SU1-bel group at 7 dpi. In 215-06 group, the expression of cCasp3 remained constant until the end of the experiment. However, no statistical differences were observed between strains, time-points and control groups (Fig.2B)

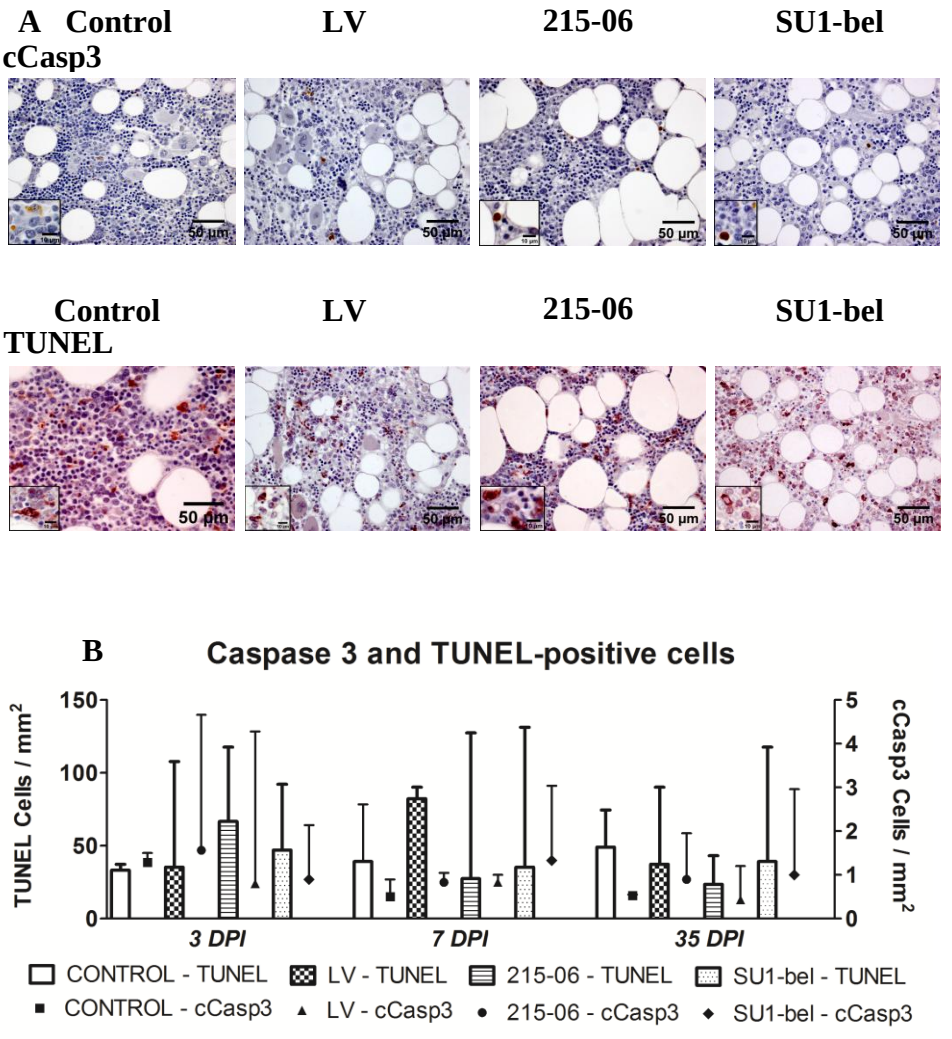

Fig. 2. Cell-death in bone marrow of PRRSV-1 infected pigs. **(A)** Representative photomicrographs of cCasp3 and TUNEL labelling in the bone marrow from a control pig and from pigs inoculated with LV and 215-06 strains and euthanized at 3 days post-infection (dpi); and from a pig inoculated with SU1-bel strains and euthanized at 7 dpi. Bar 50 µm. *Insets* detail of the difference between the pigment deposition and cell labelled with casp3. Bar, 10 µm. TUNEL labelling of apoptotic bodies inside macrophages and within the cytoplasm of different cells a control animal and all infected animal. TUNEL. Bar, 50 µm. Insets detail of apoptotic bodies inside macrophages and within the cytoplasm of different cells labelled with TUNEL method. TUNEL. Bar, 10 µm. **(B)** Graphs representing the counts for cCasp3 and TUNEL-positive cells in the bone marrow of control animals and animals infected with the three different PRRSV-1 strains included in the study. Animals were euthanized at 3, 7 and 35 dpi. Data are expressed as median ±SD. No statistical differences were observed between strains, time-points and control groups.

Expression of cytokines in the bone marrow

The expression of IL-1α, IL-6, TNF-α and IL-10 was mainly detected within the cytoplasm of macrophages and to a lesser extent in the cytoplasm of granulocytes (Fig.3A, B, D, E and insets). The expression of these cytokines in control animals was similar, with a slow decrease until the end of the experiment. Within infected groups, however, there was a wide variation in cytokines expression at all time-points.

The highest expression was observed for IL-1α and IL-6. In all infected groups, both cytokines displayed a peak at 3 dpi with a progressive decrease in the expression of IL-1α along the study. The expression of IL-1α showed statistically significant differences between all time-points ($p = 0.03$) (Fig. 3C). Contrary, the expression of IL-6 remained constant in the LV group throughout the study. In the 215-06 group, IL-6 decreased at 35 dpi. SU1-bel group showed a decrease of IL-6 at 7 and 35 dpi. Significantly different expression of IL-6 in SU1-bel at 3 dpi compared to control group and 215-06 group ($p \leq 0.04$) was observed. At 7 dpi, 215-06 group displayed significant differences when compared to control group ($p = 0.03$). Moreover, the expression of IL-6 displayed statistically significant differences between different strains and time-points ($p \leq 0.03$) (Fig. 3C).

The expression of TNF-α in the bone marrow in the LV group showed a trend to increase throughout the study, with a significant peak at 35 dpi ($p = 0.03$). The 215-06 infected animals displayed a significant increased expression of TNF-α until the end of the experiment when

compared to control group ($p \leq 0.04$). Meanwhile, in SU1-bel group there was a significant increase at 3 and 35 dpi when compared to control group ($p \leq 0.05$) (Fig. 3F). Finally, the expression of IL-10 showed a trend to decrease during different time-points in LV and 215-06 groups, whereas in SU1-bel group, the trend was to increase along the study. IL-10 displayed a lesser expression in the bone marrow in SU1-bel group at 3 dpi compared to control group ($p = 0.01$) (Fig. 3F).

Bone marrow PRRSV RNA quantification

PRRSV RNA was detected in all virus-infected animals but not within the control group. The highest RNA viral load was detected in pigs experimentally infected with the prototype PRRSV-1 LV, remaining high until the end of the experiment (35 dpi). Secondly, a high RNA viral load was also observed in 215-06 group until 7dpi, whereas SU1-bel group displayed the lowest viral load. No PRRSV RNA was detected in 215-06 and SU1-bel infected animals at 35 dpi. All PRRSV infected animals showed a significantly higher PRRSV RNA load at 3 and 7 dpi compared to 35 dpi ($p = 0.02$). However, the LV group displayed significantly higher viral RNA at 7 and 35 dpi when compared to 215-06 and SU1-bel groups ($p = 0.01$) (Fig. 4).

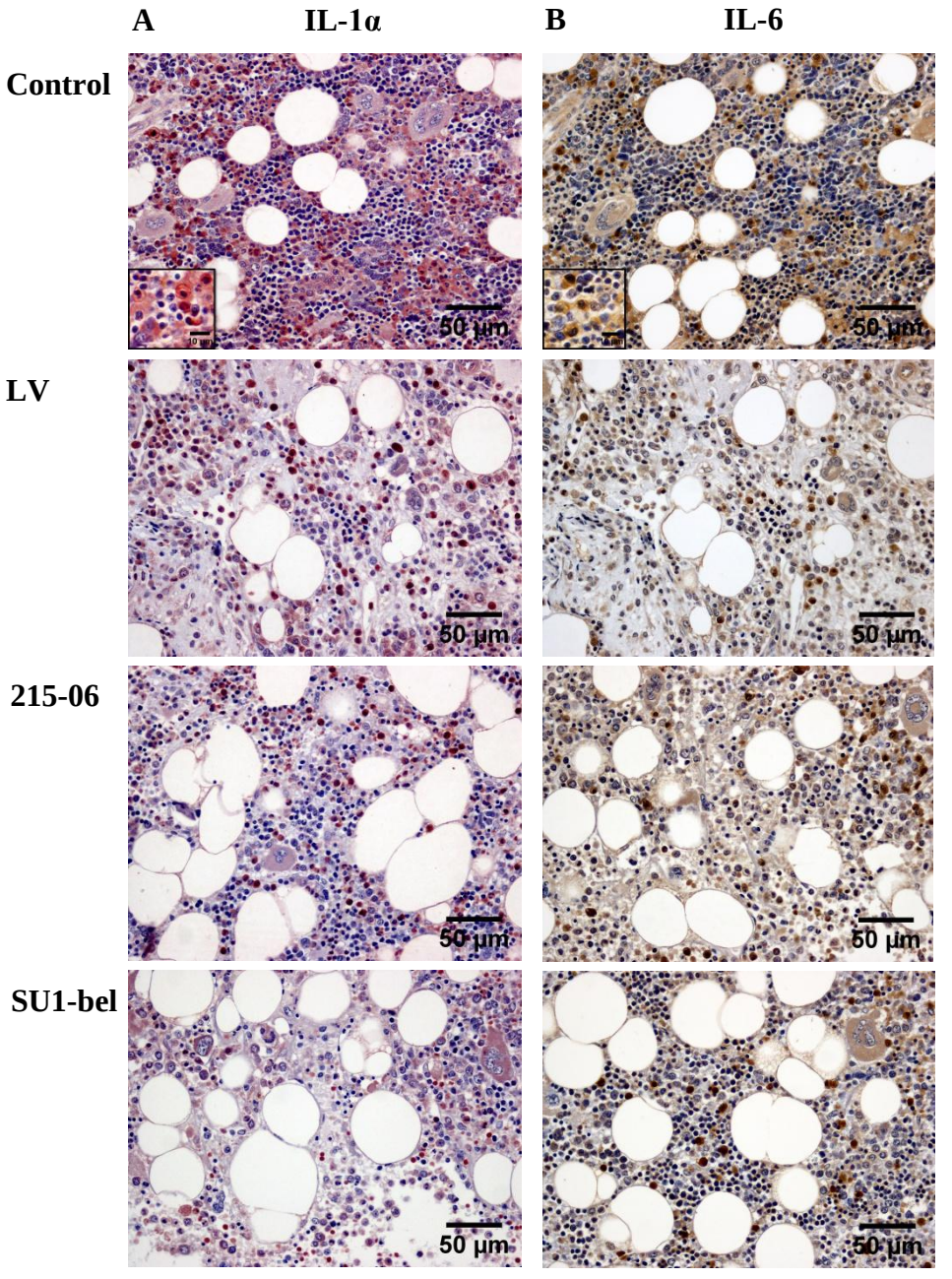

Fig. 3. Representative photomicrographs of the expression of IL-1α and IL-6 in bone marrow from a pig from control group and from pigs infected with SU1-bel, LV and 215-06 strains at 3 dpi. **(A)** Cytoplasm of macrophages and granulocytes expressing IL-1α of a pig from control group and different infected groups. IHC. Bar, 50 µm. *Insets* detail of macrophages and granulocytes expressing IL-1α. IHC. Bar, 10 µm. **(B)** Cytoplasm of macrophages expressing IL-6 of a pig from control group and different infected groups. IHC. Bar, 50 µm. *Insets* detail of macrophages expressing IL-6. IHC. Bar, 10 µm.

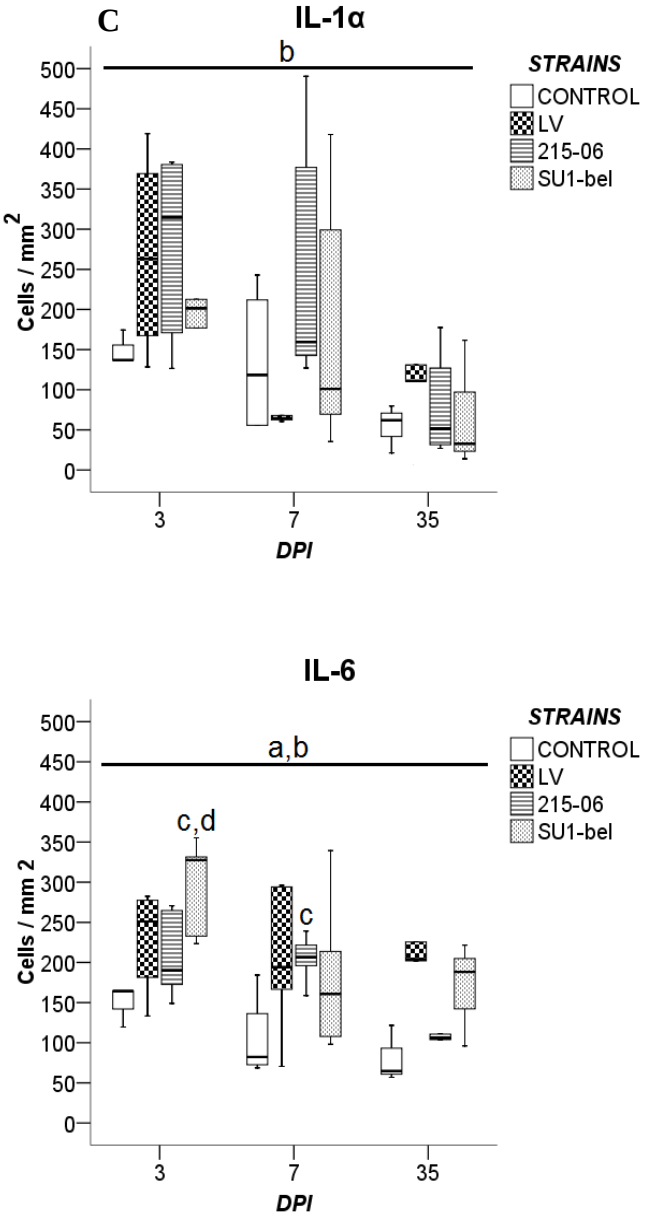

Fig. 3. **(C)** Counts for IL-1α and IL-6 positive cells in the bone marrow from control animals and pigs infected with PRRSV-1 strains varying in virulence. Animals were euthanized at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains; the letter "b" indicates significant differences between different time-points, whereas the letter "c" indicates significant differences between different strains and the control group and "d" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. Statistical significant differences were considered at p-values ≤ 0.05.

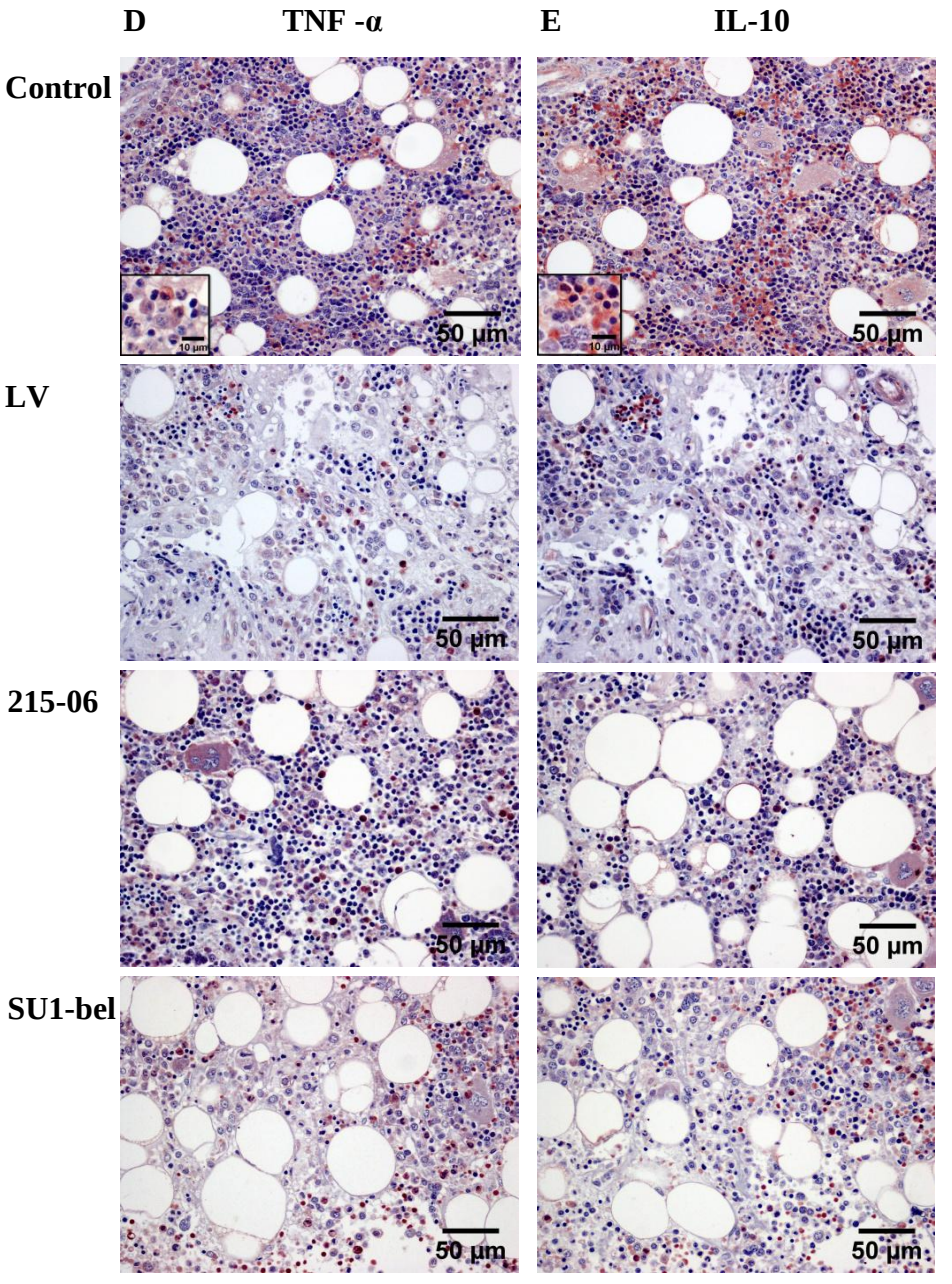

Fig. 3. Representative photomicrographs of the expression of TNF-α and IL-10 in bone marrow from a pig from control group and from pigs infected with SU1-bel, LV and 215-06 strains at 3 dpi. **(D and E)** Cytoplasm of macrophages expressing TNF-α and IL-10 of a pig from control group and different infected groups. IHC. Bar, 50 µm. *Insets* detail of macrophages expressing TNF-α and IL-10. IHC. Bar, 10 µm.

