UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

PATOGENIA DE LA FORMA RESPIRATORIA DEL SÍNDROME REPRODUCTIVO Y RESPIRATORIO PORCINO: EL PAPEL MODULADOR DE LAS CITOQUINAS EN LA RESPUESTA INMUNE

PATHOGENESIS OF THE RESPIRATORY FORM OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME: MODULATORY ROLE OF CYTOKINES IN THE IMMUNE RESPONSE

Trabajo presentado por el Licenciado en Veterinaria **D. Jaime Gómez Laguna**
para optar al Grado de Doctor por la Universidad de Córdoba

Departamento de Anatomía y Anatomía Patológica Comparadas

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TITULO: *Patogenia de la forma respiratoria del síndrome reproductivo y respiratorio porcino: el papel modulador de las citoquinas en la respuesta inmune*

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LIST OF ABBREVIATIONS / LISTA DE ABREVIATURAS

AAS	Amiloide A Sérica
ABC	Avidin-Biotin-Peroxidase complex
ANs	Anticuerpos Neutralizantes
ANOVA	analysis of variance
APPs	Acute Phase Proteins
APR	Acute Phase Response
AS ODNs	AntiSense phosphorotioate OligoDeoxyNucleotides fosforotioato de oligodesoxinucleótidos antidentido
ASF	African Swine Fever
BALCs	BronchoAlveolar Lavage Cells
BALFs	BronchoAlveolar Lavage Fluids
CD	Cluster Differentiation
CISA	Centro de Investigación en Sanidad Animal
CMSPs	Células Mononucleares de Sangre Periférica
CRP	C-reactive protein
dpi	days post-inoculation días post-inoculación
E	Envelope
ELISA	Enzyme-Linked Immunosorbent Assay

EU	European
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
Fig	Figure
FITC	Fluorescein IsoThioCyanate
GP	Glycoprotein
HIV	Human Immunodeficiency Virus
Hp	Haptoglobin
	Haptoglobina
IFN	InterFeroN
	InterFeróN
Ig	Immunoglobulins
IL	InterLeukin
	InterLeuquina
IPMA	ImmunoPeroxidase Monolayer Assay
kb	kilobase
LBP	Lipopolysaccharide Binding Protein
LV	Lelystad Virus
M	Membrane
mAb	monoclonal Antibodies
MAPs	Macrófagos Alveolares Porcinos
MHC II	class II Major Histocompatibility Complex

MLV	Modified–Lived Vaccine
mRNA	messenger ribonucleic acid
N	Nucleocapside
NAs	Neutralizing Antibodies
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ORF	Open Reading Frame
P	Protein
PAMPs	Pathogens Associated Molecular Patterns
PAMs	Pulmonary Alveolar Macrophages
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Proteína C-Reactiva
PCV2	Porcine CircoVirus type 2
PE	PhycoEritrin
PFAs	Proteínas de Fase Aguda
Pig-MAP	Pig-Major Acute Protein
PIMs	Pulmonar Intravascular Macrophages
PRCV	Porcine Respiratory CoronaVirus
PRDC	Porcine Respiratory Disease Complex
PRRS	Porcine Reproductive and Respiratory Syndrome Síndrome Reproductivo y Respiratorio Porcino

PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RFA	Respuesta de Fase Aguda
rp	recombinant porcine
r.t.	room temperature
SAA	Serum Amyloid A
SC	Secreting Cells
SD	Standard Deviation
SIV	Swine Influenza Virus
$TCID_{50}$	50 % Tissue Culture Infectious Dose
TGF	Transforming Growth Factor
	Factor Transformador de Crecimiento
TMB	TetraMethylBenzidine
TNF	Tumor Necrosis Factor
Treg	Regulatory T lymphocytes
	linfocitos T reguladores
U	Unit
US	North American
VPRRS	Virus del Síndrome Reproductivo y Respiratorio Porcino
VIH	Virus de la Inmunodeficiencia Humana
wpi	weeks post-infection

INTRODUCTION / INTRODUCCIÓN

INTRODUCTION

Porcine Reproductive and Respiratory Syndrome (PRRS) is worldwide spread and constitute one of the most significant diseases in the swine industry. This syndrome is characterised by inducing reproductive failure in sows and respiratory symptoms in growing and finishing pigs. Although there is some controversy, PRRS virus (PRRSV) is considered as able to modulate the immune response, making easier the concomitant infection with secondary bacteria, and playing a significant role in the onset of the Porcine Respiratory Disease Complex (PRDC) (Rossow, 1998). Although several studies have been carried out to elucidate the host immune response evoked against PRRSV, there are still a lot of aspects which still remain unclear.

Lung and lymphoid organs represent the target organs for PRRSV replication (Xiao *et al.*, 2004). Therefore, it is presumably that both lung and lymphoid organs play a significant role in the immune response evoked after PRRSV infection. However, nowadays few studies have been performed to determine the changes observed in these organs. Several studies have been focused on the changes observed in lymphocyte subsets of peripheral blood mononuclear cells (PBMCs), whereas other reports have been focused on the changes observed in the serum concentration of cytokines. Nonetheless, there is lack of studies which correlate the expression of cytokines with the changes observed in the immune response developed after PRRSV infection.

Cytokines are proteins which regulate the function of immune cells, participating in the cellular activation, differentiation and proliferation, and in the modulation of the synthesis of immunoglobulins. Cytokines are synthesised mainly by activated macrophages and lymphocytes, although other cells may be also involved in their production, just as neutrophils, and endothelial and epithelial cells. Several cytokines, just as interleukin-10 (IL-10), gamma interferon (IFNγ) and/or alpha interferon (IFNα), have been proposed to play a significant role in PRRS. However, no studies have been carried out to determine the changes in the expression of cytokines *in situ* in the target organs for PRRSV replication, namely the lung and lymphoid organs.

Therefore, the main goals of this thesis were to determine the relationship between the changes observed in the serum concentration of cytokines and the changes observed in the innate immune response, as well as, to study the expression of cytokines in the lung parenchyma of PRRSV-infected pigs.

The experimental studies carried out in this thesis have been founded by the Spanish Ministry of Education and Science, project number AGL2006-04146/GAN. The PhD student, Jaime Gómez-Laguna, carried out his doctoral studies supported by a scholarship from the program “Formation of University Teachers” from the Spanish Ministry of Education and Science (AP-2004-0395).

INTRODUCCIÓN

El Síndrome Reproductivo y Respiratorio Porcino (PRRS) es una enfermedad distribuida a nivel mundial y constituye una de las enfermedades más importantes de la industria porcina. Este síndrome se caracteriza por la aparición de fallo reproductivo en cerdas gestantes y de un cuadro respiratorio en cerdos en las fases de transición y engorde. El virus del (VPRRS) es considerado capaz de modular la respuesta inmune, facilitando de esta forma la aparición de infecciones concomitantes con bacterias secundarias, y desempeñando un papel importante en el establecimiento del Complejo Respiratorio Porcino (CRP). Aunque varios estudios se han llevado a cabo para elucidar la respuesta inmune del hospedador tras la infección con el VPRRS, todavía existen muchos aspectos que permanecen sin esclarecer.

El pulmón y los órganos linfoides representan los principales órganos diana para la replicación del VPRRS. De este modo, es presumible que ambos, pulmón y órganos linfoides, jueguen un papel fundamental en la respuesta inmune que se desencadena en la infección con el VPRRS. Sin embargo, hoy día pocos estudios se han realizado para determinar los cambios observados en estos órganos. Varios estudios se han centrado principalmente en los cambios observados en las subpoblaciones de linfocitos en las células mononucleares de sangre periférica (CMSPs), mientras que otros estudios se han centrado en los cambios observados en la concentración sérica de algunas

citoquinas. No obstante, no se ha estudiado la correlación entre la expresión de citoquinas y los cambios observados en la respuesta inmune desencadenada tras la infección con el VPRRS.

Las citoquinas son proteínas que regulan la función de las células inmunes, participando en la activación, diferenciación y proliferación celular, así como en la modulación de la síntesis de inmunoglobulinas. Las citoquinas son sintetizadas principalmente por macrófagos activados y linfocitos, aunque otras células pueden estar implicadas en su producción, como neutrófilos, y células endoteliales y epiteliales. Varias citoquinas, como la interleuquina 10 (IL-10), el interferón gamma (IFNγ) y/o el interferón alfa (IFNα), han sido propuestas como importantes moduladores en el PRRS. Sin embargo, no hay estudios que determinen los cambios en la expresión *in situ* de citoquinas en los órganos diana de la replicación del VPRRS, como son el pulmón y los órganos linfoides.

Por todo ello, los principales objetivos de esta tesis consistieron en determinar la relación entre los cambios observados en la concentración de citoquinas y los cambios observados en la respuesta inmune innata, así como estudiar la expresión de citoquinas en el parénquima pulmonar de cerdos infectados con el VPRRS.

Los estudios experimentales llevados a cabo en esta tesis doctoral han sido financiados por el Ministerio de Educación y Ciencia, número de proyecto AGL2006-04146/GAN. El doctorando, Jaime Gómez-Laguna, desarrolló sus

estudios de doctorado gracias a una beca de Formación de Profesorado Universitario del Ministerio de Educación y Ciencia (AP-2004-0395).

SUMMARY / RESUMEN

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is nowadays one of the most significant diseases of swine industry. This syndrome is characterised by inducing interstitial pneumonia in growing pigs and reproductive failure in gilts. PRRS is caused by a positive-stranded enveloped, RNA virus, known as PRRS virus (PRRSV), which belongs to *Arteriviridae* family, *Nidovirales* order. PRRSV replicates mainly in porcine alveolar macrophages (PAMs), and in a lesser extent in monocytes and dendritic cells.

Several studies have been carried out to decipher both the immune response against PRRSV infection and the role of cytokines in the pathogenesis of PRRS. However, nowadays several aspects remain still obscure. Therefore, the general aim of this thesis was to determine changes in the serum and tissue expression of cytokines and their relationship with the immune response evoked against a PRRSV field isolate. To assess this aim, twenty eight five weeks old, PRRSV-free pigs were inoculated by intramuscular route with PRRSV field isolate 2982. Other identical four pigs were inoculated with sterile medium and killed at the end of the study. Blood and tissue samples were collected at 0, 3, 7, 10, 14, 17, 21 and 24 days post-inoculation (dpi) for flow cytometry studies and for acute phase proteins (APPs) and cytokine expression by ELISAs and immunohisctochemical methods.

In our studies, $CD21^+$ cell counts increased in PBMCs and tracheobronchial lymph node from 17 to 24 dpi, coinciding with an increase in PRRSV-specific antibody titre in blood. $CD3^+$ T cell counts increased mainly due to an enhancement of $CD4^-CD8^{high}$ and $CD4^+CD8^+$ T cells. $CD4^-CD8^{low}$ T cells were decreased in all the organs studied, whereas $CD4^+CD8^-$ T cells decreased only in the spleen. The drop of the viraemia was correlated with an enhancement of $CD4^-CD8^{high}$ T cells and with a higher expression of interleukin-10 (IL-10) and interleukin-12 p40 (IL-12 p40). No efficient gamma interferon (IFNγ) response was detected during the acute phase of the infection and the expression of alpha interferon (IFNα) was late and reached its maximum expression once the viraemia decreased. These results pointed to IL-10 and IL-12 as cytokines which might play a significant role in PRRSV immune response, as well as $CD4^-CD8^{high}$ T cells.

Hp and Pig-MAP serum concentration displayed a moderate enhancement at 10 dpi, but CRP and SAA showed a delayed and highly variable increase. All three proinflammatory cytokines were poorly expressed, and only a mild increase in interleukin-1beta (IL-1β) was observed at 7 dpi. Although tumor necrosis factor alpha (TNFα) was expressed only in low levels, a positive correlation was observed with respect to the viral load, interleukin (IL-6) and Hp levels. The pathway used by PRRSV to modulate the innate immune response and the synthesis of proinflammatory cytokines still remains unclear, but it seems that TNFα may play a role.

Macrophages represent the first barrier against infections participating actively in the immune response, by means phagocytosis and the expression of cytokines. Cytokines may act activating or downregulating macrophage activation, depending on the cytokines synthesized. PRRS is characterised by replicating in PAMs, however, nowadays there is lack of information about the activation of macrophages and the expression of cytokines by these cells during PRRS. Thus, once the changes in the serum concentration of cytokines and their participation in the systemic immune response was studied, the last goal of this thesis was to determine changes in the different subpopulations of pulmonary macrophages and in their expression of cytokines in the lung of PRRSV-infected pigs. Interleukin-1 alpha (IL-1α), IL-6 and TNFα showed correlation with both histopathological degree of lung lesion and macrophage counts, playing a significant role in the pathogenesis of the interstitial pneumonia observed during PRRS. A significant correlation was observed between PRRSV and IL-10, IL-12 p40 and IFNγ, and TNFα and IFNγ. These results point to PRRSV modulates the immune response by the expression of IL-10, which might induce lower levels of other cytokines implied in viral clearance, just as IFNα, IFNγ, IL-12 p40 and TNFα. Moreover, these results also point to a stimulation of the expression of IFNγ by IL-12 p40 and TNFα, but not IFNα. All the cytokines studied were expressed mainly by septal macrophages and secondly by PAMs or other immune cells, just as lymphocytes or neutrophils. Our results point out that activation of septal

macrophages and PAMs differs throughout PRRSV infection, playing the first ones a main role in the synthesis and release of cytokines.

In conclusion, our results point out that both IL-10 and TNFα played a significant role in the modulation of the immune response against the PRRSV field isolate 2982 used in our studies. Therefore, efficient strategies to control PRRSV infection may be address to the modulation of both cytokines, inducing a downregulation of IL-10 and/or an upregulation of TNFα.

RESUMEN

El Síndrome Reproductivo y Respiratorio Porcino (PRRS, del inglés *Porcine Reproductive and Respiratory Syndrome*) actualmente representa uno de las enfermedades más importantes de la industria porcina. Este síndrome se caracteriza por inducir una neumonía intersticial en cerdos de transición y de engorde, así como fallo reproductivo en cerdas gestantes. El PRRS está causa por un virus ARN de cadena sencilla, con envoltura, conocido como virus del PRRS (VPRRS), que pertenece a la familia *Arteriviridae*, orden *Nidovirales*. El VPRRS se replica principalmente en macrófagos alveolares porcinos (MAPs) y, en menor medida, en monocitos y células dendríticas.

Entre los principales aspectos que quedan por conocer de la enfermedad se encuentra la alteración de la respuesta inmune y el papel que las citoquinas desempeñan en la patogenia del PRRS. Por todo ello, el objetivo general de esta tesis consistió en evaluar los cambios de la expresión sérica y tisular de diferentes citoquinas y su relación con la respuesta inmune desarrollada frente al VPRRS. Para alcanzar este objetivo se utilizaron veintiocho cerdos, libres del VPRRS, de cinco semanas de edad, que fueron inoculados intramuscularmente con el aislado de campo 2982 del VPRRS. Otros cuatro cerdos, de idénticas características a los inoculados, fueron utilizados como control, sacrificándose al final del estudio. De estos animales se tomaron muestras de sangre y de diferentes órganos a los 0, 3, 7, 10, 14, 17, 21 y 24 días post-inoculación (dpi)

para la determinación de las subpoblaciones de linfocitos y la expresión de proteínas de fase aguda (PFAs) y de citoquinas.

En nuestros estudios, se observó un aumento en el recuento de células $CD21^{+}$ tanto en células mononucleares de sangre periférica (CMSPs) como en el nódulo linfático traqueobronquial entre los 17 y los 24 dpi, coincidiendo con un incremento del título de anticuerpos específicos frente al VPRRS. El número de células T $CD3^{+}$ aumentó principalmente debido a un incremento de las células T $CD4^{-}CD8^{high}$ y $CD4^{+}CD8^{+}$. El recuento de células T $CD4^{-}CD8^{low}$ T estaba disminuido en todos los órganos examinados, mientras que las células T $CD4^{+}CD8^{-}$ disminuyeron únicamente en el bazo. La disminución de la viremia se correlacionó con un aumento de las células T $CD4^{-}CD8^{high}$, y con una mayor expresión de la interleuquina-10 (IL-10) y de la interleuquina-12 p40 (IL-12 p40). Durante la fase temprana de la infección no se detectó una respuesta eficiente de interferón gamma (IFNγ) y la expresión de interferón alfa (IFNα) fue tardía y no alcanzó su máximo nivel hasta que la viremia había disminuido. Estos resultados nos sugieren que la IL-10 e IL-12 y las células T $CD4^{-}CD8^{high}$ serían unos importantes mediadores en la respuesta inmune frente al VPRRS.

La concentración sérica de Hp y Pig-MAP mostró un aumento moderado a los 10 dpi, mientras que los niveles de CRP y SAA presentaron un incremento retardado y altamente variable. Las tres citoquinas proinflamatorias fueron detectadas en niveles bajos en suero, ya que sólo se observó un leve aumento de la interleuquina-1beta (IL-1β) a los 7 dpi. Sin embargo, aunque el factor de

necrosis tumoral alfa (TNFα) se expresó únicamente en niveles bajos, se observó una correlación positiva con la viremia, y con los niveles de interleuquina 6 (IL-6) y Hp. Por todo ello pensamos que el TNFα podría desempeñar un papel importante en la modulación de la respuesta inmune frente al VPRRS.

En el pulmón, la expresión tisular de interleuquina-1 alfa (IL-1α), IL-6 y TNFα se econtraba correlacionada tanto con el grado de lesión histopatológica de lesión pulmonar como con la cinética de los macrófagos, lo que demuestra un papel importante de estas citoquinas en la patogenia de la neumonía intersticial observada durante el PRRS. Una correlación significativa fue observada entre los niveles de expresión del virus y de la expresión de IL-10, IL-12 p40 e IFNγ, y entre TNFα e IFNγ. Nuestros resultados sugieren que el VPRRS modula la respuesta inmune a través de la expresión de IL-10, lo cual podría inducir niveles más bajos de otras citoquinas implicadas en la eliminación del virus, como IFNα, IFNγ, IL-12 p40 y TNFα. Asimismo, estos resultados también señalan a una estimulación de la expresión de IFNγ mediada por la IL-12 p40 y TNFα, pero no por IFNα. Todas las citoquinas estudiadas fueron expresadas principalmente por macrófagos del septo del pulmón y en segundo lugar por MAPs u otras células del sistema inmune, como linfocitos o neutrófilos. Estos resultados indican que la activación de los macrófagos del septo y de MAPs es diferente a lo largo de la infección con el

VPRRS, desempeñando los primeros un papel fundamental en la síntesis y liberación de citoquinas.

En conclusión, nuestros resultados señalan que tanto IL-10 como TNFα juegan un papel crucial en la modulación de la respuesta inmune frente al aislado de campo 2982 del VPRRS utilizado en nuestros estudios. De este modo, las estrategias eficaces para controlar la infección por el VPRRS pueden ir dirigidas a la modulación de ambas citoquinas, induciendo bien una inhibición de la expresión de IL-10 y/o un aumento de la expresión de TNFα.

1. BACKGROUND

1.1. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME: VIRUS, ANTIGENIC AND GENETIC DIFFERENCES, EPIDEMIOLOGY, PATHOGENESIS, CLINICAL SIGNS AND LESIONS.

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1.1. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

1.1.1. Introduction

In 1991, a virus which infection induced a disease characterised by reproductive failure in pregnant sows and respiratory symptoms in growing pigs was first isolated in The Netherlands being named as Lelystad virus (Wensvoort *et al.*, 1991). One year later, Collins and co-authors (1992) isolated in Minnesota a virus related structural and genetically to Lelystad virus. This virus was the responsible of a similar process which was named as “swine infertility and respiratory syndrome” (SIRS).

Several terminologies have been used to name the same process: “mystery swine disease”, “porcine epidemic abortion and respiratory syndrome (PEARS)”, “SIRS” or “blue ear disease”. Finally, all these names led to an international consensus and the disease is nowadays known as “porcine reproductive and respiratory syndrome (PRRS)” (Collins *et al.*, 1992).

When PRRS first appears in a farm it triggers off an acute outbreak of late term abortion and stillbirth, increased preweaning mortality and respiratory disease problems in growing and finishing pigs. Nowadays, the virus is enzootic in most of the farm from pork producing countries and its significance lied on concomitant infections with other common porcine respiratory pathogens, constituting the “porcine respiratory disease complex” (PRDC). Due to its role in

the PRDC, PRRS has been considered as one of the most important causes of economic losses in the modern swine industry (Rossow, 1998; Neumann *et al.*, 2005; REFERENCE).

1.1.2. Virus

PRRS is an infectious disease caused by a small, spherical, enveloped positive-stranded RNA virus named PRRS virus (PRRSV). PRRSV is classified within the *Arterivirus* genus, *Arteriviridae* family, which together with *Coronaviridae* family constitutes *Nidovirales* order (Meulenberg *et al.*, 1993b; Cavanagh, 1997; De Vries *et al.*, 1997). Other members of *Arteriviridae* family are equine arteritis virus, lactate dehydrogenase-elevating virus (LDV) of mice and simian hemorrhagic fever virus (Plagemann and Moennig, 1992; Conzelman *et al.*, 1993; Meulenberg *et al.*, 1993b; Cavanagh, 1997). Arteriviruses possess three main features: replication in macrophages, capacity for inducing persistent infections and high genomic variability (Snijder and Meulenberg, 1998).

PRRSV has a medium size of 62 nm (45-80 nm), with an inner icosaedric nucleocapside, ranging from 25 to 35 nm in diameter, in its inner part (Wensvoort *et al.*, 1991; Benfield *et al.*, 1992; Kim *et al.*, 1993). The nucleocapside is surrounded by a lipidic bilayer envelope which contains six structural proteins: P2b, GP2a, GP3, GP4, GP5 and M (Meulenberg *et al.*,

1995; Mardassi *et al.*, 1996; Wu *et al.*, 2001). The genome of PRRSV is approximately 15 kb containing nine open reading frames (ORFs). ORFs 1a and 1b represent 75% of the genome and encode proteins with apparent replicase and polymerase activities (Meulenber *et al.*, 1993a). ORFs 2a, 3, 4 and 5 encode for membrane glycoproteins (GP) GP2a, GP3, GP4 and GP5, respectively. GP5, or envelope (E) protein, is considered the main protein of the envelope playing a significant role in the apoptosis phenomenon triggered during PRRSV infection (Suárez *et al.*, 1996a). In addition, monoclonal antibodies (mAb) against GP5 are able to neutralise PRRSV, pointing to a role of this glycoprotein in the fixation of PRRSV to cellular receptor (Pirzadeh and Dea, 1997; Zhang *et al.*, 1998; Weiland *et al.*, 1999; Yang *et al.*, 2000). ORFs2b, 6 and 7 codes, respectively, for unglycosylated membrane protein P2b, membrane associated protein (M protein) and nucleocapside protein (N protein) (Meulenberg *et al.*, 1993b, 1995; Murtaugh *et al.*, 1995). Proteins M and N are the most abundant proteins present in the virion (Mardassi *et al.*, 1996), and the majority of the antibodies produced during the infection are specific for N protein (Loemba *et al.*, 1996).

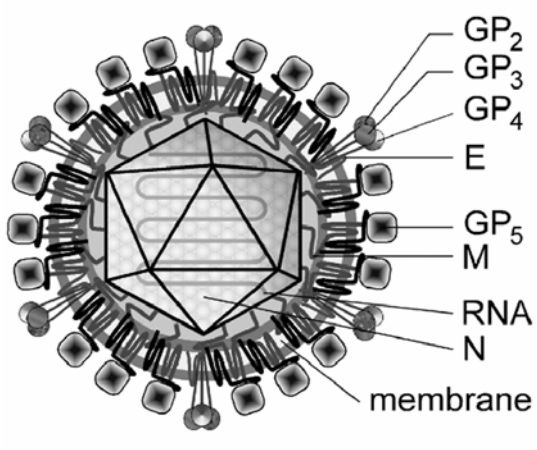


Fig. 1. Schematic representation of PRRSV which shows the icosaedric structure of the virion, the different glycoproteins and the envelope. Modified from Fields Virology 2007.

PRRSV is stable at -70 and -20 °C. At higher temperatures, the half life of PRRSV is 140 hours at 4 °C, 20 hours at 21 °C, 3 hours at 37 °C and 6 minutes at 56 °C (Benfield *et al.*, 1992; Bloemraad *et al.*, 1994).

An important characteristic of arteriviruses is their strong tropism for cells that belong to the monocyte-macrophage lineage (Tong *et al.*, 1977; Plagemann and Moening, 1992; Pol and Wagenaar, 1992; Voicu *et al.*, 1994). During the acute phase of the infection, the preferential PRRSV replication cells are porcine alveolar macrophages (PAMs) and only a limited extend of other cells, included monocytes (Molitor *et al.*, 1997; Bautista and Molitor, 1999). However, viral replication is influenced by the stage of macrophage differentiation (Duan *et al.*, 1997). PRRSV antigen is found in as much as 2 % of the alveolar macrophages during the acute phase of the infection (Mengeling *et al.*, 1995; Duan *et al.*, 1997). When PAMs are freshly isolated, the macrophage differentiation makes necessary several days of PAMs cultured for

increase their sensitivity to PRRSV infection. PRRSV can be cultured in three established non porcine lines: MARC-145 cells (Kim *et al.*, 1993), CL-2612 cells (Benfield *et al.*, 1992) and CRL-11171 cells (Meng *et al.*, 1996).

On the other hand, the virus can also be found in macrophages throughout the body tissues and organs, including secondary lymphoid tissues (Lawson *et al.*, 1997). Indeed, PRRSV persists during several weeks in lungs and lymphoid organs (Albina *et al.*, 1994; Wills *et al.*, 1997b; Allende *et al.*, 2000; Lamontagne *et al.*, 2001, 2003). Moreover, lung and lymphoid tissues, except for the spleen, appear to be the sites for viral replication in acute infection, since similar viral load has been detected in both (Xiao *et al.*, 2004). In persistent infection, however PRRSV is restricted primarily to tonsil and sternal lymph node (Wills *et al.*, 1997b; Rossow, 1998; Allende *et al.*, 2000; Xiao *et al.*, 2004).

1.1.3. Antigenic and genetic differences

There are two recognised genotypes of PRRSV: European (EU genotype, type I) and North American (US genotype, type II) genotypes (Snijder *et al.*, 2004). EU genotype is represented by Lelystad virus (LV) whereas the strain ATCC VR2332 is the prototype of the US genotype. These two genotypes are considered to come from a common ancestor, although they have important antigenic and pathogenic differences presenting only a 55-65 % of genetic similarity between them and a high genetic diversity within a given genotype

(Wensvoort *et al.*, 1992; Mardassi *et al.*, 1994; Meng *et al.*, 1995a, 1995b; Murtaugh *et al.*, 1995; Suárez *et al.*, 1996b; Drew *et al.*, 1997; Gagnon and Dea, 1998; Dea *et al.*, 2000; Forsberg *et al.*, 2002; Goldberg *et al.*, 2003; Mateu *et al.*, 2003; Stadejek *et al.*, 2006). While GP5 is conserved only in 51-58 % between different isolates, M and P2b proteins are the most conserved structural proteins (74-81 %) (Meng *et al.*, 1994; Kapur *et al.*, 1996; Wu *et al.*, 2001). In this sense, marked variability in the pathogenicity and degree of interstitial pneumonia has been described depending on PRRSV genotype (Halbur *et al.*, 1994; Shimizu *et al.*, 1996; Drew, 2000; Thanawongnuwech *et al.*, 2003).

Significant differences have also been described among several isolates from a same genotype, although they are not so marked as among different genotypes. Initially, these differences were preferentially described for the US genotypes (Meng *et al.*, 1995a; Andreyev *et al.*, 1997; Gagnon and Dea, 1998; Allende *et al.*, 1999; Dea *et al.*, 2000; Goldberg *et al.*, 2000a). However, recent publications have shown up to 18 % of divergence between EU isolates, being identified four different subtypes (Drew *et al.*, 1997; Indik *et al.*, 2000; Forsberg *et al.*, 2001; Bignotti *et al.*, 2002; Forsberg *et al.*, 2002; Stadejek *et al.*, 2002, 2006). Moreover, Forsberg and co-authors (2002) concluded that the diversity of EU isolates was higher than that from US isolates, probably due to an increase of vaccination isolates.

In addition, breed differences have also been observed respect with serum antibody titres, average daily gain and susceptibility to PRRSV induced lesions in lung, brain and heart (Halbur *et al.*, 1997). Taken together, these data suggest that allelic variation exists in disease response genes to PRRSV (Murtaugh *et al.*, 2002).

1.1.4. Epidemiology

PRRS is now present throughout the world, with the exception of Australia, New Zealand, Finland, Norway, Sweden, and Switzerland (www.oie.int, 2008). Accurate reports concerning the prevalence of the infection are only available from the United States where, overall, 40 to 60% of herds are estimated to be infected, ranging from 0 to 80%, in different States (Bautista *et al.*, 1993; Cho *et al.*, 1993). In Europe, PRRSV infection is believed to have affected more than 50% of farms (Albina, 1997a). In Spain the disease is endemic, being more than 90% of the farms positive and, although there are not available data, about 70% of breeding pigs are positive (Prieto, 2004). Anyway, the use of commercial vaccines makes more difficult to estimate the incidence and prevalence of PRRS.

PRRSV is highly infectious but not much contagious (Prieto, 2004). The virus can be transmitted by means of vertical or horizontal transmission. Horizontal transmission, and especially close pig to pig contact, is the most

important way of transmission of the virus (Albina, 1997a). Aerosols and fomites are also significant ways of horizontal transmission (De Jong *et al.*, 1991; Le Potier *et al.*, 1995; Albina, 1997a; Torremorell *et al.*, 1997; Lager and Mengeling, 2000; Kristensen *et al.*, 2002; Otake *et al.*, 2002b).

Pig to pig contact is greatly promoted by intensive animal movements (purchase of weaners and replacement breeding stock). Infection probably takes place through nose to nose contact or by contact with urine or feces (Albina, 1997a). The virus can indeed be detected in nasal and faecal swabs or in urine from pigs challenged experimentally (Rossow *et al.*, 1994). In this sense, it is important to remark that persistently infected pigs can be clinically normal although they are able to secret virus and therefore infect other pigs becoming in a significant entrance source of virus in PRRSV free herds (Bierk *et al.*, 2001).

PRRSV transmission through aerosols has been considered a significant way of transmission when the disease first appeared. However, this route of transmission is a little bit controversial since several authors have tried to demonstrate it experimentally showing contradictory results. Although PRRSV airborne spread as far as 20 km (De Jong *et al.*, 1991), transmission by air is probably more important in short distances. PRRSV transmission has been described over less than 3 km (Albina, 1997a). Le Potier and co-authors (1995) observed that many farms (45%) located in a 500 m radius around PRRS outbreaks became infected, and only few (2%) became infected in the zone 1 to

2 km from an outbreak. The transmission has also been demonstrated between pigs units separated just for 0.5 to 1 m of each other and connected by pipes (Torremorell *et al.*, 1997; Lager and Mengeling, 2000; Kristensen *et al.*, 2002). Conversely, other research groups were not successful in reproduce PRRSV airborne spreading (Wills *et al.*, 1994; Otake *et al.*, 2002a). Airborne transmission is generally enhanced during winter when the temperature is low, humidity high, and when wind speed and ultra-violet light exposure are low (Komijn *et al.*, 1991).

Although the vertical transmission via insemination is also possible, some reports show contradictory results. It is accepted that both artificial insemination (AI) with infected semen (Gradil *et al.*, 1996; Lager *et al.*, 1996; Prieto *et al.*, 1997) and sow mating with infected boars (Yaeger *et al.*, 1993; Swenson *et al.*, 1995) may lead to a venereal transmission of the disease. Several authors (Edwards *et al.*, 1992; Yaeger *et al.*, 1993; Swenson *et al.*, 1994) have shown that virus can be detected in semen from infected boars for up to 35 days post infection (dpi) and its significance in transmission to PRRSV free herds trough AI. Nevertheless, the immunity of the sow is able to avoid a transplacental infection when is exposed to a homologous strain (to a stock of the original virus) (Lager *et al.*, 1997) but not against a heterologous strain (an antigenically distinct PRRSV isolate) (Lager *et al.*, 1999). Ohlinger (1992) failed to demonstrate infectivity in semen of infected boars or contamination of farms which used AI with semen from infected herds suggesting that the risk of

PRRSV transmission via semen was probably restricted to the acute phase of infection. Anyway, it is difficult to reproduce the venereal transmission of PRRSV in field conditions due to the low seminal viral load (Prieto *et al.*, 1996, 2003) and the high viral load required to pass on PRRSV by semen compared with other routes of transmission (Benfield *et al.*, 2000).

Some avian species may be involved in the epidemiology of PRRSV acting as vectors. In this sense, it has been reported that pigs intranasally inoculated with PRRSV isolated from faeces of mallard ducks became viraemic, seroconverted and transmitted the virus to sentinel pigs (Zimmerman *et al.*, 1997). Otake and co-authors (2002c, 2002d) reported that blood-borne transmission of PRRSV can be achieved by contaminated needles and by mosquitoes. On the other hand, rodents are not susceptible to PRRSV (Hooper *et al.*, 1994).

Once infected, pigs shed virus in nasal secretions, saliva, urine, mammary gland secretions, feces and semen until at least 28 dpi (Yoon *et al.*, 1993; Rossow *et al.*, 1994; Christopher-Hennings *et al.*, 1995; Wagstrom *et al.*, 2001; Wills *et al.*, 1997a). However, the acute phase of the infection does not necessarily lead to seroconversion of all animals within the herd, being infected the remaining negative pigs at any time subsequently and therefore contribute to maintain the presence and the virus shedding (Albina, 1997a).

