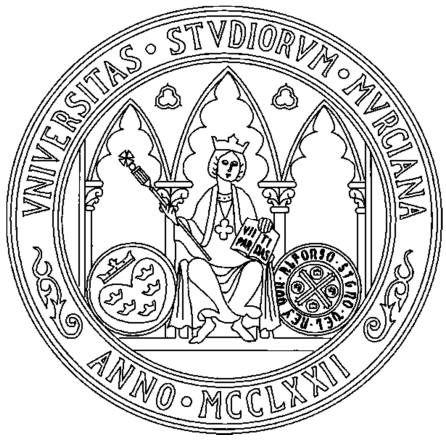

UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

In vivo and *in vitro* studies on some aspects of the innate immune response against PRRSV

Estudios *in vivo* e *in vitro* sobre algunos aspectos de la respuesta inmunitaria innata frente al virus PRRS

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A mi familia, Lorena e Isabela

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Index

List of abbreviations

Ab: antibodies

ADV: Aujeszky's Disease virus

AIR: adaptive immune response

APC: antigen presenting cells

bp: base pair

CBP: CREB binding protein

CD: Cluster of Differentiation

CPE: cytopatic effect

Ct: Cycle threshold

CV: Coefficient of variation

DAB: 3,30-diaminobenzidine tetrahydrochloride

DC: Dendritic cell

DMEM: Dulbecco's modified Eagle's medium

E: Efficiency

F: Follicle F

FD: Factor of Discrimination

fDC: follicular DC

HMC-I: histocompatibility complex class I

HMC-II: histocompatibility complex class II

HP: highly pathogenic

hpi: hours post-infection

HS: heparan sulphate glycosaminoglycans

IF: Inter-follicular

IFN: Interferon

IFN-I: type I IFN

IFN-II: type II IFN

IRF: Interferon Regulatory Factor

IHC: Immunohistochemistry

IIR: innate immune response

IKK: inhibitor-κB kinase

IL: Interleukin

IPMA: immunoperoxidase monolayer assay

IRF3: interferon regulatory factor 3

Kb: kilobase

KV: killed virus

LCM: laser capture microdissection

Lelystad virus: LV

LOD: limit of detection

mAb: monoclonal antibody

MACS: magnetic cell sorting system

mDC: myeloid DC

MDMØ: monocyte-derived MØ

Med-LN: mediastinal-LN

min: minutes

MLV: modified live virus

moDC: monocyte-derived dendritic cells

MOI: multiplicity of infection

mRNA: messenger RNA

MØ: macrophages

N: Nucleocapsid

NAb: Neutralazing antibodies

NF-κB: nuclear factor-κB

NK: Natural killer cells

nsp: non structural protein

ORF: open reading frame

PAM: porcine alveolar macrophages

PAMPs: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cells

PCV2: porcine circovirus type 2

pDC: plasmacytoid DC

pH: potential of Hydrogen

pp: polyprotein

PRRs: Pattern Recognition Receptors

PRRS: Porcine Reproductive and Respiratory Syndrome

PRRSV: PRRS virus

PRRSV-1: PRRSV genotype 1

PRRSV-2: PRRSV genotype 2

Rf-LN: retropharyngeal LN

RFS: -1 ribosomal frameshift

RPE: R-phycoerythrin

RT-qPCR: Reverse transcription quantitative polymerase chain reaction

s: seconds

SD: standard deviation

SOCS: Suppressor of cytokine signaling

SPF: specific pathogens free

s-quic: semi-quantitative immunelabelled cells

STAT-1: signal transducer and activator of transcription 1

Tb-LN: tracheobronchial lymph node

TBS: Tris buffered saline

Tc: cytotoxic T lymphocytes

TGF- β: transforming growth factor β

Th: helper T lymphocytes

TLR: Toll-Like Receptor

TNF-α: Tumor necrosis factor α

Treg: regulatory T lymphocytes

UTR: untranslated region

wt/vol: Relation weight/volume

Introduction & Objectives

Introduction

PRRS is characterized by respiratory disease in neonatal and growing pigs and reproductive failure in gilts and sows (increased number of abortions, mummified foetuses, stillbirth and weak-born piglets) (Hopper *et al*., 1992; Done and Paton, 1995; Rossow, 1998). PRRS was first described in USA in the latest 1980s as a swine infertility and reproductive syndrome or mystery swine disease (Keffaber, 1989). Later, at the beginnings of 1990s a similar syndrome was spread through Western Europe from a first detected outbreak in Germany (Ohlinger *et al*., 1991). The etiological agent that caused this syndrome, a novel virus, was isolated in 1991 in The Netherlands and it was called Lelystad virus (Wensvoort *et al*., 1991). Shortly after, in USA Benfield *et al*. (1992) characterized the virus called VR-2332 identified as etiological agent of PRRS. Nowadays the PRRS is worldwide spread with the characteristics of endemic in most of swine-producers countries (Albina, 1997; Blaha, 2000; Gao *et al*., 2004).