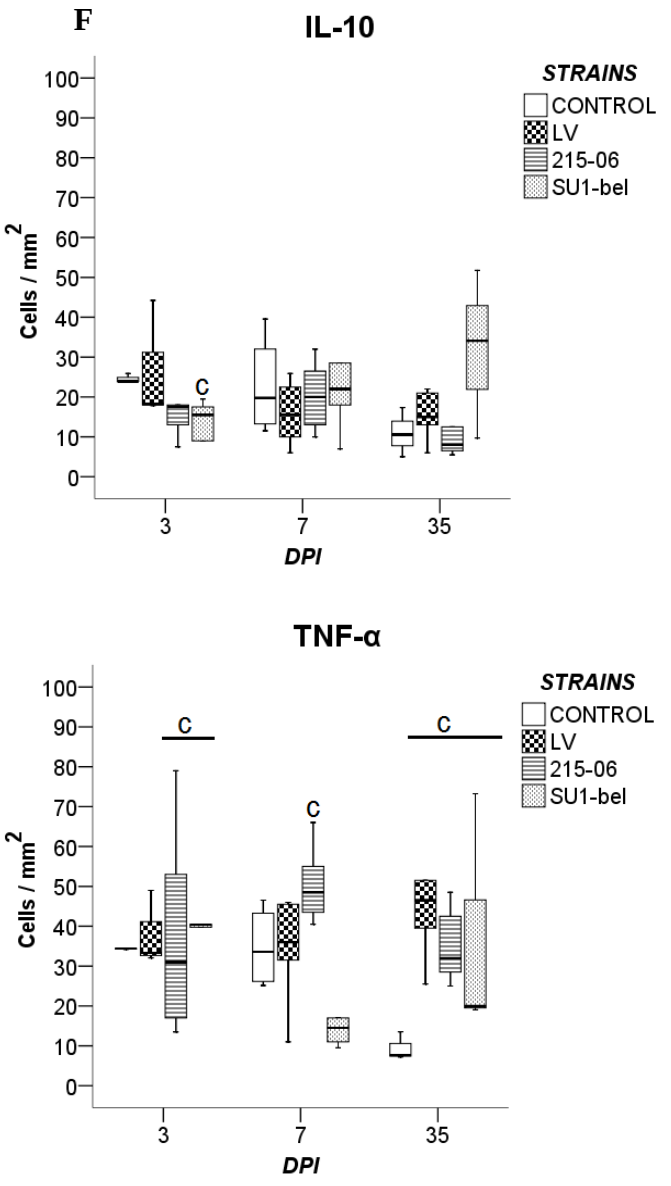

Fig. 3. **(F**) Counts for IL-1α and IL-6 positive cells in the bone marrow from control animals and pigs infected with PRRSV-1 strains varying in virulence. Animals were euthanized at 3, 7 and 35 dpi. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between different strains; the letter "b" indicates significant differences between different time-points, whereas the letter "c" indicates significant differences between different strains and the control group and "d" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. Statistical significant differences were considered at p-values ≤ 0.05.

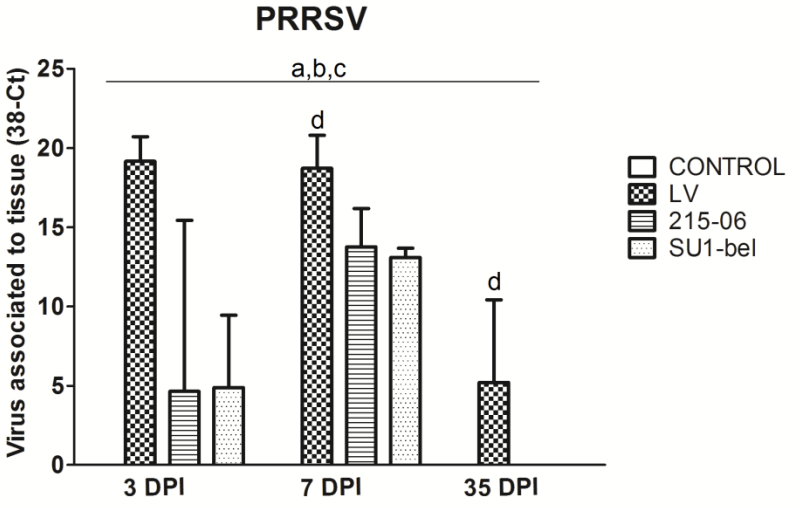

Fig. 4. PRRSV viral load in bone marrow. PRRSV RNA was quantified by RT-qPCR and data represented by changes in the cycle threshold (Ct) in the bone marrow of control animals and animals experimentally infected with three different PRRSV-1 strains (LV, 215-06 and SU1-bel strains). Animals were euthanized at 3, 7 and 35 dpi and PRRSV viral in bone marrow assessed by RT-qPCR.. Data are expressed as median ±SD. The letter "a" indicates significant differences between different strains; the letter "b" indicates significant differences between different time-points, whereas the letter "c" indicates significant differences between different strains and the control group and "d" indicates significant differences between low virulent strains (LV and/or 215-06 strains) and SU1-bel strain. Statistical significant differences were considered at p-values ≤ 0.05.

Correlation of the different measurements in different PRRSV-1 strains at different time-points

A significant negative correlation was detected in LV infected animals between the hematopoietic tissue and erythroid cells with cCasp3 (r = - 0.94; p = 0.04) at 3dpi and between erythroid cells and TUNEL (r = - 0.90; p = 0.04) at 7dpi. Meanwhile, 215-06 group showed at 7 dpi a significant positive correlation between erythroid cells and hematopoietic tissue (r = 0.97; p = 0.008). Finally, at 35 dpi the LV group revealed a significant negative correlation between the hematopoietic tissue and TUNEL (r = - 0.94; p = 0.008), and positive correlation between IL-1α and myeloid cells (r = 0.90; p = 0.04).

Discussion

The suppression of haematopoiesis in the bone marrow can be a substantial problem in viral infections *(Kolb-Maurer and Goebel, 2003)*. However, previous reports on the impact of PRRSV infection in the bone marrow of infected animals are scarce. Piglets infected *in utero* show mild to severe bone marrow hypoplasia *(Feng et al., 2001)*, and PRRSV-2 experimentally infected piglets developed non-regenerative anemia and markedly increased M:E ratio *(Halbur et al., 2002)*. In our study, we found a moderate transient hypoplasia of erythroid cells and an increased M:E ratio in piglets experimentally infected with the prototype PRRSV-1 Lelystad, the moderate virulent 215-06, and the highly virulent SU1-bel strains. Surprisingly, piglets infected with the prototype PRRSV-1 Lelystad presented a higher early erythroid hypoplasia compared to 215-06 and the highly virulent SU1-bel strains. SU1-bel strain displayed a more sustained erythroid hypoplasia when compared with the other moderate virulent strains.

The highest viral RNA level was detected in pigs experimentally infected with LV when compared with the moderate virulent 215-06 and the highly virulent SU1-bel strains. Interestingly, in other organs such as lung, secondary lymphoid organs and thymus the highest PRRSV replication was observed in SU1-bel group and secondly in LV and 215-06 groups *(Morgan et al., 2014; Amarilla et al., 2015; García-Nicolás et al., 2015)*. These data demonstrate a higher affinity of the LV strain for the bone marrow when compared to 215-06 and SU1-bel strains and importantly a higher replication of PRRSV-1 strains of lower virulence in the bone marrow.

An increase in the M:E ratio can be due to a decrease in erythroid activity and/or an increase in myeloid activity *(Halbur et al., 2002; Travlos, 2006a)*. In our study, we found increased M:E ratio in the LV and SU1-bel groups associated to both a decreased number of erythroid cells and an increased number of myeloid cells compared to control animals. These results suggest that a decrease in the erythroid activity together with an increase of the myeloid activity the increased M:E ratio in our study. In addition, the significant positive correlation detected between both the percentage of hematopoietic tissue and the number of erythroid cells in the 215-06 group supports the association between the decreased in the number of erythroid cells and a reduced percentage of hematopoietic tissue.

An increase in the myeloid activity could be explained by an increase in the leukocytic demand associated with PRRSV infection *(Halbur et al., 2002)*. A drop in counts of peripheral blood monocytes has been previously reported during the course of PRRSV-1 infection *(Gómez-Laguna et al., 2009; Morgan et at., 2013)*. In parallel, interstitial pneumonia contains increased numbers of interstitial mononuclear cells *(Gómez-Laguna et al., 2010; Balka et al., 2013; Amarilla et al., 2015)*. Further, an increase in macrophage counts has also been described in the mediastinal lymph node and in the thymus of animals infected with LV, 215-06 or SU1-bel strains *(García-Nicolás et al., 2015)*. These findings suggest an accumulation of mononuclear cells in lung and lymphoid organs associated with a decrease of peripheral blood monocytes and higher demand for myeloid cell production in PRRSV-1 infection, resulting in myeloid hyperplasia. It is well known that IL-1α and IL-6 are powerful stimulating factors for the proliferation and differentiation of CFU-GM progenitor in the bone marrow *(Gronda and Bianchi de Di Risio, 1993; Majka et*

al., 2001; Mak et al., 2005). The increased expression of IL-1α and IL-6 observed in the present study in PRRSV-1 infected animals may therefore support a bone marrow response to the increased demand of hematopoietic cells.

It has been demonstrated that PRRSV induces apoptosis in the lungs and different lymphoid organs, including the thymus *(Labarque et al., 2003; Barranco et al., 2011; Gómez-Laguna et al., 2012; Morgan et al., 2014; Rodríguez-Gómez et al., 2014; Wang et al., 2015)*. Analysis of the thymus in these pigs demonstrated changes mainly in the thymic cortex due to thymocyte apoptosis. In this study, we observed a negative correlation between the hematopoietic tissue and/or erythroid cells with the expression of cCasp3 and/or TUNEL in the bone marrow of LV infected animals at different time-points. Similar data displayed the 215-06, but with no significance ($p = 0.06$). The exact mechanisms underlying these changes in the bone marrow are not known. However, these results together with PRRSV RNA load in pigs experimentally infected suggest that PRRSV-1 strain may have an effect on erythroid cells, possibly by direct mechanisms of virus replication *(Halbur et al., 2002)*. Nevertheless, because of the significant expression of TNF-α in 215-06 and SU1-bel groups at 3dpi, TNF-α could also play a role in initiating cell death and previous studies suggested a mechanism for cytokine induced apoptosis in PRRSV infection *(Choi et al., 2002; Labarque et al., 2003; Rodríguez-Gómez et al., 2014)*. Moreover, the expression of TNF-α in bone marrow can inhibit the formation of the BFU-E and induce the synthesis of the CFU-GM *(Férnandez-Delgado, 1992; Gronda and Bianchi de Di Risio, 1993)*.

Our results demonstrate that at early stages of PRRSV infection different PRRSV-1 strains induce moderate and transient hypoplasia of erythroid cells and increased M:E ratio in the bone marrow of experimentally infected piglets, independently of the viral load and virulence. There are, however, significant different between the PRRSV-1 strains, i.e. strains of lower virulence seem to establish a better infection in the bone marrow. In addition, we can show evidences that erythroid cell hypoplasia, myeloid cell hyperplasia and increased M:E ratio are associated to a peak in the local expression of IL-1α, IL-6 and TNF-α in all infected animals. Also there are differences in the biometric of responses between different strains which warrant further investigation.

CAPÍTULO 4 / CHAPTER 4

DISCUSIÓN GENERAL

GENERAL DISCUSSION

DISCUSIÓN GENERAL

La aparición de los denominados brotes atípicos y agudos del PRRS reflejan la amplia variabilidad genética y fenotípica de los dos genotipos de este virus y de sus correspondientes subtipos y linajes *(Meng et al, 1996a; Mengeling et al, 1998; Han et al., 2006; Tian et al., 2007; Karniychuk et al., 2010; Xie et al., 2014; Chaikhumwang et al., 2015)*. Probablemente esta diversidad sea el resultado de la falta de una ARN-polimerasa ARN-dependiente que realice las pruebas de corrección en el genoma del virus y de la enorme capacidad de recombinación del mismo, dando como resultado la aparición de diferentes cepas con genomas extraordinariamente diversos y con diferentes grados de patogenicidad *(Kappes and Faaberg, 2015)*.

Varios estudios han descrito que el PRRSV puede influir y desequilibrar la expresión de diferentes citoquinas tanto pro-inflamatorias como inmunomoduladoras *(Murtaugh et al., 2002; Díaz et al., 2006; Gimeno et al., 2011; Gómez-Laguna et al., 2013; Song et al., 2013; Wang et al., 2013a; Butler et al., 2014; Huang et al., 2015)*. Este desequilibrio en la expresión de citoquinas así como las diferencias en la expresión de las mismas según el órganos evaluado *(López-Fuertes et al., 2000; Johnsen et al., 2002; Chung y Chae, 2003; Labarque et al., 2003; Suradhat et al., 2003; Gómez-Laguna et al., 2009, 2010, 2012; Barranco et al., 2012 a,b; Song et al., 2013; Weesendorp et al., 2013b; García-Nicolás et al., 2014, 2015; Amarilla et al., 2015)*, sugiere que para conocer la patogenia de este virus es necesario realizar un análisis de los efectos *in situ* de las diferentes cepas del PRRSV, especialmente centrado en el estudio de la respuesta inmune y su correlación con la virulencia de cada cepa del PRRSV.

En esta tesis doctoral hemos evaluado la expresión del antígeno vírico, el cuadro lesional y la expresión de diferentes citoquinas pro-inflamatorias e inmunomoduladoras en el pulmón y en los órganos linfoides primarios (timo y médula ósea) con el objetivo de comparar la inmunopatogenia tras la infección con cepas del PRRSV-1 de distinta virulencia (la cepa estándar del PRRSV-1, LV; dos cepas de campo, la cepa altamente virulenta SU1-bel y la cepa 215-06, de moderada virulencia; y una cepa de vacuna atenuada, DV) en el pulmón y órganos linfoides primarios de cerdos infectados experimentalmente.

Coincidiendo con otros estudios *(Halbur et al., 1996 a,b; Gómez-Laguna et al., 2013)*, hemos observado que la expresión del antígeno del PRRSV en todos los grupos infectados, a excepción del grupo DV, se detectó principalmente en macrófagos alveolares del pulmón y macrófagos del timo, con una expresión máxima a los 7 dpi. En el pulmón y el timo de los animales infectados se detectó la replicación más alta en el grupo SU1-bel, y tanto con esta cepa como con la cepa estándar del virus (LV) existió una correlación positiva entre las lesiones macroscópicas del pulmón y la presencia del antígeno vírico. Sorprendentemente, en médula ósea la cepa estándar del PRRSV-1 (LV) fue la cepa que expresó una mayor carga de RNA viral y más sostenida hasta el final del estudio (35 dpi). En cambio, con las dos cepas de campo SU1-bel, de alta virulencia, y 215-06, de moderada virulencia, sólo se detectó la expresión de RNA vírico a los 3 y 7 dpi, con una carga viral máxima a los 7 dpi.