1.1.5. Pathogenesis

Pigs of all ages are susceptible to PRRSV infection. Experimental infection can be achieved following intranasal, intratracheal, oronasal, oral, intramuscular, intrauterine, intravenous or intraperitoneal inoculations (Wensvoort *et al.*, 1991; Christianson *et al.*, 1992; Collins *et al.*, 1992; Christianson *et al.*, 1993; Rossow *et al.*, 1994; Swenson *et al.*, 1994; Wills *et al.*, 1994; Pol *et al.*, 1997; Van Reeth *et al.*, 1999; Yoon *et al.*, 1999). Under natural circumstances, the virus most frequently enters via the respiratory tract, but viraemia and dissemination throughout the body rapidly occur (Duan *et al.*, 1997; Beyer *et al.*, 2000).

Once the virus has entered in the organism, mainly macrophages (Duan *et al.*, 1997; Lawson *et al.*, 1997), but also monocytes and dendritic cells (DC) (Halbur *et al.*, 1996), become target cells for PRRSV replication. In macrophages, the virus penetrates cells by a pH dependent endocytosis phenomenon (Kreutz and Ackerman, 1996) and by a 210 kDa receptor (Duan *et al.*, 1998). Additionally, two new PRRSV receptors have been described to participate in the virus attachment: heparan sulfate, which plays a role in the attachment but probably not in virus uptake (Delputte *et al.*, 2002), and porcine sialoadhesin, which is essential for PRRSV internalization in macrophages (Vanderheijden *et al.*, 2003) and partially for attachment to these cells depending on the presence of sialic acids within the virus envelope (Delputte

and Nauwynck, 2004). The virus replicates rapidly and releases the viral progeny by infected cells lysis. Moreover, cellular infection induces apoptosis of bystander cells (Sur *et al.*, 1997, 1998; Sirinarumitr *et al.*, 1998). Apoptosis induction of bystander cells and cytokines released by infected macrophages could play a role in the development of lesions associated to PRRSV infection, as the proliferative interstitial pneumonia (Suárez, 2000; Labarque *et al.*, 2003a).

PRRSV spread from PAMs to the whole organism by haematogenous and lymphatic routes. Indeed, the virus can be constantly detected in lymphoid tissues and blood after infection (Rossow *et al.*, 1994). Haematogenous dissemination is fast enough to allow the virus to infect different organs. PRRSV has been detected in nasal turbinates, trachea, tonsils, lymph nodes, kidneys, adrenal glands, brains, liver, spleen, bone marrow and choroid plexus during different periods (Pol *et al.*, 1991; Rossow *et al.*, 1994; Halbur *et al.*, 1995a; Rossow *et al.*, 1995; Beyer *et al.*, 2000). From lungs, infectious virus has been recovered as late as 35 dpi, being infected cells mostly located in the alveolar spaces (Duan *et al.*, 1997; Beyer *et al.*, 2000). At the beginning of the infection, Halbur and co-authors (1996) evidenced PRRSV by immunohistochemistry in bronchiolar epithelial cells, arteriolar endothelial cells, mononuclear cells of alveolar septa and interstitial, intravascular and alveolar macrophages. From 3 dpi onwards, PRRSV was detected mainly in interstitial and alveolar macrophages, remaining in PAMs for longer periods (Halbur *et al.*, 1996). As

well as lung, other preferential tissue for PRRSV replication is the lymph node. In the lymph nodes, macrophages and DC from the germinal centres, which appear hyperplastic and with focal necrosis, displayed the highest immunostaining against PRRSV antigen (Halbur *et al.*, 1996), being recovered from lymph nodes infectious virus until 21 dpi (Duan *et al.*, 1997; Beyer *et al.*, 2000). PRRSV can be detected from 1 dpi to extend periods in tonsils, being detected by reverse transcriptase-polymerase chain reaction (RT-PCR) at 251 dpi although virus isolation from tonsils was possible just until 56 dpi (Wills *et al.*, 2003). PRRSV antigen distributes scattered in the tonsilar parenchyma, but especially in the epithelium of the crypts and within follicles, in macrophage-like or dendritic-like cells (Halbur *et al.*, 1996).

PRRSV replication in lymphoid tissues and its release to bloodstream could facilitate an extended viraemia (Rossow *et al.*, 1995) as well as PRRSV elimination by different routes during long periods, still without viraemia (Christopher-Hennings *et al.*, 1998; Prieto *et al.*, 2003). Thus, PRRSV infection induces an extended viraemia from 12 hours post infection (hpi) (Rossow *et al.*, 1995) to 63 dpi in sera samples (Vézina *et al.*, 1996), with half duration of 28 days. During viraemia, the virus may be distributed to various organs. In boars, the virus may infect the reproductive tract and be shed in semen (Swenson *et al.*, 1994; Christopher-Hennings *et al.*, 1995), in which is able to persist until 43-92 dpi (Swenson *et al.*, 1994; Christopher-Hennings *et al.*, 1995). In pregnant sows, PRRSV is able to cross through the placenta depending on the stage of

gestation. At early and mid gestation, transplacental infection is rarely observed (Christianson *et al*., 1993; Mengeling *et al.*, 1994). However, during the latter stages of gestation (93 days), transplacental infection occurs easily (Christianson *et al*., 1992). These differences may be explained by differences in placental permeability during gestation (Christianson *et al.*, 1993).

PRRSV can be isolated from blood samples until 23 dpi in EU genotypes infections (Prieto *et al*., 2003) and 28 dpi in US genotypes infections (Wills *et al*., 2003) although it can be detected by RT-PCR until 56 dpi (Wills *et al*., 2003). Viraemia last longer in sows than in piglets, probably due to an immature immune system and a higher proportion of susceptible cells in piglets in comparison with sows (Yoon *et al*., 1993; Mengeling *et al.*, 1994; Rossow *et al*., 1994; Prieto *et al.*, 1997).

The tropism of PRRSV for PAMs plays a function in the development of the pathogenesis of the respiratory disease. Macrophages play important roles in both innate and acquired immunity, performing a large variety of functions that include phagocytosis, inactivation of microorganisms, scavenging at sites of tissue injury, processing and presentation of antigens to lymphocytes and cytokine production. Thus, in PRRS, 50-65% PAMs are destroyed during the first week post infection (wpi) leading to a dysfunction of these cells (Molitor *et al.*, 1992; Zhou *et al.*, 1992; Molitor, 1993) and a decrease in superoxide anions and hydrogen peroxidase release by macrophages (Molitor *et al.*, 1992; Zhou *et*

al., 1992; Thanawongnuwech *et al.*, 1997; Chiou *et al.*, 2000; López-Fuertes *et al.*, 2000). The decrease in superoxide anions and hydrogen peroxidase release, leads to an impairment of the pulmonary immune response but only transiently because 4 weeks after infection PAMs recover their functions (Molitor, 1993; Done and Paton, 1995). The cytokines released during the infection, just as interleukin 1 (IL-1) or tumor necrosis factor alpha (TNFα), could mediate the inflammatory response in the lungs, being responsible of the dyspnoea and cutaneous erythema observed sometimes (Van Reeth and Nauwynck, 2000).

PRRSV has been associated to other different pathogens (Rossow, 1998) suggesting a possible immunosupresor role of the virus which would make easier concomitant and/or secondary infections. However, although the role of PRRSV in the PRDC is accepted, it has not been successful to trigger experimentally secondary bacterial infections in pigs infected previously with PRRSV (Drew, 2000). The predisposition for *Streptococcus suis* infection has been proved in both growing animals (Galina *et al.*, 1994; Halbur *et al.*, 2000; Thanawongnuwech *et al.*, 2000) and piglets infected *in utero* (Feng *et al.*, 2001). Concomitant infections of PRRS and Porcine Circovirus type 2 (PCV2) deserve special significance since lesions and piglet mortality rates displayed a great aggravation (Allan *et al.*, 2000; Harms *et al.*, 2001). During PRRSV infection has also been described an increase in the susceptibility to *Salmonella choleraesuis* (Wills *et al.*, 2000), *Bordetella bronchiseptica* (Brockmeier *et al.*,

2000), Swine Influenza virus (SIV) and porcine respiratory coronavirus (PRCV) (Van Reeth *et al.*, 1996) and *Mycoplasma hyopneumoniae* (Thacker *et al.*, 1999). In field conditions, concomitant infections between PRRS and *Streptococcus suis, Mycoplasma hyopneumoniae* or PCV2 are present in a high frequency (Segalés *et al.*, 2002).

PRRSV infection in boars is multisystemic, being possible to isolate the virus from different organs from 2 to 30 dpi (Prieto *et al.*, 2003). After infection, PRRSV spread through the whole organism, and infects the semen by replicating in organs of the reproductive tract or by the invasion of infected monocytes and macrophages from bloodstream to reproductive organs, without an obliged viral replication (Prieto *et al.*, 2003). PRRSV can be isolated only until 8 dpi from testicles, pointing that testis are not a primary viral replication place (Prieto *et al.*, 2003).

In sows, PRRSV infection takes more significance at late term gestation, since the virus has no effect until the implantation of the embryos (Prieto *et al.*, 1997). When sows are infected intranasally the percentage of embryos or foetuses infected at the two first thirds of the gestation is almost zero (Mengeling *et al.*, 1994; Prieto *et al.*, 1996), being necessary to infect directly the piglets to develop the infection (Christianson *et al.*, 1993; Lager and Mengeling, 1995). Mengeling and co-authors (1994) and Lager and co-authors (1996) reported a 100 % rate of piglets infected *in utero* at 90 days of gestation,

appearing stillbirth and weak-born piglets, which usually dye before weaning, increasing pre-weaning death ratio, or survive displaying growth retardation. Piglets with persistent infections associated to *in utero* infections present a marked dyspnoea, abdominal breathing and are more susceptible to bacterial infections (Benfield *et al.*, 1997).

1.1.6. Clinical signs

PRRS is characterised by producing both reproductive failure in breeding animals and respiratory disorders in growing and finishing pigs. However, their presentation in a naïve farm is different. While the reproductive disease is presented usually as an epizootic, developing a good protective immunity, the respiratory disease displays more characteristics typical of an endemic disease with a weak immune response and a greatly varying severity of clinical sings (Blaha, 2000; Drew, 2000).

After a severe pandemic phase, characterised by reproductive failure in pregnant sows and gilts and respiratory disease problems in pigs of all ages, but particularly in nursery pigs, the disease has become endemic in most of pig producing countries, with a majority of herds being persistently infected for several years and showing high variability and severity of clinical signs, including a subclinical course of infection (Stevenson *et al.*, 1993).

Subclinical presentation of PRRS is highly varied being also difficult to measure due to concomitant infections (Done and Paton, 1995). The clinical differences found in outbreaks are attributed to several factors, namely, different virulence and tropism of different PRRSV strains (Halbur *et al.*, 1996), immunitary status of the herd (Wensvoort, 1993), evidence of concomitant infections (Done and Paton, 1995), different management and flow of animals just as the facilities of the farm (Goldberg *et al.*, 2000b) and, finally, the size of the farm, being more severe in higher farms (Goldberg *et al.*, 2000b).

Epidemic appearance of the disease usually is limited to PRRSV negative farms being characterised by anorexia, pyrexia, depression and cyanosis of the skin, usually in the ears, vulva and limbs (Meldrum, 1991). More specific symptoms include dyspnoea and polypnea in adult animals and abdominal breathing in piglets. In the acute phase, problems appear associated to reproductive failure as a moderate increase of abortion, stillbirths, mummificated and macerated foetuses and weakborn piglets (De Jong *et al.*, 1991; Loula, 1991; Hopper *et al.*, 1992), decreasing in four the number of born alive piglets per litter in an outbreak of the disease (Polson *et al.*, 1990). In addition, weakborn piglets can suck colostrum with difficulty increasing their mortality during lactation period (Hopper *et al.*, 1992) and leading to mastitis in sows and diarrhoea in piglets. Piglets can show frequently abdominal breathing together conjunctivitis, cough, or palpebral oedema (Rossow *et al.*, 1994) and circulatory disturbances, like haemorrhages at the umbilical cord or spread

haematomas at the points of iron administration (Hopper *et al.*, 1992), cyanosis at the ears, cutaneous erythema and rough hair (Rossow, 1998). Nervous symptoms, like somnolence and anorexia in sucking piglets have been also described (Rossow *et al.*, 1999).

Boars will present, in addition to anorexia, lethargy and lack of libido, a decrease in sperm quality due to a reduction in motility, increase of abnormal acrosomes and morphological alterations (De Jong *et al.*, 1991; Feitsma *et al.*, 1992), which may lead to a decrease in the number of sperm doses between 4–7 weeks post infection (wpi) (Feitsma *et al.*, 1992).

The severity of the disease in growing and fattening pigs depends on the age of the animals, being more severe in younger animals (Rossow *et al.*, 1994), and in appearance of concomitant pathogens. However, most of the times PRRSV infection passes unnoticed in fattening pigs, displaying only fever, respiratory distress and weight loss when it is inoculated together other pathogens (Van Reeth *et al.*, 1996; Labarque *et al.*, 2002). The mean daily intake can be diminished till a 50% (Keffaber, 1989).

Chronic disease is the most common way of presentation of the disease, following an acute phase, characterised by respiratory disorders in growing pigs, and sometimes in breeding sows. During this phase also reproductive parameters are impaired leading to the greatest economical losses (Dee *et al.*, 1996) due to normal parameters will not be restored until 6 months after an outbreak of the disease, meanwhile the virus remain in growing and fattening

animals (Stevenson *et al.*, 1994). Indeed, the main characteristic of PRRS is the concomitance with secondary diseases, both bacterial and viral infections, emphasizing SIV, PRCV and specially PCV2 or bacteria like *Haemophilus parasuis*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, *Salmonella choleraesuis, Bordetella bronchiseptica* or *Pasteurella multocida*, but above all *Mycoplasma hyopneumoniae* (Galina *et al*., 1994; Van Reeth *et al*., 1996; Rossow, 1998; Thacker *et al.*, 1999; Allan *et al.*, 2000; Brockmeier *et al.*, 2000; Halbur *et al*., 2000; Thanawongnuwech *et al.*, 2000; Wills *et al*., 2000; Harms *et al*., 2001; Segalés *et al.*, 2002).

An atypical form of PRRS has been described being characterised by an acute phase with mortality higher than 5% in breeding animals and abortions ratio greater than 10%, reaching until 60%. Abortions appear along the whole gestation period, but with higher frequency at the first and the last third of the pregnancy. This form lasts only between 2 – 4 wpi. These outbreaks are related to highly virulent strains of PRRSV (Mengeling *et al*., 1998). Recently, a new outbreak of atypical PRRSV has been described associated to a deletion in the NSP2 of PRRSV (Tian *et al.*, 2007). However, further studies have to be carried out to confirm the unique involvement of an atypical PRRSV strain, due to the difficulty to discern clinically from other porcine viruses, which may be also involve in the outbreak, just as PCV2 or Hog Cholera Virus.

1.1.7. Lesions

The clinical and pathological effects of the disease depend on enzootic pathogens of each farm. Furthermore, gross lesions are not usually evidenced when PRRS is not complicated with secondary pathogens, while microscopic changes are just observed at respiratory tract (Done and Paton, 1995).

The main lesions observed in PRRS consist on tan-mottled, rubbery lungs, especially at the ventral area of medium and accessory lobules, and hyperplasia of lymph nodes (Pol *et al.*, 1991; Halbur *et al.*, 1995a, 1995b; Vézina *et al.*, 1996). The multifocal pattern of the interstitial pneumonia observed in the lungs point to a postviremic development of the lesions (Ramos *et al.*, 1992). When the virus affects pregnant sows hydrothorax, ascites and subcutaneous haemorrhages in weak born and stillbirth piglets (Plana *et al.*, 1992; Scruggs and Sorden, 2001) may be seen. In addition, mummification and maceration is common in piglets from infected litters just as oedema and haemorrhages of the umbilical cord (Lager and Halbur, 1996).

The main microscopic feature is a multifocal proliferative interstitial pneumonia, characterised by type II pneumocytes hypertrophy and hyperplasia, mononuclear cells infiltration of the alveolar septa and the presence of cellular debris and inflammatory cells in alveolar spaces (Halbur *et al.*, 1994, 1995; Rossow *et al.*, 1994, 1995). Histopathological lesions also include rhinitis, characterised by vacuolization of epithelial cells, loss of their cilia and surface

epithelium desquamation (Pol *et al.*, 1991; Collins *et al.*, 1992). These lesions appear from 3 dpi to 21 dpi (Rossow *et al.*, 1995). PAMs and epithelial cells degeneration has been ultrastructurally observed in lung and in nasal turbinate, evidenced by an excessive vacuolization of endoplasmic reticulum (Pol *et al.*, 1991). In addition, the virus is able to produce apoptosis both *in vitro* (Suárez *et al.*, 1996a) and *in vivo* in bystander cells (Sirinarumitr *et al.*, 1998; Sur *et al.*, 1997, 1998).

Moreover, hypertrophy of the germinal centres, necrosis of lymphoid follicles and an increased number of macrophages in sinusoids of lymphoid tissue, just as lymph nodes, spleen and tonsils, together subcapsular haemorrhages of the lymph nodes have been reported (Rossow *et al.*, 1994; Halbur *et al.*, 1995a). Feng and co-authors (2002) also described a marked thymic atrophy in piglets when they had been infected *in utero*.

Other microscopic lesions observed include multifocal miocarditis with mononuclear perivascular cuffing (Rossow *et al.*, 1994; Halbur *et al.*, 1995a), non suppurative diffuse encephalitis characterised by a mononuclear perivascular cuffing (Collins *et al.*, 1992) and severe meningoencephalitis (Rossow *et al.*, 1999).

Some extrapulmonary lesions attributed to PRRSV are thought to be caused by PCV2, like multisystemic vasculitis and miocarditis. As well, several authors reported PRRSV and PCV2 like the main pathogens associated to proliferative necrotizing pneumonia (PNP) (Pesch *et al.*, 2000; Grau-Roma and

Segalés, 2007). Although PRRSV has been pointed as the main responsible of PNP in the United States, PCV2 would be the main responsible of this process in Europe (Grau-Roma and Segalés, 2007).

1.1.8. Immune system, apoptosis, cytokines and their relationships: an approximation

Although it is well established that pigs develop both humoral (Yoon *et al.*, 1995; Loemba *et al.*, 1996) and cellular (Bautista and Molitor, 1997; López-Fuertes *et al.*, 1999; Meier *et al.*, 2003) immune responses in PRRSV infection, precise knowledge of the immune mechanisms induced is still incomplete. Circulating antibodies can be detected early in PRRSV infection although infected pigs may be viraemic until 6 – 12 wpi (Yoon *et al.*, 1995; Batista *et al.*, 2004; Johnson *et al.*, 2004). However, neutralizing antibodies (NAs) do not appear until 8-10 wpi and their role in PRRSV protection is not yet clear (Murtaugh *et al.*, 2002). The studies about cell mediated immune (CMI) response has been focused mainly in the study of peripheral blood mononuclear cells (PBMCs) (Shimizu *et al.*, 1996; Albina *et al.*, 1998b; López-Fuertes *et al.*, 1999; Samsom *et al.*, 2000; Feng *et al.*, 2002; Xiao *et al.*, 2004; Díaz *et al.*, 2005), meanwhile few studies have been done in peripheral lymphoid tissues (Kawashima *et al.*, 1999; Lamontagne *et al.*, 2003; Xiao *et al.*, 2004). The results obtained of the different reports are contradictory, however a

decline in the viraemia has been observed simultaneously with an increase in $CD8^+$ cells, pointing to a possible role of these cells in the clearance of the virus (Lamontagne *et al.*, 2003).

PRRSV mediated immunomodulatory effects, like interleukin 10 (IL-10) upregulation (Chung and Chae, 2003; Suradhat *et al.*, 2003) and interferon α (IFNα) suppression (Albina *et al.*, 1998a; Van Reeth *et al.*, 1999), have been suggested to play a role in delaying the host protective immune response and in favouring opportunistic infections. In this sense, one of the most controversial aspects of PRRS is the immunosuppressive capacity of the virus. In young pigs, the disease is frequently associated with secondary infection due to several pathogens, especially of the respiratory tract, suggesting that the virus reduces host defence mechanism (Stevenson *et al.*, 1993). The results obtained when the immunosuppressive state induced by PRRSV has been recreated experimentally displayed contradictory results (Galina *et al.*, 1994; Cooper *et al.*, 1995; Van Reeth *et al.*, 1996; Carvalho *et al.*, 1997; Pol *et al.*, 1997; Solano *et al.*, 1997; Wills *et al.*, 2000). On the one hand, several reviews of the literature have consistently concluded that PRRSV infection does not have an immunomodulatory or immunosuppressive effect (Beilage, 1995; Albina, 1997a, 1997b; Molitor *et al.*, 1997; Albina *et al.*, 1998b; Drew, 2000). In this sense, Albina and co-authors (1998b) suggested that the increased disease susceptibility of PRRSV infected pigs could be due to a disruption of the first lines of defence by means the replication of the virus in alveolar macrophages

and subconsequent local inflammatory reaction. Moreover, PRRSV has been shown to induce the death of alveolar macrophages, major target cells of the virus, and to reduce their functions in infected pigs (Molitor *et al.*, 1992; Zhou *et al.*, 1992). This diminishing on the number of macrophages has been related by several authors with apoptosis phenomenon linked to PRRSV infection. In this way, Feng and co-authors (2002) observed an increase in the number of apoptotic cells in the thymus of piglets infected *in utero*, Sirinarumitr and co-authors (1998) and Sur and co-authors (1998) described apoptosis in the lung and lymphoid tissues of pigs infected with PRRSV, and Suárez and co-authors (1996a) related apoptosis phenomenon with GP5 of PRRSV. Several mechanisms might be implicated in the development of apoptosis phenomena in infected animals. In this sense, high levels of IL-1β expression have been detected in alveolar macrophages in the course of PRRSV infection (Zhou *et al.*, 1992). Apoptosis could be also triggered by a decrease of the intracellular superoxide levels subsequent to infection of macrophages with PRRSV (Lin *et al.*, 1999). However, an indirect mechanism involved in PRRSV induced apoptosis has been also suggested, since apoptosis phenomena are mainly observed in non infected cells (Labarque *et al.*, 2003a).

Another striking point about PRRSV is related to the cross immunity get by means of vaccination. It has been probed that vaccination protects against homologous genotypes, however the protection against heterologous ones is only partial (Van Woensel *et al.*, 1998b; Lager *et al.*, 1999; Labarque *et al.*,

2003b), pointing to an immune response similar but not equal against different PRRSV genotypes (Díaz *et al.*, 2005). When a mixed protocol of vaccination has been followed up using both modified live vaccines (MLV) and killed vaccines (KV), only KV vaccination increased antibody levels in previously immunised (MLV or KV) sows (Bassaganya-Riera *et al.*, 2004).

1.2. IMMUNOLOGICAL RESPONSE OF SWINE TO PRRSV

Immunity to PRRSV begins with an innate antiviral response in the cytoplasm of the infected macrophage with a minimal production of type I interferon (IFNα/β) at the site of infection (Murtaugh *et al.*, 2002). Several authors have described a non significant IFNα production in PRRSV infection (Albina *et al.*, 1998a; Buddaert *et al.*, 1998; Van Reeth *et al.*, 1999; Murtaugh *et al.*, 2002) pointing to a non efficient innate immune response.

The antigen specific humoral immunity appears early after infection (5-7 dpi), although NAs are detected later in serum (Loemba *et al.*, 1996; Eichhorn and Frost, 1997; Albina *et al.*, 1998b; Meier *et al.*, 2003). Nonetheless, the efficiency of NAs in PRRS clearance is not clear, and the different reports show contradictory results (Yoon *et al.*, 1995; Murtaugh *et al.*, 2002; Batista *et al.*, 2004). The transfer of passive maternal immunity to piglets in colostrum is able to protect the piglets against the development of clinical symptoms and curtailment of viraemia (Murtaugh *et al.*, 2002).

CMI response, measured as antigen specific proliferation, is transiently induced 4–8 wpi and is restimulated for a 2–4 week window after rechallenge (López-Fuertes *et al.*, 1999). The persistence of PRRSV points to both humoral and cellular immune responses are not able to completely eliminate the virus.

Pigs infected with PRRSV show prolonged viraemia, persistent infection, and may suffer repeated episodes of the disease (Murtaugh *et al.*, 2002).

Otherwise, occasionally it has also been described a short viraemia (López-Fuertes *et al.*, 1999; Meier *et al.*, 2003; Sipos *et al.*, 2003; Díaz *et al.*, 2005). Furthermore, exposure of pigs to PRRSV induces a homologous immunity, protecting efficiently against the reexposure to the same strain (Lager *et al.*, 1997). However, further studies have to been carried out to confirm the establishment of a homologous immunity and to determine the role of a possible heterologous immunity.

Although modified-live vaccines (MLV) are able to reduce the severity of the disease, duration of viraemia, virus shedding and the frequency of PRRSV infection (Christopher-Hennings *et al.*, 1997; Nielsen *et al.*, 1997; Dee *et al.*, 1998; Van Woensel *et al.*, 1998a; Mavromatis *et al.*, 1999; Mengeling *et al.*, 1999), nowadays there is lack of a reliable vaccine which offer a total control and prevention of PRRS.

1.2.1. Innate response against PRRSV infection

In viral infections, the presence of double-stranded RNA triggers a variety of antiviral functions, of which the induction of the type I interferons (IFNα/β) is a hallmark of cellular antiviral defence (Vicek and Sen, 1996; Pfeffer *et al.*, 1998; Tizard, 2008). Virus induced IFNα/β activates or induces the synthesis of numerous proteins, including 29,59-oligoadenylate synthetase (OAS), double-stranded RNA-dependent protein kinase (PKR) and ribonuclease L (Rnase L)

(Vicek and Sen, 1996). OAS, PKR, and Rnase L suppress viral protein synthesis by a combination of RNA degradation and inhibition of protein synthesis (Chebath *et al.*, 1987; Vicek and Sen, 1996). However, there is no evidence of accelerated viral RNA degradation or reduced levels of viral protein synthesis in PRRSV-infected macrophages, accordingly with the minimal type I interferon expression observed *in vitro* (Albina *et al.*, 1998a; Buddaert *et al.*, 1998) or *in vivo* PRRSV infections (Albina *et al.*, 1998a; Buddaert *et al.*, 1998; Van Reeth *et al.*, 1999). Moreover, IFNα/β potently activates and stimulates the proliferation of natural killer (NK) cells, which comprise an important innate cellular response to viral infection (Tay *et al.*, 1998). The antiviral functions of NK cells result in the production of immunoregulatory cytokines (mainly IFNγ and TNFα) and in cytotoxicity to virus infected cells (Paya *et al.*, 1988; Orange and Biron, 1996; Tay *et al.*, 1998; Biron *et al.*, 1999; Rowland *et al.*, 2001).

In addition to IFNα production, inflammatory cytokine expression also is important in the initial response to a variety of viral respiratory infections (Van Reeth and Nauwynck, 2000). Accordingly, substantial production of IFNα, TNFα and/or IL-1 has been detected in swine influenza and porcine respiratory coronavirus, while PRRSV infection failed to elicit any significant cytokine expression by *in vivo* (Van Reeth *et al.*, 1999) and *in vitro* studies (Thanawongnuwech *et al.*, 2001). The downregulation of IFNα production seems to facilitate PRRSV replication since the increase of type I interferons level by *in vivo* stimulation or exogenous administration has been shown to

substantially reduce viral growth and to enhance humoral immune responses (Albina *et al.*, 1998a; Le Bon *et al.*, 2001).

In viral infection, TNFα and IL-1β are significant activators of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Didonato *et al.*, 1997; Regnier *et al.*, 1997; Christman *et al.*, 1998, 2000). NF-κB plays a central role in the innate response to infection by regulating the transcription of more than 100 genes, including inflammatory and immunoregulatory cytokines, antigen receptors, adhesion molecules, inhibitors of apoptosis, acute phase proteins, and innate effector molecules (Schmid and Adler, 2000). In this aspect, the mild or subclinical respiratory involvement in PRRSV infection may be due to the lack of activation of NF-κB. However, PRRSV has been reported to activate NF-κB in MARC-145 cells and alveolar macrophages (Lee and Kleiboeker, 2005), losing strength the hypothesis described above.

In summary, the initial innate immune response to PRRSV is not totally efficient. The lack of an IFNα response is significant, since IFNα-mediated events inhibit PRRSV replication *in vitro* (Albina *et al.*, 1998a; Buddaert *et al.*, 1998) and since elevation of IFNα *in vivo* by preinfection with other viruses, like PRCV, substantially attenuates subsequent PRRSV replication (Buddaert *et al.*, 1998). The alteration of the profile of cytokine expression in macrophages and DC and the modification of molecules involved in antigen presentation may be responsible of innate immune response downregulation by PRRSV (Mateu and Díaz, 2008).

1.2.2. Humoral immune response to PRRSV

Circulating antibodies against PRRSV are first detected like IgM at 5–7 dpi and then decline rapidly to undetectable levels after 2–3 weeks (Joo *et al.*, 1997; Park *et al.*, 1995; Yoon *et al.*, 1995; Loemba *et al.*, 1996). All the animals seroconvert at 14 dpi (Yoon *et al.*, 1992, 1995). Anti-PRRSV IgG antibodies are first detected at 7–10 dpi, peak at 2–7 wpi (Yoon *et al.*, 1995; Loemba *et al.*, 1996; Vézina *et al.*, 1996; Labarque *et al.*, 2000), remain constant for a period of months, and then decline to low levels by 300 dpi (Nelson *et al.*, 1994; Nielsen and Bøtner, 1997). Immunoglobulins of the IgG_1 subclass appear at 9 dpi, while IgG_2 subclass antibodies are first detected at 14 dpi with kinetics similar to that of IgG_1, but at a lower level (Labarque *et al.*, 2000). Anti-PRRSV IgA can be detected in serum at 14 dpi, reaches a maximum at 25 dpi, and remains detectable until 35 dpi. The kinetic of anti-PRRSV antibody isotypes in bronchoalveolar lavage fluid (BALF) is similar to those in serum, indicating that these antibodies extravasate from the vasculature (Labarque *et al.*, 2000).

Unlike the overall rapid production of anti PRRSV antibodies, NAs are reported to appear later in the infection and not in all inoculated animals (Loemba *et al.*, 1996; Eichhorn and Frost, 1997; Albina *et al.*, 1998b; Meier *et al.*, 2003), although some authors (Yoon *et al.*, 1994) have reported the detection of NAs as early as 9 dpi. The differences observed can be due to conventional virus neutralisation tests (VNTs) do not detect NAs in the first 4

wpi, although with some modifications of the technique they can be detected as early as 9-12 dpi (Takikawa *et al.*, 1996). NAs are consistently detected by 28 dpi or later for both EU and US genotypes (Yoon *et al.*, 1994; Meier *et al.*, 2003; Díaz *et al.*, 2005). Usually, the decline in PRRSV specific serum antibodies coincidence with the appearance of NAs (Albina *et al.*, 1998b; Molitor *et al.*, 1997; Nelson *et al.*, 1994; Shibata *et al.*, 2000; Meier *et al.*, 2003). Anti-PRRSV immunoglobulins in serum after PRRSV infection are directed primarily against PRRSV N protein (encoded by ORF 7) and M protein (encoded by ORF 6) (Dea *et al.*, 2000). Otherwise, NAs against PRRSV have been reported with specificities against GP5, GP4, and M (Halbur *et al.*, 1997; Meulenberg *et al.*, 1997; Pirzadeh and Dea, 1997, 1998; Weiland *et al.*, 1999; Yang *et al.*, 2000; Ostrowski *et al.*, 2002). GP5 seems to be the protein of major biological significance.

The role of NAs in prevention of this disease and in protection of pigs from infection is not clear. On the one hand, NAs have been correlated with a reduction in PRRSV titre in the lung and in peripheral blood, culminating in the clearance of PRRSV from circulation (Yoon *et al.*, 1995). Osorio and co-authors (2002) reported that sows with a NAs titre of 1:16 may clear PRRSV infection, when challenged at 90 days gestation, not suffering reproductive failure, which points to a protective role of NAs. Moreover, NAs titres of 1:8 have been reported to block viraemia but not peripheral tissue shedding and transmission to contact animals, whereas higher titres (1:32) could induce complete

protection (López *et al.*, 2007). On the other hand, PRRSV has been isolated from serum and different tissues in the presence of NAs (Christianson *et al.*, 1992; Rossow *et al.*, 1994; Vézina *et al.*, 1996; Wills *et al.*, 1997b; Dee *et al.*, 1998; Batista *et al.*, 2004), pointing that the level of NAs normally generated against PRRSV may not be sufficient to clearance of the virus (Murtaugh *et al.*, 2002). This fact may suggest a role of cellular immune mechanisms in the control of this viral infection (Vézina *et al.*, 1996; Wills *et al.*, 1997b). All these data together point to NAs may protect against the disease, when they are present in a sufficient quantity, but they do not play an important role in virus clearance (Mateu and Díaz, 2008).

The phenomenon of antibody-dependent enhancement (ADE) also may be involved in the reduced effectiveness of NAs response (Kurane *et al.*, 1991). Low titres of NAs may increase the association of viral particles with permissive macrophages through binding of virus antibody complexes to the Fc receptor, and thus facilitate viral binding and uptake through the macrophage PRRSV binding protein (Christianson *et al.*, 1993; Yoon *et al.*, 1996, 1997). The prolonged duration of viraemia and virus isolation from the tissues in piglets with low maternal antibodies also suggests ADE in PRRS (Shibata *et al.*, 1998).

Colostral antibody responses are closely correlated with serum antibody responses. In this way, Eichhorn and Frost (1997) showed that serum-positive sows were colostrum-positive and all colostrum-negative sows also were serum-negative. Because the concentration of IgG in colostrum exceeds that

found in serum (Pastoret *et al.*, 1998), sows may be colostrum-positive but serum-negative (Eichhorn and Frost, 1997). These maternal antibodies are reported to persist in piglets up to 6–8 weeks of age (Houben *et al.*, 1995; Chung *et al.*, 1997). However, antibodies fail to totally protect when transferred from PRRS recovered sows to 1 week old piglets (Morrison *et al.*, 1992). Pigs previously exposed to the virus are protected to reexposure to at least homologous virus challenge (Molitor *et al.*, 1997), suggesting a role for cellular immunity in protection (López-Fuertes *et al.*, 1999).