PRRS is considered one of the most economically important swine infectious diseases around the world. It has been calculated that PRRS generates $664 million annually in losses only in USA (Zimmerman *et al*., 2006; Holtkamp *et al*., 2013); in Europe it has been established a €126 mean loss per sow during PRRS outbreak (Nieuwenhuis *et al*., 2012). These economical losses are mostly due to a decrease in productivity and an increase in the morbidity/mortality in animals of all ages displaying this syndrome.

The mechaninsms of the PRRSV modulation of swine immune response are far to be totally elucidated. This knowledge is crucial for the rational design and development of more effective therapies and vaccines against PRRSV.

Objectives

The general aim of this thesis will be the evaluation of the swine immune response against PRRSV infection in the swine target cells and organs, paying special atention to the PRRSV ability to modulate the IIR and in consequence the AIR.

First. Considering the central importance of MØ for PRRS pathogenesis and the role of IFNs and cytokines in regulating MØ functions, we hypothesized that PRRSV of different virulence should differ in their interaction with cytokine-modulated MØ. To test this, IFN-γ-(M1), IL-4-(M2) and IFN-β-activated monocyte derived MØ will be evaluated for their susceptibility to different PRRSV-1 and PRRSV-2 strains as well their ability to secrete cytokines after infection.

Second. PRRSV-1 cannot proliferate in MØ belonging an effective host immune response against the virus; otherwise, the M2 MØ are susceptible to be infected by PRRSV-1 strains. The underlying mechanisms of PRRSV pathogenesis are still unclear. The analysis of transcript levels represents an alternative to immunoassays for the detection of cytokines that sometimes are difficult to detect due to their low amounts. Most of the previously published reports about swine cytokine gene expression lack information regarding the validation of the technique used, which impedes the potential implementation by new users; for this reason, we will perform a technical validation of already published RT-qPCR assays for swine proinflammatory and immunomodulatory cytokines. Once the gene expression technique is validated, we will determine the differences in the transcripts levels of proinflammatory and immunomodulatory cytokines in order to get more insights into the pathogenesis and the immune response in lung, tonsil, tracheobronchial lymph node and retropharyngeal LN of PRRSV-1 strain infected pigs.

Third. PRRSV 2982 has shown to moderate the host immune response in the site of infection, both in lung and secondary lymph organs. Swine PRRSV infection has been associated with B and T cell depletion in cortical areas of secondary lymph organs as lymph nodes; which suggest an important role of these tissues in the host immunity modulation by PRRSV. Several authors have published results on the transcriptional profiles during PRRSV infection in different organs by taking small pieces of tissue, but neither of them has shown results about transcriptional profiling in different tissues structures. The main aim of this objective will be the evaluation of the immune response against PRRSV developed in different compartments of lymphatic nodes (lymphoid follicle and inter-follicular area) of pigs infected with three different PRRSV strains within genotype 1.

Review

PRRSV taxonomy

The order *Nidovirales* includes the families *Coronaviridae*, *Roniviridae* and *Arteriviridae* (King *et al*., 2012; Snijder *et al*., 2013). *Arteriviridae* family is only composed by the genus *Arterivirus*, which includes PRRSV, equine arteritis virus, lactate dehydrogenase-elevating virus of mice, and simian hemorrhagic fever virus (Conzelmann *et al*., 1993; Meulenberg *et al*., 1993; Snijder, 1998; Gorbalenya *et al*., 2006). Arteriviruses are the smaller nidoviruses, with a genome length of 13-16 kilobases (kb), what it is the half extension compared with the 26-32 kb genome length for the rest of *Nidovirales* families (Gorbalenya *et al*., 2006).