El estudio de los órganos linfoides secundarios como ser tonsila y nódulos linfáticos mediastiníco, retrofaríngeo y esternal de estos mismos animales reveló que el mayor número de células que expresaban tanto el antígeno del PRRSV, como el RNA del virus eran los inoculados con la cepa altamente virulenta (SU1-bel) y, en segundo lugar, los inoculados con las cepas estándar (LV) y de moderada virulencia (215-06) *(Morgan et al, 2014; García-Nicolás et al, 2015)*. Al comparar los datos obtenidos en diferentes estudios, pero que fueron realizados con muestras obtenidas a partir del mismo experimento, se puede afirmar que la expresión del antígeno vírico difiere de acuerdo al órgano y al grado de virulencia de la cepa. Así, mientras que la cepa de alta virulencia (SU1-bel) tiene un mayor tropismo por varios órganos como el pulmón, órganos linfoides secundarios y timo, la cepa estándar (LV) presenta un mayor tropismo por el pulmón y la médula ósea, y la cepa de moderada virulencia (215-06) presenta una distribución más homogénea en todos los órganos. Por otro lado, independiente de la virulencia de la cepa la detección del virus en médula ósea al inicio de la infección por RT-qPCR, sugiere que este órgano es uno de los primeros en ser colonizados por el PRRSV.

En nuestro estudio el mayor grado de lesión pulmonar fue observado a los 7 dpi, siendo el grupo de animales infectados con la cepa de alta virulencia (SU1-bel) donde se observó un mayor grado de lesión. Microscópicamente, en todos los lóbulos pulmonares de los animales de los diferentes grupos se observó la existencia de una neumonía intersticial de intensidad leve a grave, con una distribución multifocal a difusa. Las citoquinas evaluadas en nuestro estudio se detectaron, principalmente, en el citoplasma de los macrófagos pulmonares septales. El papel primordial de los macrófagos septales en la producción de diferentes citoquinas en el transcurso de la infección por el PRRSV ha

sido propuesto anteriormente por nuestro grupo de investigación *(Gómez-Laguna et al., 2010, 2013)*. En el transcurso de la infección por la cepa de alta virulencia (SU1-bel) se observó una mayor expresión de IL-1α que con las otras cepas en estudio, observándose además una correlación positiva entre la expresión del antígeno vírico y la expresión de la citoquina IL-1α, lo que sugiere que esta cepa sería una buena inductora de esta citoquina que, a su vez, sería la responsable de la intensidad de la neumonía intersticial en los animales infectados con cepas de alta virulencia. Estos resultados coinciden con los de *Weesendorp et al. (2014)*, quienes señalan la existencia de un aumento de células positivas al PRRSV y de la expresión de ARNm de IL-1β en el pulmón de animales infectados con la cepa de alta virulencia Lena, en comparación con animales infectados con otras cepas del PRRSV-1, como el Lelystad (LV) o Belgium A.

En el pulmón de los animales de todos los grupos infectados se detectó una correlación positiva entre la expresión de IFN-γ y la expresión de TNF-α e IL-6, lo que sugiere que las citoquinas pro-inflamatorias desempeñan un papel muy importante en las etapas tempranas de la enfermedad. Según *Gimeno et al. (2011)*, las cepas del PRRSV-1 pueden ser clasificadas como inductoras de IL-10, TNF-α, IL-10 y TNF-α o ninguna de ellas. En nuestro estudio hemos observado que la expresión de IL-10 fue significativamente mayor en los pulmones de cerdos infectados con la cepa de moderada virulencia (215-06) y en menor medida en los cerdos infectados con la cepa de alta virulencia (SU1-bel). Adicionalmente, en todos los grupos infectados se observó la existencia de una correlación positiva entre la expresión de IL-10 y la expresión de IFN-γ e IL-6, lo que confirma el papel inmunomodulador y

anti-inflamatorio de esta citoquina en la infección por el PRRSV a nivel pulmonar *(Gómez-Laguna et al., 2013; Huang et al., 2015)*.

En el timo, las principales alteraciones se localizaron en la corteza y consistieron en una reducción del número de timocitos y de células CD3 positivas y un aumento en el número de macrófagos de cuerpo tingible, junto con un aumento de la expresión de cCasp3 o TUNEL. Los animales infectados con la cepa de alta virulencia (SU1-bel) fueron los que presentaron una mayor expresión de cCasp3 y de células positivas con la técnica TUNEL, existiendo una correlación positiva entre estos y la expresión del antígeno del PRRSV. En los animales infectados con la cepa estándar (LV) también se observó una correlación positiva entre el número de células positivas con la técnica de TUNEL y la expresión del antígeno vírico.

Diferentes estudios han asociado el grado de depleción y atrofia tímica con la capacidad del PRRSV de inducir la muerte celular y/o los fenómenos de apoptosis *(Feng et al., 2002; He et al, 2012)*. Esta reducción de la generación de células T daría lugar a un deterioro en la respuesta de las células T *(Rodríguez-Gómez et al, 2013; Salguero et al, 2015)*. La correlación entre las células que expresan algún marcador de muerte celular (TUNEL y cCasp3) y las células que expresan el antígeno vírico, así como la detección de muerte celular en un mayor número de células que en las que se detectó la expresión del antígeno del PRRSV, apuntan hacia un comportamiento dependiente de la virulencia de la cepa con relación a la inducción de la apoptosis y refuerza la hipótesis de que el PRRSV induce mecanismos de muerte celular de manera tanto directa como indirecta en el timo *(Li et al., 2014)*. Estos resultados apoyan la

hipótesis de que en el transcurso de las infecciones con el PRRSV-1 se produce un fallo y supresión del sistema inmune de los animales en crecimiento.

Las lesiones de tipo inflamatorio se caracterizan por la acumulación de plasma proteico y de leucocitos en el tejido extravascular; además, en estos procesos están involucrados varios mediadores quimiotácticos, moléculas vasoactivas y citoquinas pro-inflamatorias y anti-inflamatorias *(Ackermann, 2012)*. Contrariamente, lesiones de tipo degenerativas, como puede ser la atrofia, se caracterizan por la disminución del tamaño y número de células que componen el órgano afectado *(Myers et al., 2012)*. La ausencia de un incremento en la expresión de IL-1α junto con los cambios histopatológicos e histomorfométricos observados en el timo de todos los animales infectados con PRRSV sugieren que las lesiones observadas en nuestro estudio son de tipo degenerativo.

Además, la muerte celular de los timocitos observada estuvo precedida por un incremento en la expresión local de TNF-α y/o IL-10, principalmente, en la médula tímica de todos los animales infectados, independientemente de la cepa con la que fueron infectados. Estos resultados coinciden con aquellos que señalan una posible asociación entre los fenómenos de muerte celular observados en el pulmón y/o órganos linfoides secundarios, con la expresión de diferentes citoquinas como IL-1, IL-10, IL-6, TNF-α o TGF-β *(Choi et al., 2002; Li et al., 2014; Rodríguez-Gómez et al., 2014)*. Este hallazgo sugiere que al igual que en otros

órganos, el PRRSV podría utilizar un mecanismo indirecto para inducir los fenómenos de muerte celular en el timo.

Hemos observado el desarrollo de una hipoplasia transitoria moderada de las células eritroides, que se ha asociado con un cambio en la relación de las líneas mieloide y eritroide (M:E) en las etapas temprana de la infección (3 dpi) de los animales infectados tanto con la cepa estándar (LV) como con las cepas de campo de alta (SU1-bel) y moderada (215-06) virulencia. Sin embargo, fueron los animales infectados con la cepa estándar (LV) los que desarrollaron la hipoplasia eritroide más grave en comparación con las otras cepas en estudio. Nuestros resultados apoyan la hipótesis de que la infección con el PRRSV induce cambios en el parénquima de la médula ósea y afecta la producción cuantitativa y/o cualitativa de células progenitoras *(Feng et al., 2001)*, lo que estaría relacionado con la existencia de fallos en otros órganos y/o en la instauración de la respuesta inmune del huésped, lo que contribuiría a aumentar la susceptibilidad de los animales a sufrir infecciones secundarias *(Feng et al., 2001; Halbur et al., 2002)*.

Tanto la IL-1α como la IL-6 son poderosos factores estimulantes de la proliferación y diferenciación de CFU-GM en la médula ósea *(Gronda y Bianchi de Di Risio, 1993; Majka et al., 2001; Mak et al., 2005)*. El aumento de la expresión local de estas dos citoquinas al inicio de la infección en la médula ósea de los animales infectados con las diferentes cepas del PRRSV-1, indica que existe una respuesta del parénquima del órgano ante la pérdida inicial de las células hematopoyéticas. Por otra parte, el incremento en la expresión local de TNF-α a los 3 dpi en los grupos infectados con las cepas de campo de moderada (215-06) y alta virulencia (SU1-bel), podría ser la responsable de la inducción de los

fenómenos de muerte celular observados. Además, la expresión local de esta citoquina en la médula ósea puede inhibir la formación de la línea precursora BFU-E e inducir la síntesis de CFU-GM *(Fernández-Delgado, 1992; Gronda y Bianchi de Di Risio, 1993)*. Todos estos hallazgos sugieren que la presencia del PRRSV en médula ósea puede producir tanto de manera directa como indirecta una alteración de las líneas precursoras eritroide y mieloide.

Finalmente, tanto *Morgan et al. (2013)* como *Weesendorp et al. (2014)* sugieren que la virulencia de las cepas altamente patógenas del PRRSV-1, como las cepas SU1-bel y Lena, podrían estar asociada a una respuesta inmune inflamatoria mejorada y no con la capacidad de replicación del virus. En nuestro estudio se demuestra que en los animales infectados con la cepa de alta virulencia (SU1-bel) se desarrolla una lesión pulmonar y una depleción del timo mucho más intensa que con las cepas estándar (LV), de moderada virulencia (215-06) y atenuada (DV). Además, la hipoplasia de la médula ósea en estos animales se prolongó hasta los 7 dpi. La mayor virulencia de la cepa altamente patógena (SU1-bel) estaría relacionada en nuestro estudio con diferentes factores como ser, i) una mayor capacidad de replicación local del virus en el pulmón y timo, ii) una mayor habilidad de inducir la expresión de IL-1α en el parénquima pulmonar y iii) una mayor capacidad del SU1-bel en inducir muerte celular en el timo y producir alteraciones en el parénquima de la médula ósea de los animales infectados, comprometiendo de esta forma la generación y maduración de células inmunocompetentes y por tanto el normal funcionamiento del sistema inmune.

GENERAL DISCUSSION

The emergence of the so-called highly pathogenic PRRS showed the wide phenotypical and genotypical diversity among and within genotypes *(Meng et al, 1996a; Mengeling et al, 1998; Han et al., 2006; Tian et al., 2007; Karniychuk et al., 2010; Xie et al., 2014; Chaikhumwang et al., 2015)*. The cause of such rapid evolution may be primarily due to the lack of PRRSV RdRp proofreading and tremendous viral recombination, resulting in an extraordinary diverse composition of isolates with variable pathogenicity *(Kappes and Faaberg, 2015)*.

Several studies reported that PRRSV is able to produce an imbalance in the expression of different proinflammatory and immunomodulatory cytokines *(Murtaugh et al., 2002; Díaz et al., 2006; Gimeno et al., 2011; Gómez-Laguna et al., 2013; Song et al., 2013; Wang et al., 2013a; Butler et al., 2014; Huang et al., 2015)*. The erratic local expression of different cytokines in several organs in PRRSV infected animals *(López-Fuertes et al., 2000; Johnsen et al., 2002; Chung y Chae, 2003; Labarque et al., 2003; Suradhat et al., 2003; Gómez-Laguna et al., 2009, 2010, 2012; Barranco et al., 2012 a,b; Song et al., 2013; Weesendorp et al., 2013b; García-Nicolás et al., 2014, 2015; Amarilla et al., 2015)* points out that it is necessary to analyze *in situ* the effects of different strains on the immune response and its correlation with the degree of pathogenicity of each PRRSV strain.

In the present study, we have evaluated the expression of PRRSV-antigen, the macroscopic and microscopic lesions, and the profiles of several pro-inflammatory and immunomodulatory cytokines in the lung and primary lymphoid organs (thymus and bone marrow).

The general aim of the study was to compare the immunopathogenesis of PRRSV-1 infection with strains of different virulence (the prototype PRRSV-1, Lelystad virus, LV; two field strains, the highly virulent SU1-bel strain and the strain 215-06, of moderate virulence; and a live-attenuated vaccine, DV) in the lung and primary lymphoid organs of experimentally infected pigs.

In agreement with previous studies *(Halbur et al., 1996a,1996b; Gómez-Laguna et al., 2013)*, we showed that all animals infected with subtype 1 and subtype 3 PRRSV-1 strains, with the exception of animals infected with DV strain, expressed PRRSV antigen mainly in PAMs and macrophages in the thymus with a maximum expression at 7 dpi. The highest replication rate of PRRSV was detected in the lung and thymus of the SU1-bel group. A positive correlation was observed between the number of PRRSV-positive cells and gross pathology in the lung of SU1-bel and LV infected animals. Interestingly, the highest RNA viral load was detected in pigs experimentally infected with the prototype PRRSV-1 LV, remaining high until the end of the experiment (35 dpi). Nonetheless, both field strains (the highly virulent SU1-bel and moderately virulent 215-06) displayed RNA viral load at 3 and 7 dpi, with a high RNA viral load at 7 dpi.

The study of secondary lymphoid organs, such as tonsil, and mediastinal, retropharyngeal and sternal lymph nodes, in the same animals revealed that the highest replication rate of PRRSV and RNA viral load was present in piglets infected with the highly virulent SU1-bel strain, and secondly, in those animals infected with the prototype

PRRSV-1 LV and moderate virulent 215-06 strains *(Morgan et al, 2014; García-Nicolás et al, 2015)*. Considering these data together with those obtained in different studies with the same animals, the overall findings suggest that the expression of viral antigen differs according to the organ and the virulence of PRRSV strains. The highly virulent strain (SU1-bel) was found to possess a major tropism for the lung, secondary lymphoid organs and the thymus, while the prototype PRRSV-1 LV demonstrated a major tropism for the lung and bone marrow. Interestingly, the moderate virulent strain (215-06) displayed a homogeneous distribution in all analyzed organs. On the other hand, the RNA viral load detected by RT-qPCR in the bone marrow at the early stages of infection provided further evidence that the bone marrow represents the first organ which is targeted by PRRSV independently of the PRRSV-1 strain used,

In our study, the highest score of pulmonary pathology was observed at 7 dpi. The animals infected with the highly virulent SU1-bel strain developed severe pulmonary lesions Histologically, all pulmonary lobes from all animals of the different infected groups displayed a multifocal to diffuse, mild to severe, interstitial pneumonia. All cytokines evaluated in this study were mostly detected within the cytoplasm of pulmonary macrophages localized within the septum. The role of PIMs in the production of different cytokines in pigs infected with PRRSV has been previously proposed by our research group *(Gómez-Laguna et al., 2010, 2013)*. In this study, animals infected with the highly virulent SU1-bel strain showed higher levels of IL1-α than the moderate virulent LV and 215-06 strains. Moreover, a positive correlation was detected between the expression of PRRSV and the expression of IL-1α in SU1-bel group. These findings suggest that the SU1-bel strain is a good inducer of IL-1α in the lung, which in turn,

would be responsible for the severity of the interstitial pneumonia. Our results are in agreement with those published by *Weesendorp et al.* in 2014, who reported a higher number of PRRSV positive cells and an increase in the IL-1β mRNA expression in the lung of Lena-infected animals than in animals infected with other PRRSV-1 strains (LV, Belgium A).

A significant positive correlation between the expression of IFN-γ with respect to the expression of TNF-α and IL-6 was detected in all groups infected with PRRSV suggesting that pro-inflammatory cytokines may play a role in the induction of early lesions in PRRS. According to *Gimeno et al.* in 2011, PRRSV-1 strains may be classified as IL-10, TNF-α, both IL-10 and TNF-α inducers or inducer of none of them. In the present study, the expression of IL-10 was significantly higher in the lungs of 215-06 infected pigs and in a lesser extent in SU1-bel-infected animals. Moreover, in the 215-06 group, the expression of IL-10 was positively correlated with the expression of PRRSV antigen and the expression of TNF-α, pointing out that this strain may be an inducer of IL10 and TNF-α. In addition, all infected groups displayed significant positive correlation between the expression of IL-10 and the expression of IFN-γ and IL-6, confirming the immunomodulatory and anti-inflammatory role of this cytokine within the lung in PRRSV infection *(Gómez-Laguna et al., 2013; Huang et al., 2015)*.

In the thymus, the changes were mainly observed in the cortex and characterised by a decrease in the number of thymocytes and CD3-positive cells, and an increased number of tingible-body macrophages

associated with a decreased cCasp3 or TUNEL expression. The thymus of piglets infected with the highly virulent SU1-bel strain displayed the higher expression of cCasp3 and TUNEL, with a positive correlation between cCasp3 and TUNEL labelling and the expression of PRRSV antigen in those animals. In addition, a significant positive correlation was also detected between TUNEL and the expression of PRRSV antigen in LV-infected animals.