The impairment of host immune response in PRRSV infected animals, with a fast developed humoral response and a late and erratic cell mediate immune response, shows similarities with the immune response observe in human immunodeficiency virus (HIV) infection. In HIV most of the patients produce anti-HIV antibodies, which can be detected by means ELISA or Western blot methods, within 2 months post-infection while NAs do not appear at high titres till 25-53 wpi (Pilgrim *et al.*, 1997). It seems that PRRSV and HIV lead to a delayed induction of NAs by focusing the immune response to non neutralizing epitopes (Ho *et al.*, 1991; Ostrowski *et al.*, 2002). Thus, both PRRSV and HIV may posses a similar strategy for evading the potentially inhibitory activity of the host humoral immune response and present a similar challenge to effective vaccination (Meier *et al.*, 2003).

1.2.3. Cell-mediated immune response in PRRS

PRRSV infection induces a transient leukopenia and lymphopenia in the first week which resolves in 8–10 days (Christianson *et al.*, 1993; Nielsen and Bøtner, 1997; Feng *et al.*, 2002; Lamontagne *et al.*, 2003). However, the absence of a corresponding increase in lymphocyte numbers in the BALF at this time indicates that this change may not be a direct response to the virus (Labarque *et al.*, 2000; Samsom *et al.*, 2000), since PAMs are the target cell for viral replication.

PRRSV does elicit a late CMI response (Meier *et al.*, 2003), which usually is not organised and consistent. PRRSV infection in the lung peaks at approximately 9 dpi and foci of infection decline to near zero by 20 dpi, although virus still may be isolated from lung fluids for extended periods (Mengeling *et al.*, 1996; Labarque *et al.*, 2000; Samsom *et al.*, 2000; Tingstedt and Nielsen, 2004). Samson and co-authors (2000), using gnotobiotic and specific-pathogen-free (SPF) piglets infected with a European strain of PRRSV, identified an increase in the total number of bronchoalveolar cells (BALCs) due to an increase in $CD4^{-}CD8^{+}$ T cell population between 14 and 21 dpi, together a $CD6^{-}CD8^{+}$ and $CD6^{+}CD8^{+}$ increase at 7 dpi. The lymphocytes were classified as NK cells and cytotoxic T cells based on the cell marker phenotype $CD6^{-}CD8^{+}$ and $CD6^{+}CD8^{+}$, respectively. Both subtypes of lymphocytes regulate cellular immunity via the production of IFNγ (Trinchieri, 1995). Moreover, they found no

changes in the levels of $CD4^+CD8^-$ and $CD4^+CD8^+$ between infected and control animals neither a decrease in the number of the macrophages in the lung during the infection. Interestingly, Tingstedt and Nielsen (2004) reported a low $CD4^+$ and a high $CD2^+$, $CD3^+$ and $CD8^+$ immunohistochemical antigen expression in the lung of piglets born from PRRSV inoculated sows being correlated with the results above.

Several authors also have observed a substantial increase in $CD8^+$ T cells (Shimizu *et al.*, 1996; Albina *et al.*, 1998b; Feng *et al.*, 2002; Lamontagne *et al.*, 2003; Díaz *et al.*, 2005), whereas the decrease of $CD4^+$ T cells was not an uniform feature (Shimizu *et al.*, 1996; Feng *et al.*, 2002; Díaz *et al.*, 2005). These results led to a significantly low $CD4^+/CD8^+$ ratio in PBMCs of both naturally and experimentally infected pigs (Shimizu *et al.*, 1996; Albina *et al.*, 1998b; Feng *et al.*, 2002). Furthermore, Feng and co-authors (2002) also described an increase in $CD4^+CD8^+$ T cells in piglets infected *in utero* with PRRSV. Otherwise, these results are opposite to those reported by Zhou and co-authors (1992) and Xiao and co-authors (2004). These discrepancies are probably due to the different PRRSV isolate used in each study, since marked variability in the pathogenicity of PRRSV has been suggested (Halbur *et al.*, 1994; Shimizu *et al.*, 1996; Drew, 2000; Thanawongnuwech *et al.*, 2003). The transient nature of the PRRSV specific T cell response has been also observed in LDV infection, in which cytotoxic T cells were elicited in acute LDV infection but disappeared in the chronic phase of infection (Even *et al.*, 1995).

The mechanism involve in this low $CD4^+/CD8^+$ ratio in PRRSV infection is unknown. Shimizu and co-authors (1996) showed that PRRSV neither infects nor kills $CD4^+$ T cells and no posses a mitogenic activity for $CD8^+$ T cells, suggesting that intermediate immune effectors are necessary for the increase in $CD8^+$ T cells. These effectors could consist on helper cells or immunostimulating cytokines induced by the virus. In this sense, Paliard and co-authors (1988) reported an induction of the CD8 molecule mediated on human $CD4^+$ cells by IL4 expression, and Moore and co-authors (2001) reported $CD8^+$ T cells growth promotion and differentiation by the expression of IL-10. Anyway, the biological significance of the change in $CD8^+$ T cells in infected pigs is not well understood, although some approaches have been done. Albina and co-authors (1998b) hypothesised that the change in $CD8^+$ T cell subset could be related with the control of virus replication, due to they observed a decline of the viraemia simultaneously to the proliferation of $CD8^+$ cells.

PRRSV-specific T cell proliferative response first appears in peripheral blood at approximately 4 weeks after infection with both US either EU PRRSV isolates, once viraemia was no longer detectable (Bautista and Molitor, 1997; López-Fuertes *et al.*, 1999). *In vivo* specific cell-mediated reactivity to PRRSV antigen was confirmed by a delayed type hypersensitivity (DTH) reaction to inactivated PRRSV (Bautista and Molitor, 1997). López-Fuertes and co-authors (1999) reported a $CD4^+$ T cell proliferation in T cell response to PRRSV which was present for a period of 10 weeks after infection. Stimulated cells expressed

IFNγ and IL-2, but not IL-4 or IL-10, suggesting that the $CD4^+$ T cells possessed a Th1 cytokine expression phenotype characteristic of CMI response to intracellular pathogens (López-Fuertes *et al.*, 1999). However, *in vitro* restimulation of PBMCs with PRRSV antigen and IL-2 led to the observation that the responding cells were primarily $CD8^+$, which were represented by $CD4^+CD8^+$ double positive cells, which are known to represent antigen-specific memory T helper cells (Zuckermann and Husmann, 1996), or by $CD8^+\gamma\delta^+$ cells with constitutive cytolytic activity (NK activity), like $CD8^+$ cytolytic cells (López-Fuertes *et al.*, 1999). The delay to detect proliferative response until 4 weeks post inoculation could be due to the sequestration of specific T cells in infected organs (López-Fuertes *et al.*, 1999). Otherwise, an excess of viral load in the blood may also lead to an unresponsiveness state of specific T cells as it has been described for other viruses (Zinkernagel *et al.*, 1997).

Host IFNγ production is central to mechanisms of protection in a variety of cytopathic viral infections in murine models (Ramsay *et al.*, 1993; Zinkernagel *et al.*, 1996). IFNγ mRNA has been detected in the lymph nodes, lungs and PBMCs of PRRSV-infected pigs (López-Fuertes *et al.*, 1999; Rowland *et al.*, 2001; Thanawongnuwech *et al.*, 2003). Moreover, it has been reported that IFNγ blocks PRRSV replication in cultured cells (Bautista and Molitor, 1999) apparently by the inhibition of viral RNA synthesis via a double-stranded RNA-inducible protein kinase (Rowland *et al.*, 2001). The intensity of the IFNγ response to either wild type or attenuated PRRSV increases gradually over a

period of months, while humoral immunity decreases (Meier *et al.*, 2000). The observation that PRRSV persists in lung and lymph nodes despite the presence of NAs in serum and BALF (Albina *et al.*, 1994; Chung *et al.*, 1997; Wills *et al.*, 1997b; Zimmermann, 1999; Labarque *et al.*, 2000) argues that cell-mediated immunity is necessary for the complete elimination of the virus. Nevertheless, the existence of PRRSV persistence also suggests that cell-mediated immunity is not potent and that IFNγ production is either weak or ineffective in the early stage to the infection (Murtaugh *et al.*, 2002).

Xiao and co-authors (2004) and Díaz and co-authors (2005) suggested a hypothetical model in which the outcome of PRRSV infection is related more to the dynamic of permissive macrophages and the early events of the natural response than to the development of specific immunity. In the early events IL-10 would be released impairing the development of CMI response (Royaee *et al.*, 2004), which explained the lack of IL-2 and IL-4 producing cells and the erratic levels of IFNγ (Meier *et al.*, 2003; Díaz *et al.*, 2005). As infection progresses, the number of permissive macrophages would be decreased by the cytolytic cycles of virus replication (Xiao *et al.*, 2004). Thus, the weak CMI response would be able to confine PRRSV to specific tissues (i.e. lymph nodes) where the proportion of permissive cells could be still relatively high (Xiao *et al.*, 2004). In consequence, viraemia should cease or become inconstant and low. As far as the infection can be confined to certain tissues and permissive macrophages

are not replaced at a high rate, the number of infected cells will decline steadily and the immune response will finally be able to clear the infection. At this moment, an increase of IFNγ secreting cells (IFNγ-SCs) and the final development of NAs would take place (Díaz *et al.*, 2005). From then onwards, the pig would be protected against homologous challenge (Mengeling *et al.*, 2003). Such a model would explain why no clear correlation between the immune response and the clearance of infection can be determined, as well as making sense of the delayed T cell responses observed in PRRSV infection (Díaz *et al.*, 2005).

1.2.4. Lymphocyte subsets in peripheral lymphoid tissue during PRRSV infection

While an extensive study on PBMCs in PRRSV infection has been performed, the available information about lymphoid T cell subsets in lymphoid tissues is scarce and controversial. Moreover, peripheral blood contains only 2% of the total T cells of the body (Westermann and Pabst, 1992) and is the site of neither viral replication nor antigen presentation. Therefore, it is possible that the main T cell dependent response to PRRSV might be occurring in lymphoid tissues, analogous to T cell response to simian immunodeficiency virus and human immunodeficiency virus (HIV) (Kuster *et al.*, 2000; Sopper *et al.*, 2003). A significant increase of $CD2^{+}CD8^{high}$ T cells in spleen from 10 to 45 dpi by

means immunohistochemistry (Kawashima *et al.*, 1999) or flow cytometry (Lamontagne *et al.*, 2003) techniques, has been correlated with a rapid elimination of the virus from blood and spleen (Lamontagne *et al.*, 2003). However, a decrease or no significant changes in $CD8^{high}$ cells were observed in the rest of lymphoid organs studied (Kawashima *et al.*, 1999; Lamontagne *et al.*, 2003), which may lead to the persistence of the virus. Xiao and co-authors (2004) did not find any change in the percentage of $CD4^+$ or $CD8^+$ T cells in lung, blood or lymphoid tissues. On the other hand, Kawashima and co-authors (1999) described an increase of $CD4^+$ cells in spleen and a decrease of this subset in the rest of lymphoid tissues studied. Lamontagne and co-authors (2003) did not find significant changes in the percentages of $CD4^+$ cells in any lymphoid tissue studied, suggesting that this lack of changes may be due to a depletion of all the lymphoid subsets in the lymphoid organs analyzed.

The increase in the percentage of $CD2^+CD8^{high}$ cells was reflected in a decrease of $CD4/CD8^{high}$ ratio in the spleen and $CD8^{low}/CD8^{high}$ ratio in the spleen and mediastinal lymph node, while no significant changes were detected in tonsils (Lamontagne *et al.*, 2003). $CD2^+CD8^{low}$ cells only increase transiently in tonsils at 3 dpi. NK cells, characterised as $CD2^+CD8^{low}MIL4^+$, were not significantly modified in PRRSV infection indicating that the innate immune response was not stimulated in peripheral lymphoid organs (Lamontagne *et al.*, 2003) or that newly NK cells produced were recruited in lungs (Samson *et al.*, 2000), facilitating viral persistence in lymphoid organs. $CD2^+CD8^{low}MIL4^-$

memory cell subset only displayed a significant decrease in the spleen at 3 dpi (Lamontagne *et al.*, 2003). Conversely, Xiao and co-authors (2004) did not find any change in the percentage of $CD4^+CD8^+$ T cells, although they reported a decrease in $\gamma\delta$ T cells population in all examined tissues, especially in lung and lymph nodes. The decline of $\gamma\delta$ T cells would contribute to the impairment of CMI response to PRRSV (Murtaugh *et al.*, 2002) and may be due to a low production of proinflammatory cytokines (Van Reeth *et al.*, 1999).

Although Lamontagne and co-authors (2003) initially suggested a possible role of $CD8^{high}$ cells in the clearance of the virus due to the coincidence between the increase of this subset and the decrease of viral titre in serum and spleen, later they also observed RNA viral persistence in the blood and the spleen indicating an impaired immune mediated viral elimination. Moreover, the persistence of the virus in tonsils and mediastinal lymph nodes indicate both the absence of T cell immune stimulation, observed by the low levels of $CD8^{high}$ cells in these tissues, or a fast death of activated lymphoid cells (Lamontagne *et al.*, 2003). Lamontagne and co-authors (2001, 2003) conclude from their results that the CMI response evoked against PRRSV is not efficient and that lymphoid cells could be susceptible to PRRSV mediated apoptosis.

The only information available about B cell levels in PRRSV infected lymphoid tissues is that reported by Kawashima and co-authors (1999). They reported an enhancement in the number of B cells in tonsil, Peyer's patches and lymphoid patches of the ileocecal junction, and in superficial inguinal,

mandibular and tracheobronchial lymph nodes during PRRSV infection while the number of B cells decrease in thymus.

1.2.5. Actual and future approaches in PRRSV vaccination

Several approaches have been done to understand the host immune response against PRRSV vaccination, since different reports show contradictory results. Therefore, Sipos and co-authors (2003) reported no significant changes neither in cytokine expression nor in lymphocyte subsets, although a trend towards $CD8^+$ T cells and TNFα and IL-6 enhancement was observed. Although most of the authors report a delayed onset of the CMI response (Bautista and Molitor, 1997; López-Fuertes *et al.*, 1999; Xiao *et al.*, 2004), Piras and co-authors (2005) reported a fast onset of PRRSV specific T cell response in 6-8 weeks old pigs inoculated with the highly virulent European PRRSV strain 120. They observed that PRRSV specific IFNγ response was mainly due to an increase in $CD8^{high}$ T cells in challenged animals and to an increase in $CD4^+CD8^+$ and $CD8^{high}$ T cell subsets in vaccinated animals with an inactivated vaccine.

In vaccinated sows has been described a decrease in the proliferation of $CD8^+$ and $CD4^+CD8^+$ T cells against PRRSV, regardless of the type of vaccine used, which was partially overcome when the sows were immunised with a heterologous PRRSV vaccine (Bassaganya-Riera *et al.*, 2004). This finding

points to immunization with combined vaccines (MLV and KV) would supply more antigen-specific T lymphocytes proliferation against PRRSV than single vaccination. Conversely, no improve in the levels of NAs were found using a heterologous immunization (Bassaganya-Riera *et al.*, 2004). An increase in the antibody response to GP5 has been observed in pigs vaccinated with a MLV using cholera toxin as adjuvant (Foss *et al.*, 2002).

Meier and co-authors (2003) reported a fast enhancement of non neutralizing antibodies and a delayed expression of both NAs and IFNγ after immunization with a MLV of PRRS. Moreover, these authors also found no changes in the immune response when adding the adjuvant Imugen(R) to the MLV, which showed a marked enhancement in both NAs titre and IFNγ response in pigs vaccinated against pseudorabies virus (Meier *et al.*, 2003).

Since IFNγ is known to protect macrophages from PRRSV infection, but low levels of this cytokine are produced during the disease, some vaccine adjuvants have been used to increase the expression of IFNγ. Thus, a plasmid encoding either porcine IL-12 or IFNα has been co-administered during vaccination with a MLV (Meier *et al.*, 2004; Royaee *et al.*, 2004). Both plasmids displayed an increase in the expression of IFNγ, however, whereas the IFNγ response was maintained when the plasmid encoding IFNα was used, a decrease in the number of IFNγ-SC was observed after the second week post-immunization with the plasmid encoding IL-12 (Meier *et al.*, 2004). Although a correlation between the number of IFNα-SC and IFNγ-SC was observed in the

immunization with the plasmid encoding IFNα (Royaee *et al.*, 2004), the immunization with both plasmids, encoding either IL-12 or IFNα, induced no improvement in the titres of PRRSV-specific antibodies or NAs (Meier *et al.*, 2004). Therefore, the use of these plasmids induces to a Th1 polarization of the immune response, although no improvement in the humoral immune response was observed with respect to the vaccination alone without any plasmid as adjuvant.

An increase in the expression of IFNγ has been reported in PRRSV-infected animals treated with recombinant porcine IL-12 (rpIL-12) (Foss *et al.*, 2002; Carter and Curiel, 2005), being also observed a decrease in PRRSV titres and in the expression of IL-10 by PAMs isolated form these animals (Carter and Curiel, 2005). However, when rpIL-12 was used as a MLV adjuvant, no changes in the expression of IFNγ neither in the titres of PRRSV-specific antibodies or NAs were observed among the control group, immunized only with the MLV, and the group immunized with the MLV together rpIL-12 (Meier *et al.*, 2004).

Although the use of plasmids and recombinant porcine cytokines, may represent useful tools in the improvement of the immune response evoked after PRRSV vaccination, there is lack of a precise knowledge of how these mediators modulate the immune response. Moreover, different PRRSV genotypes should be included in future studies to confirm a homogeneous effect of the tested vaccine.

All these reports point to a different immunological behaviour of PRRSV vaccines. This fact, has been also reported by Díaz and co-authors, who observed different IFNγ-SC frequencies when different MLV against PRRSV were used. Therefore, new vaccines need to be developed to reach a better control against this disease. These new vaccines must accomplish four essential characteristics: efficacy, universality, safety and ability to differentiate vaccinated from infected animals (Mateu and Díaz, 2008).

1.3. CYTOKINES PROFILE IN PRRSV INFECTION: INTERFERONS (IFNα, IFNγ) AND INTERLEUKINS (IL-1, IL-6, IL-10, IL-12, TNFα)

1.3.1. Interferons (IFNs)

1.3.1.1. *IFNα*

Type I IFNs are a superfamily which includes seven subfamilies: IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNδ and IFNτ (Pestka *et al*., 2004). IFNα and IFNβ are the best characterised, being identified more than 12 subtypes of IFNα in humans and pigs, and one subtype of IFNβ in humans and multiple subtypes in pigs and ruminants (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). IFNα and IFNβ were originally named leukocyte and fibroblast IFN, respectively, according to their main producer cells, but nowadays it is known that these IFNs can be produced by many other cell types (Van Reeth and Nauwynck, 2000). IFNα and IFNβ subtypes are closely related, share a common receptor and have similar effects (Van Reeth and Nauwynck, 2000). They constitute one of the two pathways involved in the innate cytokine response, inducing an antiviral state in target cells (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). Some of the main functions of type I IFNs are inhibition of IL-12 expression, activation of macrophages and NK cell cytotoxicity, *in vivo* induction of CD8 T cell proliferation and stimulation of T cell differentiation into IFNγ-SCs during

viral infections (Cousens *et al.*, 1999; Cella *et al.*, 2000; Kadowaki *et al.*, 2000; Biron and Sen, 2001; Tizard, 2008). Correlations between the frequencies of virus specific IFNα-SCs and virus specific IFNγ-SCs have been described in PRRSV infected pigs (Royaee *et al.*, 2004).

In PRRSV infection IFNα response by porcine alveolar macrophages (Albina *et al.*, 1998a; Buddaert *et al.*, 1998; Van Reeth *et al.*, 1999) or PBMCs (Albina *et al.*, 1998a) is not significant, being detected at low levels from 2 dpi to 9 dpi (Albina *et al.*, 1998a; Chung *et al.*, 2004; Van Gucht *et al.*, 2004). When compared with other viral diseases, PRRSV induce much lower levels of IFNα in the lungs than SIV or PRCV (Van Reeth *et al.*, 1999; Van Reeth *et al.*, 2002). Therefore, PRRSV would be able to inhibit IFNα response, since the virus can be actively replicating in the lung where this cytokine can not be detected (Albina *et al.*, 1998a; Buddaert *et al.*, 1998; Chung *et al.*, 2004). However, the mechanism used by PRRSV to inhibit IFNα remains still unknown.

Lee and co-authors (2004) reported different abilities to induce or inhibit IFNα by different PRRSV isolates. Thus, the absence or the very low level of IFNα production combined with the initially inadequate viral inhibitory immunity could contribute to the prolonged infection and the elicitation of a weak initial IFNγ response observed in PRRSV infection (Meier *et al.*, 2003).

1.3.1.2. *IFNγ*

IFNγ, type II IFN or immune IFN, in contrast to type I IFNs, is produced exclusively by cells of the immune system (Boehm *et al.*, 1997) and represent an important regulator of the adaptative immunity (Braciale *et al.*, 2007). It is initially produced by NK cells in the innate immune response being later also produced by T cells during the adaptative immune response (Biron and Sen, 2001; Domeika *et al.*, 2002; Rodríguez-Carreño *et al.*, 2002; Biron and Sen, 2007). IFNγ promotes and mediates a Th1 immune response and suppresses Th2 cell development (Abbas *et al.*, 1996). Although some functions of IFNγ overlap with those of IFNα/β there are some specific functions for IFNγ. Thus, IFNγ (1) stimulates monocytic cells to an antimicrobial defence mechanism, mainly by means of nitric oxide synthetase 2 (NOS2 or iNOS) induction; (2) enhances inflammatory response, increasing the expression of TNF receptors and also (3) enhances the production of several cytokines, like IL-12 by macrophages and DC (Biron and Sen, 2007; Braciale *et al.*, 2007). Reciprocally, several cytokines may also induce an enhancement of IFNγ expression. IL-12, especially in combination with IL-18, is a potent inducer of IFNγ (Biron and Sen, 2001; Domeika *et al.*, 2002; Biron and Sen, 2007; Braciale *et al.*, 2007), however, T cell IFNγ response has been detected in absence of IL-12 in several viral infections, probably due to viruses elicit specific CD8 T cells response primed to produce high levels of IFNγ (Biron and Sen, 2001;

Biron and Sen, 2007; Braciale *et al.*, 2007). Porcine $CD4^{+}CD8^{low}$, $CD4^{-}CD8^{high}$ and $\gamma\delta$ T cells are reported as the main T cells subsets implicated in IFNγ production (Olin *et al.*, 2005; Charerntantanakul and Roth, 2007).

Because IFNγ plays a key role in CMI response, factors that increase its expression may enhance anti-PRRSV cell-mediated response. IL-12 and IFNα, in particular, are involved in the differentiation of naïve T cells into antigen-specific IFNγ-SCs (Tough *et al.*, 1999; Cousens *et al.*, 1999; Banyer *et al.*, 2000; Cella *et al.*, 2000; Kadowaki *et al.*, 2000; Biron and Sen, 2007), being reported correlations between the frequencies of virus specific IFNα-SCs and virus specific IFNγ-SCs (Royaee *et al.*, 2004). Therefore, PRRSV vaccination with IL-12 or IFNα accelerates the development of a virus-specific IFNγ response (Foss *et al.*, 2002; Meier *et al.*, 2004). Nevertheless, PRRSV is a poor inducer of proinflammatory cytokines (Albina *et al.*, 1998b; Buddaert *et al.*, 1998; Van Reeth *et al.*, 1999), leading also to a poor early expression of IFNγ.

IFNγ in PRRSV infection requires of macrophage activation for its expression, and its effect is time and dose dependent (Bautista and Molitor, 1999). Moreover, porcine IFNγ has been demonstrated to block PRRSV replication in cell cultures (Bautista and Molitor, 1999), apparently by the inhibition of viral RNA synthesis via double stranded RNA inducible protein kinase (Rowland *et al.*, 2001). An enhanced expression of IFNγ has been reported in both *in vitro* (López-Fuertes *et al.*, 1999) and *in vivo* (Choi *et al.*, 2002; Johnsen *et al.*, 2002; Feng *et al.*, 2003; Meier *et al.*, 2003; Xiao *et al.*,

2004; Díaz *et al.*, 2005; Olin *et al.*, 2005) experimental infections. However, PRRSV specific IFNγ-SCs appear late after infection, at 14 dpi, increasing in waves until 4-6 months post infection when them still remain (Meier *et al.*, 2003; Xiao *et al.*, 2004; Díaz *et al.*, 2005, 2006; Olin *et al.*, 2005). Correlations between the level of PRRSV-specific IFNγ-SCs and the detection of viraemia in infected (Díaz *et al.*, 2005) and vaccinated pigs (Díaz *et al.*, 2006), and the protection against reproductive failure (Lowe *et al.*, 2005) have been proved suggesting that IFNγ may develop an important role in virus clearance.

Differences in IFNγ expression depending on PRRSV strain have been also reported, being described a peak in IFNγ expression in the lungs at 7-10 dpi when a high virulent PRRSV strain was inoculated (Choi *et al.*, 2002; Thanawongnuwech *et al.*, 2003), being the expression of IFNγ lower when a low virulent strain was used (Thanawongnuwech *et al.*, 2003). The progressive increase of IFNγ production observed along PRRSV infection could be explained by the differentiation of naïve T cells into PRRSV-specific IFNγ-SCs, stimulated by IFNγ release by PRRSV-specific IFNγ-SCs. This initial increase of IFNγ would lead to a microenvironment which facilitates T cell differentiation into IFNγ-SCs, resulting in an eventual enhancement of this type of immune response (Meier *et al.*, 2003). However, other authors have described no changes in mRNA expression of IFNγ after vaccination with a European MLV strain of PRRSV (Sipos *et al.*, 2003).

1.3.2. Interleukins

1.3.2.1. *IL-1*

IL-1, together IL-6 and TNFα, constitute an alternative pathway involved in the innate immune response (Biron and Sen, 2001). IL-1 is synthesised by both non immune cells (i.e. fibroblasts, vascular endothelium) and immune cells (i.e. monocytes-macrophages) (Chamberlain *et al.*, 1999; Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). There are two forms of IL-1: IL-1α and IL-1β (Biron and Sen, 2001). Although both forms of IL-1 required to be cleaved for acquiring function, IL-1α precursor may have some biologic activity (Biron and Sen, 2001). IL-1α and β proteins have only 25 % homology, however, they bind to the same receptors and have identical biological effects (Murtaugh *et al.*, 1996; Biron and Sen, 2001). Functions of IL-1 overlap to those of TNFα and when they are secreted simultaneously some of their effects can synergise (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). These cytokines, known as proinflammatory cytokines, may enhance IFNγ response, induce adherence of leukocytes to endothelial cells, increase the microvascular permeability, induce bronchoconstriction and act as endogenous pyrogen (Murtaugh *et al.*, 1996; Biron and Sen, 2001). Moreover, IL-1 can be a major contributor of IL-6 release (Biron and Sen, 2001).

During PRRSV infection only a mild increase in IL-1α and IL-1β mRNAs expression have been reported in *in vitro* studies (Thanawongnuwech *et al.*, 2001, 2004). *In vivo* experiments showed an increase in IL-1α and IL-1β in BALF from 1 to 52 dpi (Labarque *et al.*, 2003a; Van Gucht *et al.*, 2003, 2004; Thanawongnuwech *et al.*, 2004), although IL-1β was not enhanced in serum until 42 dpi (Thanawongnuwech *et al.*, 2004). However, no significant changes in IL-1α mRNA were observed in pigs vaccinated with a European MLV strain of PRRSV (Sipos *et al.*, 2003). Furthermore, PRRSV is able to downregulate the expression of IL-1 and TNFα in PAMs infected *in vitro* (López-Fuertes *et al.*, 2000). Thus, further researches about IL-1 expression in PRRS infection and vaccination are required for a better understanding of the role of this cytokine in the immunopathogenesis of the disease.

1.3.2.2. *IL-6*

IL-6 is a multifunctional cytokine which plays an important role in host defence, acute phase reactions and immune response. This cytokine may be produced by lymphoid and non lymphoid cell types (Biron and Sen, 2001). The main porcine cell types reported to express IL-6 include PBMCs, alveolar and intravascular macrophages, fibroblasts and endothelial cells (Murtaugh *et al.*, 1996; Scamurra *et al.*, 1996). Whereas IL-1 and TNFα may upregulate IL-6 production, IL-6 downregulates in contrast IL-1 and TNFα levels by means of

the production of IL-1 receptor antagonists and soluble TNFα receptors (Murtaugh *et al.*, 1996; Van Gucht *et al.*, 2003). IL-6, just as IL-1 and TNFα, is also considered like an early cytokine which may be inhibited by the expression of inhibitory cytokines such as IL-4, IL-10 or IL-13 (Cavaillon, 1994).

IL-6 is mainly characterised by activation of hepatocytes inducing the synthesis of acute phase proteins during the acute inflammatory response, and by promotion of B cell growth and differentiation into immunoglobulin-secreting plasma cells, playing an important role in the humoral response (Murtaugh *et al.*, 1996; Biron and Sen, 2001). Finally, IL-6 may be also involved in the antiinflammatory response inhibiting the release of IL-1 and TNFα and reducing the influx of inflammatory cells to the site of inflammation (Murtaugh *et al.*, 1996).

Low levels of IL-6 protein have been detected from 3 to 21 dpi after PRRSV infection (Asai *et al.*, 1999; Van Gucht *et al.*, 2003), whereas results of IL-6 mRNA amplification are less uniform being detected from 0 to 28 dpi (Feng *et al.*, 2003; Thanawongnuwech *et al.*, 2004). When pigs were vaccinated with a European MLV strain of PRRSV IL-6 trended to increase at 22 and 44 dpi (Sipos *et al.*, 2003). The low response of IL-6 after inoculation or vaccination points to a probably no significant role of this cytokine in PRRS.

1.3.2.3. *TNFα*

Tumour necrosis factors (TNFs) are differentiated into two groups: TNFα, produced mainly by activated monocytes/macrophages and NK cells, and TNFβ, also known as lymphotoxin (LT) and produced by lymphocytes after antigenic stimulation (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). Production of TNFα generally occurs at an earlier stage than TNFβ during infection, being more closely associated to proinflammatory effects, although both bind to common receptors and share several biological activities (Van Reeth and Nauwynck, 2000). Enhancement of vascular permeability and adhesion properties of endothelial cells, cell death, activation of monocytes and neutrophils, induction of IFNγ responses, DC migration, upregulation of class I MHC expression and activation of antiviral state, killing virus infected cells in an IFN independent way, are among the responses to TNFα expression (Murtaugh *et al.*, 1996; Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). Additionally, TNFα is related to the induction of fever, sleepiness, loss of appetite and acute phase effects in liver (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001).

TNFα is absent or poorly expressed during PRRSV infection. Although its expression has been reported *in vitro* (Thanawongnuwech *et al.*, 2001, 2004), *in vivo* studies have shown a lack (Asai *et al.*, 1999) or a weak TNFα response, between 7-28 dpi, in both infected (Choi *et al.*, 2002; Johnsen *et al.*, 2002;

Labarque *et al.*, 2003a; Van Gucht *et al.*, 2003; Thanawongnuwech *et al.*, 2004) and vaccinated pigs (Sipos *et al.*, 2003). López-Fuertes and co-authors (2000) demonstrated a downregulation on TNFα and IL-1 expression in PRRSV infected PAMs and a reduction of viral replication after recombinant porcine TNFα addition although its effect did not synergise with those of IFNα. Therefore, vaccines which induce an enhancement on TNFα response may represent an useful tool for PRRSV control.

1.3.2.4. *IL-10*

IL-10 is produced mainly by cells of monocyte/macrophage lineage, regulatory T cells or, less frequently B cells (Biron and Sen, 2001; Moore *et al.*, 2001). IL-10 is a potent antiinflammatory cytokine capable to modify a broad spectrum of activated monocyte/macrophages functions just as inhibition of cytokine, chemokine, prostaglandin (PG) E2 and class II major histocompatibility complex (MHC) antigen presentation and toll-like receptor (TLR) 4 expression, downregulating both Th1 and Th2 responses (Moore *et al.*, 2001; Pestka *et al.*, 2004). The main cytokines suppressed by IL-10 include IL-1α, IL-1β, IL-6, IL-12, IFNγ and TNFα (Cavaillon, 1994; Biron and Sen, 2001; Moore *et al.*, 2001; Pestka *et al.*, 2004), being especially significant for its antiinflammatory activities the inhibition of IL-1 and TNFα, due to the synergism observed between these two cytokines (Moore *et al.*, 2001). IL-10 expression

has a strongly inhibition effect on $CD4^+$ T cells proliferation whereas stimulates NK and $CD8^+$ T cells and induces their recruitment, cytotoxicity and proliferation (Moore *et al.*, 2001; Pestka *et al.*, 2004).

IL-10 may be a crucial cytokine in the development of the impaired immune response against PRRSV, playing a significant role in the pathogenesis of the disease. *In vitro* and *in vivo* PRRSV infections with both genotypes, EU and US genotypes, displayed an increase in protein and genomic levels of IL-10 expression from 0 to 14 dpi (Johnsen *et al.*, 2002; Chung and Chae, 2003; Feng *et al.*, 2003; Labarque *et al.*, 2003a; Suradhat and Thanawongnuwech, 2003; Suradhat *et al.*, 2003; Thanawongnuwech and Thacker, 2003; Thanawongnuwech *et al.*, 2004; Díaz *et al.*, 2005, 2006). However, other authors have described no changes in the expression of IL-10 (López-Fuertes *et al.*, 1999; Sipos *et al.*, 2003). These differences may be due to the different PRRSV strains or the different health status of pigs used in each study.