PRRSV is classified in two genotypes, type 1 (European genotypes or PRRSV-1), represented by LV prototype, and type 2 (American genotypes or PRRSV-2), represented by VR-2332 strain prototype (Nelsen *et al*., 1999). There is a high inter- and intra-genotype genetic variability (Stadejek *et al*., 2008), with an average homology of only 60% of genome, and existing a high variability for non-structural (55% to 63%) and structural proteins (61% to 81%) (Murtaugh *et al*., 2010; Shi *et al*., 2010). In consequence different PRRSV isolates present high antigenic and pathogenic variability (De Baere *et al*., 2012). Therefore, PRRSV genotypes are divided in several subtypes depending on virulence and genetic differences. PRRSV-1 has been divided into 3 subtypes: pan-European subtype 1 and East European subtypes 2 and 3 (Stadejek *et al*., 2008), and it has been suggested the possibility of a fourth subtype (Stadejek *et al*., 2013); the HP-PRRS-1 strains belong to subtype 3 (Karniychuk *et al*., 2010). Whereas PRRSV-2 has been divided into at least nine lineages, including the HP-PRRSV which emerged in 2006 in China; during this outbreak killed more than 2.000.000 of pigs (Shi *et al*., 2010). Despite these significant genetic differences between both PRRSV

genotypes and also intra-genotype, both genotypes are considered the same virus (Murtaugh *et al*., 2010).

PRRSV structural biology

The PRRSV virions electron microscopy studies have shown a spherical to oval like shape about 50-65 nm diameter with a 40 nm diameter core enveloped by smooth lipid bilayered (Spilman *et al*., 2009). PRRSV has a positive-stranded RNA genome of 15.7 kb, it has a 5'-UTR, divided into 10 ORFs, and 3'-UTR (Meulenberg *et al*., 1998; Johnson *et al*., 2011) (Fig. 1).

ORF1 is in the 5'-proximal and it is divided into ORF1a and ORF1b, these are the precursors of two large polyproteins, pp1a and pp1ab. The ORF1b is only expressed by a RFS when it is on the region between ORF1a and ORF1b (Meulenberg *et al*., 1998). Both ppt1a and ppt1ab are splitted into at least 14 nsp mainly involved in replication and transcription, ORF1a encoded for 10 nsps (nsp1α, nsp1β, nsp2 to nsp6, nsp7α, nsp7β and nsp8) and ORF1b encodes for 4 nsps (nsp9 to nsp12) (Dokland *et al*., 2010; Johnson *et al*., 2011). The nsp1α, nsp1β, nsp2 and nsp4 are the nsps that carried out the ppt1a and ppt1b proteolysis (Snijder *et al*., 1996; Siddell *et al*., 2005).

The ORF2-ORF7 encode information for 8 structural proteins. These 3' proximal ORFs are translated from a nested set of six major subgenomic messenger RNAs (mRNAs). This ORFs are overlapping, so diferent part of PRRSV structural proteins are encoded by the same genome information. In the PRRSV double-lipid envelope six estructural proteins are located: two unglicosylated proteins (M and E) and four glicosylated proteins (GP2, GP3, GP4 and GP5) (Fig. 2). GP5 and M are associated into a disulfide-linked heterodimer, and these are the main envelope proteins (Kimman *et al*., 2009). This asssosiation happens between the secondary glycoproteins GP2, GP3 and GP4 that

form a covalently associated heterotrimeric complex (Wissink *et al*., 2005). The secondary envelope unglicosylated protein E has the properties of an ion-channel protein (Du *et al*., 2010). Recently, it has been described a small and hydrophobic ORF5a protein encoded by an overlapping region between ORF4 and ORF5 (Johnson *et al*., 2011). It has been reported that ORF5a protein is essential for PRRSV viability but it has no capacity to induce an effective hummoral immune response againts PRRSV infection in pigs (Robison *et al*., 2013; Sun et al., 2013). The ORF 7 encodes information for N, which was suggeted to have icosaedral shape in the firsts studies (Wootton and Yoo, 2003). Contrary to this idea, Spilman *et al*. (2009) using cryo-electron tomographic analysis showed a not isometric structure for the PRRSV N. They suggested that N protein dimers organized to interact through the N-terminal domain with the viral RNA causing the formation of chain with the RNA in the centre (Fig. 1B). The structural proteins are not essential for the virus replication (Molenkamp *et al*., 2000), but thesy are neccesary for the ensambling of infecting virions (Wissink *et al*., 2005).