In several studies, the degree of depletion and atrophy in the thymus and the ability of PRRSV to induce cell death or apoptosis have been closely associated *(Feng et al., 2002; He et al., 2012)*. The reduced generation of T cells could be responsible for the impaired T cell response *(Rodríguez-Gómez et al., 2013; Salguero et al., 2015)*. The correlation between the expression of some markers for cell death (TUNEL and cCasp3) and the expression of PRRSV antigen, point towards a strain-dependent behavior in the induction of apoptosis, reinforcing the hypotheses that PRRSV-induced cell death could be mediated by direct or indirect mechanisms in the thymus *(Li et al., 2014)*. These results support the fact that PRRSV-1 infection is able to produce a failure or suppression in the immune system of growing piglets.

Inflammatory lesions were characterized by accumulation of fluid and plasma proteins, as well as leukocytes in extravascular tissue. Such a process is usually a well-ordered cascade mediated by chemoattractants, vasoactive molecules and proinflammatory and antiinflammatory cytokines *(Ackermann, 2012)*. On other hand, degenerative lesions such as atrophy are characterized by reduction in the number and/or size of cells *(Myers et al., 2012)*. The absence of an

increase in the expression of IL-1α together with histopathological and histomorphometrical changes in the thymus of all PRRSV-1 infected animals point towards a more degenerative process within this organ.

In addition, and independently of the PRRSV-1 strain virulence, apoptosis of thymocytes was preceded by a peak in the local expression of TNF-α and/or IL-10 principally within the medulla of all infected animals. These data are in agreement with previously reported studies in which there is an association between apoptosis in the lungs and lymphoid tissues with the expression of several cytokines such as IL-1, IL-10, IL-6, TNF-α and TGF-β *(Choi et al., 2002; Labarque et al, 2003; Rodríguez-Gómez et al., 2014)*. Similarly to what take place in other analyzed organs, the present data suggest that PRRSV may also use an indirect mechanism to induce apoptosis in the thymus.

In the present study, we found a moderate, transient hypoplasia of erythroid cells associated with an increased M:E ratio in piglets experimentally infected with the prototype PRRSV-1 Lelystad, the moderate virulent 215-06, and the highly virulent SU1-bel strains at the early stages of infection (3 dpi). Surprisingly, piglets infected with the prototype PRRSV-1 strain Lelystad presented the highest and earliest erythroid hypoplasia compared to 215-06 and the highly virulent SU1-bel strains. Our data support the hypothesis that PRRSV-infection could produce quantitative and/or qualitative changes in the parenchyma of the bone marrow *(Feng et al., 2001)*. This fact could be related to the existence of an immune system failure in other organs and may therefore

contribute to an increased susceptibility to secondary infections in infected animals *(Feng et al., 2001; Halbur et al., 2002)*.

Both IL-1α and IL-6 are powerful stimulating factors for the proliferation and differentiation of CFU-GM progenitors in the bone marrow *(Gronda and Bianchi de Di Risio, 1993; Majka et al., 2001; Mak et al., 2005)*. The increase in the local expression of IL-1α and IL-6 at early stages of infection by PRRSV-1 may indicate a tissue response to the initial loss of hematopoietic cells. On the other hand, the increase of the expression of TNF-α in piglets infected with the moderately virulent 215-06 strain and the highly virulent SU1-bel strain at 3dpi, could play a role in cell death. Moreover, the expression of TNF-α in the bone marrow can inhibit the formation of the BFU-E and induce the synthesis of the CFU-GM *(Fernández-Delgado, 1992; Gronda y Bianchi de Di Risio, 1993)*. Taking all these facts into account, these data suggest that PRRSV could produce direct or indirect changes in erythroid and myeloid cells in bone marrow.

Finally, both *Morgan et al.* in 2013 and *Weesendorp et al.* in 2014 suggested that the virulence of the highly pathogenic PRRSV-1 strains including the Lena and the SU1-bel strains, are associated to a better inflammatory immune response and do not associate with viral replication ability. In our study, we found that the highly virulent SU1-bel strain displayed the most severe pulmonary lesion and thymic depletion than the prototype PRRSV-1 LV, moderately virulent 215-06 and attenuated DV strains. In addition, those animals showed a moderate and transient hypoplasia of the bone marrow that remained until 7 dpi. Thus, the higher virulence of the SU1-bel strain used in our study might

be related first to its great ability to replicate in the lung and thymus; secondly, to its ability in inducing IL-1α production in the lung; and thirdly, to its higher capacity to induce cell death in the thymus and changes in the bone marrow parenchyma. These alterations might all together compromise the normal process of formation and maturation of immunocompetent cells and thus, impair the normal functioning of the immune system.

CAPÍTULO 5 / CHAPTER 5

CONCLUSIONES

CONCLUSIONS

CONCLUSIONES GENERALES

Primera: correspondiente al estudio "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"

En el pulmón, la cepa altamente virulenta SU1-bel del subtipo-3 PRRSV-1, se replica de manera más eficiente en el pulmón de cerdos infectados induciendo una mayor expresión de citoquinas pro-inflamatorias, principalmente IL-1α; en cambio la cepa moderadamente virulenta 215-06 del subtipo-1 PRRSV-1 induce una mayor expresión de IL-10 en comparación con otras las cepas del PRRSV-1. Lo que demuestra que la capacidad de inducir la expresión de diferentes citoquinas a nivel pulmonar está relacionada con el grado de virulencia de los diferentes aislados de PRRSV-1, relacionándose de forma directa con el desarrollo de los signos clínicos y las lesiones pulmonares.

Segunda: correspondiente al estudio "Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains"

Las diferentes cepas del PRRSV-1 estudiadas, independientemente de su virulencia, inducen la existencia de fenómenos de apoptosis de los timocitos. La cepa de alta virulencia (cepa SU1-bel) induce una mayor depleción del

timo correlacionada con la mayor expresión del antígeno vírico en comparación con las cepas estándar (cepa LV) y de moderada virulencia (cepa 215-06). Además, la apoptosis de los timocitos está precedida por un aumento de la expresión local, en la médula tímica, de TNF-α y/o IL-10.

Tercera: correspondiente al estudio "Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the viral load and virulence"

Las diferentes cepas de PRRSV-1 son capaces de inducir, independientemente de la virulencia de la cepa y la carga viral, una hipoplasia moderada y transitoria de la línea eritroide, con un aumento de la relación M:E al inicio de la infección. Adicionalmente, la hipoplasia eritroide, la hiperplasia mieloide y el aumento de la relación M:E están asociados con un aumento de la expresión local de IL-1α, IL-6 y TNF-α.

GENERAL CONCLUSIONS

First: corresponding the study "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"

The subtype 3 European SU1-bel strain replicates more efficiently in the lung of infected animals, as well as induces a higher expression of proinflammatory cytokines, mainly IL-1α, than the other strains under study (LV, 215-06, DV strains). On the other hand, the subtype 1 European 215-06 strain induces the highest expression of IL-10 in comparison with the other examined PRRSV-1 strains. These findings suggest that the differential expression of cytokines at lung level may have a key role in the virulence and the development of the onset of the clinical signs.

Second: corresponding the study "Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains"

The different examined PRRSV-1 strains induce, independently of their virulence, changes in the cortex of the thymus due to apoptosis of thymocytes. Highly virulent PRRSV-1 (SU1-bel) shows the most severe depletion of the thymus with the highest expression of PRRSV antigen compared to the moderate virulent strains of the pan European

subtype 1 (the prototype LV strains and the field strain 215-06). In addition, apoptosis of thymocytes is preceded by a peak in the local expression of TNF-α and/or IL-10 chiefly in medulla of all infected animals.

Third: corresponding the study "Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the viral load and virulence"

At early stages of infection, independently of the viral load and virulence, different PRRSV-1 strains are capable to induce moderate and transient hypoplasia of erythroid cells and increased M:E ratio in the bone marrow of experimentally infected piglets. In addition, we provide evidences that erythroid cell hypoplasia, myeloid cell hyperplasia and increased M:E ratio are associated to a peak in the local expression of IL-1α, IL-6 and TNF-α in all infected animals.

CAPÍTULO 6 / CHAPTER 6

ASPECTOS FUTUROS

FUTURE ASPECTS

ASPECTOS FUTUROS

Está ampliamente aceptado por la comunidad científica que, independientemente de la variabilidad genética o fenotípica, el PRRSV provoca una desregulación de la respuesta inmune innata o adaptativa del hospedador y que las medidas actuales para su control son ineficaces. *(Forsberg et al., 2002; Gómez-Laguna et al., 2013; Butler et al., 2014; Huang et al., 2014; Kappes y Faaberg, 2015)*.

Nuestros resultados han puesto de manifiesto que la cepa de alta virulencia (SU1-bel) del subtipo-3 del PRRSV-1, provoca un cuadro clínico y lesional mucho más grave que las otras cepas de menor virulencia (LV, 215-06 y DV) del subtipo-1 del PRRSV-1 estudiadas. Además, concuerdan con los obtenidos por *Karniychuk et al. (2010)* y *Weesendorp et al. (2014)* que también compararon una cepa del subtipo-3 (Lena) con dos cepas del subtipo-1 del PRRSV-1 (LV y Belgium A). Sin embargo, si comparamos ambas cepas del subtipos-3 (Lena *versus* SU1-bel) se observa que animales infectados con la cepa Lena presentan una carga viral más alta que los cerdos infectados con la cepa SU1-bel y que estos animales presentan un cuadro clínico más grave que los infectados con la cepa Lena. Ambos grupos de animales infectados con las cepas SU1-bel y Lena mostraron un cuadro lesional similar *(Karniychuk et al 2010; Morgan et al., 2013)*.

Morgan et al. (2013) y *Weesendorp et al. (2014)* sugieren que la mayor virulencia de ambas cepas del subtipo-3 del PRRSV-1, Lena y SU1-bel, es debida a que estas cepas son capaces de producir una respuesta inmune mejorada. Sin embargo, en nuestro estudio se ha

puesto de manifiesto que en la infección por la cepa SU1-bel se produce en el pulmón una correlación positiva entre el antígeno vírico, el cuadro lesional y la expresión local de IL-1α. Una infección que, además, induce la apoptosis de los timocitos y, por tanto, una depleción del timo, así como una hipoplasia moderada y transitoria de la médula ósea, lo que sugiere que la respuesta inmune estaría comprometida. Recientemente, *Lu et al. (2015)* han realizado la secuenciación completa de la cepa SU1-bel, demostrando que tan sólo tiene una similitud del 79% con la cepa LV y del 88% con la cepa Lena, y que, aunque tiene un genoma más corto y una alta variabilidad en ciertos grupos de genes, presenta múltiples nuevos sgRNA que codifican proteínas aún no descritas para el PRRSV-1.

Aunque el PAM es considerado la célula diana del PRRSV, el virus es capaz también de replicarse en otros macrófagos y subpoblaciones de macrófagos de diferentes órganos *(Halbur et al., 1996b; Duan et al., 1997a; Thanawongnuwech et al., 2000; Gómez-Laguna et al., 2010)*. Incluso, cepas HP-PRRSV, como JXwn06 presenta un tropismo a células epiteliales *(Li et al., 2007; Hu et al., 2013)*. Esta interacción virus/célula aún no está completamente definida, a pesar de que se reconocen al menos seis moléculas diferentes como potenciales receptores del PRRSV *(Kim et al., 2006; Calvert et al., 2007; Shanmukhappa et al., 2007; Huang et al., 2009)*. En este sentido, la mayoría de los estudios coinciden en que el receptor de la membrana celular CD163 podría ser el receptor primario del PRRSV *(Calvert et al., 2007; Van Gorp et al., 2008; Patton et al., 2009; Wang et al., 2013c)* y la sialoadesina (Sn) podría funcionar como una proteína accesoria para la entrada del virus *(Vanderheijden et al., 2003; Delputte y Nauwynck, 2004; De Baere et al., 2012)*. Sin embargo, *Frydas et al. (2013)* han descrito un fenotipo celular $CD163^{-}Sn^{-}$ susceptibles a la

infección por la cepa HP-PRRSV Lena. Estos hallazgos ponen de manifiesto la necesidad de estudiar la interacción entre las diferentes células susceptibles a la infección y la expresión de receptores del virus con cepas del PRRSV de diferente grado de patogenicidad.

Por otra parte, las infecciones víricas dan lugar a la activación de los receptores de reconocimiento de patrones (PRRs), gracias al reconocimiento de los patrones moleculares asociados a patógenos (PAMPs, del inglés *Pathogen-associated molecular patterns*); juntos inician una cascada de señalización antiviral con la producción de varias citoquinas, que son esenciales para la activación e interacción de la respuesta inmune innata y adaptativa del hospedador. Entre los PRRs destacan por su interés dos tipos de receptores intracelulares, el RIG-I (de inglés, *retinoic acid inducible gene-I*) y el MDA5 (del inglés, *melanoma differentiation associated gene 5*) que actúan como centinelas para la detección del ARN viral en el citoplasma. La estimulación de estos receptores provoca el ensamblaje e iniciación de la cascada de señalización, lo que resulta en la transcripción de IFNs tipo I *(Akira et al., 2006; Honda et al., 2006; Takeuchi y Akira, 2008; Loo y Gale, 2011; Huang et al., 2014)*. En la actualidad se carece de información sobre la evolución en la expresión de estos receptores en los órganos de interés en la patogenia del PRRS, tras la infección con cepas del PRRSV-1 de diferente grado de patogenicidad; por lo que sería de interés el estudio de estos receptores considerando que los IFNs tipo I son importantes citoquinas antivirales que limitan la replicación y propagación viral.

En resumen, estos antecedentes nos indican la necesidad de profundizar en el conocimiento de los mecanismos de interacción entre

el sistema inmune del cerdo y el PRRSV, para determinar los mecanismos moleculares utilizados por el virus y sus proteínas que modulan las respuesta inmune del huésped y vías de señalización según la virulencia de la cepa y el tipo de órgano afectado. Así como sobre las características de los macrófagos infectados y no infectados, ya que estos actúan como APCs en los órganos linfoides primarios y secundarios. *Butler et al. (2014)* señalan que **"*la belleza genética del PRRRV es su gran número de variantes disponibles y otras que pueden ser diseñadas*"**. Estamos convencidos de que la secuenciación completa de las diferentes cepas facilitará establecer comparaciones entre ellas e identificar las secuencia comunes que, podrían servir de base para la creación de mutantes que ayuden a determinar con mayor precisión las características genéticas del PRRSV que provocan la desregulación de las vías moleculares del hospedador, sin olvidar que estos mutantes podrían servir como nuevos candidatos vacunales.

Finalmente, debemos de ser cautelosos con los resultados obtenidos a nivel celular y/o molecular, ya que varios estudios han demostrado claramente que podemos estar frente a una enfermedad con dos modelos biológicos diferentes cuando nos referimos a respuestas inmunológicas: los animales adultos y los animales recién nacidos *(Butler et al., 2007, 2008; Sinkora et al., 2014)*.

FUTURE ASPECTS

It is widely accepted by the scientific community that PRRSV, independently of its genetic and phenotypic variability, is able to produce a dysregulation of the innate and adaptive immune responses, and that the methods applied to time are not effective to control this disease *(Forsberg et al., 2002; Gómez-Laguna et al., 2013; Butler et al., 2014; Huang et al., 2014; Kappes y Faaberg, 2015).*

The results of the present study point out that the subtype-3 highly virulent strain (SU1-bel) of the PRRSV-1 display the most severe clinical signs and morphologic lesions comparing to others subtype-1 PRRSV-1 strains, such as the moderate virulent strains LV, 215-06 and DV. These data are in agreement with those obtained in studies conducted by *Karniychuk et al.* in 2010 and *Weesendorp et al.* in 2014 in which they compared a subtype-3 strain (Lena) with two other PRRSV-1 subtype-1 strains (LV and Belgium A). Nevertheless, animals infected with the Lena strain displayed higher viral load and more severe clinical symptoms than piglets infected with SU1-bel strain. However, both groups of infected animals (Lena and SU1-bel) showed similar lesions *(Karniychuk et al 2010; Morgan et al, 2013).*

Morgan et al. in 2013 and *Weesendorp et al. in 2014* suggested that the high virulence of both subtype-3 PRRSV-1 strains, Lena and SU1-bel, is related to the ability of these strains to induce an enhanced early immune response. However, our results indicate a positive correlation between viral antigen, pathology and expression of IL-1α in the lung parenchyma of piglets infected with highly virulent SU1-bel

strain. In addition, these animals displayed an increased presence of thymocyte apoptosis with consecutive depletion of the thymus, and a moderate and transient bone marrow hypoplasia, suggesting that SU1-bel strain infected piglets possess a compromised immunologic response. Recently, *Lu et al.* in 2015 sequenced the whole genome of the SU1-bel strain genome and evidenced that the SU1-bel strain has 79% and 88% similarity with the prototypical Lelystad (LV) and Lena strains, respectively. Moreover, the SU1-bel genome is the shortest among these three strains and has a higher variability in some gene clusters, as well as new several sgRNA, with some of them encoding novel proteins not previously described in other PRRSV-1 strains.