On the other side, peaks in IL-10 production have been reported to coincide with lower levels of IFNγ-SCs in PBMCs from pigs vaccinated with different EU PRRSV genotypes, inducing a strong IL-10 response (Díaz *et al.*, 2006). Moreover, several studies have described a non significant production of TNFα and IFNα (Albina *et al.*, 1998a; Buddaert *et al.*, 1998; Van Reeth *et al.*, 1999; Van Reeth and Nauwynck, 2000; Van Gucht *et al.*, 2004), together a mild clinical course and respiratory signs (Van Reeth *et al.*, 1999; Van Reeth and Nauwynck, 2000; Van Gucht *et al.*, 2003) following PRRSV infection which

could be due to the increase in IL-10 expression mentioned above. All these findings together support the hypothesis that IL-10 may interfere actively in PRRSV infection through the alteration of the cascade of proinflammatory cytokines and the inhibition of antigen presenting cells (APCs), leading to a delayed protective immunity against PRRSV.

1.3.2.5. *IL-12*

IL-12 is a key cytokine in both innate and adaptative immunity and in regulation of mucosal immunity, being produced by activated DC and macrophages (Braciale *et al*., 2007). IL-12 is a heterodimer constituted by two subunits, p35, expressed constitutively, and p40, which is expressed in response to challenge (Biron and Sen, 2001). However, both subunits are required to trigger off its biological activity (Biron and Sen, 2001). The main function of IL-12 consists on NK cell IFNγ production after a challenge (Biron and Sen, 2001; Biron and Sen, 2007). Other functions of this cytokine are promotion of CD8 T cell cytolytic differentiation and CD4 T cells activation to effector CD4 T cells (Chan *et al*., 1992; Cesano *et al*., 1993; Biron and Sen, 2007; Braciale *et al*., 2007), and promotion of type I immunity against intracellular pathogens (Hsieh *et al*., 1993; Macatonia *et al*., 1995).

The information about the expression of this cytokine during PRRS is scarce and contradictorial. *In vitro* studies showed no IL-12 mRNA expression by

PAMs infected with a highly virulent PRRSV (VR-2385) (Thanawongnuwech *et al.*, 2001), however, PAMs isolated from piglets infected with the same strain showed an increased expression of IL-12 mRNA at 10, 24 and 48 dpi (Thanawongnuwech and Thacker, 2003). Chung and Chae (2003) observed an enhanced mRNA expression of both IL-12 p35 and IL-12 p40 from 1 to 7 dpi in the lung of inoculated pigs, suggesting a possible role of IL-12 in the pulmonary defence against PRRSV. Otherwise, other authors observed only a weak increase in mRNA IL-12 expression in BALCs (Johnsen *et al.*, 2002) or no increase in PBMCs (Feng *et al.*, 2003) from *in utero* infected piglets. Moreover, an enhancement in the IL-10/IL-12 ratio has been described pointing to a role of the imbalance between these two cytokines in the immunomodulation observed during PRRSV infection (Feng *et al.*, 2003).

The efficiency of IL-12 as adjuvant has been also studied in the immune response against PRRS displaying a marked increase in the expression of IFNγ (Foss *et al.*, 2002; Carter and Curiel, 2005) but no improvement in antibody titres (Foss *et al.*, 2002).

1.4. ACUTE PHASE PROTEINS (APPS) AND THEIR EXPRESSION IN PRRS

The acute phase response (APR) is characterised by the disturbance of the normal homeostasis by several stimuli like infection, inflammation, stress, trauma or tissue damage (Eckersall, 2000; Ceciliani *et al.*, 2002; Gruys *et al.*, 2005). This APR is triggered by the synthesis of proinflammatory cytokines, namely IL-1, IL-6 and TNFα, at the local site of the injury. These cytokines are released into the bloodstream, reach the liver and induce the production of acute phase proteins (APPs) by the hepatocytes (Eckersall, 2000; Petersen *et al.*, 2004). APPs have been classified as “positive” or “negative” depending on the increase or decrease of their serum concentration, respectively (Ceciliani *et al.*, 2002; Petersen *et al.*, 2004). Haptoglobin (Hp), C-reactive protein (CRP) and serum amyloid A (SAA) are considered as main APPs in pigs. The classification of the pig-major acute protein (Pig-MAP) is controversial, and it is considered as a major or moderate APP depending on the study (Parra *et al.*, 2006).

It is generally accepted that APPs are inductors of a proinflammatory reaction and fever, but their overexpression can lead to an anti-inflammatory response (Ceciliani *et al.*, 2002; Petersen *et al.*, 2004). Thus, APPs are used today as potential biological markers for monitoring animal welfare and the health status of swine herds and of individual pigs at slaughter (Eckersall, 2000; Petersen *et al.*, 2004; Gruys *et al.*, 2005). Moreover, APPs may be used to

determine the virulence of different isolates of the same bacteria or virus, or the efficacy of vaccines (Heegaard *et al*., 1998).

Hp is considered a diagnostically useful APP in most species. Hp may show a more than 10 times increase in serum, and marked differences have been observed between herds whereas no differences have been observed between breeds (Petersen *et al.*, 2004). The main biological function of Hp consists on prevention of iron loss by the formation of haemoglobin-iron complexes (Ceciliani *et al*., 2002; Petersen *et al*., 2004). Therefore, Hp also develops a bacteriostatic effect reducing the level of available iron for the microorganisms (Petersen *et al*., 2004). Furthermore, Hp seems to play a major role in modulating immune responses through a complex network of interactions. The expression of Hp has been related to the secretion of antiinflammatory cytokines, particularly IL-10, through the interaction with CD163, a haemoglobin scavenger receptor that is solely present in cells of monocyte/macrophage lineage (Moestrup and Moller, 2004; Philippidis *et al*., 2004). However, the exact mechanism used by Hp as modulator of the immune response is not clear, acting as suppressor of lymphocyte proliferation in bovine (Murata and Miyamoto, 1993), and as supporter of B and T lymphocytes proliferation and differentiation in Hp-deficient C57BL/6J mice (Huntoon *et al.*, 2008).

CRP was discovered in the serum of patients which suffered a pneumococcal infection, as a substance which reacted with C polysaccharide (Petersen *et al*., 2004). In the acute phase response CRP increases more

moderately than Hp, showing between 1 to 10 times increase (Petersen *et al.*, 2004). Although some authors consider CRP as an useful tool to differentiate between a bacterial or a viral infection, other authors could not detect such differences because of the individual variability (Petersen *et al.*, 2004). CRP participates in the innate immune response removing bacteria and damaged cells by complement activation and opsonisation, activating monocyte/macrophage to inflammatory cytokines production, and preventing neutrophils migration (Ceciliani *et al.*, 2002; Petersen *et al.*, 2004). Since CRP is a component of the innate response, it may be considered as an early bioindicator of health status in swine herds (Stevenson *et al.*, 2006).

SAA shows more than 10 times increase after any injury which triggers off the APR (Petersen *et al.*, 2004). SAA carries out several functions related with the inflammatory response, just as cholesterol removal from the local site of inflammation and transport to hepatocytes; chemotaxis of monocytes, polymorphonuclear leukocytes and T cells; inhibitory effect on fever, oxidative burst, platelet activation and *in vitro* immune response (Ceciliani *et al.*, 2004; Petersen *et al.*, 2004). Secondary amyloidosis is triggered by a conformational change of SAA into an insoluble peptide, AA, which takes place when there is a marked high expression of SAA (Ceciliani *et al.*, 2004).

Generally it is accepted that Pig-MAP enhanced more than 10 times in the APR (Petersen *et al.*, 2004). However, Parra and co-authors (2006) suggested that the changes observed in this APP sometimes are moderate and not as

exacerbated as those observed for the other APPs named above. Pig-MAP is a relatively novel APP and its specific functions still remain unclear.

APPs have been tested in pigs after exposure to stress (Salamano *et al.*, 2008) and after natural (Chen *et al.*, 2003; Segalés *et al.*, 2004; Parra *et al.*, 2006) or experimental infections (Francisco *et al.*, 1996; Asai *et al.*, 1999; Magnusson *et al.*, 1999; Knura-Deszczk *et al.*, 2002; Van Gucht *et al.*, 2005; Stevenson *et al.*, 2006). Increased levels of Hp, CRP and/or Pig-MAP have been reported in porcine viral and bacterial respiratory infections, like porcine circovirus type 2 (PCV2) (Segalés *et al.*, 2004; Parra *et al.*, 2006; Stevenson *et al.*, 2006), swine influenza virus (SIV) (Barbé and Van Reeth, 2006), Aujeszky's disease virus (Parra *et al.*, 2006), *Actinobacillus pleuropneumoniae* (Heegaard *et al.*, 1998), *Mycoplasma hyopneumoniae* (Parra *et al.*, 2006), *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* type D (Francisco *et al.*, 1996).

Nowadays just few studies have been focused on the expression of APPs during PRRS. Moreover, these studies are usually limited to only one APP or to a single time-point or short timeframe of the infection. Elevated serum concentrations of Hp, CRP and SAA have been reported in pigs naturally infected with PRRSV, sometimes earlier than the development of specific PRRSV antibodies (Parra *et al.*, 2006). Enhanced serum Hp concentration has also been described after experimental infection with a European strain of

PRRSV from 7 to 21 dpi, being correlated with an increase on IL-6 but not TNFα (Assai *et al.*, 1999) or IL-10 (Díaz *et al.*, 2005) production.

AIMS OF THE THESIS / OBJETIVOS DE LA TESIS

AIMS OF THE THESIS

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most significant porcine diseases of the modern swine industry, being the responsible of important economic losses. Nonetheless, although numerous studies have been carried out, nowadays it is still no clear the immune pathways involved in the immune compromise observed after PRRS virus (PPRSV) infection. Therefore, the general aims of the present thesis were to extend the knowledge on the immune response evoked at lymphoid tissue level to determine the role of lymphocyte subsets and evaluate the systemic and local expression of cytokines and their role in the immune response.

The specific aims may be defined as follows:

1. To characterise the changes observed in lymphocytes subsets in blood and lymphoid tissues in an acute experimental infection with a European PRRSV field isolate and their relationship with the serum expression of IL-10, IL-12, IFNα, and IFNγ.
2. To study the relationship between the serum expression of acute phase proteins and proinflammatory cytokines during an early infection with PRRSV.
3. To determine the expression of IL-1, IL-6, IL-10, IL-12, TNFα, IFNα, and IFNγ in the lung of pigs experimentally infected with a PRRSV field

isolate, the main cell types involved in their expression and their role in the pathogenesis of the respiratory form of the disease.

OBJETIVOS DE LA TESIS

El Síndrome Reproductivo y Respiratorio Porcino es una de las enfermedades más importantes de la industria porcina moderna, siendo el responsable de importantes pérdidas económicas. No obstante, aunque se han llevado a cabo numerosos estudios, hoy día no se conoce con claridad cuáles son los mecanismos inmunes involucrados en el compromiso inmunitario observado tras la infección con el VPRRS. De este modo, el objetivo general de esta tesis consistió en determinar la expresión de citoquinas tanto a nivel sistémico como local así como su papel en la respuesta inmune.

Los objetivos específicos se detallan a continuación:

1. Caracterizar los cambios observados en las subpoblaciones de linfocitos en sangre y órganos linfoide durante una infección aguda experimental con un aislado de campo del VPRRS y su relación con la expresión sérica de IL-10, IL-12, IFNα, e IFNγ.
2. Estudiar la relación entre la expresión sérica de proteínas de fase aguda y citoquinas proinflamatorias durante una infección aguda con el VPRRS.
3. Determinar la expresión de IL-1, IL-6, IL-10, IL-12, TNFα, IFNα, e IFNγ en el pulmón de credos infectados experimentalmente con un aislado de campo del VPRRS, así como las principales células involucradas en su

expresión y su papel en la patogenia de la forma respiratoria de la enfermedad.

3.

EXPERIMENTAL STUDIES

3.1. COMMON EXPERIMENTAL DESIGN.

3.2. CHANGES IN LYMPHOCYTE SUBSETS AND CYTOKINES DURING EUROPEAN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME: INCREASED EXPRESSION OF IL-12 AND IL-10 AND PROLIFERATION OF $CD4^{-}CD8^{HIGH}$.

3.3. ACUTE PHASE RESPONSE IN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS).

3.4. *IN SITU* EXPRESSION OF CYTOKINES BY MACROPHAGES IN THE LUNG OF PRRSV-INOCULATED PIGS.

3.1. COMMON EXPERIMENTAL DESIGN

To assess all the aims of this thesis a common experiment was carried out, using different techniques for the different approaches. The common experimental design is the following:

3.1.1. Virus

The third passage of the PRRSV field isolate 2982 (kindly provided by Dr. E. Mateu) was used in this study. The virus was initially isolated in porcine alveolar macrophages (PAMs) from serum of a naturally infected piglet during an outbreak of PRRS affecting a Spanish farm where piglets displayed respiratory signs. Viral stock was adjusted to a titre of $10^{3.0}$ $TCID_{50}$/ml as determined by means of an immunoperoxidase monolayer assay (IPMA) (Weensvoort *et al.*, 1991) in PAMs. PRRSV strain 2982 belonged to EU sub-genotype 1 and shared a 93 % similarity to LV based on ORF5 sequences. The viral stock was free of aerobic and anaerobic bacterial contamination as determine after bacteriological culture.

3.1.2. Animals and experimental design

A total of thirty-two, male, five-week-old piglets from a high-healthy farm historically seronegative for PRRSV were used for the experimental infection. Pigs were clinically healthy and were housed in biocontainment level III animal facilities at “Centro de Investigación en Sanidad Animal” (CISA-INIA, Valdeolmos, Madrid, Spain). Pigs were allowed to stay housed in the biocontainment level III facilities 10 days prior to challenge. Twenty eight pigs were randomly distributed in batches of four and inoculated by the intramuscular route, behind the right ear in the neck with 1 ml the viral inoculum. The four animals of each batch were killed at 3, 7, 10, 14, 17, 21 and 24 days post-inoculation (dpi), respectively. The four remaining pigs, used as controls, were inoculated with 1 ml of sterile RPMI 1640 medium (BioWhitaker) following the same procedure and humanely killed at the end of the study (24 dpi). Euthanasia was performed by initial anesthesia with tiletamine-zolazepam (ZOLETIL, Virbac) followed by a lethal dose of 5 % sodium thiopental (THIOVET, Vet Limited). Tissue samples were subjected to *in situ* hybridization and were proved as negative to PCV2. This experiment was carried out under the guidelines of the European Union (Directive 86/609/EEC) and was approved by Cordoba University Ethical Review Committee.

3.2. CHANGES IN LYMPHOCYTE SUBSETS AND CYTOKINES DURING EUROPEAN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME: INCREASED EXPRESSION OF IL-12 AND IL-10 AND PROLIFERATION OF CD4-CD8^{HIGH}

Porcine Reproductive and Respiratory Syndrome (PRRS) is known to persist during several weeks in lungs and lymphoid organs (Wills *et al*., 1997; Albina *et al*., 1998a; Allende *et al*., 2000; Lamontagne *et al*., 2001, 2003). Lung and lymphoid tissues appear to be sites for viral replication in acute infection, since similar viral load has been detected in these organs (Xiao *et al*., 2004). Pigs develop both humoral (Yoon *et al*., 1995; Loemba *et al*., 1996; Lamontagne *et al*., 2003) and cellular (Bautista and Molitor, 1997; López-Fuertes *et al*., 1999; Meier *et al*., 2003) immune responses against PRRSV infection. However, precise understanding of the immune mechanisms induced is still incomplete. Moreover, several studies have been focused mainly in the changes observed in the lymphocyte subsets of peripheral blood mononuclear cells (PBMCs) (Shimizu *et al*., 1996; Albina *et al*., 1998b; López-Fuertes *et al*., 1999; Samsom *et al*., 2000; Lamontagne *et al*., 2001; Xiao *et al*., 2004; Díaz *et al*., 2006), meanwhile few studies have been carried out in lymphoid organs (Kawashima *et al*., 1999; Lamontagne *et al*., 2001; Xiao *et al*., 2004).

The delayed onset of the host protective immune response after PRRSV infection has been related with the upregulation of interleukin-10 (IL-10) expression (Chung and Chae, 2003; Suradhat and Thanawongnuwech, 2003;

Díaz *et al*., 2005, 2006), erratic levels of gamma interferon (IFNγ) (Díaz *et al*., 2005, 2006) and alpha interferon (IFNα) suppression (Albina *et al*., 1994; Van Reeth *et al*., 1999). Interleukin-12 (IL-12) has been detected in both *in vitro* either *in vivo* studies (Chung and Chae, 2003; Royaee *et al*., 2004; Thanawongnuwech and Thacker, 2003), but in low levels. IL-12 has been also used as a vaccine adjuvant candidate inducing a moderate enhancement in IFNγ production (Carter and Curiel, 2005; Meier *et al*., 2004).

Thereby, the main goal of this study, which represents the first aim of this thesis, was to characterise the changes observed in lymphocyte subsets in blood and lymphoid organs during an acute experimental infection with a EU PRRSV field isolate and correlate them with the expression of IL-10, IL-12/23 p40, IFNα and IFNγ.

Materials and Methods

Virus

The inoculum used in this experiment has been described above in the section “Common experimental design”, page 55.

Animals and experimental design

The animals and experimental design used in this experiment have been described above in the section “Common experimental design”, page 56.

Clinical signs, total leukocyte counts and leukocyte formula

Pigs were monitored daily for rectal temperature and a clinical respiratory score (ranging from 0 to 6) was calculated all throughout the experiment, as described previously (Halbur *et al.*, 1995).

Blood samples were taken at 0, 3, 7, 10, 14, 17, 21 and 24 dpi. Samples were taken from eight animals at the different time points but at 24 dpi only the four remaining animals were bled ante mortem. Blood samples were diluted in acetic acid solution and stained with Türk liquid and the total number of leukocytes was visually counted in a modified Neubauer counting-chamber, as described previously (Schalm *et al.*, 1975). Blood smears were fixed in methanol for 5 minutes and stained with May-Grünwald-Giemsa staining for 30 minutes. The relative quantities of the different leukocytes populations were assessed counting a total of 500 leukocytes per sample.

Viraemia and specific antibody detection in serum samples

Viraemia and specific antibody detection in serum samples were determined as described previously (Labarque *et al*., 2000). Briefly, the virus titration was carried out cultivating 50 µl of tenfold serial dilutions of serum samples on PAMs, from PRRSV-negative pigs, and incubating at 37 ºC for 1 hour. The samples were replaced by medium, and the PAMs were incubated for 72 hours at 37 ºC for developing cytopathic effect, washed once with PBS and further stained using an immunoperoxidase monolayer assay (IPMA) (Wensvoort *et al*., 1991). For specific antibody detection, 50 µl of serial fourfold dilutions of serum samples were incubated on MARC-145 cells for 1 hour at 37 °C and next stained using an IPMA (Wensvoort *et al*., 1991).

Leukocyte isolation

Swine PBMCs were separated from whole blood by Ficoll-Paque (Roche) gradient centrifugation. Samples from lymphoid organs, medial retropharyngeal and tracheobronchial lymph nodes and spleen were taken and freshly preserved in RPMI 1640 medium (BioWhitaker); tissue samples were manually disaggregated in RPMI 1640 medium and filtered through 70 µm pore-size cell strainer membranes (BD Pharmingen). Erythrocytes were lysed adding 1 ml of NH_4Cl to both cell preparations from tissue sources either peripheral blood

followed by serial rinses in RPMI 1640 medium. The resulting mononuclear cell preparations were washed twice with RPMI 1640 (10 % fetal calf serum, FCS) by centrifugation and resuspended. Cell viability was determined using trypan blue vital staining. A total of 10^6 leukocytes were seeded per well in 96-wells plates.

Flow cytometry

Isolated leukocytes were transferred to U-bottom microtiter plates ($1x10^6$ cells in a volume of 100 µl) and centrifuged (3 min at 1,600 x *g*). Then, cells were stained using 50 µl per well of monoclonal antibodies (mAb), diluted 1 in 125. Porcine anti-CD3-FITC (BD Pharmingen, clone BB23-8E6-8C), anti-CD4-FITC (BD Pharmingen, clone 74-12-4), anti-CD4-PE (BD Pharmingen, clone 74-12-4), anti-CD8-PE (BD Pharmingen, clone 76-2-11), and anti-CD21 (VMRD Inc., clone BB6-11C9) were added and incubated on ice and in the dark for 20 minutes. Cells were double-stained for CD4 and CD8 and single stained for CD3, CD21. Goat anti-mouse IgG_1-Alexa 488 (Molecular Probes) diluted 1 in 250 was used as secondary antibody for anti-CD21 labelling. After antibody incubation, cells were washed with 150 µl of PBS-staining buffer, centrifuged and fixed with 4 % paraformaldehyde for 10 minutes at room temperature in the darkness. Cells were washed four times with PBS-staining buffer prior to analysis in a FACS analyser (Facscalibur, Becton-Dickinson). The results

presented in this study were based on lymphocyte gating on a forward scatter-versus-side scatter diagram.

Serum detection of cytokines (IL-10, IL-12/23 p40, IFNγ and IFNα) by ELISAs.

Serum samples were analysed for cytokines expression by means of commercial ELISA kits for IL-10, IL-12/23 p40 and IFNγ, following manufacturer's instructions (Swine IL-10 and Swine IFNγ ELISA kits, Biosource; Porcine IL-12/IL-23 p40 Immunoassay, R&D Systems). All the ELISA kits were carried out using a species specific monoclonal antibody and their sensitivity thresholds were 3 pg/ml, 9 pg/ml, and 2 pg/ml, respectively. All samples were analysed in duplicate. Cytokine concentrations were calculated by using the linear-regression formula from optical densities of the cytokine standards provided by the manufacturer.

IFNα was determined by a sandwich ELISA carried out as previously described by Diaz de Arce *et al*. (1992) with modifications. Microtitre plates were coated overnight at room temperature with F17 IFNα mAb (kindly provided by Dr. K. Van Reeth) at 1 µg/ml in 50 mM Tris-HCl buffer (pH 9.5), then blocked for 1 h at 37 ºC in PBS containing 5 % BSA and 0.05 % Tween 20. After five washes in PBS containing 0.05 % Tween 20, plates with duplicate samples and recombinant IFNα protein (R&D Systems) standard range were incubated for 2 h at room temperature. After five washes, biotinylated-K9 IFNα mAb (kindly

provided by Dr. K. Van Reeth) (0.88 µg/ml) was added for 1 h at room temperature. Following five washes, streptavidin-horseradish peroxidase complex (Amersham) (dilute 1 in 500) was then added for 1 h at room temperature. Reaction was developed with 100 µl per well of tetramethylbenzidine (TMB). After 30 minutes, the reaction was stopped adding 100 µl per well of 1M H_2SO_4. The absorbance was measured at 450nm wavelength.

Statistical analysis

All the values are expressed as the mean ± SD. Since control animals were bled at 0, 7, 14, 21 and 24 dpi, blood values of inoculated animals at 3, 10 and 17 dpi were analysed with the mean value of the control animals at the prior- and post-time points. The values of all the studied parameters were evaluated for approximate normality of distribution by using Kolmogorov-Smirnov statistic. The difference between means was assessed by ANOVA test followed by a Mann-Whitney-U non-parametric test (GraphPad Instat 3.05). $P<0.05$ was considered significant.

Results

Clinical signs, total leukocyte counts and leukocyte formula

No differences were observed in the respiratory score between the control group and the inoculated animals throughout the study, however, from 3 dpi more than 20 % of the inoculated animals presented dullness and moderate growth retardation. The rectal temperature was always between the normal physiological ranges, although it was mildly elevated at 3 and 10 dpi ($P<0.05$), and decreased at the end of the experiment ($P<0.05$) (Fig. 2).

No significant changes were observed in the total leukocytes counts throughout the experiment in either control or inoculated animals (Fig. 3A). The average percentage of monocytes suffered a progressive decrease from 3 to 17 dpi, being statistically significant at 14 dpi ($P<0.05$) (Fig. 3B). The average percentage of lymphocytes displayed an undulant profile with higher percentages at 7 dpi and at the end of the study with respect to the control group ($p<0.05$) and decreasing significantly at 14 dpi ($P<0.05$) (Fig. 3C). The drop observed in monocytes and lymphocytes population at 14 dpi was accompanied by an increase of neutrophils at this time-point ($P<0.05$) (Figs. 3B, 3C and 3D). In addition, a decrease in the average percentage of neutrophils was also observed at 7 and 24 dpi matching the increase of the percentage of lymphocytes at those dates ($P<0.05$) (Figs. 3B, 3C and 3D).

Viraemia and specific antibody detection in serum samples

Neither virus nor PRRSV-specific antibodies were detected in control animals throughout the study. Virus was detected in blood samples from 3 dpi until the end of the study, showing a progressive titre increase from 3 to 10 dpi and decreasing by the end of the study (Fig. 4). PRRSV-specific antibodies were first detected at 7 dpi. Antibody titres increased progressively until 17 dpi remained constant afterwards (Fig. 4).

Flow cytometry

$CD3^+$ and $CD21^+$ cells subsets

Anti-CD3 and anti-CD21 monoclonal antibodies were used to stain αβ T cells and B cells, respectively. The percentages of $CD21^+$ cells in both inoculated and control animals were always higher in the lymph nodes studied than in PBMCs or spleen (Tables I and II). Medial retropharyngeal lymph node displayed no changes in $CD3^+$ cells throughout the study, while $CD3^+$ cells underwent an increase until 10 dpi in PBMCs ($P<0.05$) and spleen ($P<0.05$), decreasing at 17 and 21 dpi in PBMCs ($P<0.05$) (Tables I and II). Tracheobronchial lymph node described a similar trend to that one observed in PBMCs and spleen, however, the differences with the control group were considered not statistically significant (Table II). $CD21^+$ cells subset was

increased from 17 until 24 dpi in PBMCs and tracheobronchial lymph node ($P<0.05$) (Tables I and II). Medial retropharyngeal lymph node and spleen presented only a mild increase, no statistically significant (Table II).

$CD4^+CD8^-$, $CD4^-CD8^{high}$, $CD4^-CD8^{low}$ and $CD4^+CD8^+$ T cells subsets

$CD4^+CD8^-$ T cells did not display significant changes in PBMCs and lymph nodes, however, their percentage decreased significantly at 14 and 17 dpi in the spleen ($P<0.05$) (Figs. 5 and 6). At the end of the study a decrease of $CD4^-CD8^{low}$ T cells together with an increase in $CD4^-CD8^{high}$ and $CD4^+CD8^+$ T cells were observed in the tracheobronchial lymph node and the spleen ($P<0.05$) (Figs. 6B, 6C and 6D). PBMCs showed similar changes, although the increase in $CD4^-CD8^{high}$ T cells was not statistically significant (Fig. 5D). Moreover, the same kinetics in the medial retropharyngeal lymph node were observed but without statistically significant differences with respect to the control group (Fig. 6).

Cytokine expression in serum samples

Inoculated animals showed higher levels than control animals for all the cytokines analysed in serum from 3 to 24 dpi (Fig. 7), however, these differences were considered statistically significant only for IL-12 p40 at 3, 7, 17 and 24 dpi. IL-10, IL-12 p40 and IFNα increased displaying a peak at 10 (IL-10

and IL-12 p40) and at 14 dpi (IFNα), respectively, and decreased at the end of the study (Figs. 7A, 7B and 7C). IFN-γ expression was erratic and increased towards the end of the study (Fig. 7D).

Discussion

The changes observed in the different lymphocyte subsets at blood and lymphoid tissue levels together with the expression of different cytokines during an experimental infection with a EU PRRSV field isolate are discussed in the present report. Few studies have been carried out about the significance of the lymphocyte subsets changes in lymphoid organs, being mainly focused on US genotypes.

Although the oronasal via represent the most common way of natural infection (Albina, 1997), in our study we carried out an intramuscular inoculation of the virus to guarantee the same infective dose to every animal. The experimental infection with the PRRSV field isolate 2982 developed no changes in respiratory signs and only mild hyperthermia in inoculated animals. Difficulties to trigger respiratory symptoms after experimental infection with different PRRSV strains have been also previously reported (Van Reeth *et al.*, 1999; Van Reeth and Nauwynck, 2000; Foss *et al.*, 2002; Sipos *et al.*, 2003; Van Gucht *et al.*, 2003).

US PRRSV genotypes are characterised by an early and prolonged viraemia (Rossow *et al.*, 1995; Vézina *et al.*, 1996), whereas EU genotypes usually induce a shorter period of viraemia (Albina *et al.*, 1998b; López-Fuertes *et al.*, 1999;Díaz *et al.*, 2005, 2006). In this study the virus was isolated from serum samples from 3 dpi, peaking at 10 dpi and decreasing by the end of the experiment. The peak of the viraemia was followed by a decrease in the number of monocytes and lymphocytes at 14 dpi. These changes may be explained to an increase in neutrophils at the same time point, probably due to chemo-attractive agents released during the infection mainly in the lung (Van Reeth *et al.*, 1999).

Medial retropharyngeal and tracheobronchial lymph nodes and spleen were the lymphoid organs selected for this study, since they deal with the lymphatic drainage from the oronasal mucosa, lung and systemic level, respectively. Díaz *et al.* (2005) reported no changes of $CD21^+$ cells subset in pigs infected with a EU PRRSV genotype. Conversely, a rapid polyclonal activation of B cells has been reported in pigs inoculated with a PRRSV US isolate together with an early increase of B cells, mainly in the tonsils (Lamontagne *et al.*, 2001). Interestingly, in our study, using a EU isolate as inoculum, $CD21^+$ cell counts were also increased from 3 dpi onwards in all the studied organs. Moreover, this increase was considered significant in PBMCs and tracheobronchial lymph node, which seems logical due to the viral replication is focused in the lung during acute PRRSV infection. This is in agreement with the lack of significant

changes in the counts of $CD21^+$ cells observed in the spleen, since PRRSV replicates in the spleen in a lesser extent than in lymph nodes during an acute infection (Xiao *et al.*, 2004).

The global changes observed in αβ T lymphocytes were analysed by means of the use of the mAb $CD3^+$. The dynamic of $CD3^+$ cells was similar to the curve of viraemia, showing a peak also at 10 dpi. The changes in αβ T lymphocytes were studied more in depth by means of a double staining with anti-porcine CD4 and anti-porcine CD8. The most significant changes in lymphocyte subsets consisted on a generalised increase of $CD4^+CD8^+$ and $CD4^-CD8^{high}$ T cells together with a decrease of $CD4^-CD8^{low}$ T cells in all the studied organs at the end of the study. Moreover, $CD4^+CD8^-$ T cell counts did not show any significant change in all the studied organs but the spleen, in which a significant decrease in $CD4^+CD8^-$ T cell was observed at 14 and 17 dpi. At this time, $CD4^-CD8^{high}$ T cells were significantly enhanced with respect to the control animals, pointing to a marked imbalance in helper/cytotoxic activity in the spleen, as it has been previously suggested for US genotypes (Lamontagne *et al.*, 2003). Porcine $CD4^+CD8^+$ T cells are considered as memory cells that induce cell proliferation and immunoglobulins production, as well as production of the cytokines IL-2, INFα and IFNγ (Charerntantanakul and Roth, 2007). Interestingly, the percentages of $CD4^+CD8^+$ T cells presented a significant increase at 24 dpi, coinciding with an enhancement of both $CD21^+$ cells and PRRSV-specific antibody titre, pointing to a continuous exposure to PRRSV

antigen. The dynamic of αβ T lymphocyte subsets in lymphoid organs during PRRSV infection is still uncertain. Xiao *et al*. (2004) described no changes for both $CD4^+$ and $CD8^+$ T cells in an infection with a US genotype, meanwhile, Lamontagne et al. (2003) reported an increase in $CD4^-CD8^{high}$ T cells in the mediastinal lymph node and spleen of pigs infected with a US isolate. In our study, $CD4^-CD8^{high}$ T cells were increase from 7 until 24 dpi in PBMCs, tracheobronchial lymph node and spleen, and in the medial retropharyngeal lymph node at the end of the study. PBMCs presented a higher number of $CD4^-CD8^{high}$ T cells at 10 dpi, when the viraemia began to decrease. Since $CD4^-CD8^{high}$ T cells are characterised by presenting cytotoxic activity, the enhancement of $CD4^-CD8^{high}$ T cells and the drop of the viraemia at the same time-point suggest a possible role of the cytotoxic activity of this T cell subset in the virus clearance, as it has been suggested before for US (Lamontagne *et al*., 2003) and EU genotypes (Nielsen *et al*., 2003; Tingsted and Nielsen, 2004). However, when pigs were treated with an anti-CD8 mAb, no changes in the ability to clear PRRSV were observed (Lohse *et al*., 2004), pointing to another mechanism involved in PRRSV clearance. The antiviral state induced by IFNα during the innate immune response, as well as, the neutralization or clearance of the virus by neutralizing antibodies (NAs) or antigen-specific IFNγ-SC might be some of the mechanisms involved in PRRSV clearance.

Shimizu *et al*. (1996), suggested that immune effectors may be necessary to induce such enhancement in $CD8^+$ T cells, since PRRSV fails to induce *in vitro*

$CD8^+$ T cells proliferation in PBMCs. IL-12 and IL-10 are regulatory cytokines able to stimulate natural killer (NK) and $CD8^+$ T cells, inducing their recruitment, cytotoxicity and proliferation (Wolf *et al.*, 1994; Moore *et al.*, 2001; Pestka *et al.*, 2004). Moreover, IL-10 also has strong inhibitory effects on $CD4^+$ T cells proliferation (Moore *et al.*, 2001; Pestka *et al.*, 2004). Interestingly, in our study IL-12 p40 and IL-10 displayed a peak at 10 dpi, just when the increase in $CD4^-CD8^{high}$ T cells was observed. Furthermore, the expression of IL-10 may be related also with the absence of changes observed in $CD4^+CD8^-$ T cells in our study. However, further studies are required to determine which cells involve in the synthesis of IL-10 and IL-12, and if these cytokines are able to modulate T cell subsets during PRRSV infection. On the other hand, $CD4^-CD8^{low}$ T cells decreased just after the peak of viraemia and at the end of the study. How $CD4^-CD8^{low}$ T cell subset responses against antigen exposure remains unclear nowadays.

IFNα and IFNβ constitute one of the two pathways involved in the innate cytokine response, inducing an antiviral state in target cells (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). We observed a higher expression of IFNα from 10 to 14 dpi, coinciding with the drop of the viraemia. However, the lower levels of IFNα expression in PRRS than in other porcine respiratory viral diseases, such as Swine Influenza Virus or Porcine Respiratory Coronavirus infections (Van Reeth and Nauwynck, 2000), reflects that IFNα expression is insufficient for PRRSV clearance. A possible role of NAs has been also

suggested in PRRSV clearance, correlating the level of NAs with a reduction in PRRSV titre in the lung and in peripheral blood (Yoon *et al.*, 1995). Nonetheless, PRRSV has been isolated from serum and different tissues in the presence of NAs (Vézina *et al.*, 1996; Wills *et al.*, 1997; Batista *et al.*, 2004), pointing to NAs normally generated against PRRSV may not be sufficient to the resolution of the viraemia (Murtaugh *et al.*, 2002). In our study, the detection of viraemia until the end of the study confirms an inefficient virus clearance.

IL-12 and IFNα are involved in the differentiation of naïve T cells into antigen-specific IFNγ-SCs. Correlations between the frequencies of virus specific IFNα-SCs and virus specific IFNγ-SCs has been described in pigs vaccinated with an attenuated, modified–lived vaccine (MLV) of PRRSV (Royaee *et al.*, 2004). Moreover, IFNγ protects macrophages *in vitro* against PRRSV replication (Bautista and Molitor, 1999). However, the inhibitory effect of IL-10 and the poor expression of IFNα compared with other porcine viral infections (Van Reeth and Nauwynck, 2000), contributed to the minimal expression of IFNγ in the acute phase of our experimental PRRSV infection. Indeed, the expression of IFNγ was downregulated when the expression of IL-10 was higher, increasing once the expression of IL-10 dropped. These results point to IL-10 plays a role in the development of the immune response against PRRSV.

We described here the main changes in lymphocytes subsets and cytokines in pigs inoculated with a EU PRRSV field isolate. An enhancement of IL-12 and

IL-10 was correlated with a peak of $CD4^{-}CD8^{high}$ T cells and with the drop of the viraemia. The possible role of these cytokines stimulating the cytotoxic activity, and if such cytotoxic activity is also involved in PRRSV clearance, is aspects which need to be clarified. IL-10 expression might be also the responsible of the low levels of IFNγ detected, in spite of the expression of IL-12 and IFNα. Further studies should be conducted to determine the role of IL-12 and IL-10 in PRRS, and the pathways involved in their expression during the disease to develop efficient measures of control against PRRSV infection.

TABLE I. Percentages of CD3 and CD21 lymphocyte subsets in PBMCs of control and inoculated animals analysed by flow cytometry.

Data are expressed as means ± SD. *$P<0.05$.

	PBMCs (%) ± SD							
	0 dpi	**3 dpi**	**7 dpi**	**10 dpi**	**14 dpi**	**17 dpi**	**21 dpi**	**24 dpi**
CD3+								
Controls	46.79±5.40	ND	62.24±8.84	ND	66.38±7.84	ND	72.06±4.51	62.94±6.40
Inoculated	43.53±4.89	61.65±11.83	63.40±6.17	78.61±8.53*	63.55±13.18	60.25±6.25*	54.30±1.60*	65.84±4.78
CD21+								
Controls	15.55±5.32	ND	19.21±6.82	ND	20.38±2.39	ND	22.67±3.57	15.09±2.30
Inoculated	16.47±4.46	17.59±10.87	18.83±3.41	18.71±6.96	20.80±10.44	27.68±4.24*	30.84±7.50	28.24±9.73*

ND: Not determined.

TABLE II. Changes in CD3 and CD21 lymphocyte subsets studied by flow cytometry in medial retropharyngeal and tracheobronchial lymph nodes and spleen of inoculated animals respect with control animals (killed at 24dpi). Data are expressed as means ± SD.

*$P<0.05$.

	Lymphoid organs cells (%) ± SD							
	Control Animals	**3 dpi**	**7 dpi**	**10 dpi**	**14 dpi**	**17 dpi**	**21 dpi**	**24 dpi**
CD3+								
LN Med R	66.11±7.95	73.21±8.72	65.55±6.60	70.31±4.09	70.69±17.75	67.85±7.13	61.95±9.71	74.20±4.13
LN TB	66.15±0.90	61.55±9.11	68.17±7.47	68.80±6.93	58.59±11.66	60.83±6.05	60.93±13.80	68.22±9.20
Spleen	71.07±6.57	69.74±14.89	78.40±7.10	82.62±3.03*	78.68±10.29	70.76±4.06	71.21±6.57	82.89±8.77
CD21+								
LN Med R	32.88±5.80	33.63±9.09	31.38±6.75	40.46±13.37	ND	38.80±11.52	36.36±1.45	31.58±4.39
LN TB	24.13±6.57	ND	ND	35.60±8.17	ND	48.79±9.39*	33.81±11.70	31.34±4.44*
Spleen	14.87±4.26	16.67±4.54	ND	14.95±3.62	ND	ND	16.09±4.21	18.21±3.21

LN Med R.: Lymph node medial retropharyngeal. LN TB: Lymph node tracheobronchial. ND: Not determined.

FIGURES LEGENDS

Figure 2. Rectal temperature (ºC) of control (○) and inoculated (■) animals throughout the study. Data expressed as the mean ± SD. *$P<0.05$.

Figure 3. (3A) Absolute numbers of total leukocytes counts (x10^6/ml), and differential percentage of monocytes (3B), lymphocytes (3C), and neutrophils (3D) from control (white columns) and inoculated (black columns) pigs. Data are expressed as mean ± SD. *$P<0.05$.

Figure 4. Viraemia (■, expressed as log10) and PRRSV-specific antibody (▲, expressed as log2) titres from serum of the pigs inoculated with the PRRSV field isolate 2982.

Figure 5. Double colour flow cytometric analysis of CD4 expression versus CD8 expression in PBMCs. 5A and 5B show the changes observed in $CD4^+CD8^-$, $CD4^-CD8^{high}$, $CD4^-CD8^{low}$ and $CD4^+CD8^+$ T cells subsets in PBMCs between a control and an inoculated animal at the end of the study (24 dpi). 5C to 5F show the changes observed for the same T cell subsets in control (white columns) and inoculated (black columns) animals throughout the study. Data are expressed as means ± SD. *$P<0.05$.

Figure 6. Flow cytometric analysis of CD4 expression versus CD8 expression in lymphoid organs. 6A to 6D show the changes observed in $CD4^+CD8^-$, $CD4^-CD8^{high}$, $CD4^-CD8^{low}$ and $CD4^+CD8^+$ T cells subsets in inoculated animals respect with control animals (killed at 24 dpi) throughout the study. Data are expressed as means ± SD. *$P<0.05$.

Figure 7. Expression of IL-10, IL-12 p40, IFN-γ and IFN-α in serum samples of inoculated pigs throughout infection with 2982 PRRSV field isolate. Serum concentrations of IL-10, IL-12 p40 and IFN-γ are expressed as pg/ml (7A, 7B and 7C), and serum concentration of IFN-α is expressed as U/ml (6D). Data are expressed as means ± SD.

Fig.2

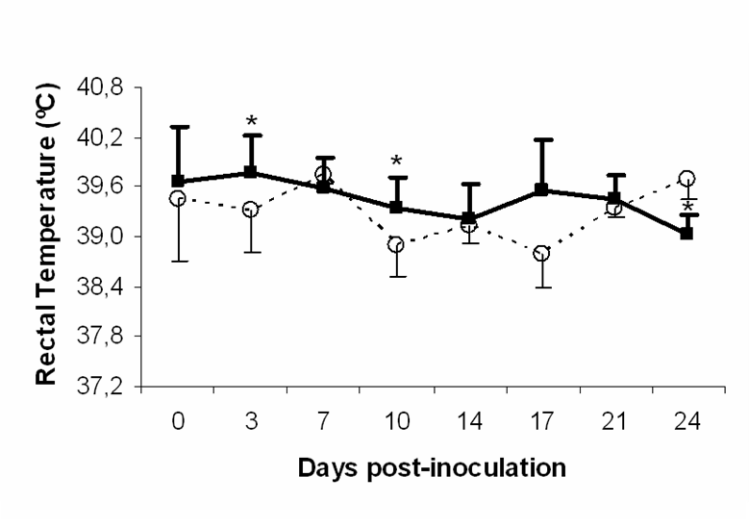

Rectal Temperature (ºC)
40,8
40,2
39,6
39,0
38,4
37,8
37,2
0
3
7
10
14
17
21
24
Days post-inoculation

Fig.3

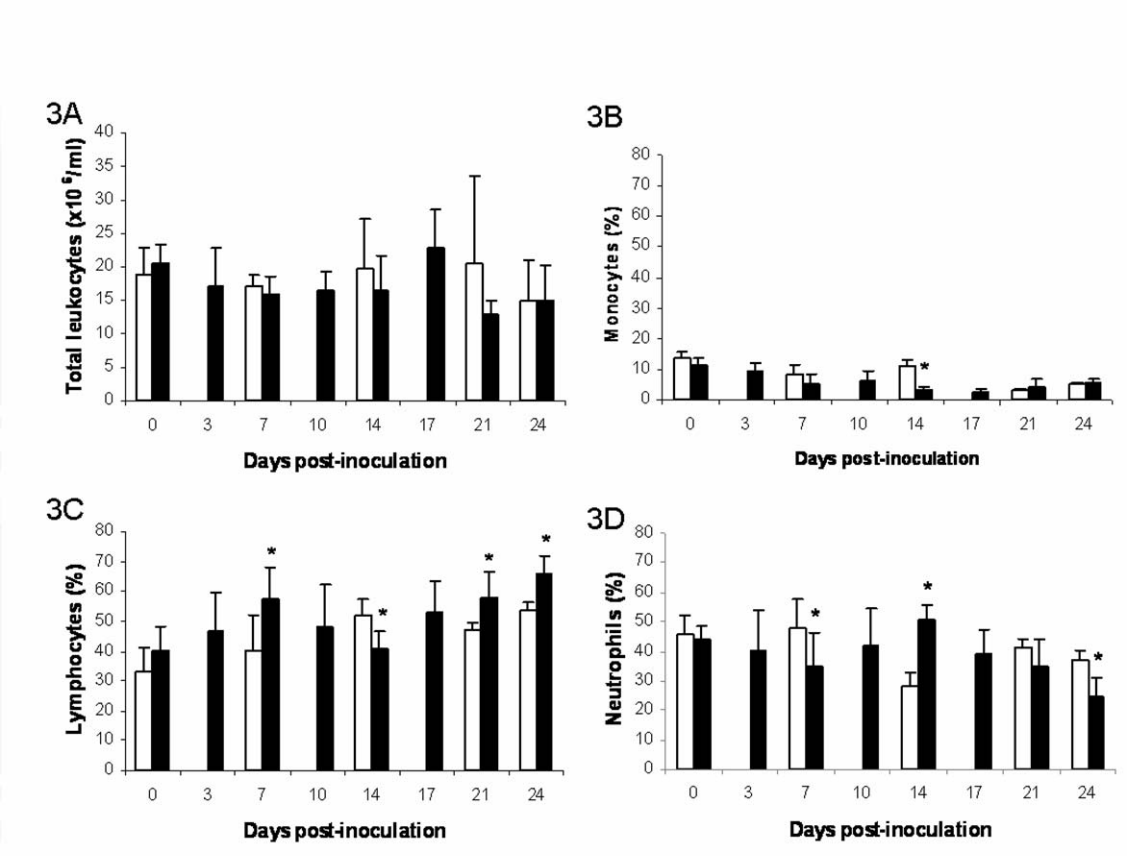

3A
Total leukocytes (x10 6/ml)
Days post-inoculation
3B
Monocytes (%)
Days post-inoculation
3C
Lymphocytes (%)
Days post-inoculation
3D
Neutrophils (%)
Days post-inoculation

Fig.4

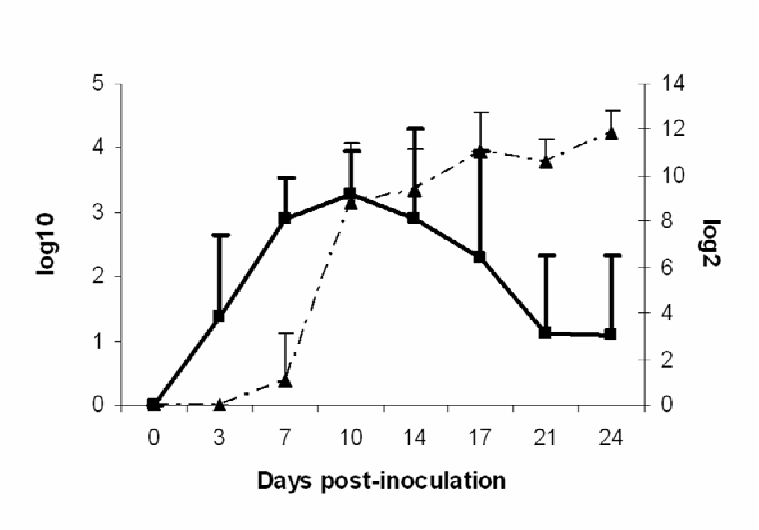

log10
log2
Days post-inoculation
0
3
7
10
14
17
21
24
0
1
2
3
4
5
0
2
4
6
8
10
12
14

Fig.5

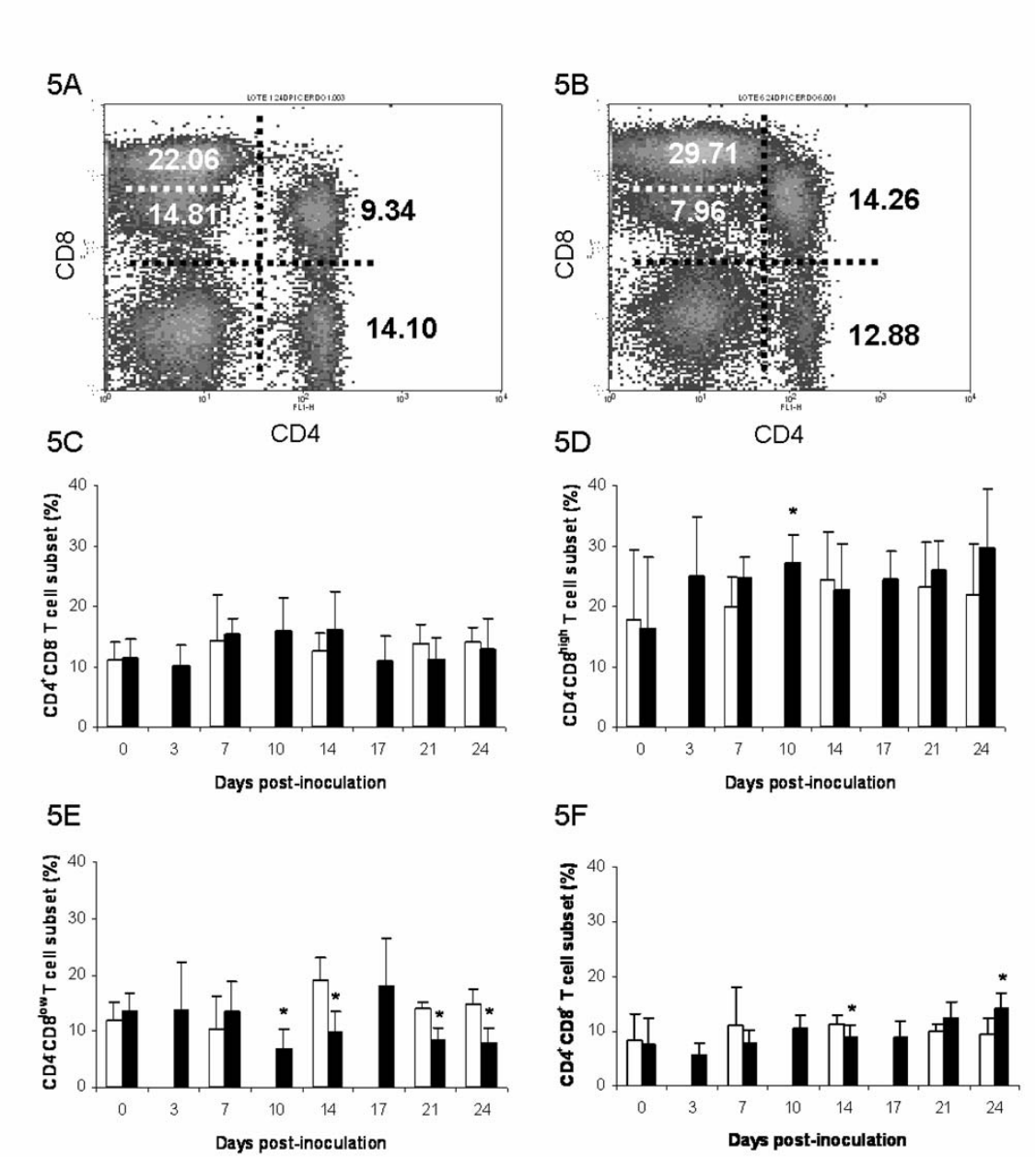

5A
22.06
14.81
9.34
14.10
CD8
CD4
5B
29.71
7.96
14.26
12.88
CD8
CD4
5C
CD4+CD8+ T cell subset (%)
Days post-inoculation
5D
CD4-CD8high T cell subset (%)
Days post-inoculation
5E
CD4-CD8low T cell subset (%)
Days post-inoculation
5F
CD4+CD8+ T cell subset (%)
Days post-inoculation

Fig.6

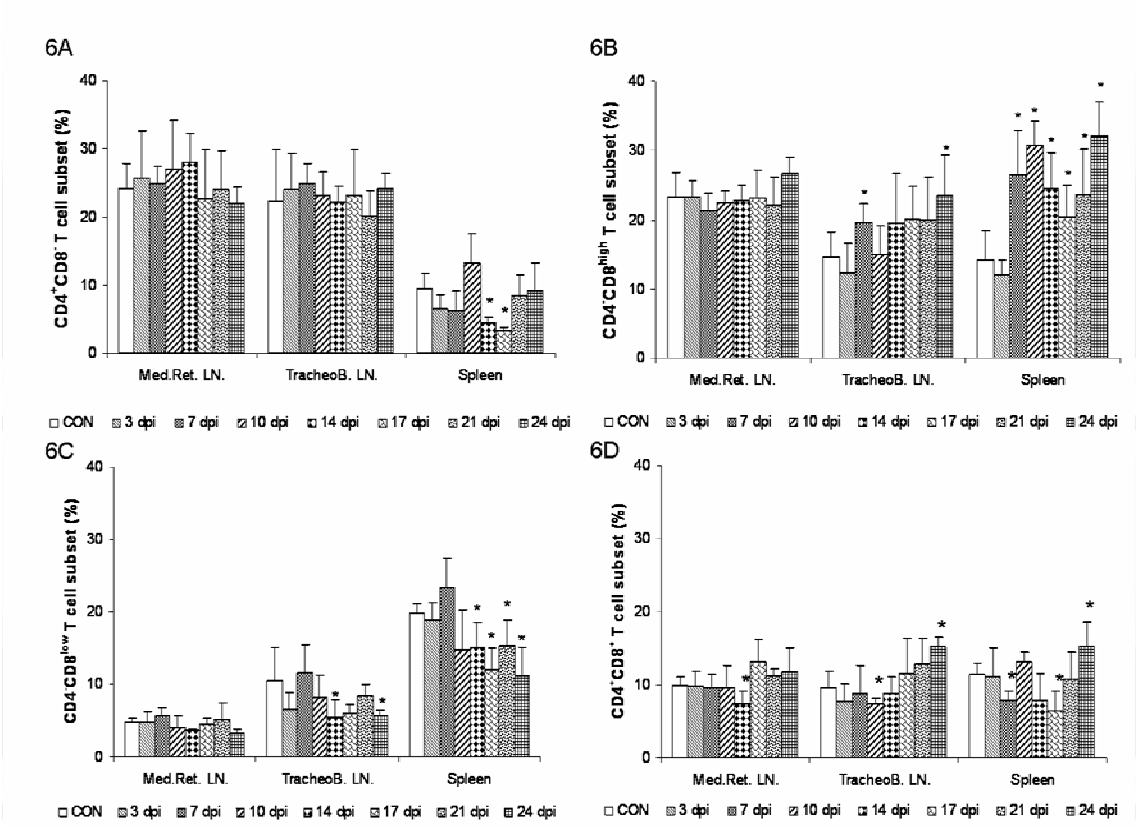
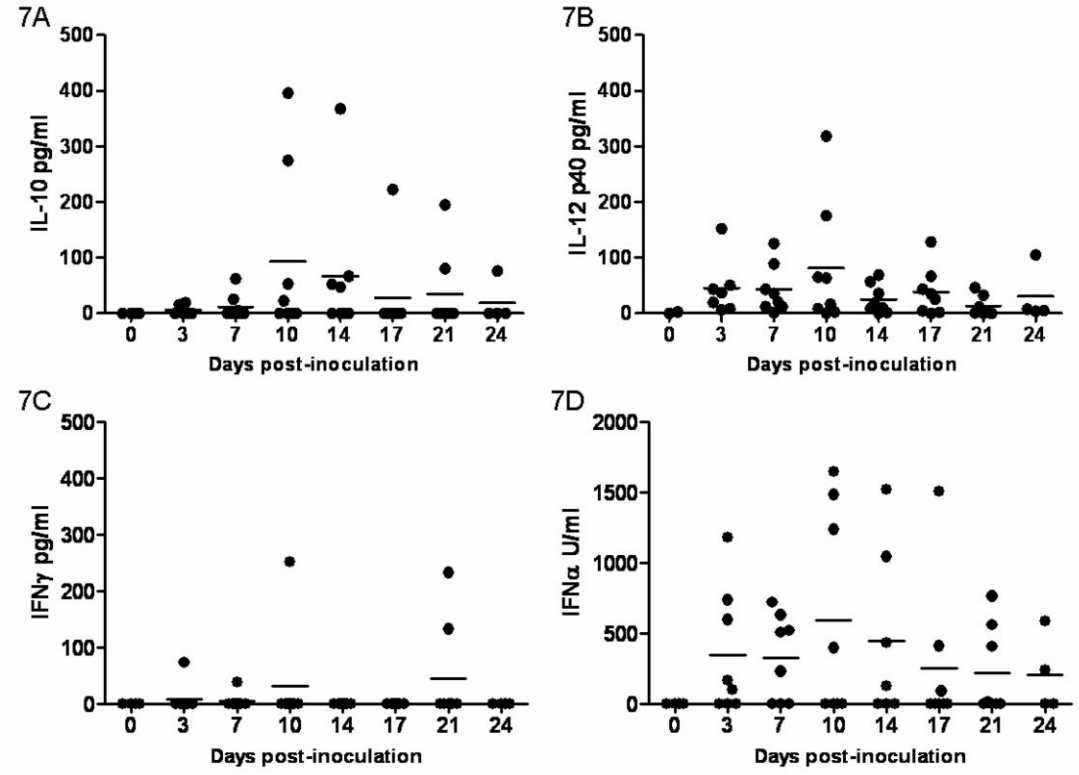

6A
6B
6C
6D
CD4+CD8+ T cell subset (%)
CD4-CD8high T cell subset (%)
CD4-CD8low T cell subset (%)
CD4-CD8+ T cell subset (%)
Med.Ret. LN.
TracheoB. LN.
Spleen
CON
3 dpi
7 dpi
10 dpi
14 dpi
17 dpi
21 dpi
24 dpi

Fig.7


7A
7B
7C
7D
IL-10 pg/ml
IL-12 p40 pg/ml
IFNγ pg/ml
IFNα U/ml
Days post-inoculation

3.3. ACUTE PHASE RESPONSE IN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (PRRS)

The APR is characterised by the disturbance of the normal homeostasis by several stimuli like infection, inflammation, stress, trauma or tissue damage (Eckersall, 2000; Ceciliani *et al.*, 2002; Gruys *et al.*, 2005). This APR is triggered by the synthesis of proinflammatory cytokines, namely IL-1, IL-6 and TNFα, at the local site of the injury. These cytokines are released into the bloodstream, reach the liver and induce the production of APPs by the hepatocytes (Eckersall, 2000; Petersen *et al.*, 2004).

APPs are used today as potential biological markers for monitoring animal welfare and the health status of swine herds and of individual pigs at slaughter (Eckersall, 2000; Petersen *et al.*, 2004; Gruys *et al.*, 2005). Moreover, APPs may be used to determine the virulence of different isolates of the same bacteria or virus, or the efficacy of vaccines (Heegaard *et al.*, 1998). To our knowledge, there are few studies on the expression of APPs during PRRS. Moreover, these studies are usually limited to only one APP or to a single time-point or short timeframe of the infection. Enhanced serum Hp concentration has been reported after experimental infection from 7 to 21 dpi (Asai *et al.*, 1999; Díaz *et al.*, 2005), and this was associated with an elevated expression of IL-6 but not TNFα (Asai *et al.*, 1999). Lipopolysaccharide binding protein (LBP) is an APP known to transfer bacterial lipopolysaccharide to CD14, a macrophage and

B cell surface receptor (Ceciliani *et al.*, 2002). LBP concentrations in bronchoalveolar lavage fluids (BALFs) were elevated between 7 and 14 dpi, whereas CD14 expression increased from 3 to 9 dpi in PRRSV infected pigs (Van Gucht *et al.*, 2005). Elevated serum concentrations of Hp, CRP and SAA have also been reported in pigs naturally infected with PRRSV, sometimes before the development of specific PRRSV antibodies (Parra *et al.*, 2006).

The main aim of this study, which coincides with the second aim of the thesis, was to analyse the kinetics of APPs and proinflammatory cytokines in the blood stream during the APR in pigs infected with a European PRRSV field isolate.

Materials and methods

Virus, animals and experimental design

The inoculum, animals and experimental design used in this experiment have been described above in the section “Common experimental design”, pages 55-56.

Virus titre in serum samples

The viral titration technique has been described above in the page 58.

Clinical signs, gross pathology and histopathology of the lungs

The pigs were monitored daily for clinical signs, i.e. rectal temperature and a clinical respiratory score, as described previously (Halbur *et al.*, 1995). Respiratory scores ranged from 0 to 6. Post mortem examination was carried out following standard operational procedures and any observed lesion was recorded. Macroscopic lung lesions were evaluated by visual inspection following the scoring system described by Halbur *et al.* (1995). Samples from the right lung (cranial lobe, medial lobe, accessory lobe, caudal dorsal lobe and caudal ventral lobe) were fixed in 10 % buffered formaldehyde and embedded in paraffin-wax. Four µm sections were stained with haematoxylin and eosin for histopathological examination. Microscopic lesion scores from 0 to 4 were assigned as previously described (Halbur *et al.*, 1995).

Detection of APPs in serum

Serum samples were analyzed for APPs by means of commercial kits, previously validated in our laboratory (Tecles *et al.*, 2007). Porcine serum Hp concentrations were quantified by using a non-species specific spectrophotometric method with commercial kit (Phase™ Range Haptoglobin Assay; Tridelta Development Ltd). The assay presented a detection limit of 0.02 mg/ml and was performed according to the manufacturer's instructions on an

automated analyser (Cobas Mira Plus; ABX Diagnostics, Montpellier, France). Serum CRP and Pig-MAP levels were assessed with porcine specific ELISA kits based on monoclonal antibodies (Phase™ Range; Tridelta Development Ltd, Maynooth, Ireland; PigCHAMP Pro Europa S.A., Segovia, Spain). Their detection limits were determined as 2.00 µg/ml and 0.18 mg/ml, respectively. SAA concentration was determined by using a commercial non-species specific ELISA kit based on a monoclonal antibody (Phase™ Range; Tridelta Development Ltd, Maynooth, Ireland). The detection limit for this ELISA test was 3.06 µg/ml. All samples were analysed in duplicate.

Detection of cytokines in serum

Sera samples were analyzed for cytokines concentration by means of commercial ELISA kits for IL-1β, IL-6 and TNFα, following manufacturer's instructions (Swine IL-1β and Swine TNFα ELISA kits, Biosource; Porcine IL-6 Immunoassay, R&D Systems). Swine IL-1β and Swine TNFα ELISA kits were carried out using a non-species specific polyclonal antibody and a species-specific porcine monoclonal antibody, respectively. Porcine IL-6 Immunoassay determines the serum IL-6 concentration by means of a species-specific porcine polyclonal antibody. The detection limits of the different ELISA kits used were 15 pg/ml, 3 pg/ml and 10 pg/ml, respectively. All samples were analysed in duplicate. Cytokine concentrations were calculated by using the linear-

regression formula from optical densities of the cytokine standards provided by the manufacturer.

Statistical analysis

Microscopic lung lesion scores, serum APPs and proinflammatory cytokines concentrations are expressed as means ± SD. Values of gross pulmonary lesions are expressed as percentages (%). Since control animals were bled at 0, 7, 14, 21 and 24 dpi, blood values of inoculated animals at 3, 10 and 17 dpi were analysed with the mean value of the control animals at the prior- and post-time points. The values were evaluated for approximate normality of distribution by using Kolmogorov-Smirnov test. Differences between the means of control and inoculated animals were assessed by a Kruskal-Wallis test followed by a Mann-Whitney-U non-parametric test (GraphPad Instat 3.05), and differences between means of control animals throughout the study were assessed by a Friedman test followed by a Wilcoxon matched pairs test (GraphPad Instat 3.05). Correlation between viral load, lung lesions and APP and cytokine serum concentrations was assessed by a Pearson test (GraphPah Instat 3.05). $P<0.05$ and $P<0.01$ were considered as significant and very significant statistical differences, respectively.

Results

Virus titre in serum samples

No virus was detected in control animals throughout the study. Viraemia was detected in blood samples from 4/8 inoculated pigs at 3 dpi, and in 8/8 inoculated pigs at 7 and 10 dpi. From 10 dpi onwards the viraemia decreased, and was detected in 7/8 inoculated pigs at 14 dpi, in 6/8 inoculated pigs at 17 dpi, and in 4/8 and 2/4 of inoculated pigs at 21 and 24 dpi (Fig.8).

Clinical signs, gross pathology and histopathology

Control animals displayed no clinical signs throughout the study. No differences were observed in the respiratory score between the control group and the inoculated animals, however, from 3 dpi more than 20 % of the inoculated animals presented dullness and weight loss. The rectal temperature was mildly elevated at 3 and 10 dpi, and remained normal at the end of the experiment (data not shown). Gross lesions displayed a significant increase from 7 dpi until the end of the study ($P<0.05$), when almost 50 % of the lung parenchyma was affected. Infected animals developed significant microscopic lung lesions compared to the control group ($P<0.01$) (Fig.9). The most severe lesions were observed in the

cranial and medial pulmonary lobes. Gross and microscopic lesions are summarised in Table III.

APPs expression in serum

Serum APPs concentrations displayed no significant changes in control animals throughout the study (Figs. 10A and 10B). Comparing serum APPs expression between control and inoculated animals, APPs values presented high variability due to interindividual variation. In the PRRSV inoculated pigs, mean levels of SAA and CRP were decreased at 7 dpi; while Hp and Pig-MAP levels were increased at 10 and/or 14 dpi. Serum Hp concentration peaked at 3 and 10 dpi (1.34-fold, and 2.42-fold, respectively), being statistically significant at 10 dpi ($P<0.01$) (Fig. 10A). Pig-MAP levels were enhanced from 3 dpi to 21 dpi (from 1.27-fold to 2.60-fold, respectively), showing a peak at 10 and 14 dpi ($P<0.05$) (Fig.3A). CRP and SAA serum concentrations significantly decreased at 7 dpi ($P<0.01$) in inoculated animals with respect to the control group, and were increased at 14, 17 and 21 dpi (1.59-fold, 2.55-fold, and 2.37-fold, respectively) for CRP, and from 17 to 21 dpi (5.29-fold, and 2.30-fold, respectively) for SAA (Fig. 10B). No correlation was found between viral load, lung lesions and APPs serum concentration throughout the study.

Cytokines expression in serum

Serum concentration of proinflammatory cytokines displayed no significant changes in control animals throughout the study (Fig. 11A). Comparing between control and inoculated animals, serum levels of cytokines showed high variability due to individual variability (Figs. 11A and 11B), not being detected significant changes in the serum concentration of proinflammatory cytokines between control and inoculated groups. Nevertheless, IL-1β levels showed a moderate increase at 7 dpi decreasing at 10 dpi, whereas serum IL-6 and TNFα levels were mildly enhanced at 10 dpi decreasing by the end of the study (Fig. 11B). The expression of TNFα showed a significant correlation with respect to the viraemia ($r = 0.70$; $P<0.05$), and to the serum levels of IL-6 ($r = 0.75$; $P<0.05$) and Hp ($r = 0.81$; $P<0.05$).

When all the three proinflammatory cytokines were considered together, their serum concentration was significantly correlated with the extent of the gross lung lesions ($r = 0.71$; $P<0.05$).

Discussion

The main goal of this study was to determine the changes and the relationship between serum levels of APPs and proinflammatory cytokines as

well as their role in PRRSV pathogenesis. Our experimental infection with PRRSV field isolate 2982 displayed no respiratory symptoms and only a mild increase in the rectal temperature in PRRSV inoculated animals, although lesions of the pulmonary parenchyma were evident throughout the study. IL-1, IL-6 and TNFα are considered as “early” cytokines which are involved in the development of local inflammatory injuries (Van Reeth and Nauwynck, 2000). Nonetheless, PRRSV is known to induce poor levels of proinflammatory cytokines compared with other porcine viral respiratory diseases, like SIV and porcine respiratory coronavirus (Van Reeth and Nauwynck, 2000; Van Reeth *et al.*, 2002). The infection with the field isolate used in our study induced a poor expression of all three proinflammatory cytokines in serum, being observed only a mild (IL-6, TNFα) or moderate (IL-1β) increase of these cytokines. Previous studies have reported an enhancement in IL-1 concentrations in PRRSV-infected pigs (Van Reeth *et al.*, 1999). Asai and co-authors (1999) reported an increase in IL-6 but not in TNFα expression in the serum of pigs infected with a US genotype of PRRSV. Both IL-1 and TNFα may induce the synthesis of IL-6 (Van Reeth and Nauwynck, 2000), but in our study the expression of IL-6 was

correlated only with the expression of TNFα. These results might be related with a different pattern of expression of proinflammatory cytokines between different PRRSV genotypes.