Although PAMs are target cells for PRRSV, the virus is able to replicate in macrophages and other subpopulations of macrophages from different organs *(Halbur et al., 1996b; Duan et al., 1997a; Thanawongnuwech et al., 2000; Gómez-Laguna et al., 2010)*. Recently, it has been reported that HP-PRRSV strains such as the JXwn06-81c has a cellular tropism for epithelial cells *(Li et al., 2007; Hu et al., 2013)*. Although, there are six different molecules recognized as potential entry receptors for PRRSV, the relation between the viral/cell interaction remains still unclear *(Kim et al., 2006; Calvert et al., 2007; Shanmukhappa et al., 2007; Huang et al., 2009)*. Several studies agree with the fact that the CD163 receptor may be the primary receptor for PRRSV *(Calvert et al., 2007; Van Gorp et al., 2008; Patton et al., 2009; Wang et al., 2013c)* and sialoadhesin could act as an accessory protein for PRRSV entry *(Vanderheijden et al., 2003; Delputte y Nauwynck, 2004; De Baere et al., 2012)*. However, *Frydas et al.* reported in 2013 that the HP-PRRSV Lena strain is able to replicate in $CD163^{-}Sn^{-}$ cells. These data provide further insights in the necessity of studying the relation

between receptors and susceptible cells with different virulent PRRSV strains.

On other hand, viral infections are the result of the activation of pattern recognition receptors (PRRs) by recognizing specific pathogen-associated molecular patterns (PAMPs). Together, PRRs and PAMPs initiate antiviral signaling cascades which trigger the production of several cytokines which are essential for the activation and interaction of the host innate and adaptive immune responses. Two intracellular PRRs, the retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) act as sentinels for detecting viral RNA in the cytoplasm. Their stimulation triggers the assembly and initiation of signaling cascades resulting in the transcription of type I IFNs *(Akira et al., 2006; Honda et al., 2006; Takeuchi y Akira, 2008; Loo y Gale, 2011; Huang et al., 2014)*. The lack of information about the distribution and the expression of these receptors in the main organs of interest after infection with different virulent PRRSV-1 strains justifies the development of new studies with this aim, taking also into account that type I IFNs are important antiviral cytokines that limit viral replication and spread.

In summary these findings point out that there is a necessity to investigate in depth the interaction between the host immune system and PRRSV aiming to better understand the molecular mechanisms used by PRRSV, the proteins that modulate the host immune responses and the signaling pathways depending on the strain virulence and the affected lymphoid organ. Moreover, it is noteworthy to elucidate important characteristics of PRRSV-infected and uninfected macrophages taking into account that such cells act as APCs in primary and secondary

lymphoid organs. *Butler et al. in 2014* stated that ***"the beauty of PRRRV genetics is that a large number of variant are available and others can be engineered"***. We believe that the complete sequencing will facilitate comparisons between different strains and provide the identity of common sequences. In addition, it will serve as a basis for the creation of PRRSV mutants that could help to determine more precisely the genetic modifications of PRRSV which are responsible for the dysregulations of the molecular pathways in the specific host, and more importantly, could be use as novel vaccine candidates.

Finally, we are aware that all results at the cellular/molecular level should be interpreted carefully considering that several studies demonstrated that we may be dealing with a disease with two different biologic models regarding the triggered immune response: one for adult animals and one for neonates *(Butler et al, 2007, 2008; Sinkora et al, 2014)*.

CAPÍTULO 7 / CHAPTER 7

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Research paper

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

Shyrley P. Amarilla[a,*], Jaime Gómez-Laguna[b], Librado Carrasco[a], Irene M. Rodríguez-Gómez[a], José M. Caridad y Ocerín[c], Sophie B. Morgan[d], Simon P. Graham[d], Jean-Pierre Frossard[d], Trevor W. Drew[d], Francisco J. Salguero[d,e]

[a] Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, Campus Universitario de Rabanales, 'International Excellence Agrifood Campus, CeiA3', 14071 Cordoba, Spain
[b] CICAP – Food Research Centre, 14400 Pozoblanco, Córdoba, Spain
[c] Department of Statistics, Econometrics, Operations Research, Business Organization and Applied Economics, Faculty of Law and Economics, 14071 Cordoba, Spain
[d] Department of Virology, Animal Health and Veterinary Laboratories Agency-Weybridge, Addlestone, Surrey KT15 3NB, UK
[e] Department of Pathology and Infectious Diseases, School of Veterinary Medicine, University of Surrey, Guildford GU2 7TE, UK

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ABSTRACT

Porcine reproductive and respiratory syndrome viruses (PRRSV) show high genetic differences both among and within genotypes. Recently, several highly pathogenic PRRSV (HP-PRRSV) strains have been described. This study compares and characterizes the production of cytokines by pulmonary macrophages in pigs experimentally infected with four different PRRSV-1 strains: two low-virulent strains, Lelystad (LV) and a British field strain (215-06); a HP strain (SU1-bel) from Belarus and the attenuated vaccine strain DV (Porcilis® PRRS). Animals were clinically monitored and post-mortem examinations were performed at 3, 7 and 35 days post-infection (dpi). Lung samples were processed for histopathological and immunohistochemical studies by using specific antibodies against PRRSV, IL1-α, IL-6, TNF-α, IL-10 and IFN-γ. SU1-bel infected animals presented the highest mean scores for clinical observations, gross and microscopic lesions as well as for PRRSV expression compared with the other infected groups ($p \leq 0.027$). These animals displayed the highest expression of IL1-α at 7 dpi, together with the highest score for lung pathology, whereas LV, 215-06 and DV inoculated animals only showed a transient enhancement in some of these cytokines. SU1-bel-infected pigs showed a positive correlation between the amount of PRRSV antigen and IL-1α expression ($r = 0.645$, $p < 0.001$). The highest expression of IL-10 was detected in 215-06-infected animals ($p \leq 0.004$), with a positive correlation with the numbers of virus-infected cells ($r = 0.375$, $p \leq 0.013$). In conclusion, the HP-PRRSV SU1-bel strain replicated more efficiently in the lung of infected animals and induced a higher expression of IL-1α than the other PRRSV-1-infected groups, which may have played a key role in the onset of the clinical signs and interstitial pneumonia.

* Corresponding author. Tel.: +34 957218162.
E-mail address: ashyrleypaola@yahoo.com (S.P. Amarilla).

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single stranded RNA virus belonging to the order *Nidovirales*, family *Arteriviridae*, genus Arterivirus (Meulenberg, 2000; Snijder et al., 2013). PRRSV strains are classified into two distinct genotypes, type 1 or European PRRSV (PRRSV-1) and type 2 or North American PRRSV (PRRSV-2), with significant genetic differences among them. The European type strains can be further divided into at least three subtypes: Pan-European subtype 1 and Eastern European subtypes 2 and 3 (Stadejek et al., 2008). Several authors have described differences in virulence among European PRRSV subtypes, finding that subtype 3 strains, such as Lena, are significantly more pathogenic than subtype 1 strains (Karniychuk et al., 2010; Morgan et al., 2013, 2014; Weesendorp et al., 2013, 2014; García-Nicolás et al., 2014a). Numerous studies have demonstrated that PRRSV-2 strains induce more severe respiratory disease comparing to PRRSV-1 strains (Martinez-Lobo et al., 2011; Han et al., 2013a, 2013b, 2014). Moreover, it has been proved that several isolates of PRRSV-2 possess different virulence and pathogenic characteristics (Halbur et al., 1996; Shang et al., 2013).

The increased clinical and pathological effect of the SU1-bel strain, an Eastern European subtype 3 strain, has recently been associated to an enhanced inflammatory immune response rather than higher levels of virus in blood or broncho-alveolar lavage fluid (BALF) (Morgan et al., 2013). The mechanisms by which a PRRSV strain exerts its virulence are not fully understood, but it has been suggested to be related to the *in vivo* replication capacity, tissue distribution, or immunomodulatory properties (Haynes et al., 1997; Johnson et al., 2004; Loving et al., 2008; Forsberg et al., 2002; Rodriguez-Gomez et al., 2013a,b). In addition, the immune response varies in pigs infected with different European PRRSV strains. Whereas, Morgan et al. (2013) evidenced significantly higher numbers of PRRSV-specific IFN-γ producing cells in SU1-bel infected animals when compared with subtype 1 strains, Weesendorp et al. (2013) detected a lower level of IFN-γ secreting cells in Lena infected-pigs. In this latter study higher cytokine mRNA levels in blood, mainly TNF-α, were observed in pigs infected with the Lena strain than in animals infected with Belgium A and LV strains during the first week post-infection. Besides, $CD8^{-}$ γδ T cells displayed a significant decreased in Lena-infected animals along the first month post-infection as long as Natural Killer (NK) and cytotoxic T cell subsets were enhanced in the animals infected with other PRRSV-1 strains (Weesendorp et al., 2013).

Cytokines play an important role in the induction and regulation of immune responses, and their production by macrophages and other immune or non-immune cells plays a key role in the induction of pathology or protective immunity (Van Reeth and Nauwynck, 2000; Barranco et al., 2012a). PRRSV replicates primarily in pulmonary alveolar macrophages (PAMs), and the expression of proinflammatory cytokines has been observed mainly in septal macrophages, suggesting that they are activated indirectly by the replication of PRRSV in bystander cells (Gomez-Laguna et al., 2010, 2013). Under most conditions the degree of pathological damage in respiratory viral infections does not correlate with the number of virus particles in the infected tissues, rather aberrant induction and imbalance of cytokines may induce a severe systemic inflammatory response syndrome (Kimura et al., 2013). Cytokines, such as IL-1α, IL6, and TNF-α, secreted by macrophages and other immune cells have various biological effects on the organism that may protect against infection. However, these cytokines may also induce inflammation and tissue damage when they are over-expressed (Liu et al., 2010; Kimura et al., 2013).

Gimeno et al. (2011) found that different PRRSV-1 strains are able to induce *in vitro* different patterns of expression of IL-10 and TNF-α and classified four possible phenotypes based on their ability to induce IL-10 and/or TNF-α. Moreover, the expression of IL-1α, IL-6 and TNF-α in the lungs of pigs infected with PRRSV-1 has been correlated with the development of the interstitial pneumonia typical of this disease (Gomez-Laguna et al., 2010). The variability in cytokine profiles induced by different PRRSV strains, as well as the emergence of HP-PRRSV in Asia and Eastern Europe, highlight the need to evaluate differences in the immunobiology among PRRSV strains of differing virulence (Gomez-Laguna et al., 2013). This study aimed to compare and characterize the production of different cytokines by subpopulations of pulmonary macrophages in pigs experimentally infected with different PRRSV-1 strains.

2. Materials and methods

2.1. Animal selection and housing

The experimental design is described in detail by Morgan et al. (2013). In short, sixty-five 5-weeks old male piglets, negative for PRRSV were obtained from an isolated, specific-pathogen-free pig farm in the Netherlands. All pigs were confirmed negative against PRRSV, porcine circovirus type 2 (PCV-2) and *Mycoplasma hyopneumoniae* by ELISA and PCR. Besides *M. hyopneumoniae* culture from all animals at the end of the study yielded negative results. The animals were randomly allocated into five groups and housed in separate pens of a containment facility at the AHVLA. In this study we analysed five animals within the infected groups and two animals within the control group per dpi (Table 1). This experiment was approved by the

Table 1
The organization of all pigs experimentally infected with four different PRRSV-1 strains: LV, 215-06, SU1-bel and DV. Animals were euthanized at 3, 7 and 35 dpi.

DPI	Control	LV[a]	215-06[b]	SU1-bel[c]	DV[d]
3	2	4	5	5	5
7	2	5	5	5	5
35	2	5	5	5[e]	5

[a] LV: Lelystad virus-Ter Huurne. The prototype PRRSV-1 strain.
[b] 215-06: British field strain.
[c] SU1-bel: highly pathogenic strain.
[d] DV: attenuated vaccine strain.
[e] Two animals in the SU1-bel group displayed a prolonged fever along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi.

AHVLA ethical review committee, and all procedures were carried out under the Animals (Scientific Procedures) Act, 1986, UK.

2.2. Viruses

Four PRRSV-1 strains were used in this study, as previously reported (Morgan et al., 2013): Lelystad virus-Ter Huurne (LV), the prototype PRRSV-1 strain; strain 215-06, isolated from the serum of a post-weaning piglet showing signs of wasting and poor condition in 2006; strain SU1-bel, isolated from lung tissue homogenate from a 30-day old piglet from a farm in Belarus in 2010; and, the attenuated strain DV (Porcilis® PRRS, Intervet BV, Boxmeer, The Netherlands). LV, 215-06 and SU1-bel strains were propagated in PAMs and cultured in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (Autogen Bioclear, Calne, UK) and 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies) (cRPMI) for 3 days in a humidified incubator at 37 °C with 5% CO_2. Both SU1-bel and 215-06 strains were used at the 4th passage and the LV strain was used at the 8th passage (Morgan et al., 2013).

2.3. Experimental design and animal monitoring

Pigs were randomly allocated and statistically blocked by weight as follows: the control group consisted of six pigs while each virus-infected group contained 14–15 animals. At 7 weeks of age, after acclimatizing for 14 days, the piglets were inoculated intranasally with 10^5 $TCID_{50}$ of the respective virus (either LV, 215-06 or SU1-bel) in 1.5 ml of cRPMI. The group infected with strain DV was inoculated with 10^4 $TCID_{50}$. Controls were inoculated with 1.5 ml of PAM cryolysate diluted in cRPMI. Every day, from 3 days before experimental inoculation until the end of the experiment (35 dpi) rectal temperatures and clinical signs were recorded. Clinical signs associated with PRRSV infection were scored between 0 and 3, where 0 was normal and a score between 1 and 3 represented increasing severity of each observation as previously described (Weesendorp et al., 2013). No equipment was shared and staff changed in between rooms to prevent virus transmission between the groups. At days 3, 7 and 35 post-infection (dpi), two pigs from the control group and four to five pigs from each infected group were euthanized by administration of an intravenous lethal dose of pentobarbitone, followed by exsanguinations.

2.4. Gross and histopathological lesions

At necropsy, gross pathology scores of the lungs were performed based on the method developed by Halbur et al. (1995). After gross pathology examination, samples of the right lung (including apical, cardiac and diaphragmatic lobes) were collected and fixed by immersion in 10% neutral buffered formalin (Fisher Scientific Ltd., Leicestershire, UK) and in Bouin's solution (Fisher Scientific Ltd.) and routinely processed, for the histopathological and immunohistochemical studies. Four μm sections were stained with haematoxylin and eosin (HE) for microscopical examination. Lung sections were blindly examined by two pathologists and scored for the estimated severity and distribution of the interstitial pneumonia as previously reported (Halbur et al., 1995).

2.5. Immunohistochemistry

The Avidin–Biotin–Peroxidase complex technique (ABC) was used for the detection of PRRSV, in formalin-fixed tissues, and cytokines, in Bouin-fixed tissues, as previously described (Gomez-Laguna et al., 2010), with the following modifications: the Labelled Streptavidin Biotin (LSAB) method was used specifically for the detection of IL-6. Briefly, 4 μm sections were dewaxed in xylene and acetone, and rehydrated through graded ethanol solutions. Endogenous peroxidase activity was quenched by incubation in a 3% H_2O_2 solution in methanol for 30 minutes (min). The primary antibodies used were monoclonal anti-PRRSV (clones SDOW-17 and SR-30; Rural Technologies Inc., Brookings, USA) diluted 1 in 1000; polyclonal anti-pig IL-1α (Endogen, Woburn, USA) diluted 1 in 100; monoclonal anti-human TNF-α (clone 68B6A3 L1; Invitrogen, Camarillo, USA) diluted 1 in 75; polyclonal anti-pig IL-6 (Thermo Scientific, Rockford, USA) diluted 1 in 100; polyclonal anti-pig IL-10 (R&D Systems, Abingdon, United Kingdom) diluted 1 in 20; and polyclonal anti-pig IFN-γ (R&D Systems) diluted 1 in 20. The antigen retrieval method used for all the cytokines studied was permeabilisation with Tween 20 diluted 0.01% in PBS during 10 min, but for monoclonal anti-PRRSV antibodies high temperature antigen retrieval with tri-sodium citrate dihydrate buffer pH 6.0 was used. Primary antibodies were incubated overnight at 4 °C in a humid chamber, but IL-6 which was incubated 1 h at 37 °C. In each case, the corresponding biotinylated secondary antibody was incubated for 30 min at room temperature. An avidin-peroxidase complex (Vector Laboratories, Burlingame, USA) was applied for 1 h at room temperature. Labelling was "visualized" by application of the NovaREDTM substrate kit (Vector Laboratories). Dako LSAB + System-HRP and liquid DAB + Substrate Chromogen System were used for the antibody polyclonal anti-pig IL-6 visualization. All sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Primary antibody-omitted negative controls (replacing the primary antibody by blocking solution) and isotype-matched reagents or negative control rabbit immunoglobulin fraction (DAKO) of irrelevant specificity were used in each immunohistochemistry run.

The number of labelled cells was determined as previously described (Salguero et al., 2005). Briefly, the labelled cells were counted in 50 non-overlapping and consecutively selected high magnification fields of 0.20 mm^2. Results were expressed as the number of cells per mm^2. Immunolabelled cells were identified and counted morphologically as macrophages, lymphocytes or neutrophils. Pulmonary intravascular macrophages and interstitial macrophages were grouped together and described as 'septal macrophages'. The results are expressed as the mean of the immunolabelled cells in the three lung lobes of each animal at each time-point.

2.6. Statistical analysis

Several univariate models were estimated for different response variables, and also a multivariate analysis of variance, for the macroscopic and histopathological lung lesions, PRRVS antigen and the expression of cytokines, depending on the factors that may be cause of variation: time-point, lung lobes and strains. The control group was only included in the study as a reference of the basal level and was not included in the statistical analysis because it had no sufficient number of animals at each time-point. Since vaccinated animals (DV group) presented a similar evolution than control animals along the study, all infected groups were compared among them and with the DV group and the differences were assessed by Dunnet, *t*-student and Mann Whitney tests, according to the parameters under study. Correlation between gross and histopathological lung lesions and the expression of virus and cytokines was assessed by the Pearson and Spearman test. Differences with p-values ≤ 0.05 were considered to be statistically significant (SPSS 22.0, IBM SPSS Statistics, Chicago, USA).

3. Results

3.1. Clinical signs and gross pulmonary lesions

The clinical signs and gross pulmonary lesions have previously been described elsewhere (Morgan et al., 2013, 2014). Briefly, SU1-bel infected animals had mean temperatures above 40 °C and were significantly higher than controls at 3 and between 6 and 10 dpi. These temperatures were higher than controls at 3 and between 6 and 10 dpi. Mean temperatures of the LV, 215-06 and DV groups were not different from controls. Clinical observations showed that the SU1-bel group had higher mean clinical scores between 5 and 17 dpi compared to the other groups. Two animals in the SU1-bel group displayed a prolonged hyperthermia along with high clinical scores and were euthanized for welfare reasons at 12 and 13 dpi. The mean scores of the other groups did not increase above 3 for the duration of the study.

At post-mortem, a score dependent on the percentage of the lung surface affected by gross lesions was given, with a maximum of 100 points. At 3 dpi gross lesions, consistent with mottled tan and rubbery pulmonary parenchyma which failed to collapse, were only detected in three and two animals of the 215-06 and SU1-bel groups, respectively, with the latter displaying the highest average score. Statistically significant differences were detected in the score for gross lesions at different time-points ($p = 0.001$) and between different strains ($p < 0.001$), with a significant interaction between time-point and strain ($p < 0.001$). SU1-bel ($p = 0.027$) and 215-06 groups ($p < 0.001$) displayed the highest score for gross lesions when compared with DV group. Moreover, at 7 dpi SU1-bel group displayed statistically significant higher score for gross lesions than LV and 215-06 groups ($p \leq 0.004$ in each group) (Fig. 1A). The SU1-bel animals euthanized at 12 and 13 dpi presented high gross pathology scores of 66 and 39, respectively. No gross pathology was seen in animals from the DV group along the study (Morgan et al., 2014).

3.2. Microscopic lung lesions

The microscopic lesions were characterized by thickened alveolar septa with increased numbers of interstitial mononuclear cells (macrophages and lymphocytes), hyperplasia and hypertrophy of type II pneumocytes and alveoli filled with necrotic/apoptotic cell debris and macrophages. These lesions when present were multifocal to diffuse, mild to severe. Mild to moderate perivascular lymphohistiocytic infiltrate was observed in some animals, mostly in apical, cardiac and diaphragmatic lung lobes, from 3 dpi onwards. Microscopic lung lesion scores are summarized in Fig. 1B. All infected groups displayed a similar kinetics with a peak of microscopical lesions at 7 dpi. No remarkable microscopic lung lesions were observed in control animals throughout the study. Statistically significant differences were observed at different time-points and between strains ($p < 0.001$), but not among different lung lobes. In addition, a significant interaction between time-point and strain was detected ($p < 0.001$). Statistically significant differences were observed between LV, SU1-bel and 215-06 groups with respect to DV group ($p < 0.001$). SU1-bel and 215-06 strains showed statistically significant higher score for microscopic lesions than LV group at 3 dpi ($p < 0.001$ in each group), with SU1-bel group showing the highest score at 7 dpi ($p < 0.001$ with respect to LV; and, $p = 0.010$ with respect to 215-06) (Fig. 1B). At 35 dpi 215-06 infected animals displayed the highest score for microscopic lung lesion when compared with SU1-bel ($p = 0.033$) and LV ($p = 0.018$) groups (Fig. 1B). Animals from DV group displayed minimal lesions throughout the study. The SU1-bel animals euthanized at 12 and 13 dpi had the highest microscopic lung lesion score (3.3 and 3.5, respectively).

3.3. Labelling of PRRSV antigen

PRRSV antigen was detected from 3 dpi until the end of the study in all virus-infected animals except for the animals infected with DV strain and control group. Statistical analysis of expression of PRRSV antigen demonstrated significant differences between different time-points and different strains ($p < 0.001$), with a significant interaction between both variables ($p < 0.001$). Differences in the expression of PRRSV antigen were detected for each strain at different time-points. Statistically significant differences were detected between LV, 215-06 and SU1-bel groups with respect to DV group ($p < 0.010$). In addition, SU1-bel and 215-06 groups showed a statistically significant higher expression of viral antigen than LV group ($p < 0.001$ and $p = 0.005$, respectively), with SU1-bel group displaying the highest expression of PRRSV antigen at 7 dpi ($p = 0.002$ with respect to LV group; and, $p = 0.004$ with respect to 215-06 group) (Fig. 1C).

3.4. Tissue expression of proinflammatory cytokines

The expression of proinflammatory cytokines was mainly observed in the cytoplasm of septal macrophages and PAMs, and for IL-1α also in the cytoplasm of neutrophils. All proinflammatory cytokines were mostly detected in the cytoplasm of septal macrophages compared

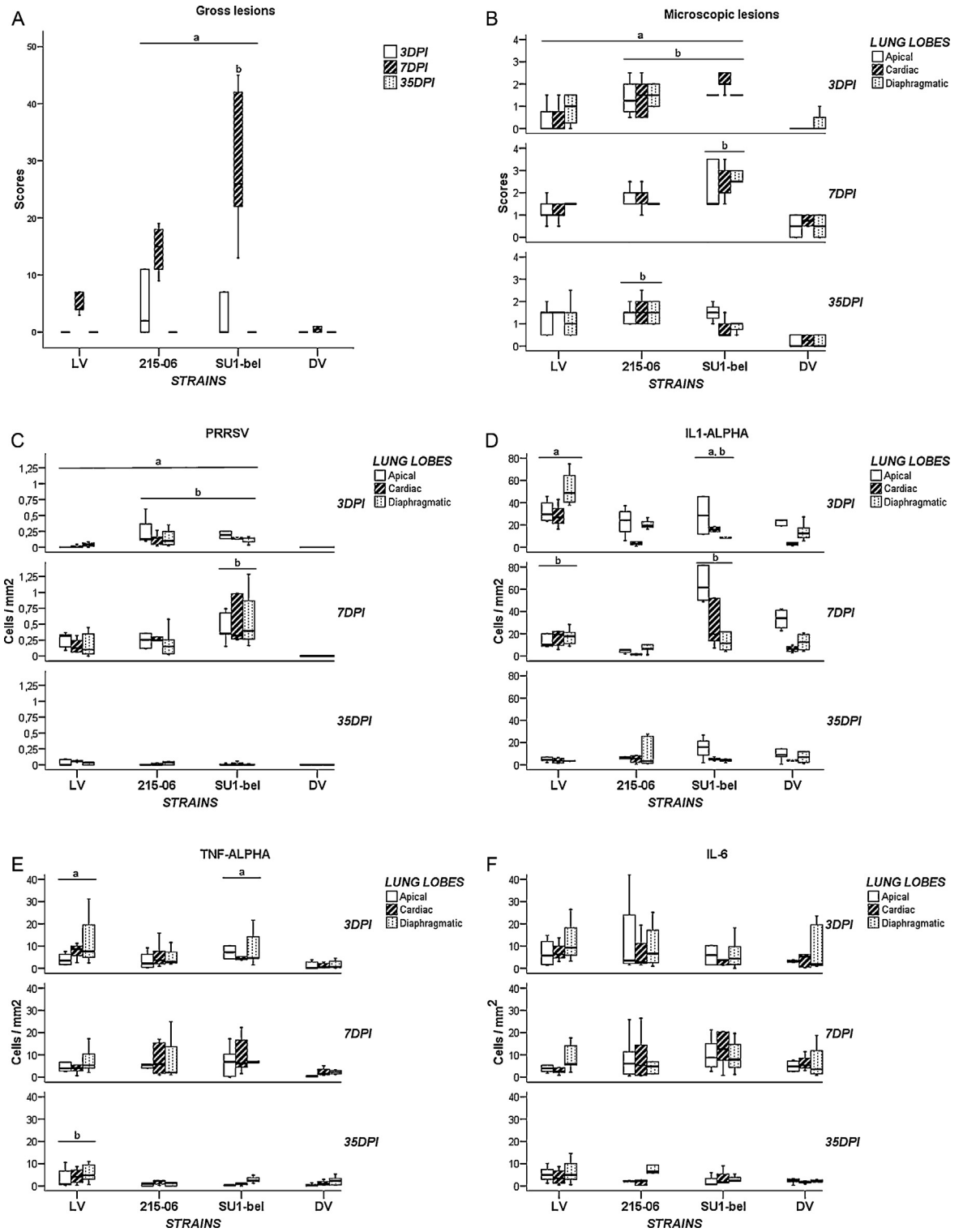

Fig. 1. Gross pathology (A) and microscopic lesions (B) scores, and counts for PRRSV, IL-1α, TNF-α and IL-6 positive cells (C–F) in lungs from pigs experimentally infected with four different PRRSV-1 strains: LV, 215-06, SU1-bel and DV and compared to animals from the control group. Animals were euthanized at 3, 7 and 35 dpi. Two animals from SU1-bel group were euthanized at 12 and 13 dpi due to welfare considerations. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between the inoculated group(s) and DV group at one time-point, whereas the letter "b" indicates significant differences between a specific inoculated group and the remaining groups (both inoculated and DV groups) at one time-point ($p < 0.05$).

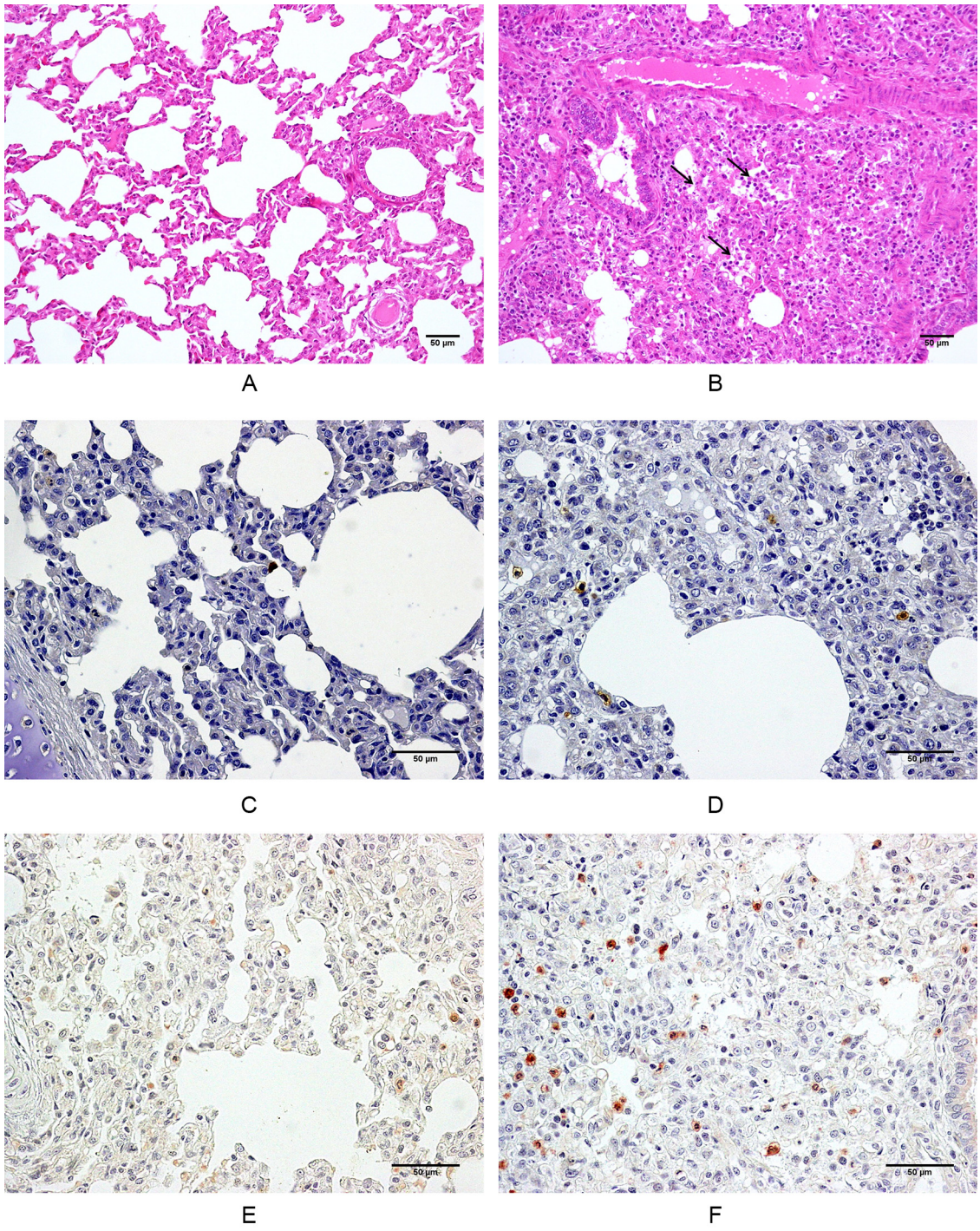

Fig. 2. Representative photomicrographs of the apical lobe of the right lung from a pig from control group (A) and from a pig inoculated with SU1-bel strain and euthanized at 7 dpi (B). Interstitial pneumonia was characterized by thickened alveolar septa with increased numbers of interstitial mononuclear cells, hyperplasia and hypertrophy of type II pneumocytes and alveoli filled necrotic/apoptotic cell debris and macrophages (black arrow). HE. Bar, 50 µm. Alveolar and septal macrophages expressing PRRSV antigen in pigs from 215-06 and SU1-bel strains, respectively, euthanized at 7 dpi. IHQ. Bar, 50 µm (C and D). Few septal macrophages and neutrophils expressing IL-1α in the lung of a pig from LV group euthanized at 7 dpi. IHQ. Bar, 50 µm (E). Marked infiltration of macrophages and neutrophils expressing IL-1α in the lung parenchyma of a pig from SU1-bel euthanized at 7 dpi (F). IHQ. Bar, 50 µm.

to PAMs and neutrophils (Fig. 2E and F). Differences in the expression of IL-1α were statically significant for different time-points, lung lobes and PRRSV strains ($p < 0.001$ in each case), with a significant interaction between all of them. SU1-bel and LV groups showed statistical significant differences in the expression of IL-1α with respect to DV group ($p < 0.006$ and $p < 0.001$, respectively). Furthermore, the animals infected with SU1-bel showed statistically

significant higher levels of IL1-α than LV and 215-06 groups at 3 dpi ($p < 0.001$ and $p < 0.001$, respectively), and both SU1-bel and LV groups displayed a significantly enhanced expression of IL1-α with respect to 215-06 group at 7 dpi ($p < 0.001$ and $p < 0.001$, respectively) (Fig. 1D).

The expression of TNF-α yielded significant differences for the evaluated variables: time-point ($p \leq 0.001$), lung lobe ($p = 0.057$) and PRRSV strain ($p \leq 0.001$), but no significant interaction was detected among any of them. SU1-bel and LV groups showed a significantly higher expression of TNF-α than DV group ($p < 0.001$), but no significant differences were observed for 215-06 group. At 35 dpi LV group showed higher levels of TNF-α when compared with SU1-bel ($p = 0.018$) and 215-06 group ($p = 0.018$) (Fig. 1E).

The expression of IL-6 was only significantly enhanced for the variable time-point ($p = 0.005$). In animals infected with SU1-bel strain an increase in the expression of IL-6 was detected at 7 dpi ($p = 0.068$) (Fig. 1F).

The immunolabelling of proinflammatory cytokines was observed mainly in areas of lung with moderate to severe, diffuse, interstitial pneumonia (Fig. 2F). Fewer immunolabelled cells were observed in areas with mild to moderate, multifocal, interstitial pneumonia or in areas without lesions. Inoculated animals showed always higher counts of cells expressing proinflammatory cytokines than DV group at 3 and 7 dpi. The apical lung lobe displayed statistically significant higher levels for the expression of IL1-α than cardiac and diaphragmatic lobes ($p < 0.001$ and $p < 0.005$, respectively); however, these lobes showed higher levels of expression of TNF-α than the apical lobe ($p = 0.024$ and $p = 0.003$, respectively).