Instead of the poor expression of proinflammatory cytokines observed in our study, a correlation between the extent of pulmonary lesion observed in inoculated animals and the expression of all the three proinflammatory cytokines was observed. The lack of significant changes in serum levels of proinflammatory cytokines during our study contrast with the inflammatory response observed at lung level. This inflammatory response might be related with higher amounts of cytokines detected at the pulmonary parenchyma, whereas the serum concentration of these cytokines may remain generally low or undetectable (Baarsch *et al.*, 1995; Conn *et al.*, 1995). This statement indicates that proinflammatory cytokines would show a paracrine synthesis, not being observed a significant increased in the serum levels of these cytokines, which is supported by the results obtained in a parallel study carried out by our group, which reports a significant enhancement in the *in situ* expression of

proinflammatory cytokines by macrophages in the lung of PRRSV-infected pigs (Gómez-Laguna *et al.*, 2009).

On the other side, the mild expression of TNFα observed in our study showed a correlation with respect to the viraemia. Besides its role in the inflammatory response, TNFα may act as an antiviral cytokines, protecting cells from viral infection, or killing selectively virus infected cells in an interferon-independent way (Van Reeth and Nauwynck, 2000). The poor expression of TNFα observed in our study, point to impairment in the regulation of the host immune response, which makes unable the induction of an efficient PRRSV clearance.

The lower expression of proinflammatory cytokines during PRRSV infection than in other viral infections (Van Reeth and Nauwynck, 2000; Van Reeth *et al.*, 2002) is also related with the expression of lower levels of APPs (Parra *et al.*, 2006), since proinflammatory cytokines are known to induce APPs production by hepatocytes (Eckersall, 2000; Petersen *et al.*, 2004). The results obtained from our study are in accordance with data from a field study (Parra *et al.*, 2006), although in our study the fold-increase was lower than in the latter. The

use of an experimental model, with an exhaustively controlled environment, instead of field conditions may have influenced in the lower levels observed in this study.

APPs were only moderately increased in their expression from 7 dpi, just when lung lesions started to be observed. Hp and Pig-MAP were the earliest APPs synthesised after the mild enhancement observed in proinflammatory cytokines concentrations. Hp and Pig-MAP expression were increased at 10 dpi, coinciding with the highest titre of viraemia. Serum Hp concentration enhancement has been reported previously related to an increase in IL-6 but not TNFα expression during PRRSV infection (Asai *et al*., 1999). Otherwise, in our study the changes observed in Hp concentration were correlated with the mild expression of TNFα but not with IL-1β or IL-6 expression. No correlation was found between the serum concentration of the other APPs and cytokines analysed in this study, however, individual inoculated pigs showing higher values for any of the APPs presented also higher level of any or several of the cytokines analysed, higher viral load and/or higher score of lung lesion (data not shown). The differences observed in the proinflammatory cytokines profile in

our study with respect to previous reports (Asai *et al.*, 1999; Van Reeth *et al.*, 1999), suggest that the expression of proinflammatory cytokines, and the subsequent expression of APPs, may become in useful tools to determine differences in the pathogenicity between PRRSV isolates.

The serum concentrations of CRP and SAA showed a delayed increase at 17 dpi. SAA was the APP which showed a higher increase in the present study, being enhanced 5.29-fold at 17 dpi. In spite of the enhancement observed in both SAA and CRP concentrations in inoculated animals with respect to the control group, the differences were not statistically significant. This fact may be due to the high interindividual variability for SAA and CRP values between different animals, confirmed by the wide standard deviation, as previously suggested for postweaning multisystemic wasting syndrome (Segalés *et al.*, 2004). In further studies, the same batch of animals should be bled at the different time-points to avoid the interindividual variability.

The lack of increased levels of proinflammatory cytokines secretion during PRRSV infection is a question which nowadays remains without an answer. One possible explanation for the low serum levels of IL-1β, IL-6, and TNFα may

be that these cytokines are mainly expressed at the local place where the inflammatory response is triggered, as it has been suggested above. Furthermore, some studies have been addressed to determine the pathways used by PRRSV to modulate the innate immune response. Several factors may be involved in the inhibition of proinflammatory cytokines, inducing, therefore, an inhibition of the APRS and a decreased and/or delayed expression of APPs, as it was observed in our study for both CRP and SAA. NF-κB is a transcription factor which activation induces the synthesis of several immune molecules, as proinflammatory cytokines. The inhibition of NF-κB activation might be one mechanism involved in the poor expression of proinflammatory cytokines observed during PRRSV infection. However, PRRSV has been reported to activate NF-κB in MARC-145 cells and alveolar macrophages (Lee and Kleiboeker, 2005). IL-10 is an immunomodulatory cytokine which potently inhibit the production of IL-1, IL-6, TNF, and several other cytokines (Moore *et al.*, 2001). An enhancement in IL-10 expression has been previously reported after PRRSV-infection or vaccination, showing an inverse correlation with the expression of IFNγ (Díaz *et al.*, 2005; 2006). Indeed, IL-10 may inhibit the

production of proinflammatory cytokines following the same pathway triggered to inhibit IFNγ synthesis. Otherwise, the expression of transforming growth factor-β (TGF-β), other regulatory cytokine able to inhibit the expression of proinflammatory cytokines, seems to be downregulated after PRRSV immunization with a modified live virus vaccine (Royaee *et al*., 2004). Finally, PRRSV is also able to interfere the antigen presentation process, inducing a downregulation of the innate immune response and the cytokine expression (Mateu and Díaz, 2008).

In conclusion, this study reports a poor expression of both APPs and proinflammatory cytokines during the APR in pigs inoculated with 2982 PRRSV field isolate. Only a mild enhancement of proinflammatory cytokines was observed together with a moderate increase in Hp and Pig-MAP serum concentrations. Moreover, TNFα was correlated with viraemia, IL-6 and Hp expression, pointing to a role of this cytokine in the modulation of the immune response against PRRSV. The impairment of the innate immune response may be related with the onset of a regulatory response, able to inhibit the synthesis of proinflammatory cytokines and consequently the synthesis of APPs . Further

studies should be conducted to determine the exact pathway used by the virus to downregulate the immune response as well as the utility of APPs to establish differences between PRRSV isolates.

TABLE III: Gross and microscopic lesions of control and inoculated animals throughout the study. Gross lesions are expressed as % of pulmonary parenchyma with lesion. Data are expressed as the mean ± SD.

	Control	3 dpi	7dpi	10dpi	14dpi	17dpi	21dpi	24dpi
Gross lesions	1.67±1.53	13.25±15.44	47.75±17.52*	31.50±12.01*	45.25±23.21*	20.50±16.11*	28.50±17.14*	47.25±17.52*
Microscopic lesions								
Cranial lobe	0.25±0.50	0.75±0.96	1.75±0.50*	2.00±0.00*	2.25±0.96*	1.50±1.00	2.00±1.41	1.75±1.26
Medial lobe	0.25±0.50	0.75±0.96	2.25±0.50*	1.75±0.50*	2.50±0.58*	1.25±0.96	2.50±0.58*	2.25±0.50*
Accessory lobe	0.25±0.50	0.75±0.96	1.50±0.58*	1.00±0.82	2.00±0.82*	2.25±0.50**	1.50±1.29	1.75±1.26*
Caudal ventral lobe	0.25±0.50	0.75±0.96	1.75±0.50*	1.75±0.96*	1.50±1.00	1.25±0.96	1.75±0.50*	2.00±0.82*
Caudal dorsal lobe	0.25±0.50	1.25±0.96	1.25±1.50	0.75±0.96	1.75±0.50*	1.50±1.00	2.00±0.82*	2.25±0.50*
Mean total microscopic lesions	0.25±0.50	0.85±0.96**	1.70±0.72**	1.45±0.65**	2.00±0.77**	1.55±0.88**	1.95±0.92**	2.00±0.87**

Microscopic scores: 0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; 4, severe interstitial pneumonia. *,** Indicate significant differences between control and inoculated animals ($P<0.05$, $P<0.01$, respectively).

FIGURES LEGENDS

Figure 8. Virus titre (●, expressed as log10) from serum of the pigs inoculated with the PRRSV field isolate 2982. Each point represents one different animal and the horizontal line shows the mean value at each time point.

Figure 9. Photomicrograph of the cranial lobe of the right lung from a control pig (killed at 24 dpi) (9A) and from a pig inoculated with PRRSV field isolate 95/05 at 14 dpi (9B). Interstitial pneumonia is characterised by a severe septal infiltration with mononuclear cells and type 2 pneumocyte hypertrophy and hyperplasia (Haematoxylin and eosin; original magnification 10x).

Figure 10. (10A) Hp and Pig-MAP serum concentrations in control and inoculated animals. The median is marked with a line, the box shows the 25th to 75th percentile, the whiskers show maximum and minimum values. *,** Indicate significant differences between control and inoculated animals at one time-point ($P<0.05$ and $P<0.01$, respectively). (10B) SAA and CRP serum concentrations in control and inoculated animals. The median is marked with a line, the box shows the 25th to 75th percentile, the whiskers show maximum and minimum values. ** Indicate significant differences between control and inoculated animals at one time-point ($P<0.01$).

Figure 11. IL-1β (■), IL-6 (●) and TNF-α (▲) concentrations in serum samples from control (white symbols; 11A) and inoculated (black symbols; 11B) animals. Data are expressed as means ± SD.

Fig.8

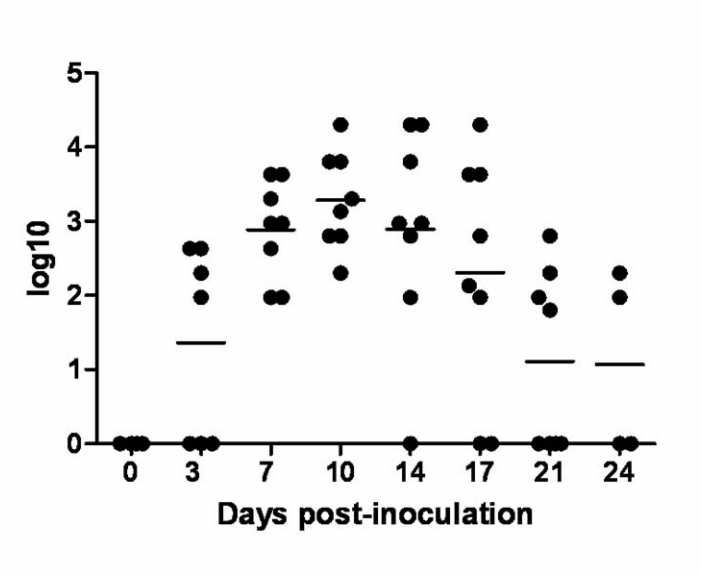


Fig.9

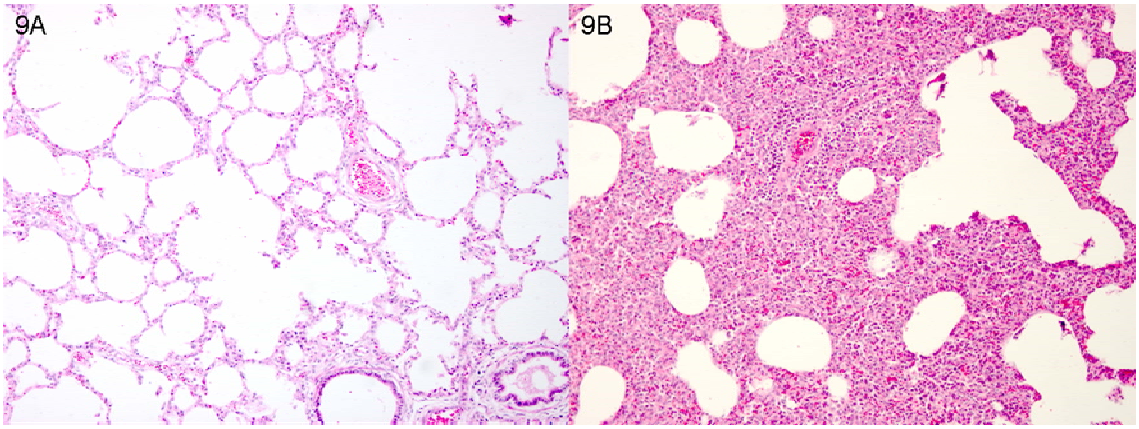
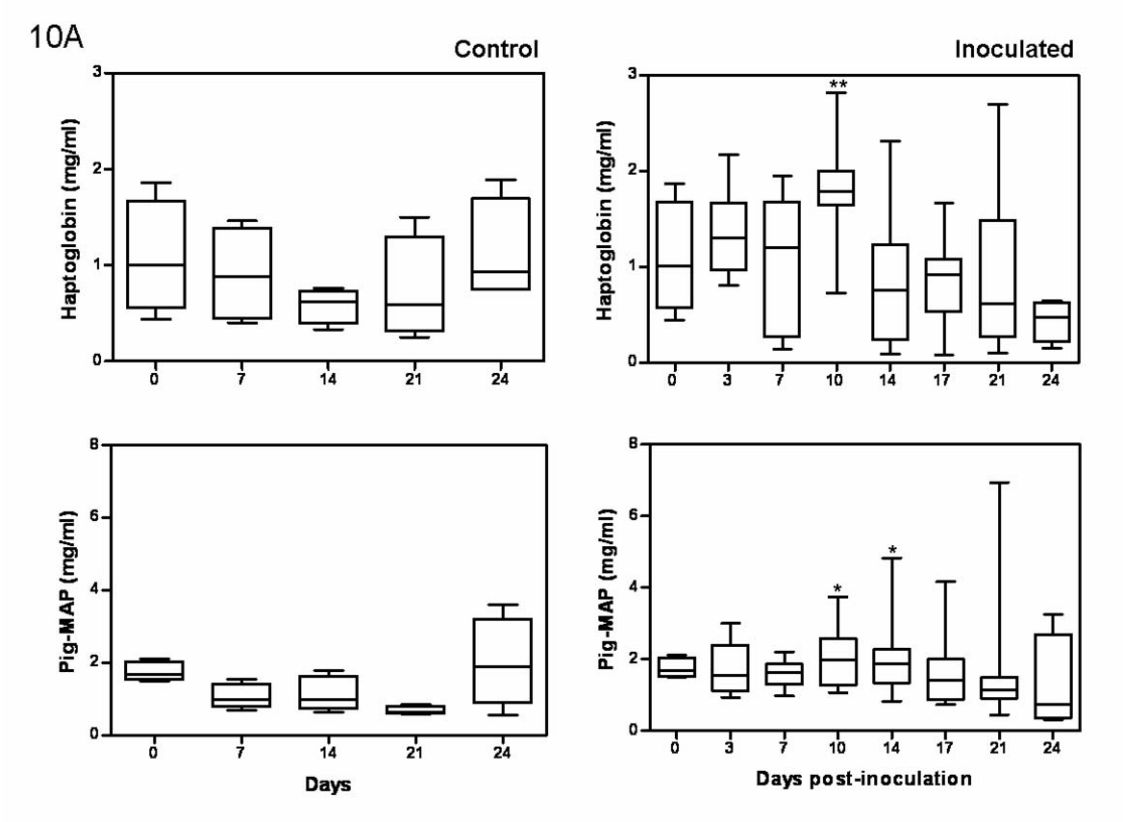


Fig.10


10A
Control
Inoculated
Haptoglobin (mg/ml)
Pig-MAP (mg/ml)
Days
Days post-inoculation

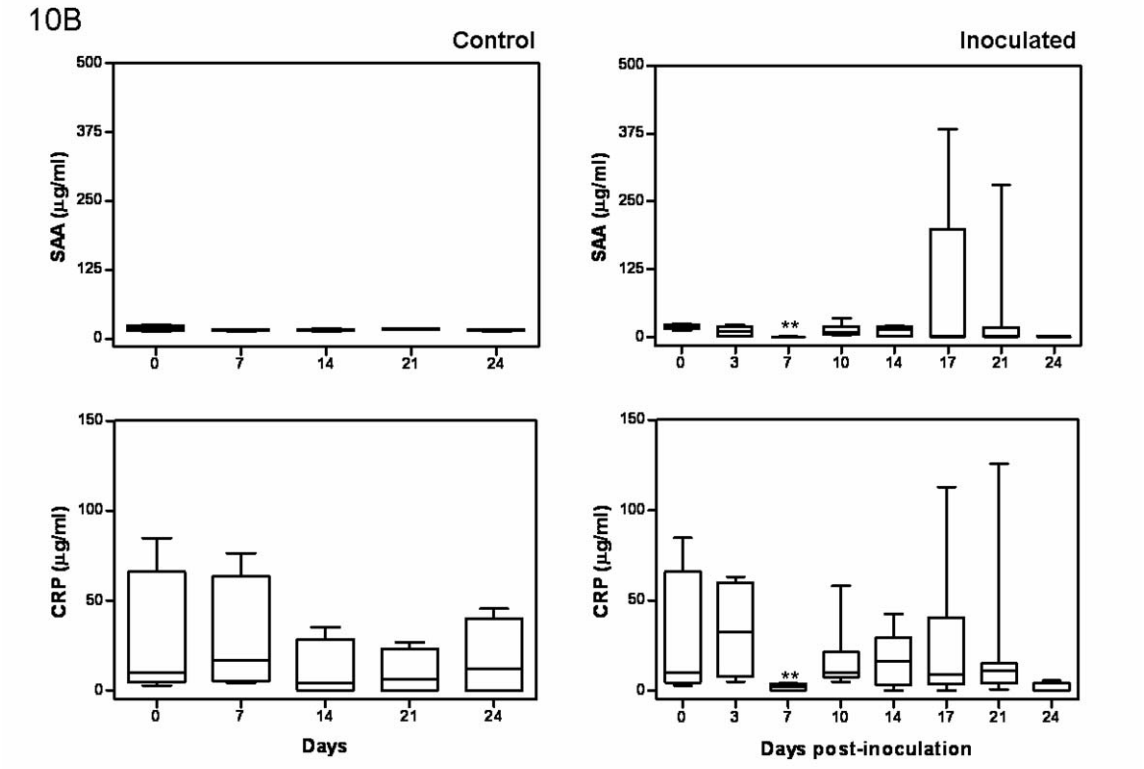
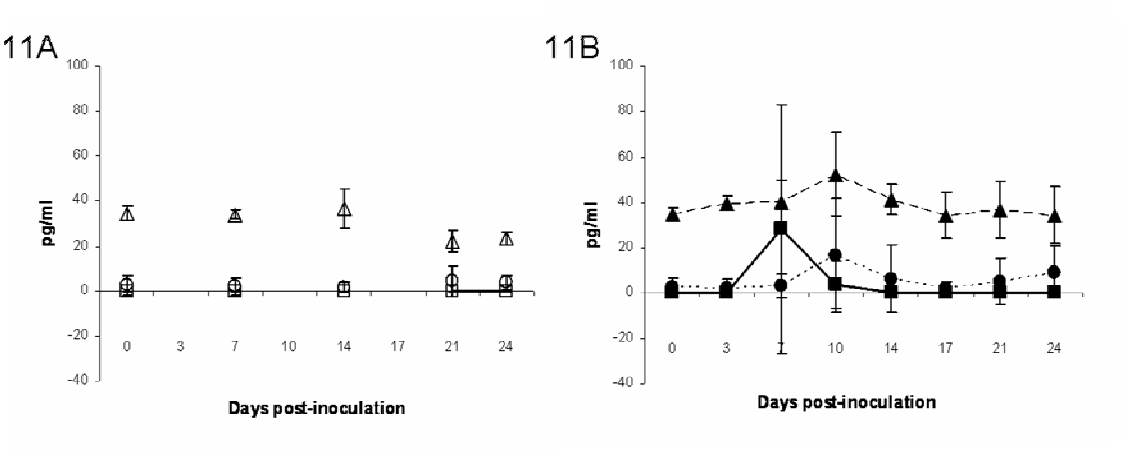

10B
Control
Inoculated
SAA (µg/ml)
CRP (µg/ml)
Days
Days post-inoculation

Fig.11


11A
100
80
60
40
20
0
-20
-40
pg/ml
0
3
7
10
14
17
21
24
Days post-inoculation
11B
100
80
60
40
20
0
-20
-40
pg/ml
0
3
7
10
14
17
21
24
Days post-inoculation

3.4. *IN SITU* EXPRESSION OF CYTOKINES BY MACROPHAGES IN THE LUNG OF PRRSV-INFECTED PIGS

Macrophages represent the first barrier against infections by pathogens, taking part in the immune response in several aspects: phagocyting invading pathogens through pattern recognition receptors, expressing molecules of the class II major histocompatibility complex (MHC II) and/or synthesizing cytokines (Mitchell and Kumar, 2004). Cytokines may be also synthesised by several other immune or non immune cells, as lymphocytes, neutrophils or fibroblasts. The expression of both cytokines either “pathogens associated molecular patterns” (PAMPs) constitute the main pathways involved in the macrophages activation (Zhang and Mosser, 2008). Furthermore, cytokines may act as promoters or inhibitors of macrophage activation, depending on which cytokines are expressed. Indeed, IL-12, TNFα, IFNα and IFNγ act as potent activators of macrophages, whereas, IL-10 is able to inhibit their activation (Mitchell and Kumar, 2004).

IFNγ and IL-12 are cytokines involved in the Th1 immune response, and both of them stimulate the synthesis of the other one (Biron and Sen, 2001). On the other hand, proinflammatory cytokines are the main cytokines involved in the inflammatory changes observed during the innate response (Biron and Sen, 2001). IFNα is another cytokine which participates in the innate response, developing an antiviral activity, inducing the differentiation of naïve T cells into

IFNγ-SCs and downregulating the expression of IL-12 (Biron and Sen, 2001; Tizard, 2008). Finally, IL-10 is considered as an immunosuppressive cytokine, which downregulates the expression of several cytokines (Biron and Sen, 2001; Moore *et al*., 2001).

Several studies have been carried out to determine the role of cytokines in the pathogenesis of PRRS (Van Reeth and Nauwynck, 2000). However, it is not clear how cytokines participate in macrophages activation during PRRSV infection neither their role in the development of the immune response. Moreover, the interactions between different cytokines make even more difficult the understanding of the mechanisms trigger off during PRRSV infection. Thanawongnuwech *et al*. (2003) suggested that the expression of IFNγ by macrophages and lymphocytes may be related with an inhibitory effect in PRRSV replication. Nonetheless, IL-10 expression was associated with a lower number of IFNγ-SCs in PBMCs during a PRRS-modified live vaccine trial (Díaz *et al*., 2006). In addition, the role of cytokines in the interstitial pneumonia described in PRRS has not been determined.

The main aim of this study, which represents the third goal of the thesis, was to determine the changes observed in both PAMs and septal macrophages in the lung of PRRSV-infected pigs, as well as, the changes observed in the expression of cytokines by macrophages *in situ* in the lung parenchyma.

Materials and Methods

Virus, animals and experimental design

The inoculum, animals and experimental design used in this experiment have been described above in the section “Common experimental design”, pp. 55-56.

Clinical signs, gross pathology and histopathology of the lungs

The clinical signs and gross pathology were evaluated as described above in the page 70.

During post mortem examination, macroscopic lung lesions were evaluated by visual inspection and samples from the medial lobe of the right lung were fixed in 10 % buffered formaldehyde and Bouin solution, and embedded in paraffin-wax. Four µm sections were stained with haematoxylin and eosin for histopathological examination.

Immunohistochemical examinations

Since PRRSV is most frequently detected in the apical and medial lung lobes (Halbur *et al.*, 1996), the medial lobe was selected for

immunohistochemical examinations. The Avidin-Biotin-Peroxidase complex technique (ABC) was used for the detection of PRRSV, macrophages and cytokines antigens as described previously (Hsu *et al.*, 1981). Briefly, the sections were deparaffinised and dehydrated in a graded series of ethanol; the endogenous peroxidase activity was quenched in a 3 % H_2O_2 solution in methanol during 30 minutes. The sections were washed with PBS (pH 7.4, 0.01 M) and incubated (30 minutes, room temperature (r.t.)) with 100 µl per slide of blocking solution in a humid chamber. Table IV describes the primary antibodies and antigen retrieval methods used. Primary antibodies were incubated overnight at 4 ºC in a humid chamber. In each case, the correspondent secondary antibody was incubated at r.t. for 30 min. An avidin-peroxidase complex (Vector Laboratories) was applied for 1 hour at r.t. The immunoenzymatic reaction was developed using NovaRED Substrate kit (Vector Laboratories). Sections were counterstained with Mayer's haematoxylin, dehydrated and routinely mounted. The specific primary antibody was replaced by blocking solution, normal serum and isotype-specific reagents for each primary antibody in the negative controls.

Semiquantitative analysis

The number of positive cells labelled against the different antibodies in medial lobe of the right lung was counted using a method described previously

(Salguero *et al.*, 2005). Briefly, cells immunolabelled were counted in 50 no overlapping consecutive selected, high magnification fields of 0.20 mm^2 for each inoculated or control animal. Results are expressed as number of cells per mm^2. Immunolabelled cells were identified and counted morphologically as macrophages, lymphocytes or neutrophils. Due to in lung tissue sections pulmonar intravascular macrophages (PIMs) are hardly differentiable from macrophages from the septa, we use the term “septal macrophages” to refer to both PIMs and interstitial macrophages.

Statistical analysis

Values of macrophages, PRRSV and cytokines antigens counts are expressed as the mean ± SD. The values were evaluated for approximate normality of distribution by using Kolmogorov-Smirnov statistic. Differences between the means of control and inoculated animals were assessed by a Kruskal-Wallis test followed by a Mann-Whitney-U non-parametric test (GraphPad Instat 3.05). Correlation between the histopathological lung lesion and the expression of virus, macrophages and cytokines antigens was assessed by a Spearman test (GraphPad Instat 3.05). $P<0.05$ was considered as significant statistical difference.

Results

Clinical signs, gross pathology and histopathology of the lungs

Animals from the control group showed neither clinical signs nor significant gross or microscopic lung lesions. Although inoculated animals displayed no significant respiratory changes, dullness, weight loss, and a mild hyperthermia were evident from 3 dpi. From 7 dpi until the end of the study, almost 50 % of the pulmonary parenchyma of inoculated animals showed interstitial pneumonia, which was confirmed by histopathological examination of the tissue samples (Figs. 12A and 13A).

Tissue expression of macrophage and PRRSV antigens

MAC387 antibody was used to detect changes in the counts of macrophages in the lung throughout the study. The number of macrophages was increased in inoculated animals from 7 dpi onwards (Fig. 12B). This enhancement was mainly due to an increase in the number of septal macrophages, and secondly PAMs, being more significant at 7, 14, and at the end of the study (Figs. 12B and 13B). PAMs were significantly decreased at 7 dpi recovering normal values onwards (Fig. 12B). The expression of the

antibody MAC387 was significantly correlated with the microscopic score of lung lesion (r = 0.85; P<0.05) (Table V).

Control animals were negative for PRRSV antigen labelling. PRRSV antigen was detected in the lung of PRRSV-infected pigs from 3 dpi until the end of the study, reaching a peak at 7 dpi (P<0.05) (Fig. 12C). The antigen expression was detected mainly in the cytoplasm of macrophages, being statistically significant higher in porcine alveolar macrophages (PAMs) than in septal macrophages (P<0.05) (Figs. 12C and 13C). Immunolabelled cells were observed in both areas of interstitial pneumonia and lung parenchyma without lesion in the inoculated animals.

Tissue expression of proinflammatory cytokines antigens

IL-1α antigen was observed in the cytoplasm of PAMs, septal macrophages and neutrophils, playing the latter a significant role in the expression of this cytokine (Fig. 12D). The expression of IL-1α was always higher in inoculated animals than in control pigs, and displayed a mild to marked enhancement at 7 and 14 dpi (P<0.05) (Fig. 12D), respectively. The increase observed in IL-1α at 14 dpi was associated to a severe infiltrate of IL-1α-secreting neutrophils (P<0.05) (Figs. 12D and 13D).

The expression of IL-6 and TNFα antigens peaked at 7 and 14dpi (P<0.05) (Figs. 12E and 12F). IL-6 immunolabelling still remained enhanced at the end of

the study (Fig. 12E), but the expression of TNFα was no significant with respect to control animals at 21dpi (Fig. 12F). Septal macrophages were the main cell population involved in the expression of both IL-6 and TNFα antigens ($P<0.05$) (Figs. 12E, 12F, 13E and 13F). PAMs and lymphocytes were also related with the expression of these cytokines, but in a lesser extent (Figs. 12E and 12F).

The immunostaining against proinflammatory cytokines antigens was observed mainly in areas of interstitial pneumonia, with a moderate to severe thickening of the alveolar septa, and only few immunolabelled cells were observed in non pathological areas of the lung (Figs. 13D, 13E and 13F). The correlations between the histopathological lung lesion, macrophages counts and the expression of proinflammatory cytokines is shown in Table V. In addition, Table VI shows the correlation between the expression of TNFα and IFNγ.

Tissue expression of IFNα, IFNγ, IL-10 and IL-12 p40 antigens

IFNα antigen was expressed in the cytoplasm of PAMs, septal macrophages and lymphocytes. Septal macrophages were the main cell type involve in the expression of this cytokine, which displayed a significant increase at 3 dpi ($P<0.05$) and decreased onwards (Figs. 14A and 15A). The number of IFNα-expressing PAMs was also enhanced at 3 dpi ($P<0.05$). IFNα expression was always higher in inoculated animals respect with control animals (Fig. 14A). The

expression of IFNα showed a significant correlation with respect to virus expression (r = 0.86; $P<0.05$) (Table VI).

The kinetics of both IFNγ and IL-12 p40 was similar throughout the study (r = 0.95; $P<0.05$) (Table VI), displaying a peak at 7 dpi and decreasing onwards (Figs. 14B and 14C). These cytokines were expressed mainly by septal macrophages, but also by PAMs and lymphocytes (Figs. 15B and 15C). Inoculated animals showed always higher counts of IFNγ-expressing cells than control animals.

The expression of the cytokine IL-10 displayed an increase at 7 dpi decreasing onwards (Fig. 14D). The antibody against porcine IL-10 was observed mainly in the cytoplasm of septal macrophages (Fig. 15D). The kinetics shown by this cytokine was significantly correlated with that one of the virus (r = 0.77; $P<0.05$) (Table VI).

The number of septal macrophages expressing any of these cytokines was statistically higher than the number of PAMs (Fig. 14). The immunolabelling against IFNα, IFNγ, IL-12 p40 and IL-10 was associated to areas of mild to moderate interstitial pneumonia, and in a lesser extent to areas of pulmonary parenchyma without lesion (Fig. 15). The correlations between the expression of PRRSV, IFNα, IFNγ, IL-10, IL-12 p40 and TNFα in the lung of PRRSV-infected pigs are shown in Table VI.

Discussion

Several reports have studied the changes in cytokines during PRRSV infection, nonetheless, there is lack of study of the main cell-types involved in their expression. In this study the expression of cytokines by macrophages has been examined to determine their activation during PRRS and their role in the inflammatory reaction and modulation of the immune response.

Our experimental infection displayed no respiratory symptoms but dullness, weight loss, mild hyperthermia and lesions of the pulmonary parenchyma were observed. A peak of PRRSV replication was observed at 7 dpi, mainly located in PAMs, considered as the target cell for viral replication (Molitor *et al.*, 1997; Bautista and Molitor, 1999). No correlation was observed between viral replication and the degree of histopathological lung lesion. However, the microscopic lung lesion was significantly correlated with a marked infiltrate of the septa and the counts of macrophages. Moreover, lung lesion showed a significant correlation with the expression of both IL-1α and IL6, but not TNFα, and macrophages counts were correlated with the expression of IL-1α and TNFα, but not IL-6. These results point to a significant role of IL-1α in the development of the interstitial pneumonia during PRRS. Nonetheless, when all the three proinflammatory cytokines were considered a highly significant correlation was observed with respect to both histopathological pulmonary lesion and macrophage counts, which demonstrates the role of joint

proinflammatory cytokines in the inflammatory response evoked in PRRSV infection.

Although PRRSV replicated mainly in PAMs, proinflammatory cytokines were expressed mainly by septal macrophages, especially for IL-6 and TNFα from 14 dpi onwards. This fact points to an activation of septal macrophages, which may be induce by the synthesis of cytokines itself (Zhang and Mosser, 2008). Similar findings have been reported for other porcine viral diseases, like African Swine Fever (ASF), which triggers an activation of interstitial macrophages expressing IL-1α and TNFα after viral replication (Carrasco *et al.*, 2002).

In our study, a severe intra-alveolar neutrophil infiltration expressing IL-1α was observed at 14 dpi. The increase of both IL-1α and TNFα at earlier time-points may induce such inflammatory infiltrate and activation of neutrophils since these cytokines are considered as neutrophils-chemoattractant and stimulant agents (Van Reeth and Nauwynck, 2000). Furthermore, IL-1 and TNFα may induce the synthesis of IL-6 (Van Reeth and Nauwynck, 2000; Mitchell and Kumar, 2004), however, in our study no correlation was observed between the expression of these cytokines, although the maximum expression of IL-6 temporally coincided with a higher expression of IL-1α and/or TNFα.

IFNs are cytokines known for playing a significant role in the host immune response against viruses (Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). IFNα participates in the innate immune response and is able to induce

an enhancement in IFNγ levels (Biron and Sen, 2001; Tizard, 2008). A significant correlation was observed in our study between PRRSV replication and IFNα expression, pointing out that PRRSV directly modulate macrophages towards the expression of IFNα. However, PRRSV induces lower levels of IFNα when compared with other porcine respiratory viral diseases, such as Swine Influenza Virus or Porcine Respiratory Coronavirus infections (Van Reeth and Nauwynck, 2000), which indicates that IFNα expression is not enough for PRRSV clearance.