3.5. Tissue expression of IFN-γ and IL-10

IFN-γ was expressed mainly in the cytoplasm of septal macrophages and lymphocytes. The kinetics of expression of IFN-γ was similar in LV, 215-06 and SU1-bel infected animals. Statistically significant differences in the expression of IFN-γ were only detected for the variant time-point ($p = 0.001$), without significant differences among any of the PRRSV strains evaluated in this study (Fig. 3A).

IL-10 was expressed in the cytoplasm of PAMs and septal macrophages (Fig. 3B). Statistical analysis of the expression of IL-10 demonstrated significant differences between different time-points ($p = 0.001$), lung lobes ($p < 0.001$) and different strains ($p = 0.035$), but no interaction was detected among them. A significantly enhanced expression of IL-10 was observed in 215-06 infected animals when compared with DV group ($p = 0.027$). In addition, a higher number of IL-10 immunolabelled cells in 215-06 group than in LV group was evidenced at 7 dpi ($p = 0.034$) (Fig. 3C and D).

Immunolabelling for IFN-γ and IL-10 was mostly observed in areas of mild to moderate interstitial pneumonia. The apical lung lobe displayed a statistically significant higher expression of IL-10 than cardiac and diaphragmatic lobes ($p < 0.001$ and $p = 0.002$, respectively) and in turn the expression of this cytokine trended to increase in the diaphragmatic lung lobe when compared with the cardiac lobe ($p = 0.055$).

3.6. Correlation study

LV and SU1-bel-infected pigs showed a statistically significant positive correlation between the expression of PRRSV antigen and gross lesions (Table 2). In addition, SU1-bel-infected pigs displayed a statistically significant positive correlation between the expression of PRRSV antigen and both microscopic lesions ($r = 0.645$, $p < 0.001$) and the expression of IL1-α ($r = 0.517$, $p < 0.001$) and also between gross lesions and both the expression of IL1-α and IL10 ($r = 0.731$, $p = 0.005$; and, $r = 0.575$, $p = 0.040$, respectively). A significant positive correlation was detected in all groups between the expression of IL-6 and IFN-γ, and also between the expression of IL-6 and IL-10 for 215-06 and DV groups (Table 3). In all groups, but LV-infected pigs, the expression of IFN-γ was positively correlated with the expression of IL-10. In LV and SU1-bel groups the expression of TNF-α displayed a significant positive correlation with the expression of IL-6 and IFN-γ (Table 2).

4. Discussion

An up regulation of proinflammatory cytokines has been correlated with lung pathology and disease during different porcine viral respiratory infections (Van Reeth and Nauwynck, 2000). The measurement of the expression of cytokines in the lung of infected animals may contribute to elucidate the mechanisms or pathways used by different PRRSV strains for developing respiratory disease. The immune response against PRRSV infection has previously been analysed by determining the changes observed in different immune mediators, such as cytokines in blood (Loving et al., 2008; Wang et al., 2011; Guo et al., 2013a, 2013b; Morgan et al., 2013; Weesendorp et al., 2013), whereas other studies characterize the expression of cytokines *in situ* in target organs, such as lung or lymphoid organs, in PRRSV-infected pigs (Choi et al., 2002; Chung and Chae, 2003; Thanawongnuwech et al., 2003; Chung et al., 2004; Gomez-Laguna et al., 2010; Barranco et al., 2012a, 2012b). Differences in pathogenicity have been reported between strains belonging to subtypes 1–3 of the European genotype (Karniychuk et al., 2010; Han et al., 2013a; Morgan et al., 2013, 2014; Weesendorp et al., 2013, 2014). Thus the present manuscript compared the expression of cytokines in the lung of pigs infected with a highly pathogenic subtype 3 PRRSV-1 strain (SU1-bel strain) with low virulent strains of the pan-European subtype 1 (LV, 215-06 and DV strains). In our study, the animals infected with the SU1-bel strain developed the highest clinical score and more severe gross and microscopic lesions compared to the LV, 215-06 and DV groups. Besides, in this group two animals were euthanized because they had prolonged fever and high clinical scores.

All animals infected with subtype 1 and subtype 3 PRRSV-1 strains expressed PRRSV antigen in the lung throughout the study with a maximum expression at 7 dpi. The highest replication of PRRSV was detected in the SU1-bel group and a positive correlation was observed between the number of PRRSV-positive cells and gross pathology in the lung of SU1-bel and LV infected animals. Moreover,

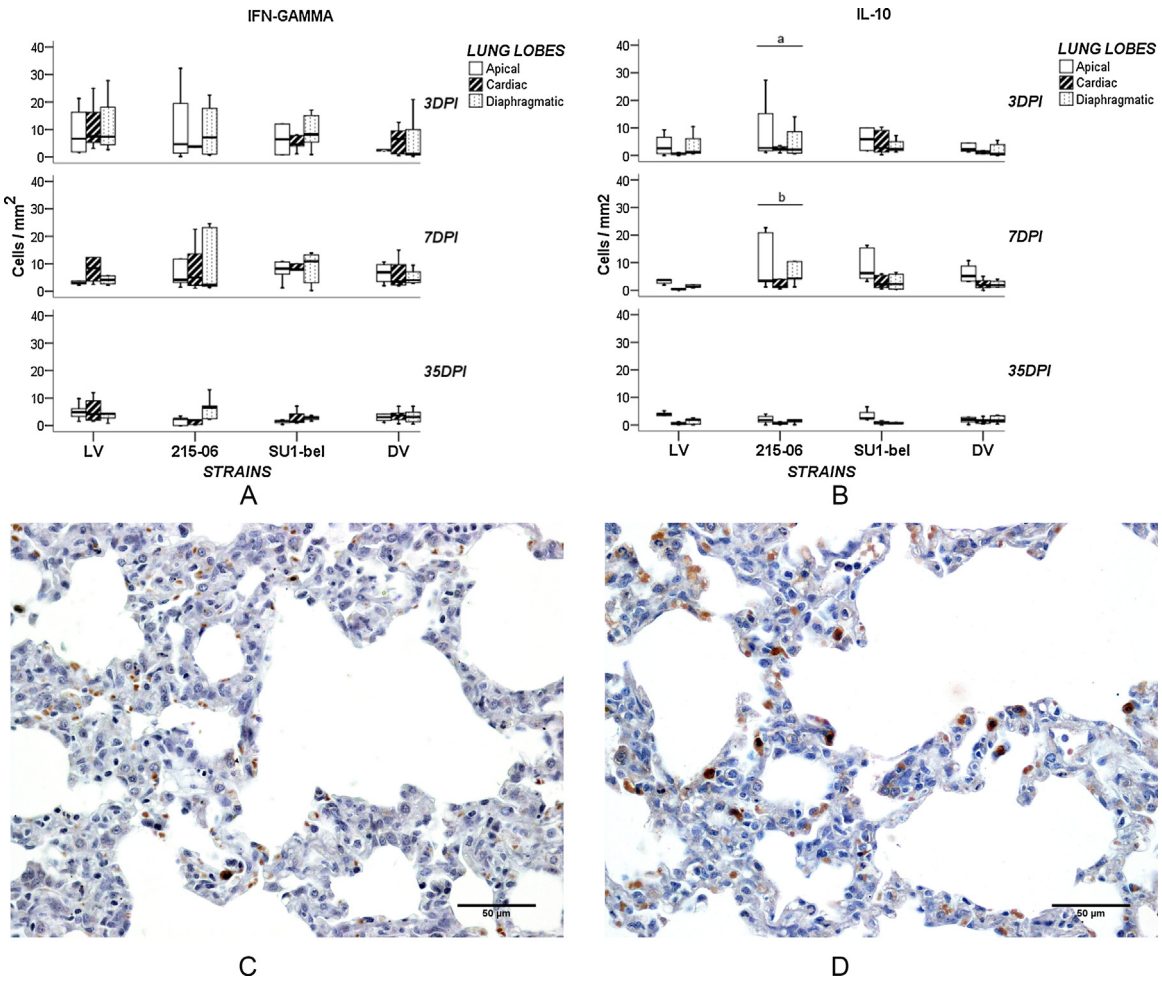

Fig. 3. Counts for IFN-γ and IL-10 in the animals infected with four different PRRSV-1 strains: (A and B) Lelystad virus, 215-06 a British field strain, SU1-bel from Belarus and strain DV (Porcilis® PRRS) and in animals from the control group. Animals were sacrificed at 3, 7 and 35 dpi. Two animals from SU1-bel group were euthanized at 12 and 13 dpi due to welfare considerations. The median is marked with a line, the box shows the 25th–75th percentile, the whiskers show maximum and minimum values. The letter "a" indicates significant differences between the inoculated group(s) and DV group at one time-point, whereas the letter "b" indicates significant differences between a specific inoculated group and the remaining groups (both inoculated and DV groups) at one time-point ($p < 0.05$). Photomicrograph of the apical lobe of the lung from pigs from LV and 215-06 strains euthanized at 7 dpi showing few alveolar and septal macrophages expressing IL-10 (C) and numerous cells expressing IL-10 in the pulmonary parenchyma (D). IHQ. Bar, 50 μm.

a positive correlation was detected between the expression of PRRSV and the expression of IL-1α in SU1-bel group, which suggests that the virus induces the expression of IL-1α in the lung of PRRSV-infected animals inducing the typical interstitial pneumonia of this disease. Our results are in agreement with those recently published by Weesendorp et al. (2014) who reported a higher number of PRRSV positive cells and an increase in the IL-1β mRNA expression in the lung of Lena-infected animals than in animals infected with other PRRSV-1 strains (LV, Belgium A).

PRRSV replicated mainly in PAM and all cytokines were mostly detected in the cytoplasm of septal macrophages and for IL-1α also in the cytoplasm of neutrophils. Similar

Table 2
Correlation detected between the expression of PRRSV antigens and gross lesions, microscopic lesions and cytokines in animals infected with each one of PRRSV-1 strains (LV, 215-06 and SU1-bel) throughout the study.

	PRRSV/gross		PRRSV/microscopic		PRRSV/IL-1α		PRRSV/TNF-α		PRRSV/IL-6		PRRSV/IL-10	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
LV	0.838	<0.001	0.414	0.008	NS		NS		NS		NS	
215-06	NS		0.322	0.035	NS		NS		NS		0.375	0.013
SU1-bel	0.694	0.008	0.645	<0.001	0.517	0.001	0.369	0.045	0.336	0.045	NS	

NS: not statistically significant.

Table 3
Correlation detected between the expression of different cytokines and gross lesions and microscopic lesions in animals infected with each one of PRRSV-1 strains (LV, 215-06, SU1-bel and DV) throughout the study.

	IL-1α/gross		IL-1α/microscopic		TNF-α/IL-6		TNF-α/IFN-γ		TNF-α/IL-10		IL-6/IFN-γ		IL-6/IL-10		IL-10/gross		IL-10/IFN-γ	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
LV	0.838	<0.001		NS	0.766	<0.001	0.637	<0.001		NS	0.761	<0.001	0.437	0.005		NS	0.312	0.050
215-06		NS		NS		NS	0.464	0.002	0.305	0.047	0.715	<0.001	0.518	<0.001		NS	0.594	<0.001
SU1-bel	0.694	0.008	0.517	0.001	0.652	<0.001	0.713	<0.001		NS	0.741	<0.001	0.445	0.007	0.575	0.040	0.593	<0.001
DV		NS		NS	0.408	0.005	0.404	0.006		NS	0.576	<0.001	0.510	<0.001		NS	0.503	<0.001

NS: not statistically significant.

results have previously been published by our research group (Gomez-Laguna et al., 2010) and support the hypothesis that septal macrophages play a key role in the synthesis of cytokines in pigs infected with PRRSV (Gomez-Laguna et al., 2013). In the present study the profile of lung expression of TNF-α and IL-6 was similar for each infected-group, nonetheless the expression of IL-1α differed significantly among groups. Animals infected with the SU1-bel strain trended to display the highest expression of all three proinflammatory cytokines at 7 dpi compared to the other PRRSV-infected groups. Our results evidence a main role of IL-1α in the onset of the local inflammatory response during PRRS in the lung, which may synergistically act together with other proinflammatory mediators in the development of the lesions in the lung and the clinical signs in infected pigs (Van Reeth and Nauwynck, 2000; Kimura et al., 2013). This idea is supported by the significant positive correlation between the levels of TNF-α and IL-6 in LV and SU1-bel groups, which is suggestive of the synergistic action among proinflammatory cytokines (Van Reeth et al., 2002). The data presented in this manuscript show that the highly virulent subtype 3 SU1-bel PRRSV strain replicates more efficiently and induce more severe lesions in the lung of infected animals, mainly associated to a higher expression of IL-1α, than other moderately virulent subtype 1 PRRSV strains, such as LV and 215-06 strains, or the attenuated vaccine strain DV.

In a parallel study carried out by Morgan et al. (2013) a significantly higher viral load in serum and BALF in LV-infected animals when compared to SU1-bel and 215-06 infected animals at 3 and 7 dpi was reported. These authors suggested that the virulence of the SU1-bel strain could be associated to an enhanced inflammatory immune response and not to a higher viral replication capability. Our results highlight that the higher virulence reported for the SU1-bel strain could be associated both to a higher local PRRSV replication rate and to an increased expression of IL-1α in the lung parenchyma.

In our study, the kinetics of IFN-γ was similar in all PRRSV infected groups showing only a mild to moderate enhancement at 3 dpi. In a previous study carried out by our group an enhancement in the expression of IFN-γ in pigs infected with the strain 2982, which presents a 93% of homology with LV, was already detected at 3 dpi, coinciding with the results observed in the present study (García-Nicolás et al., 2014b). Furthermore, the results of the present study are partially in agreement with the PRRSV-specific IFN-γ response in the blood of SU1-bel-infected animals previously reported by Morgan et al. (2013), who found an enhancement only between 14 and 28 dpi with a peak at 21 dpi. This increase in the expression of IFN-γ has also previously been reported in infections with a HP-PRRSV-2 strain, from 10 to 28 dpi (Thanawongnuwech et al., 2003), whereas other studies with the subtype 3 PRRSV strain Lena could not evidence changes in the expression of this cytokine (Weesendorp et al., 2013). IFN-γ participates in regulating the immune response and its production by pulmonary macrophages is induced by the expression of other cytokines including TNF-α and IFN-α (Gomez-Laguna et al., 2010). Interestingly, a significant positive correlation between the

expression of IFN-γ with respect to the expression of TNF-α and IL-6 was detected in all groups infected with PRRSV in our study, which suggests that proinflammatory cytokines may play a role in the early induction of IFN-γ in PRRS.

PRRSV strains may induce the production of IL-10 (Suradhat et al., 2003; Diaz et al., 2006; Gimeno et al., 2011; Wang et al., 2011), which has been proposed as a mechanism which may prolong viraemia and inhibition of cell mediated immunity at an early stage of infection (Diaz et al., 2006; Wang et al., 2011). According to Gimeno et al. (2011), PRRSV-1 strains may be classified as IL-10, TNF-α, both IL-10 and TNF-α inducers or none of them. In the present study the expression of IL-10 was significantly higher in the lungs of 215-06 infected pigs and in a lesser extent in SU1-bel-infected pigs. In the 215-06 group the expression of IL-10 was positively correlated with the expression of PRRSV antigen and the expression of TNF-α, pointing out that this strain may be a strain inducer of IL10 and TNF-α. The 3262 PRRSV-1 strain, classified also as IL-10^{+}TNF-α^{+} by Gimeno et al. (2011), was characterized by a down-regulation of the co-stimulatory molecules CD80/86 which may be a mechanism of PRRSV strains with this cytokine profile to avoid an efficient host immune response. The higher expression of IL-10 detected in the animals infected with the PRRSV strain 215-06 than LV and DV strains was associated with a lower expression of IL-1α which may be the cause of the moderate virulence of this strain. In addition all infected groups had a significant positive correlation between the expression of IL-10 with the expression of IFN-γ and IL-6, confirming the role of this cytokine as an immunomodulatory and anti-inflammatory cytokine in PRRSV infection.

The absence of clinical signs, gross lesions and PRRSV replication in all animals inoculated with DV strain suggests that there was no infection in these animals, but a mild interstitial pneumonia associated to a mild expression of IL-1α and IL-10 at 7 dpi was found. Modified-live DV vaccines are known to induce a relatively weak cell mediated immunity (Charerntantanakul, 2012), which is in agreement with the lack of significant changes in the expression of IFN-γ in the lung.

In conclusion, the results of the present study indicate that subtype 3 European SU1-bel strain replicated more efficiently in the lung of infected animals, as well as induced a higher expression of IL-1α than what LV, 215-06 and DV strains which may have had a key role in the increased pathogenicity and the development of the onset of the clinical signs.

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CURRICULUM VITAE

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Publicaciones

Los resultados de esta tesis doctoral han sido publicados en forma de los siguientes artículos:

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad, Y.O.J.M., Morgan, S.B., Graham, S.P., Frossard, J.P., Drew, T.W., Salguero, F.J. **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. (2015). doi:10.1016/j.vetimm.2015.02.003.

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad, Y.O.J.M., Graham, SP., Frossard, J-P., Steinbach F., Salguero FJ. **Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains**. En revisión desde el 16 de junio de 2015, en la revista *Veterinary Microbiology*.

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad, Y.O.J.M., Graham, SP., Frossard, J-P., Steinbach F., Salguero FJ. **Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the**

viral load and virulence. En revisión desde octubre de 2015 en la revista *Frontiers in immunology*.

Comunicaciones

Los resultados de esta tesis doctoral han sido presentados en los siguientes congresos nacionales e internacionales:

- ✓ Amarilla SP; Gómez-Laguna J y Carrasco L **INMUNOPATOGENIA DEL PRRS: Variabilidad de la respuesta inmune dependiente de la patogenicidad de las cepas. Estudio relacionado con el pulmón**. Presentado como comunicación de tipo oral, en el I Congreso Científico de Investigadores en Formación en Agroalimentación de la eidA3 y II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba, celebrado en Córdoba, España el 08 de mayo de 2012.

- ✓ Amarilla SP; Morgan S; Gómez-Laguna J; Graham S; Frossard J-P; Steinbach F; Carrasco L y Salguero FJ. **A virulent eastern European PRRSV strain induces high levels of IL-1alpha in the lung**. Presentado como comunicación de tipo oral, en el 30th Meeting of the Eurpean Society of Veterinary Pathology, Annual Meeting of the European College of Veterinary Pathologists and 24th Annual Meeting of the Spanish Society of Veterinary Pathology, celebrado en León, España del 05 al 08 de septiembre de 2012.

- ✓ Amarilla SP; Gómez-Laguna J y Carrasco L **INMUNOPATOGENIA DEL PRRS: Variabilidad de la respuesta inmune dependiente de la patogenicidad de las cepas. Estudio relacionado con el Pulmón**. Presentado como comunicación de tipo oral, en el III Congreso Científico de Investigadores en Formación de la Universidad de Córdoba y II Congreso Científico de Investigadores en Formación en Agroalimentación, celebrado en Córdoba, España el 09 de abril de 2013.

- ✓ Amarilla SP, Gómez-Laguna J, Rodríguez-Gómez IM, Barranco I, Morgan SB, Drew TW, Carrasco L y Salguero FJ. **Variabilidad en la expresión de TNF-alpha, IL1-alpha e IL-10 en pulmones de cerdos infectados con diferentes cepas del genotipo Europeo del PRRSV**. Presentado como comunicación de tipo oral, en el XXV Reunión de la Sociedad Española de Anatomía Patológica Veterinaria, celebrado en Toledo, España del 19 al 21 de junio de 2013.

- ✓ Amarilla SP, Gómez-Laguna J, Rodríguez-Gómez IM, Barranco I, Carrasco L, Morgan SB, Drew TW y Salguero FJ. **Pulmonary macrophages as primary source of cytokines in response to infection with different PRRSV-1 strains**. Presentado como comunicación de tipo póster, en el 31st Meeting of the European Society of Veterinary Pathology and the Annual Meeting of the European College of Veterinary Pathologists, celebrado en London, Reino Unido del 04 al 07 de septiembre de 2013.

- ✓ Amarilla SP; Gómez-Laguna J; Rodríguez-Gómez IM; Morgan SB; Graham SP; Frossard J-P; Drew TW; Carrasco L y Salguero FJ. **Highly virulent strain induces sustained levels of proinflammatory cytokines compared to other PRRSV-1 strains in the lung**. Presentado como comunicación tipo póster, en el 6TH European Symposium of porcine health management, celebrado en Sorrento, Italia del 07 al 09 de mayo de 2014.

- ✓ Amarilla SP; Gómez-Laguna J; Rodríguez-Gómez IM; Morgan SB; Graham SP; Frossard J-P; Drew TW; Carrasco L y Salguero FJ. **Diferentes cepas del genotipo Europeo del virus del PRRS produce depleción y atrofia tímica**. Presentado como comunicación tipo oral, en el XXVI Reunión de la Sociedad Española de Anatomía Patológica Veterinaria, celebrado en Zaragoza, España del 18 al 20 de junio de 2014.

- ✓ Amarilla SP; Gómez-Laguna J; Carrasco L y Salguero FJ. **INMUNOPATOGENIA DEL PRRS: Variabilidad de la respuesta inmune dependiente de la patogenicidad de las cepas. Estudio relacionado con el timo**. Presentado como comunicación tipo oral, en el IV Congreso Científico de Investigadores en Formación de la Universidad de Córdoba y III Congreso Científico de Investigadores en Formación en Agroalimentación, celebrado en Córdoba, España el 19 de noviembre de 2014**.**

- ✓ Amarilla SP; Gómez-Laguna J; Carrasco L; Rodríguez-Gómez IM; Frossard J-P; Graham SP y Salguero FJ. **Thymocyte depletion in experimental highly virulent PRRSV-1 infection**. Presentado como comunicación tipo póster, en el 7TH European Symposium of

porcine health management, celebrado en Nantes, Francia del 22 al 24 de abril de 2015.

Otras comunicaciones relacionadas con el tema de la tesis

- ✓ Amarilla SP; Gómez-Laguna J; Barranco I; Rodríguez-Gómez IM; Pallarés FJ; García-Nicolás y Carrasco L. **FADD and DAXX Markers to study apoptosis pathways in porcine paraffin-embedded tissues**. Presentado como comunicación tipo oral, en el II Iberic Meeting of Veterinary Pathology and XVI Annual Meeting of the Portuguese Society of Veterinary Anatomical Pathology, celebrado en Lisboa, Portugal del 01 al 03 de junio de 2011.

- ✓ Barranco I; Gómez-Laguna J; Rodríguez-Gómez IM; Amarilla SP; Salguero FJ; García-Nicolás O; Quereda JJ; Ramis G; Pallarés FJ y Carrasco L. **Differential expression of proinflammatory cytokines in the lymphoid organs of porcine reproductive and respiratory syndrome virus-infected pigs**. Presentado como comunicación tipo póster, en el 6^{TH} International symposium on Emerging and Re-Emerging pig diseases, celebrado en Barcelona, España del 12 al 15 de junio de 2011.

- ✓ Rodriguez-Gómez IM; Barranco I; Amarilla SP; Gómez-Laguna J; García-Nicolás O; Ramis G; Pallarés FJ y Carrasco L. **Activation of the extrinsic pathway of apoptosis during PRRS**. Presentado como comunicación tipo oral, 31st Meeting of the European Society of Veterinary Pathology and the Annual Meeting of the European College of Veterinary Pathologists, celebrado en London, Reino Unido del 04 al 07 de septiembre de 2013.

- ✓ Amarilla SP; Rodríguez-Gómez IM; Gómez-Laguna J; Garcia-Nicólas O; Barranco I; Molleda C; Graham S. P; Frossard J-P; Steinbach F. y Salguero FJ. **Expresión local de citoquinas pro-inflamatorias en el nódulo linfático mediastínico de cerdos infectados con diferentes cepas del genotipo Europeo del PRRSV**. Presentado como comunicación tipo póster, XXVII Reunión de la Sociedad Española de Anatomía Patológica Veterinaria, celebrado en Barcelona, España del 17 al 19 de junio de 2015.

- ✓ Amarilla SP; Rodríguez-Gómez IM; Gómez-Laguna J; Carrasco L; Graham SP; Frossard JP; Steinbach F y Salguero FJ. **Cytokine expression in tonsils in response to infection with different PRRSV-1 strains**. Presentado como comunicación tipo póster, en el 33rd Meeting of the European Society of Veterinary Pathology and the Annual Meeting of the European College of Veterinary Pathologists, celebrado en Helsinki, Finland del 02 al 05 de septiembre 2015.

Estancia

Departamento de Patología y Enfermedades Infecciosas, de la Facultad de Veterinaria y Medicina de la Universidad de Surrey, UK; desde el 22 de junio al 22 de septiembre de 2014. Durante esta estancia se realizó la caracterización de la expresión local de células positivas a CD3 y MAC378 en el timo mediante la utilización de un software de análisis de imágenes NIS Elements BR (4.20.00 Build 967, 64 bits).

CURRICULUM VITAE

Publications

The results of the present PhD Thesis have been published as:

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad, Y.O.J.M., Morgan, S.B., Graham, S.P., Frossard, J.P., Drew, T.W., Salguero, F.J. **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. (2015). doi:10.1016/j.vetimm.2015.02.003.

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad, Y.O.J.M., Graham, SP., Frossard, J-P., Steinbach F., Salguero FJ. **Thymic depletion of lymphocytes is associated with the virulence of PRRSV-1 strains**. Under review in Veterinary Microbiology. VETMIC-D-15-10699. Submitted 16th June 2015.

Amarilla, S.P., Gómez-Laguna, J., Carrasco, L., Rodríguez-Gómez, I.M., Caridad, Y.O.J.M., Graham, SP., Frossard, J-P., Steinbach F., Salguero FJ. **Porcine reproductive and respiratory syndrome viruses induce hypoplasia of erythroid cells and myeloid cell hyperplasia in the bone marrow of experimentally infected piglets independently of the**

viral load and virulence. Frontiers in immunology. Submitted October 2015

Communications

The results of the present PhD Thesis have been presented in the following international and national meetings:

- ✓ Amarilla, SP., Gómez-Laguna, J and Carrasco, L. **IMMUNOPATHOGENESIS OF PRRS: Variability of immune response dependent pathogenic strains. Study related to the lung**. Presented as oral communication, in the I Congreso Científico de Investigadores en Formación en Agroalimentación de la CeidA3 y II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba. Córdoba - Spain. 8th, May 2012.

- ✓ Amarilla, SP., Morgan, S., Gómez-Laguna, J., Graham, S., Frossard, J.P., Steinbach, F., Carrasco, L and Salguero, FJ. **A virulent eastern European PRRSV strain induces high levels of IL-1alpha in the lung.** Presented as oral communication, in the 30th Meeting of the Eurpean Society of Veterinary Pathology, Annual Meeting of the European College of Veterinary Pathologists and 24th Annual Meeting of the Spanish Society of Veterinary Pathology. León - Spain. 5th to 8th, September 2012.

- ✓ Amarilla, S.P., Gómez-Laguna, J and Carrasco, L. **IMMUNOPATHOGENESIS OF PRRS: Variability of immune response dependent pathogenic strains. Study related to the lung**. Presented as oral communication, in the III Congreso Científico de

Investigadores en Formación de la Universidad de Córdoba y II Congreso Científico de Investigadores en Formación en Agroalimentación. Córdoba - Spain. 9th, April 2013.

- ✓ Amarilla, SP., Gómez-Laguna, J., Rodríguez-Gómez, IM., Barranco, I., Morgan, SB., Drew, TW., Carrasco. L and Salguero, FJ. **Variability in expression of TNF-alpha, IL1-alpha and IL-10 in the lungs of pigs infected with different PRRSV genotype European strains**. Presented as oral communication, in the XXV Reunión de la Sociedad Española de Anatomía Patológica Veterinaria. Toledo -Spain. 19th to 21st, June 2013.

- ✓ Amarilla, SP., Gómez-Laguna, J., Rodríguez-Gómez, IM., Barranco, I., Carrasco, L., Morgan, SB., Drew, TW and Salguero, FJ. **Pulmonary macrophages as primary source of cytokines in response to infection with different PRRSV-1 strains**. Presented as poster communication, in the 31st Meeting of the European Society of Veterinary Pathology and the Annual Meeting of the European College of Veterinary Pathologists. London - United Kingdom. 4th to 7th, September 2013.

- ✓ Amarilla, SP., Gómez-Laguna, J., Rodríguez-Gómez, IM., Morgan, SB., Graham, SP., Frossard, JP., Drew, TW., Carrasco, L and Salguero, FJ. **Highly virulent strain induces sustained levels of proinflammatory cytokines compared to other PRRSV-1 strains in the lung**. Presented as poster communication, in the 6th European Symposium of porcine health management. Sorrento - Italy. 7th to 9th, May 2014.

- ✓ Amarilla SP; Gómez-Laguna J; Rodríguez-Gómez IM; Morgan SB; Graham SP; Frossard J-P; Drew TW; Carrasco L and Salguero FJ. **Different European genotype PRRS strains produced to depletion and atrophy of the thymus**. Presented as oral communication, in the XXVI Reunión de la Sociedad Española de Anatomía Patológica Veterinaria. Zaragoza - Spain. 18th to 20th, June 2014.

- ✓ Amarilla SP; Gómez-Laguna J; Carrasco L and Salguero FJ. **IMMUNOPATHOGENESIS OF PRRS: Variability of immune response dependent pathogenic strains. Study related to the thymus**. Presented as oral communication, in the IV Congreso Científico de Investigadores en Formación de la Universidad de Córdoba y III Congreso Científico de Investigadores en Formación en Agroalimentación. Córdoba - Spain. 19th, Novembre 2014**.**

- ✓ Amarilla SP; Gómez-Laguna J; Carrasco L; Rodríguez-Gómez IM; Frossard J-P; Graham SP and Salguero FJ. **Thymocyte depletion in experimental highly virulent PRRSV-1 infection**. Presented as poster communication, in the 7th European Symposium of porcine health management. Nantes - France. 22th to 24th, April 2015.

Other communications related with the thesis

- ✓ Amarilla SP; Gómez-Laguna J; Barranco I; Rodríguez-Gómez IM; Pallarés FJ; García-Nicolás and Carrasco L. **FADD and DAXX Markers to study apoptosis pathways in porcine paraffin-embedded tissues**. Presented as oral communication, in the II Iberic Meeting of Veterinary Pathology and XVI Annual Meeting of the

Portuguese Society of Veterinary Anatomical Pathology. Lisboa - Portugal.1st to 3rd, June 2011.

- ✓ Barranco I; Gómez-Laguna J; Rodríguez-Gómez IM; Amarilla SP; Salguero FJ; García-Nicolás O; Quereda JJ; Ramis G; Pallarés FJ and Carrasco L. **Differential expression of proinflammatory cytokines in the lymphoid organs of porcine reproductive and respiratory syndrome virus-infected pigs**. Presented as oral communication, in the 6th International symposium on Emerging and Re-Emerging pig diseases. Barcelona - Spain. 12nd to 15th, June 2011.

- ✓ Rodriguez-Gómez IM; Barranco I; Amarilla SP; Gómez-Laguna J; García-Nicolás O; Ramis G; Pallarés FJ and Carrasco L. **Activation of the extrinsic pathway of apoptosis during PRRS**. Presented as oral communication, in the 31st Meeting of the European Society of Veterinary Pathology and the Annual Meeting of the European College of Veterinary Pathologists. London - United Kingdom. 4th to 7th, September 2013.

- ✓ Amarilla S.P; Rodríguez-Gómez IM; Gómez-Laguna J; Garcia-Nicólas O; Barranco I; Molleda C; Graham S. P; Frossard J-P; Steinbach F and Salguero FJ. **Local expression of pro-inflammatory cytokines in the mediastinal lymph node of pigs infected with different PRRSV genotype European strains.** Presented as poster communication, in the XXVII Reunión de la Sociedad Española de Anatomía Patológica Veterinaria. Barcelona - Spain. 17th to 19th, June 2015.

- ✓ Amarilla SP; Rodríguez-Gómez IM; Gómez-Laguna J; Carrasco L; Graham SP; Frossard JP; Steinbach F and Salguero FJ. **Cytokine expression in tonsils in response to infection with different PRRSV-1 strains**. Presented as poster communication, in the 33rd Meeting of the European Society of Veterinary Pathology and the Annual Meeting of the European College of Veterinary Pathologists. Helsinki - Finland. 2nd to 5th, September 2015.

Research stay:

Department of pathology and infectious diseases, School of Veterinary Medicine, University of Surrey, United Kingdom; from 22th, June to 22th, September 2014. During this stay the PhD Student SP Amarilla characterized the local expression of CD3 and MAC378 positive cells in the thymus by using the image analysis software NIS Elements BR (4.20.00 Build 967, 64 bits).

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AGRADECIMIENTOS

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Shyrley Paola Amarilla.

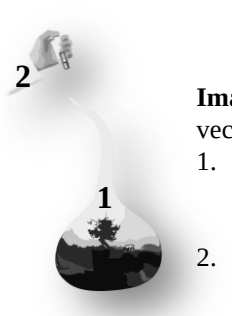

Imagen de los capítulos y contratapa. (1) Fotografía e **(2)**imágen vectorizadas en CorelDraw®X7 2015 por SP Amarilla.

1. Vista Este desde el laboratorio del Dpto. de Anatomía Patológica Comparada. Facultad de Veterinaria, Universidad de Córdoba – España.
2. Micropipeta monocanal. Finnpipette™ F1, tipo variable. Thermo Scientific.

SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

CÓRDOBA – NOVIEMBRE 2015