The expression of IFNγ by macrophages and lymphocytes has been previously reported in the lung of PRRSV-infected pigs (Thanawongnuwech *et al.*, 2003). In this study, an increase in the expression of IFNγ was observed at 10 dpi for high virulent strains whereas low virulent strains displayed a higher expression at the end of the study (28 dpi). In our study, the expression of IFNγ was undulating, showing a peak at 7 dpi, just when PRRSV replication displayed a maximum. IFNγ is known to protect macrophages *in vitro* against PRRSV replication (Bautista and Molitor, 1999), however, the viral replication observed still at the end of our study supports that the expression of IFNγ is insufficient to avoid a prolonged PRRSV replication.

The activation of pulmonary macrophages to produce IFNγ is induced by the expression of other cytokines, just as IL-12, TNFα or IFNα (Nguyen and Benveniste, 2002; Mitchell and Kumar, 2004; Tizard, 2008). In our study a tight correlation was observed with respect to IL-12 p40 and TNFα but not to IFNα.

Therefore, IL-12 p40 and TNFα was the most significant cytokines involved in the synthesis of IFNγ in our study, being less important the contribution of IFNα. Royaee *et al*. (2004) have reported a correlation between virus specific-IFNα-SCs and virus specific-IFNγ-SCs in pigs vaccinated with an attenuated, modified–lived vaccine (MLV) of PRRSV. High antigenic and pathogenic differences have been attributed to European and North American PRRSV genotypes, and within a given genotype (Halbur *et al*., 1995; Mateu *et al*., 2003; Stadejek *et al*., 2006), which may be the cause of the discrepancies observed between our study and that one of Royaee *et al*. (2004).

Instead of the expression of IFNα, IFNγ, IL-12 p40 and TNFα observed in our study, PRRSV was still replicating in the lung of PRRSV-infected pigs at the end of the study. IL-10 is an immunomodulatory cytokine which is able to inhibit the synthesis and release of other several cytokines (Biron and Sen, 2001; Moore *et al*., 2001). Therefore, the expression of IL-10 observed in our study might be the responsible for lower levels of cytokines, such as IFNα, IFNγ, IL-12 p40 and TNFα, which may avoid a prolonged viral replication in the lung of infected animals. Interestingly, the expression of IL-10 was significantly correlated with PRRSV replication. These results point out that PRRSV acts inducing the expression of IL-10, and so inhibiting the expression of other cytokines allowing a prolonged viral replication in the lung. This idea is supported by the correlation observed in our study between the expression of IL-10 and IFNα.

Our results indicate that activation of septal macrophages and PAMs differs throughout PRRSV infection, playing the first ones a main role in the synthesis and release of cytokines. Proinflammatory cytokines play a significant role in the pathogenesis of the interstitial pneumonia observed during PRRS, being directly correlated with the infiltration of the septa by macrophages. Additionally, PRRSV seems to modulate the immune response by the expression of IL-10 by macrophages, which may be the responsible of lower levels of other cytokines implied in viral clearance, just as IFNα, IFNγ, IL-12 p40 and TNFα.

TABLE IV: Antibodies source and immunohistochemical techniques used for the immunocharacterisation of PRRSV, macrophages and cytokines antigens expression.

Specificity	*Type of antibody*	*Source*	*Commercial origin*	*Fixative*	*Dilution*	*Antigen retrieval*
Anti-PRRSV (clone SDOW-17/SR-30)	Monoclonal	Mouse myeloma cells	Rural Technologies Inc.	Bouin	1:1.000	HTAR
Anti-human MΦ (clone MAC387)	Monoclonal	NS1 Mouse myeloma cell line	Chemicon Europe	Formaldehyde 10 %	1:750	Protease 10'
Anti-human IL-1α	Polyclonal	Rabbit serum	Endogen	Bouin	1:100	Tween 20 0.01 %
Anti-pig IL-6	Polyclonal	Rabbit serum	Endogen	Bouin	1:10	Tween 20 0.01 %
Anti-human TNFα (clone 68B6A3)	Monoclonal	NSO Mouse myeloma cell line	Biosource	Bouin	1:25	Tween 20 0.01 %
Anti-IFNα (clone F17)	Monoclonal	Mouse myeloma cells	Prof. K. Van Reeth	Bouin	1:300	Tween 20 0.01 %
Anti-pig IFNγ	Polyclonal	Goat serum	RnD Systems	Bouin	1:20	Tween 20 0.01 %
Anti-pig IL-10	Polyclonal	Goat serum	RnD Systems	Bouin	1:20	Tween 20 0.01 %
Anti-pig IL-12	Polyclonal	Goat serum	RnD Systems	Bouin	1:20	Tween 20 0.01 %

MΦ: macrophages. pAb: Polyclonal Antibody. HTAR: High Temperature Antigen Retrieval with citrate buffer ph 6.0. Protease 10': protease digestion for 10'.

Tween 20 0.01 %: Tween 20 diluted 0.01 % in PBS during 10'.

TABLE V: Correlations between the histopathological lung lesion, the counts of macrophages and the expression of proinflammatory cytokines.

	Microscopic lesion	MΦ	IL-1α	IL-6	TNF-α	IL-1α + IL-6 + TNF-α
Microscopic lesion	-	0.85*	0.85*	0.80*	0.54	0.87*
MΦ		-	1.00*	0.69	0.74*	0.98*
IL-1α			-	0.69	0.74*	0.98*
IL-6				-	0.62	0.79*
TNF-α					-	0.76*
IL-1α + IL-6 + TNF-α						-

MΦ: macrophages. *P<0.05

TABLE VI: Correlations between the expression of PRRSV, IFNα, IFNγ, IL-10 and IL-12 p40 in the lung of PRRSV-infected pigs.

	PRRSV	IFNα	IFNγ	IL-10	IL-12 p40	TNF-α
PRRSV	-	0.86*	0.54	0.77*	0.42	0.31
IFNα		-	0.57	0.93*	0.52	0.43
IFNγ			-	0.60	0.95*	0.71*
IL-10				-	0.64	0.60
IL-12 p40					-	0.74*
TNF-α						-

*P<0.05

FIGURES LEGENDS

Figure 12. (A) Histopathological score for lung lesions throughout the infection with PRRSV field isolate 2982. (B, C, D, E and F) Counts for MAC387, SDOW17/SR30, IL-1α, IL-6 and TNF-α, respectively. * Indicate statistical significant differences ($P<0.05$) of the inoculated group with respect to the control group. ** Indicate statistical significant differences ($P<0.05$) between the counts of PAMs and septal macrophages within a given time point.

Figure 13. (A) Photomicrograph of the medial lobe of the right lung from a pig inoculated with PRRSV field isolate 2982 and killed at 7 dpi. Interstitial pneumonia is characterized by a severe septal infiltration with mononuclear cells and type 2 pneumocyte hypertrophy and hyperplasia. HE. Bar, 100μm. (B) Marked infiltrate of macrophages in the alveolar septa in the lung of a pig killed at 7 dpi. IHC. Bar, 100μm. (C) PAMs and septal macrophages immunolabelled against SDOW17/SR30 antibody in a pig killed at 7 dpi (ABC complex method) . IHC. Bar, 25μm. (D) Numerous IL-1α-expressing-septal macrophages and neutrophils in the pulmonary parenchyma of a pig killed at 7 dpi, which shows a marked thickening of the alveolar septa. IHC. Bar, 100μm. (E) PAMs and septal macrophages expressing IL-6 in their cytoplasm in the lung of a pig killed at 7 dpi. IHC. Bar, 25μm. (F) Pulmonary parenchyma of a pig killed at 7 dpi, with a mild interstitial pneumonia, showing septal macrophages immunolabelled against TNF-α antibody. IHC. Bar, 30μm.

Figure 14. (A, B, C, and D) Counts for IFNα, IFNγ, IL-12 p40 and IL-10, respectively, in the lung of pigs infected with PRRSV field isolate 2982. * Indicate statistical significant differences ($P<0.05$) of the inoculated group with respect to the control group. ** Indicate statistical significant differences ($P<0.05$) between the counts of PAMs and septal macrophages within a given time point.

Figure 15. (A) Septal macrophages and a PIMs immunolabelled against IFNα in the lung of a pig killed at 3 dpi with a mild thickening of the alveolar septa. IHC. Bar, 20µm. (B) A focus of mild interstitial pneumonia in the lung of a pig killed at 14 dpi, which shows septal macrophages expressing IFNγ (ABC complex method). IHC. Bar, 15µm. (C) Septal macrophages showing cytoplasmic immunostaining against porcine IL-12 p40, in the lung of a pig killed at 7 dpi with a marked thickening of the alveolar septa. IHC. Bar, 25µm. (D) IL-10 immunolabelling in the cytoplasm of septal macrophages in the pulmonary parenchyma of a pig killed at 7 dpi, which shows thickening of the alveolar septa. IHC. Bar, 20µm.

Fig.12

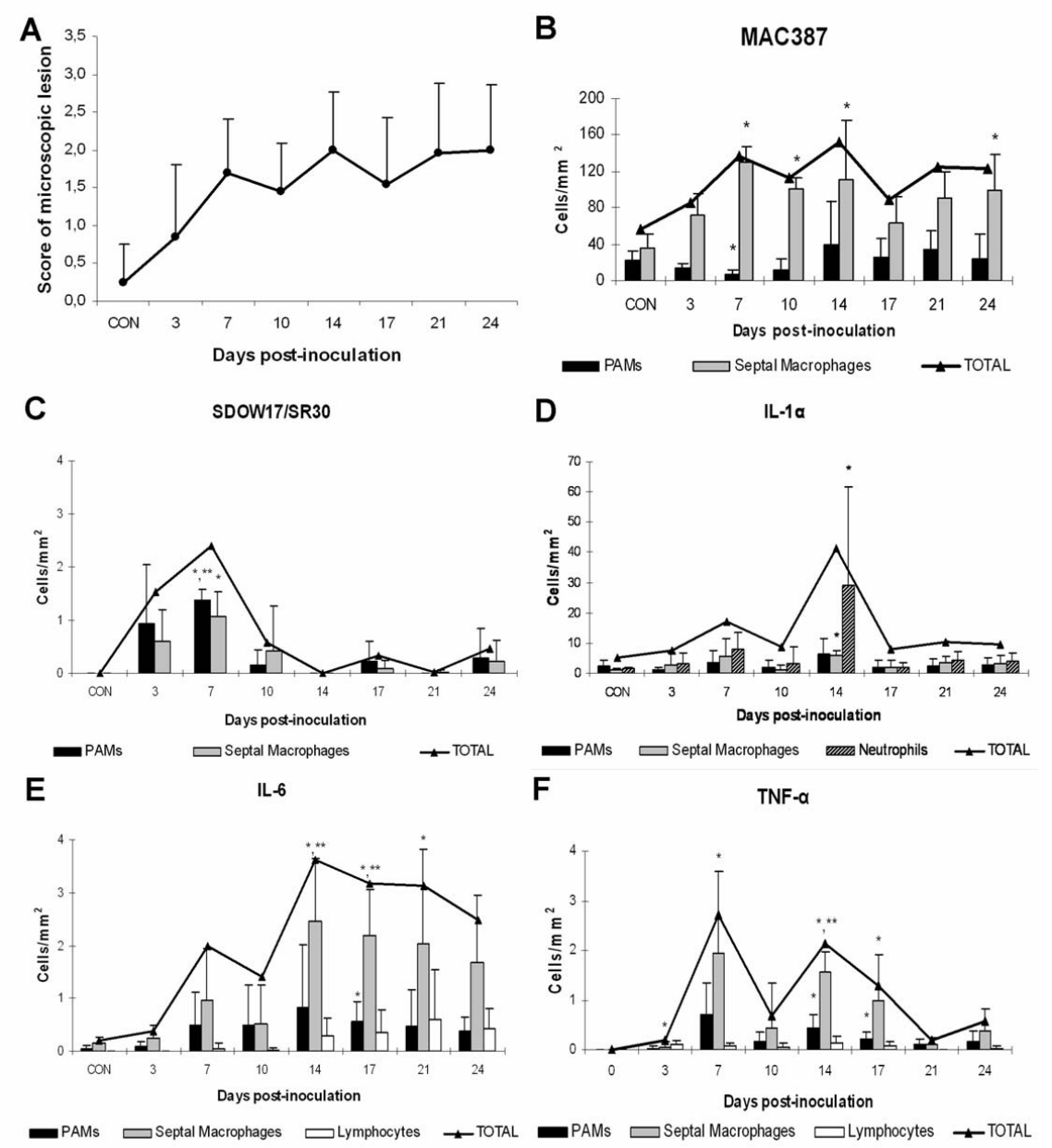

A
Score of microscopic lesion
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
CON 3 7 10 14 17 21 24
Days post-inoculation
B
MAC387
Cells/mm 2
200
160
120
80
40
0
CON 3 7 10 14 17 21 24
Days post-inoculation
PAMs
Septal Macrophages
TOTAL
C
SDOW17/SR30
Cells/mm2
4
3
2
1
0
CON 3 7 10 14 17 21 24
Days post-inoculation
PAMs
Septal Macrophages
TOTAL
D
IL-1α
Cells/mm2
70
60
50
40
30
20
10
0
CON 3 7 10 14 17 21 24
Days post-inoculation
PAMs
Septal Macrophages
Neutrophils
TOTAL
E
IL-6
Cells/mm2
4
3
2
1
0
CON 3 7 10 14 17 21 24
Days post-inoculation
PAMs
Septal Macrophages
Lymphocytes
TOTAL
F
TNF-α
Cells/mm2
4
3
2
1
0
0 3 7 10 14 17 21 24
Days post-inoculation
PAMs
Septal Macrophages
Lymphocytes
TOTAL

Fig.13

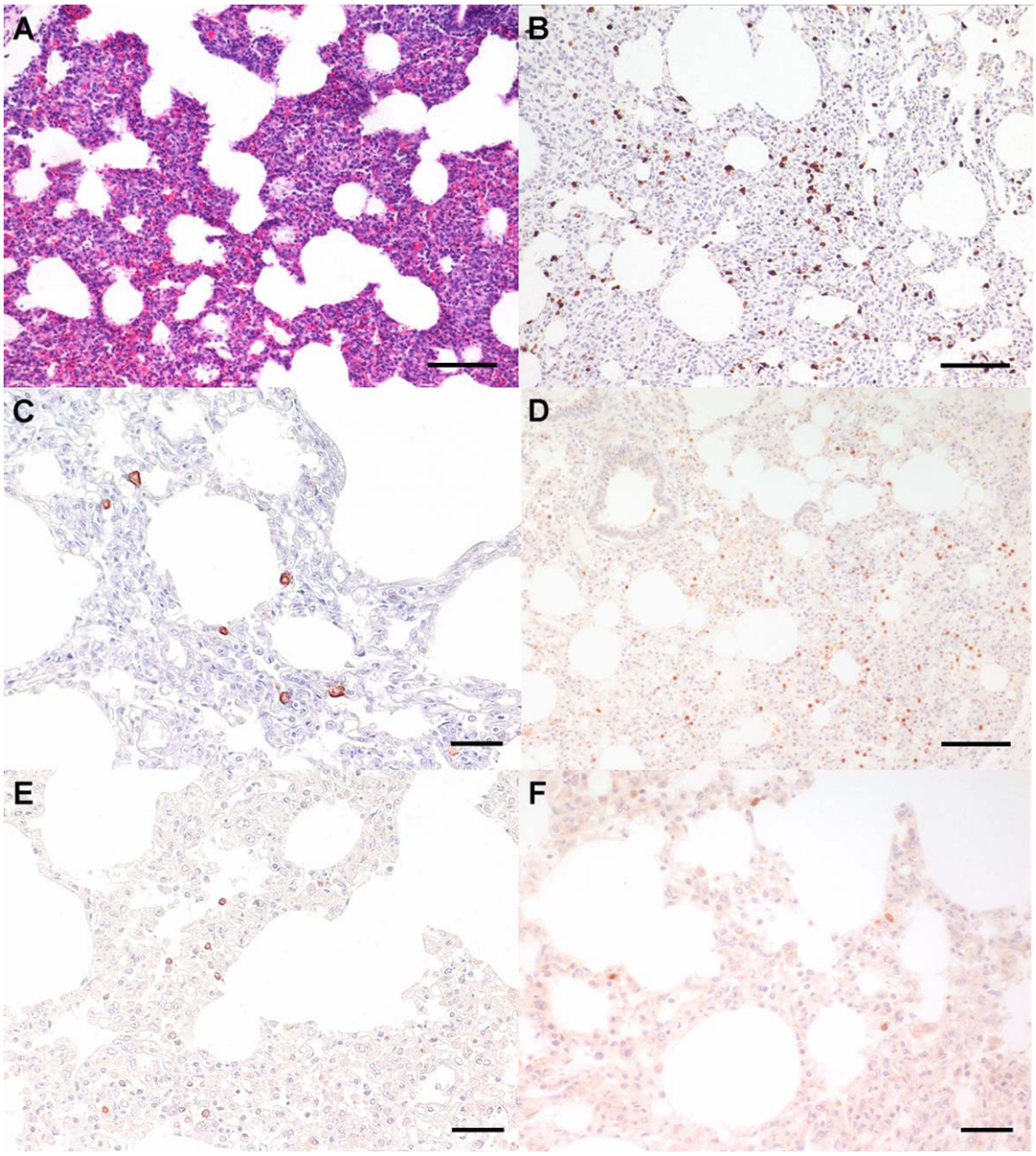


Fig.14

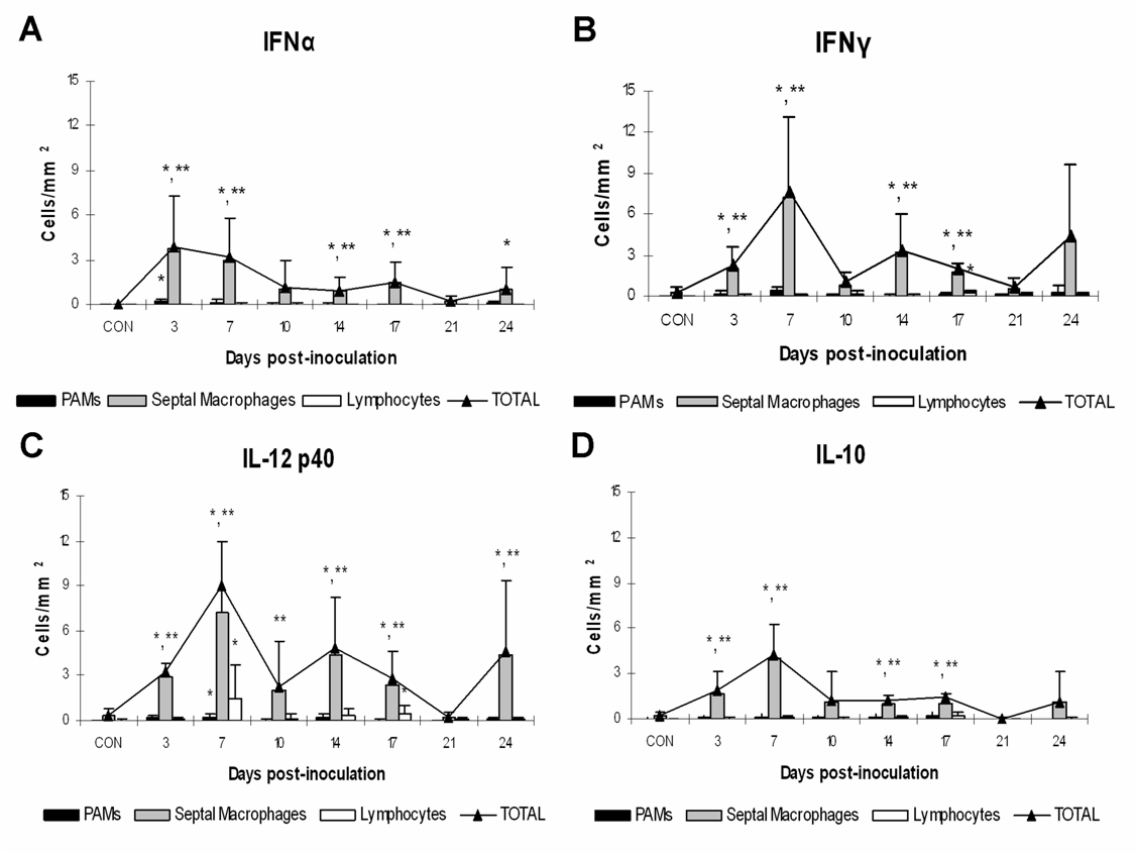
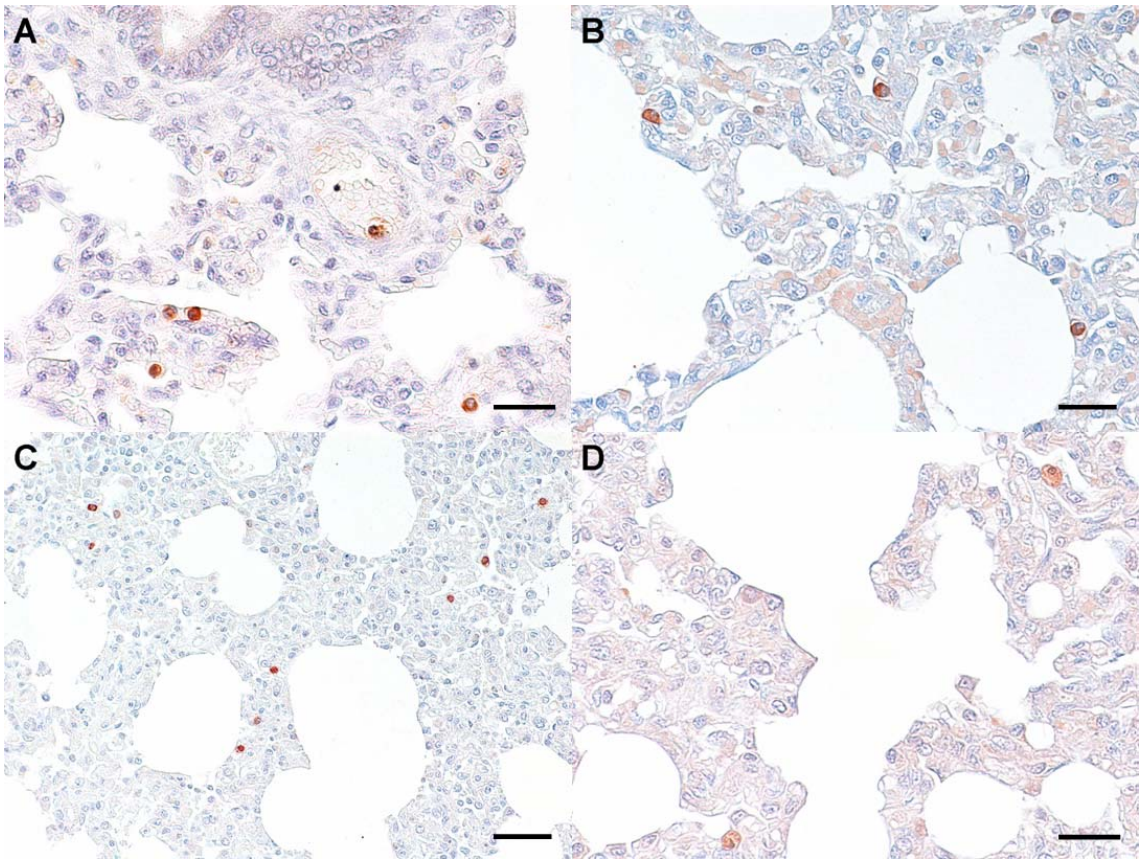

A
IFNα
B
IFNγ
C
IL-12 p40
D
IL-10
Cells/mm 2
Days post-inoculation
CON
3
7
10
14
17
21
24
PAMs
Septal Macrophages
Lymphocytes
TOTAL

Fig.15



4.

GENERAL DISCUSSION

GENERAL DISCUSSION

Deciphering how PRRSV modulates the host immune response is one of the most striking points nowadays in swine research. In this way, several reports have tried to elucidate how the host immune response is set up after PRRSV infection (Yoon *et al*., 1995; Loemba *et al*., 1996; Shimizu *et al*., 1996; Bautista and Molitor, 1997; Wills *et al*., 1997; Albina *et al*., 1998a, 1998b; Kawashima *et al*., 1999; López-Fuertes *et al*., 1999; Allende *et al*., 2000; Samsom *et al*., 2000; Lamontagne *et al*., 2001, 2003; Meier *et al*., 2003; Xiao *et al*., 2004; Díaz *et al*., 2006), although there is not yet conclusive results.

The genomic or protein expression of some cytokines has been studied by several authors after PRRSV infection or vaccination (Albina *et al*., 1994; Asai *et al*., 1999; Van Reeth *et al*., 1999; Van Reeth and Nauwynck, 2000; Chung and Chae, 2003; Suradhat and Thanawongnuwech, 2003; Thanawongnuwech and Thacker, 2003; Thanawongnuwech *et al*., 2003; Meier *et al*., 2004; Royaee *et al*., 2004; Carter and Curiel, 2005; Díaz *et al*., 2005, 2006). Nonetheless, few reports have related the expression of cytokines with their role in the immune response in PRRS. Therefore, the main goal of this thesis was to correlate the changes observed in serum concentration of cytokines in an early infection with a European PRRSV field isolate and the changes observed in lymphocyte subsets and in the inflammatory response evoked during the innate immune response.

Some reports have studied the CMI against PRRSV by determining the changes in the levels of IFNγ. In this sense, some authors have reported a correlation between a downregulation of IFNγ together with an overexpression of IL-10 (Díaz *et al*., 2005, 2006), other authors have found no significant changes in both or any of these cytokines (López-Fuertes *et al*., 1999; Sipos *et al*., 2003). On the other hand, the CMI response has also been studied determining the changes observed in lymphocyte subsets observed after PRRSV infection (Shimizu *et al*., 1996; Albina *et al*., 1998b; López-Fuertes *et al*., 1999; Samsom *et al*., 2000; Lamontagne *et al*., 2001; Xiao *et al*., 2004; Díaz *et al*., 2006). The high antigenic and pathogenic variability observed between PRRSV strains is considered as one of the main factors which may mediate the contradictory results obtained in these reports.

Changes in lymphocyte subsets of PBMCs have been reported by several authors throughout PRRSV infection (Shimizu *et al*., 1996; Albina *et al*., 1998b; López-Fuertes *et al*., 1999; Samsom *et al*., 2000; Lamontagne *et al*., 2001; Xiao *et al*., 2004; Díaz *et al*., 2006), otherwise, few studies have been carried out to determine these changes in lymphoid organs (Kawashima *et al*., 1999; Lamontagne *et al*., 2001; Xiao *et al*., 2004). The most significant changes observed in our experimental infection in PBMCs consisted on a generalised increase of $CD4^{+}CD8^{+}$ and $CD4^{-}CD8^{high}$ T cells together with a decrease of $CD4^{-}CD8^{low}$ T cells, pointing to a marked imbalance in helper/cytotoxic activity, which has been also previously suggested for US genotypes (Lamontagne *et*

al., 2003). Moreover, an enhancement in the counts of $CD4^{-}CD8^{high}$ T cells was observed at 10 dpi, when the viraemia began to decrease, suggesting a possible role of the cytotoxic activity of this T cell subset in the virus clearance.

The kinetics of lymphocyte subsets of lymphoid organs has been scarcely studied, displaying controversial results. Thus, after inoculating different US PRRSV strains, it has been pointed that either no changes for both $CD4^{+}$ and $CD8^{+}$ T cells (Xiao *et al*., 2004) or an increase in $CD4^{-}CD8^{high}$ T cells (Lamontagne *et al*., 2003) were observed in the examined lymphoid organs. In our study, a generalised increased in $CD4^{-}CD8^{high}$ T cells was observed from 7 dpi in all the studied lymphoid organs, but no cytotoxic response seems to be evoked after PRRSV infection (Lohse *et al*., 2004). Thus, the role of $CD4^{-}CD8^{high}$ T cells in the pathogenesis of the disease should be clarified.

PRRSV has been shown to fail in inducing *in vitro* $CD8^{+}$ T cells proliferation in PBMCs (Shimizu *et al*., 1996). Thus, any other immune effectors may be necessary to induce such enhancement in $CD8^{+}$ T cells. In our study IL-12 p40 and IL-10 displayed a peak at 10 dpi, just when the increase in $CD4^{-}CD8^{high}$ T cells was observed. Both IL-12 and IL-10 are regulatory cytokines known for stimulating $CD8^{+}$ T cells (Wolf *et al*., 1994; Moore *et al*., 2001; Pestka *et al*., 2004) and inhibiting $CD4^{+}$ T cells proliferation (Moore *et al*., 2001; Pestka *et al*., 2004). Therefore, the expression of both may be involved in the changes observed in the lymphocyte subsets during PRRSV infection.

The antiviral state induced by IFNα during the innate immune response, as well as, the neutralization or clearance of the virus by NAs or antigen-specific IFNγ-SC might be some of the mechanisms involved in PRRSV clearance (Yoon *et al*., 1995; Bautista and Molitor, 1999; Van Reeth and Nauwynck, 2000; Biron and Sen, 2001). Nonetheless, the poor expression of IFNα in PRRS with respect to other porcine respiratory viral diseases (Van Reeth and Nauwynck, 2000), the isolation of PRRSV in the presence of NAs (Vézina *et al*., 1996; Wills *et al*., 1997; Batista *et al*., 2004), and the inhibitory effect of IL-10 in the expression of IFNγ (Moore *et al*., 2001) points out that these mechanisms are not efficient inducing PRRSV clearance (Murtaugh *et al*., 2002).

The expression of INFα together with the detection of viraemia until the end of our study confirms an inefficient virus clearance. Moreover, in our studies IL-10 was expressed when lower levels of IFNγ were detected, increasing once the expression of IL-10 dropped, which shows the regulatory role of IL-10 on IFNγ during PRRSV infection.

Besides IFNγ, IL-10 is also able to inhibit the production of IL-1, IL-6, TNFα, as well as other cytokines (Moore *et al*., 2001). Therefore, it would be a reason for the poor serum expression of all three proinflammatory cytokines observed in our study, which has been previously reported (Van Reeth and Nauwynck, 2000; Van Reeth *et al*., 2002). Moreover, Van Reeth and co-authors (1999) reported an enhancement in IL-1 levels but not in TNFα in pigs infected with a EU genotype of PRRSV, whereas Asai and co-authors (1999) observed an

enhancement in IL-6 but not in TNFα expression in pigs infected with a US genotype. These differences observed between different genotypes point out that that the serum profile of proinflammatory cytokines may be useful in determining differences in the pathogenicity between PRRSV isolates.

Proinflammatory cytokines are the main immune mediators of the APR inducing the synthesis of APPs after a tissue injury (Eckersall, 2000; Ceciliani *et al.*, 2002; Petersen *et al.*, 2004; Gruys *et al.*, 2005). APPs are considered as biomarkers in monitoring animal welfare and determining the health status (Eckersall, 2000; Petersen *et al.*, 2004; Gruys *et al.*, 2005). However, rather limited studies have been focused on the expression of APPs during PRRS.

An increased in serum Hp concentration has been related to an enhancement in IL-6 but not TNFα expression during PRRSV infection (Asai *et al.*, 1999). However, our results showed an enhancement in the levels of Hp correlated with respect to the serum expression of TNFα but not to IL-1β or IL-6 serum expression. Moreover, the serum levels of TNFα were also correlated with the viraemia and with the expression of IL-6, although the mild expression of TNFα observed support the theory that this cytokine is inefficient in PRRSV clearance.

APPs showed a different kinetics throughout the infection with the PRRSV field isolate 2982. Hp and Pig-MAP were the earliest APPs synthesised after the mild enhancement observed in proinflammatory cytokines concentrations, coinciding with the highest titre of viraemia at 10 dpi. Otherwise, CRP and SAA

showed a delayed enhancement at 17 dpi. The delayed expression of some APPs may be due to the participation of regulatory factors, which also may affect the synthesis of proinflammatory cytokines. NF-κB is a transcription factor which activation is needed to the synthesis of cytokines. Therefore, a inhibition of NF-κB activation might be the responsible of the poor level of proinflammatory cytokines observed in PRRS, but PRRSV has been reported to activate NF-κB (Lee and Kleiboeker, 2005). Other mechanism which may be involved in the poor expression of proinflammatory cytokines may be the synthesis of regulatory cytokines, as IL-10 or TGFβ, which has been described above. PRRSV has been also reported to avoid the antigen presentation process, which may downregulate the synthesis of cytokines (Mateu and Díaz, 2008).

The lack of significant changes in serum levels of proinflammatory cytokines during our study contrast with the inflammatory response observed at lung level. This inflammatory response might be related with higher amounts of cytokines detected at the pulmonary parenchyma, whereas the serum concentration of these cytokines are generally low or undetectable (Baarsch *et al.*, 1995; Conn *et al.*, 1995). These statements indicate that proinflammatory cytokines would show a paracrine synthesis, not being observed a significant increased in the serum levels of these cytokines. To confirm this hypothesis, we carried out a last study to determine the expression *in situ* of cytokines in the lung of PRRSV-infected pigs.

PRRSV replicated mainly in PAMs of the lung of infected pigs displaying a peak at 7 dpi, just earlier than the peak of viraemia observed at 10 dpi. The lungs of infected pigs showed a marked thickening of the alveolar septa due to a severe infiltrate of macrophages. The role of PAMs and septal macrophages seems to differ a lot in PRRS, since PAMs were the main cells where PRRSV replicated and septal macrophages were mainly involved in the synthesis of cytokines.

Although a poor expression of proinflammatory cytokines was observed in serum, the expression IL-1α, IL6, and TNFα in the pulmonary parenchyma was significantly enhanced, showing a correlation with the degree of lung lesion and with the counts of macrophages, what point out to an activation of these cells. IL-1α was the cytokine mainly involved in the development of the interstitial pneumonia, whereas both IL-1α and TNFα induced an intra-alveolar neutrophils infiltrate and their activation. Serum expression of TNFα was correlated, in our study, with the serum expression of IL-6. However, no correlation was observed between the tissue expression of IL-1α and/or TNFα with respect to the tissue expression of IL-6, although the maximum expression of IL-6 temporally coincided with a higher expression of IL-1α and/or TNFα.

The antigen expression of PRRSV was correlated with the expression of IFNα antigen. PRRSV antigen was detected until the end of the study, pointing to a persistent viral replication which may induce the persistent viraemia characteristic of the disease. The persistent viral replication in the lung is also

indicative of an insufficient expression of IFNs to induce PRRSV clearance, which has been also proposed from the serum expression of IFNs. An enhanced expression of IFNγ in the lung of PRRSV-infected pigs has been previously documented (Thanawongnuwech *et al*., 2003). In our study, the expression of IFNγ antigen showed a peak at 7 dpi, coinciding with the of PRRSV replication in the lung. Therefore, IFNs are not expressed enough to induce viral clearance although may mediate a decrease in viral replication, which supports the findings shown *in vitro* by Bautista and Molitor (1999).

The expression of IFNγ may be induced by many other cytokines, namely IL-12, TNFα or IFNα (Nguyen and Benveniste, 2002; Mitchell and Kumar, 2004; Tizard, 2008). Both the expression of IL-12 p40 and TNFα, but not IFNα, was correlated in our study with the expression of respect to. Thus, IL-12 p40 and TNFα represented in our study the main stimulus for the synthesis of IFNγ. A previous study reported a correlation between virus specific-IFNα-SCs and virus specific-IFNγ-SCs (Royaee *et al*., 2004). The discrepancy shown with respect to our study may be due to the high antigenic and pathogenic differences observed between PRRSV genotypes (Halbur *et al*., 1995; Mateu *et al*., 2003; Stadejek *et al*., 2006).

The persistent viral replication observed in our study point to a not enough expression of IFNα, IFNγ, IL-12 p40 and TNFα to induce viral clearance. IL-10 was expressed in the lung of PRRSV-infected pigs showing a peak at 7 dpi, which coincided with the peak of viral replication in the lung. These results point

to a modulation of the expression of IL-10 by PRRSV, which leads to lower levels of IFNα, IFNγ, IL-12 p40 and TNFα (Moore *et al.*, 2001), as it has been also proposed above to the serum expression of these cytokines.

DISCUSIÓN GENERAL

Uno de los puntos más importantes en la investigación en sanidad animal consiste en elucidar como el VPRRS modula la respuesta inmune del hospedador. En este sentido, diversos estudios han intentado determinar como se activa la respuesta inmune del hospedador tras la infección con el VPRRS (Yoon *et al*., 1995; Loemba *et al*., 1996; Shimizu *et al*., 1996; Bautista y Molitor, 1997; Wills *et al*., 1997; Albina *et al*., 1998a, 1998b; Kawashima *et al*., 1999; López-Fuertes *et al*., 1999; Allende *et al*., 2000; Samsom *et al*., 2000; Lamontagne *et al*., 2001, 2003; Meier *et al*., 2003; Xiao *et al*., 2004; Díaz *et al*., 2006), aunque todavía no existen resultados concluyentes.

La expresión génica y proteica de algunas citoquinas ha sido estudiada por varios autores tras la infección o vacunación frente al VPRRS (Albina *et al*., 1994; Asai *et al*., 1999; Van Reeth *et al*., 1999; Van Reeth and Nauwynck, 2000; Chung y Chae, 2003; Suradhat y Thanawongnuwech, 2003; Thanawongnuwech y Thacker, 2003; Thanawongnuwech *et al*., 2003; Meier *et al*., 2004; Royaee *et al*., 2004; Carter y Curiel, 2005; Díaz *et al*., 2005, 2006). No obstante, son escasos los estudios que han intentado relacionar directamente la expresión de citoquinas y su papel en la respuesta inmune en el PRRS. De este modo, el principal objetivo de esta tesis consistió en correlacionar los cambios observados en la concentración sérica de citoquinas durante la infección temprana con un aislado de campo del VPRRS con los

cambios observados en las subpoblaciones de linfocitos y en la respuesta inflamatoria desencadenada durante la respuesta inmune innata.

Varios estudios se han centrado en determinar la respuesta inmune de base celular desarrollada tras la infección con el VPRRS a través de los cambios observados en los niveles de IFNγ. En este sentido, algunos autores han descrito una correlación entre una bajo regulación de la expresión de IFNγ con una sobre expresión de la IL-10 (Díaz *et al*., 2005, 2006). Sin embargo, otros autores no han observado cambios significativos en estas citoquinas (López-Fuertes *et al*., 1999; Sipos *et al*., 2003). Por otro lado, la respuesta inmune de base celular mediada también ha sido estudiada determinando los cambios observados en las subpoblaciones de linfocitos tras la infección con el VPRRS (Shimizu *et al*., 1996; Albina *et al*., 1998b; López-Fuertes *et al*., 1999; Samsom *et al*., 2000; Lamontagne *et al*., 2001; Xiao *et al*., 2004; Díaz *et al*., 2006). La elevada variabilidad antigénica y patogénica entre distintas cepas del VPRRS es considerada como uno de los principales factores implicados en la obtención de resultados contradictorios en diferentes estudios.

Los cambios en las subpoblaciones de CMSPs a lo largo de la infección por el VPRRS han sido descritos por varios autores (Shimizu *et al*., 1996; Albina *et al*., 1998b; López-Fuertes *et al*., 1999; Samsom *et al*., 2000; Lamontagne *et al*., 2001; Xiao *et al*., 2004; Díaz *et al*., 2006), por el contrario, pocos estudios se han centrado en determinar dichos cambios en los órganos linfoides (Kawashima *et al*., 1999; Lamontagne *et al*., 2001; Xiao *et al*., 2004).

Los cambios más significativos observados en nuestra infección experimental en CMSPs consistieron en un aumento generalizado de linfocitos T $CD4^{+}CD8^{+}$ y $CD4^{-}CD8^{high}$ junto a un descenso de los linfocitos T $CD4^{-}CD8^{low}$, indicando un marcado desequilibrio entre los linfocitos colaboradores y los linfocitos citotóxicos, lo cual también ha sido previamente sugerido para genotipos americanos (Lamontagne *et al.*, 2003). Además, se observó un aumento en el recuento de linfocitos T $CD4^{-}CD8^{high}$ a los 10 dpi, justo cuando la viremia comenzó a descender, sugiriendo un posible papel de la actividad citotóxica de estos linfocitos en la eliminación del virus.

La cinética de las subpoblaciones de linfocitos en los órganos linfoides ha sido escasamente estudiada, dando lugar a resultados contradictorios. Así, tras la inoculación de diferentes cepas americanas del VPRRS, se ha señalado que en los órganos linfoides analizados o bien no se producen cambios en las subpoblaciones de linfocitos T $CD4^{+}$ y $CD8^{+}$ (Xiao *et al.*, 2004) o bien se produce un aumento de los linfocitos T $CD4^{-}CD8^{high}$ (Lamontagne *et al.*, 2003). En nuestro estudio, se observó un aumento generalizado de los linfocitos T $CD4^{-}CD8^{high}$ T desde los 7 dpi en todos los órganos linfoides estudiados, pero no se desencadena una respuesta citotóxica tras la infección por el VPRRS (Lohse *et al.*, 2004), por lo que debería estudiarse qué papel desempeñan estas células en la patogenia de la enfermedad.

El VPRRS es incapaz de inducir *in vitro* la proliferación de linfocitos T $CD8^{+}$ (Shimizu *et al.*, 1996). De este modo, sería necesario algún otro efector

inmunitario para inducir el aumento de linfocitos T $CD8^+$ observado en la enfermedad. En nuestro estudio la IL-12 p40 y la IL-10 mostraron un incremento a los 10 dpi, coincidiendo con un aumento en el recuento de linfocitos T $CD4^-CD8^{high}$. Tanto la IL-12 como la IL-10 son citoquinas conocidas por estimular la proliferación de linfocitos T $CD8^+$ (Wolf *et al*., 1994; Moore *et al*., 2001; Pestka *et al*., 2004) e inhibir la proliferación de linfocitos T $CD4^+$ (Moore *et al*., 2001; Pestka *et al*., 2004). Por lo tanto, la expresión de ambas citoquinas puede estar implicada en los cambios observados en las subpoblaciones de linfocitos a lo largo de la infección con el VPRRS.

El estado antiviral inducido por el IFNα durante la respuesta inmune innata, al igual que la neutralización o eliminación del virus a través de anticuerpos neutralizantes (ANs) o de células secretoras de IFNγ antígeno específicas podrían ser algunos de los mecanismos involucrados en la eliminación del VPRRS (Yoon *et al*., 1995; Bautista y Molitor, 1999; Van Reeth y Nauwynck, 2000; Biron y Sen, 2001). Sin embargo, la pobre expresión de IFNα observada en el PRRS en comparación con otras enfermedades víricas respiratorias porcinas (Van Reeth y Nauwynck, 2000), el aislamiento del VPRRS en presencia de ANs (Vézina *et al*., 1996; Wills *et al*., 1997; Batista *et al*., 2004), y el efecto inhibidor de la IL-10 sobre la expresión de IFNγ (Moore *et al*., 2001) señalan que estos mecanismos no son suficientemente eficientes para inducir la eliminación del VPRRS (Murtaugh *et al*., 2002).

La pobre expresión de IFNα junto a la detección de la viremia hasta el final de nuestro estudio, confirma una eliminación ineficaz del virus. Asimismo, en nuestro caso la IL-10 se expresó cuando se observaron niveles más bajos de IFNγ, los cuales aumentaron una vez que la expresión de IL-10 disminuyó, lo cual demuestra el papel regulador de la IL-10 sobre la síntesis de IFNγ a lo largo de la infección con el VPRRS.

Aparte del IFNγ, la IL-10 es capaz de inhibir la producción de IL-1, IL-6, TNFα, así como otras citoquinas (Moore *et al*., 2001). De este modo, se justificaría la pobre expresión de las tres citoquinas proinflamatorias observada en nuestro estudio a nivel sérico, lo que coincide con estudios previos (Van Reeth y Nauwynck, 2000; Van Reeth *et al*., 2002). Además, Van Reeth y colaboradores (1999) describieron un aumento en los niveles de IL-1 pero no de TNFα en cerdos infectados con un genotipo europeo del VPRRS, mientras que en el mismo año, Asai y colaboradores (1999), describieron un aumento en la expresión de IL-6 pero no de TNFα en cerdos infectados con un genotipo americano del virus. Estas diferencias observadas entre diferentes genotipos señalan que el seroperfil de las citoquinas proinflamatorias puede representar una herramienta útil para determinar diferencias en la patogenicidad entre diferentes aislados del VPRRS.

Las citoquinas proinflamatorias son los principales mediadores inmunes de la RFA induciendo la síntesis de PFA tras un daño tisular (Eckersall, 2000; Ceciliani *et al*., 2002; Petersen *et al*., 2004; Gruys *et al*., 2005). Las PFAs son

consideradas como biomarcadores útiles para el monitorizaje del bienestar animal y para determinar el estatus sanitario de un individuo o granja (Eckersall, 2000; Petersen *et al.*, 2004; Gruys *et al.*, 2005). Sin embargo, muy pocos estudios se han centrado en la expresión de PFAs en el transcurso del PRRS.

Un aumento en la concentración sérica de la Hp se ha relacionado con un incremento en la expresión de IL-6 pero no de TNFα a lo largo de la infección con el VPRRS (Asai *et al.*, 1999). Sin embargo, nuestros resultados demuestran un aumento en los niveles de Hp correlacionado con la expresión sérica de TNFα pero no con los niveles en suero de IL-1β o de IL-6. Además, la expresión sérica de TNFα también estaba correlacionada con el título del virus y con la expresión de IL-6, aunque los bajos niveles de expresión de TNFα observada apoya la teoría de que esta citoquinas es ineficaz en la eliminación del VPRRS.

Las PFAs desarrollaron una cinética diferente en el transcurso de la infección con el aislado de campo 2982 del VPRRS. La Hp y la Pig-MAP fueron las PFAs que se sintetizaron de manera más temprana tras el leve aumento observado en la concentración sérica de las citoquinas proinflamatorias. Tanto la Hp como la Pig-MAP estaban aumentadas a los 10 dpi coincidiendo con el pico observado en la viremia. Por el contrario, la PCR y la AAS mostraron un aumento tardío a los 17 dpi. La expresión tardía de algunas PFAs podría deberse a la participación de factores reguladores, que podrían afectar

igualmente a la síntesis de citoquinas proinflamatorias. El NF-κB es un factor de trascripción cuya activación es necesaria para la síntesis de citoquinas. De este modo, la inhibición del NF-κB podría ser el responsable de los bajos niveles de citoquinas proinflamatorias observados en el PRRS, no obstante, se ha demostrado que el VPRRS es capaz de activar el NF-κB (Lee y Kleiboeker, 2005). Otro mecanismo que podría estar involucrado en la pobre expresión de citoquinas proinflamatorias podría ser la síntesis de citoquinas reguladoras, como la IL-10 o el factor transformador del crecimiento beta (TGFβ). También se ha descrito que el VPRRS es capaz de alterar el proceso de presentación antigénica, lo cual puede inducir una bajo regulación de la síntesis de citoquinas (Mateu y Díaz, 2008).

La ausencia de cambios significativos en los niveles séricos de citoquinas proinflamatorias durante nuestro estudio contrasta con la observación de una respuesta inflamatoria a nivel pulmonar. Esta respuesta inflamatoria local estaría relacionada con mayores niveles de citoquinas detectados en el parénquima pulmonar, y la concentración sérica de las citoquinas es generalmente baja o indetectable (Baarsch *et al*., 1995; Conn *et al*., 1995). Estos resultados indicarían que las citoquinas proinflamatorias se expresarían de forma paracrina, no observándose un aumento marcado de los niveles séricos de dichas citoquinas. Para confirmar esta hipótesis, se llevó a cabo un último estudio para determinar la expresión *in situ* de citoquinas en el pulmón de cerdos infectados con el VPRRS.

El VPRRS se replicó principalmente en MAPs del pulmón de cerdos infectados presentando un pico a los 7 dpi, justo antes del pico de viremia observado a los 10 dpi. Los pulmones de los cerdos infectados mostraron un marcado engrosamiento de los septos alveolares debido a un intenso infiltrado de macrófagos. El papel de los MAPs y de los macrófagos septales en el PRRS parece ser muy diferente, ya que mientras que en los MAPs se produce principalmente la replicación del virus, en los septales se observa principalmente la expresión de citoquinas.

Aunque en nuestro estudió se observó una pobre expresión sérica de las citoquinas proinflamatorias, la expresión de IL-1α, IL6, y TNFα en el parénquima pulmonar estaba significativamente incrementada, mostrando una correlación con el grado de lesión pulmonar y con el recuento de macrófagos, considerado como indicativo de activación de estas células. La IL-1α fue la citoquina principalmente implicada en el desarrollo de la neumonía intersticial, mientras que tanto la IL-1α como el TNFα fueron las responsables del infiltrado intra-alveolar de neutrófilos observado y de su activación. La expresión sérica de TNFα estaba correlacionada, en nuestro estudio, con la expresión sérica de IL-6. Sin embargo, no se observó ninguna correlación entre la expresión tisular de IL-1α y/o TNFα con respecto a la expresión de IL-6, aunque la expresión máxima de IL-6 coincidió temporalmente con una mayor expresión de IL-1α, TNFα o ambos.

La expresión del antígeno del VPRRS estaba correlacionada con la expresión del antígeno del IFNα. El antígeno del VPRRS se detectó hasta el final del estudio, indicando la presencia de una replicación vírica persistente que podría inducir la viremia persistente carcterística de la enfermedad. Esta replicación vírica persistente en el pulmón es indicativa de una expresión insuficiente de IFNs para lograr la eliminación del VPRRS, lo cual ya se había observado en la expresión sérica de los IFNs. Un aumento en la expresión de IFNγ en el pulmón de cerdos infectados con el VPRRS ha sido descrita previamente (Thanawongnuwech *et al*., 2003). En nuestro estudio, la expresión del antígeno del IFNγ mostró un pico a los 7 dpi, coincidiendo con el pico observado en el pulmón para la replicación del virus, por lo que aunque se producen IFNs, su expresión no sería suficiente como para inducir la eliminación del virus, aunque sí que podrían mediar en una disminución de la replicación del virus, lo cual apoya los hallazgos previos observados *in vitro* por Bautista y Molitor (1999).

La expresión de IFNγ se puede inducir por muchas otra citoquinas, como IL-12, TNFα o IFNα (Nguyen y Benveniste, 2002; Mitchell y Kumar, 2004; Tizard, 2008). Tanto la expresión de IL-12 p40 como la de TNFα, pero no la de IFNα, estaban correlacionadas en nuestro estudio con la expresión de IFNγ. Así, IL-12 p40 y TNFα representaron el principal estímulo para la síntesis de IFNγ. En un estudio previo se describe una correlación entre las células secretoras de IFNα y las células secretoras de IFNγ (Royaee *et al*., 2004). Las

diferencias mostradas entre este estudio y el nuestro pueden deberse a la elevada variabilidad antigénica y patogénica observada entre distintos genotipos del VPRRS (Halbur *et al*., 1995; Mateu *et al*., 2003; Stadejek *et al*., 2006).

La replicación vírica persistente observada en nuestro estudio indica una expresión insuficiente de IFNα, IFNγ, IL-12 p40 y TNFα para inducir la eliminación del virus. La IL-10 se expresó en el pulmón de cerdos infectados con el VPRRS mostrando un pico a los 7 dpi, lo que coincidió con el pico de replicación del virus observado en el pulmón. Estos resultados señalan una modulación de la expresión de la IL-10 por el VPRRS, lo cual conduce a niveles más bajos de IFNα, IFNγ, IL-12 p40 y TNFα (Moore *et al*., 2001), como también habíamos propuesto anteriormente para la expresión sérica de estas citoquinas.

5.

CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

- Porcine Reproductive and Respiratory Syndrome Virus field isolate 2982 induces an enhancement of interleukin-12 and interleukin-10, which is correlated with a peak of $CD4^{-}CD8^{high}$ T cells and with the drop of the viraemia.
- Interleukin-10 serum expression coincides with low serum levels of gamma interferon, in spite of the expression of interleukin-12 and alpha interferon, therefore this cytokine play an immunomodulatory role inhibiting gamma interferon.
- Porcine Reproductive and Respiratory Syndrome Virus field isolate 2982 induces a poor serum expression of both acute phase proteins and proinflammatory cytokines during the acute phase response to the infection, being detected only a mild enhancement of interleukin-1β serum levels together with a moderate increase in haptoglobin and Pig-MAP serum concentrations.
- Serum concentration of tumor necrosis factor-alpha is correlated with viral load, interleukin-6 and haptoglobin expression in Porcine Reproductive and Respiratory Syndrome, thus, this cytokine plays a significant role in the modulation of the immune response against Porcine Reproductive and Respiratory Syndrome Virus.

- Activation of septal macrophages and pulmonary alveolar macrophages differs throughout Porcine Reproductive and Respiratory Syndrome Virus infection, playing the first ones a main role in the synthesis and release of cytokines at the local area of tissue injury, whereas the second ones are mainly involved in the viral replication.
- Proinflammatory cytokines are mainly expressed in the lung parenchyma but not in the serum of Porcine Reproductive and Respiratory Syndrome Virus-infected pigs, displaying a paracrine synthesis, which displayed mild serum levels of these cytokines.
- Proinflammatory cytokines play a significant role in the pathogenesis of the interstitial pneumonia observed during Porcine Reproductive and Respiratory Syndrome, being directly correlated their expression with the infiltration of the alveolar septa by macrophages.
- Porcine Reproductive and Respiratory Syndrome Virus seems to modulate the immune response by the expression of interleukin-10 by macrophages, which may be the responsible of lower levels of other cytokines implied in viral clearance, just as alpha interferon, gamma interferon, interleukin-12 p40 and tumor necrosis factor-alpha.

CONCLUSIONES

- El aislado de campo 2982 del virus del Síndrome Reproductivo y Respiratorio Porcino induce un aumento de los niveles séricos de interleuquina-12 e interleuquina-10, coincidiendo con un pico en el recuento de células T $CD4^{-}CD8^{high}$ y con una disminución de la viremia.
- La expresión sérica de interleuquina-10 coincide con bajos niveles séricos de interferón gamma, a pesar de la expresión de interleuquina-12 e interferón alfa, por lo que esta citoquina jugaría un papel inmunomodulador inhibiendo la síntesis de interferón gamma.
- El aislado de campo 2982 del virus del Síndrome Reproductivo y Respiratorio Porcino induce una pobre expresión sérica tanto de proteínas de fase aguda como de las citoquinas proinflamatorias durante la respuesta de fase aguda a la infección, siendo detectado únicamente un leve aumento de la concentración sérica de interleuquina-1β junto a un moderado incremento en los niveles séricos de haptoglobina y Pig-MAP.
- Los niveles séricos del factor de necrosis tumoral-alfa están correlacionados con la viremia, y con la expresión sérica de interleuquina-6 y haptoglobina en el PRRS, por lo que esta citoquina tiene un importante papel en la modulación de la respuesta inmune frente al virus del Síndrome Reproductivo y Respiratorio Porcino.

- La activación de los macrófagos septales y de los macrófagos alveolares difiere a lo largo de la infección con el virus del Síndrome Reproductivo y Respiratorio Porcino, desempeñando los primeros un papel fundamental en la síntesis y liberación de citoquinas a nivel local en las áreas de daño tisular, mientras que los segundos son las células en las que principalmente se replica el virus.
- Las citoquinas proinflamatorias se expresan principalmente a nivel del parénquima pulmonar pero no en el suero de cerdos infectados con el virus del Síndrome Reproductivo y Respiratorio Porcino, representando una síntesis paracrina, que dará lugar a niveles séricos bajos de dichas citoquinas.
- Las citoquinas proinflamatorias desempeñan un papel importante en la patogenia de la neumonía interstiticial observada durante Síndrome Reproductivo y Respiratorio Porcino, estando su expresión directamente correlacionada con el infiltrado de macrófagos observado a nivel de los septos alveolares.
- El virus del Síndrome Reproductivo y Respiratorio Porcino parece modular la respuesta inmune a través de la expresión de interleuquina-10 por los macrófagos, la cual podría ser la responsable de niveles más bajos de otra citoquinas implicadas en la eliminación del virus, como interferón alfa, interferón gamma, interleuquina-12 p40 y factor de necrosis tumoral-alfa.

6.

FUTURE ASPECTS

FUTURE ASPECTS

Future studies should include different strains from both EU and US genotypes, due to the variability observed between genotypes, to try deciphering common pathways involved in the establishment of the immune response, with the aim to establish a more versatile and efficient control of the disease.

In our studies, both IL-10 and TNFα played a significant role in the modulation of the immune response against the PRRSV field isolate 2982. The use of tools which allow the blockade of the expression of IL-10 and an upregulation in the expression of TNFα, would lead to get new knowledge about how these cytokines acts in the immune response evoked against PRRSV. Thus, the use of specific antibodies would block the expression of IL-10, and therefore avoid the inhibition induced by IL-10 of the expression of several cytokines. In this sense, Charentantanakul *et al*. (2006) observed an enhancement in the expression of IFNγ and TNFα in PBMCs inoculated with several isolates of PRRSV after a neutralization assay of IL-10, by means a specific monoclonal antibody. Nonetheless, nowadays there is lack of a similar study in vivo, which may confirm the immunoregulatory effect of IL-10 in the expression of other cytokines, after its blockade by means specific antibodies.

A strategy developed by pathogens to evade the host immune response consists on the induction of a regulatory response associated to a suppression

of the host effector immune response. This regulatory response may be induced either directly by immunomodulatory cytokines produced by cells of the innate immune response, as IL-10 or TGF-β, or indirectly by regulatory cells (Belkaid, 2007). Regulatory T cells (Treg) ($CD4^+CD25^+FOXP3^+$) are included among these cells, and may be classified as natural or induce Treg, if they are originated before or after the exposition to the pathogen, respectively (Belkaid, 2007). Treg are able to interact with dendritic cells form lymph node and inhibit the activation of effector T cells, leading to an inhibition of the CMI response (Tang *et al.*, 2006). The suppressive activity of Treg may be helped by the production of specific cytokines, just as IL-10 and TGF-β (von Boehmer, 2005).

Some viral diseases, just as the infection by human immunodeficiency virus (HIV), an increase in the counts of induced Treg has been reported together with a decrease in circulatory natural Treg(Andersson *et al.*, 2005). The role of Treg in the pathogenesis of HIV infection has been observed in *in vitro* studies, which showed an enhancement of the specific immune response against this virus after blocking $CD4^+CD25^+$ T cells in PBMCs (Kinter *et al.*, 2004). Moreover, when a *in vitro* depletion of Treg in the blood stream of patients infected by hepatitis C virus was carried out an increase in antigen-specific $CD8^+$ T cells (Sugimoto *et al.*, 2003).

Among the results obtained in this thesis, a lack of changes in $CD4^+$ T cells, as well as, a moderate increase in $CD8^{high}$ and $CD4^+CD8^+$ T cells, together with the expression of IL-10 and IL-12 was observed. The lack of an efficient CMI

response against PRRSV might be associated to an increase in Treg subset. The increase in the counts of Treg, might be related with the expression of IL-10, representing therefore one of the main mediators involved in the inhibition of an efficient immune response. For these reasons, the study of the role of Treg throughout the infection by PRRSV, as well as their relationship with the profile of cytokines expressed during the disease represent an interesting field for future studies.

Antisense phosphorotioate oligodeoxynucleotides (AS ODNs), also known as RNA interference (RNAi), is able to inhibit protein synthesis by a specific inhibition of the gen expression which codifies a determined protein, using two different mechanisms, by means the target messenger RNA (mRNA) degradation by the ribonuclease Rnase-H and by the blockade of translation (Chiang *et al.*, 1991). In this sense, Sidahmed and Wilkie (2007) observed a decreased in the genomic and protein expression of both IL-10 and IFNγ when a specific AS ODNs corresponding to the AUG initiation codon of both IL-10 and IFNγ mRNA. However, these authors did not evaluate the effect of specific AS ODNs for IL-10 on the expression of other cytokines. Due to the significant role that IL-10 seems to play in the pathogenesis of PRRS, the use of specific AS ODNs for this cytokines might represent an useful tool to diminish the expression of IL-10 and therefore induce a higher expression of antiviral cytokines, just as IFNs.

ASPECTOS FUTUROS

Debido a la variabilidad existente entre distintos genotipos, sería de interés que en futuros estudios se incluyeran diferentes cepas de ambos genotipos, europeo y americano, para intentar descifrar mecanismos comunes que conduzcan a la instauración de la respuesta inmune, con el objetivo de poder establecer un control más amplio y eficaz frente a la enfermedad.

En nuestros estudios, tanto la IL-10 como el TNFα desempeñaron un papel importante en la modulación de la respuesta inmune frente al aislado de campo 2982 del VPRRS. El empleo de distintas herramientas que permitan bloquear la expresión de IL-10 e inducir una sobre expresión del TNFα, permitiría obtener nuevos conocimientos sobre cómo estas citoquinas participan en la respuesta inmune desencadenada frente al VPRRS. Así, el empleo de anticuerpos específicos permitiría bloquear la expresión de IL-10, y de esta forma evitar la inhibición en la síntesis de diversas citoquinas inducida por la IL-10. En este sentido, Charentantanakul *et al*. (2006) observaron un aumentó en la expresión de IFNγ y de TNFα en CMSPs inoculadas con distintos aislados del VPRRS tras la neutralización de la IL-10, utilizando un anticuerpo monoclonal específico frente a la IL-10 porcina. No obstante, hoy día no se ha llevado a cabo ningún estudio similar *in vivo*, en el que se confirme el efecto inmunomodulador de la IL-10 sobre la expresión de otras citoquinas, tras bloquear su expresión mediante anticuerpos específicos.

Una de las estrategias utilizadas por los patógenos para evadir la respuesta inmune del hospedador consiste en la inducción de una respuesta reguladora asociada con una supresión de la respuesta inmune efectora del hospedador. Esta respuesta reguladora se puede inducir directamente a través de citoquinas inmunomoduladoras producidas por células de la respuesta inmune innata, como la IL-10 o el TGF-β, o indirectamente a través de la generación de células reguladoras (Belkaid, 2007). Entre estas células se encuentran los linfocitos T reguladores (Treg) ($CD4^+CD25^+FOXP3^+$), que se pueden clasificar como Treg naturales o como Treg inducidos, según se originen antes o después de la exposición al patógeno, respectivamente (Belkaid, 2007). Los Treg son capaces de interaccionar con las células dendríticas de los nódulos linfáticos inhibiendo la activación de células T efectoras por estas últimas, lo que conduciría a una inhibición de la respuesta inmune de base celular (Tang *et al.*, 2006). La acción supresora de los Treg también se puede ver favorecida por la producción de determinadas citoquinas, como la IL-10 y el TGF-β (von Boehmer, 2005).

En determinadas enfermedades víricas, como la infección con el virus de la inmunodeficiencia humana (VIH), se ha descrito un incremento en el número de Treg inducidos en tejidos linfoides junto a una disminución de Treg naturales en circulación (Andersson *et al.*, 2005). El papel de los Treg en la patogenia de la infección con el VIH se ha puesto de manifiesto en estudios *in vitro*, en los que se observó un aumento de la respuesta inmune específica frente al virus al

inhibir los linfocitos T $CD4^+CD25^+$ en leucocitos de sangre periférica (Kinter *et al.*, 2004). Asimismo, al inducir *in vitro* una depleción en el número de Treg en células sanguíneas de individuos infectados con el virus de la hepatitis C se observó un aumento de células T $CD8^+$ antígeno-específicas (Sugimoto *et al.*, 2003).

Entre los resultados obtenidos de esta tesis se ha observado una ausencia de cambios en linfocitos T $CD4^+$, así como un ligero incremento de los linfocitos T $CD8^{high}$ y $CD4^+CD8^+$, junto con un aumento de la expresión de interleuquina (IL-10) e interleuquina (IL-12). La ausencia de una respuesta inmune de base celular eficaz frente al VPRRS, podría estar asociada con un aumento de las poblaciones de Tregs. El incremento en el número de Tregs, podría estar a su vez relacionado con la expresión de IL-10, siendo así esta interleuquina uno de los principales mediadores involucrados en la inhibición de una respuesta inmune eficaz. Por ello, el estudio del papel de los Treg en la infección con el VPRRS, así como su relación con el perfil de citoquinas observado en la enfermedad representa un área de interés a tener en cuenta en futuros estudios.

El fosforotioato de oligodesoxinucleótidos antisentido (AS ODNs), también llamado ARN de interferencia (RNAi), es capaz de inducir la inhibición de la síntesis de una proteína inhibiendo de forma específica la expresión del gen correspondiente que la codifica, mediante dos mecanismos, la degradación del ARN mesanjero (ARNm) diana por la ribonucleasa H-Rnasa y mediante el

bloqueo de la translación (Chiang *et al.*, 1991). En este sentido, Sidahmed y Wilkie (2007) observaron que al utlizar AS ODNs correspondientes al codón de inciación AUG tanto del ARNm de la IL-10 como del IFNγ se produjo una disminución en la expresión tanto del ARNm como de la proteína de ambas citoquinas, respectivamente. Sin embargo, estos autores no estudiaron el impacto de AS ODNs específicos para la IL-10 sobre la expresión de otras citoquinas. Teniendo en cuenta el importante papel que parece jugar la IL-10 en la patogenia del PRRS, el empleo de AS ODNs específicos frente a esta citoquina podría representar una herramienta útil para conseguir disminuir la expresión de la IL-10 y de esta forma conseguir una mayor expresión de determinadas citoquinas antivirales, como pueden ser los IFNs.

8.

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The results obtained form the studies carried out in this thesis are being nowadays reviewed for its publication in journals belonging to the fields of virology and veterinary science, and have been presented as communications to international and national meetings. The most significant contribution are the following:

Gómez-Laguna J., Salguero F.J., Fernández de Marco M., Pallarés F.J., Medina A., Bernabé A., Carrasco L. **IFN gamma expression in an early infection with a European pathogenic PRRS strain.** Poster. *25th Annual Meeting of the European Society of Veterinary Pathology (ESVP)*. Munich, Germany. 29th August to 1st September 2007.

Barranco I., *Gómez-Laguna J.*, Salguero F.J., Pallarés F.J., Fernández de Marco M., Bernabé A., Carrasco L. **Proinflammatory cytokines expression in the lung of Porcine Reproductive and Respiratory Syndrome Virus-infected pigs.** Poster. *XX Meeting of Sociedad Española de Anatomía Patológica (SEAPV)*. Breña Baja (La Palma), Canary Islands, Spain. 18th-21st June 2008.

Gómez-Laguna J., Salguero F.J., Pallarés F.J., González A., Fernández de Marco M., Bernabé A., Carrasco L. **Correlation between changes in lymphocyte subsets in blood and peripheral lyumphoid tissues in pigs infected experimentally with a European field strain of Porcine**

Reproductive and Respiratory Syndrome Virus. Oral Communication. *20th International Pig Veterinary Society Congress (IPVS)*. Durban, South Africa. 22nd-26th, June 2008.

Gómez-Laguna J., Salguero F.J., Pallarés F.J., Barranco I., Martínez-Subiela S., Cerón J.J., Carrasco L. **Acute phase proteins expression un pigs experimentally infected with a European field strain of Porcine Reproductive and Respiratory Syndrome Virus and their relationship with cytokines expression.** Poster. *20th International Pig Veterinary Society Congress (IPVS)*. Durban, South Africa. 22nd-26th, June 2008.

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CURRICULUM VITAE

Los resultados obtenidos de los estudios llevados a cabo en esta tesis doctoral están siendo actualmente revisados para su publicación en revistas indexadas pertenecientes a los campos de virología y ciencias veterinarias, y han sido presentados como comunicaciones en congresos nacionales e internacionales. Las contribuciones más importantes se detallan a continuación:

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