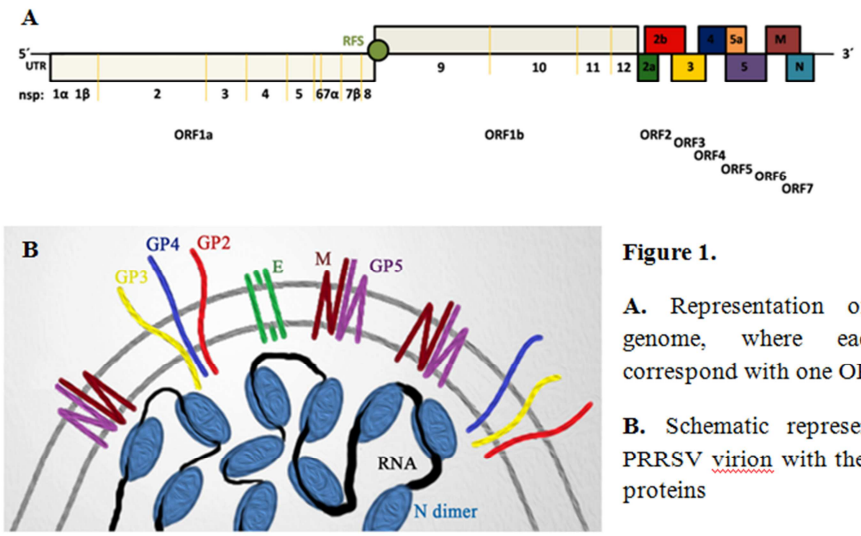

Figure 1.

A. Representation of PRRSV genome, where each block correspond with one ORF.

B. Schematic representation of PRRSV virion with the structural proteins

Cell tropism and PRRSV replication

PRRSV shows a restricted cell tropism for cells from the monocyte/macrophage lineage, the main *in vivo* target cells for replication being PAM, and other tissue MØ (Van Breedam *et al*., 2010). It has been proposed that PRRSV can infect and replicatein $CD163^+$ and $CD169^+$ endometric and placental cells of infected sows (Karniychuck *et al*., 2011). PRRSV is able to infect and/or replicate in monocyte-derived cells as MØ and DC generated *in vitro* (Wang *et al*., 2007). Beyond to MØ, it has been described the PRRSV presence in other swine cells during the *in vivo* infection, such as testicular germ cells, heart endothelial cells, thymus interdigitating cells, and DC in secondary lymphatic organs (Halbur *et al*., 1995a; Sur *et al*., 1997).

Considering that PAM are the main PRRSV target cells *in vivo*, they represent an appropriate model for *in vitro* studies to identify viral and cellular factors of importance for virus replication and host responses at cellular level. Nevertheless, it is necessary to sacrifice pigs (better from SPF animals) to collect sufficient PAM from lung lavages. Alternatively to PAM, certain monkey kidney derived cells are permissive to the infection by PRRSV leading its replication, which belongs CL2621 (subclones of MA104 cell line) and MARC-145 cells are the most used cells line used in research (Bautista *et al*., 1993; Provost *et al*., 2012). Recently, SJPL cell line (monkey origin) has been reported as PRRSV infection and replication susceptible, and interestingly these cells differ phenotypically from MARC-145 (Provost *et al*., 2012). Nevertheless, these monkey-derived cells are not susceptible for all PRRSV strains, especially for PRRS-1 subtype 3 strains. Nowadays, PAM and MARC-145 cells lineage are commonly used as primary cells for *in vitro* studies, being MARC-145 extensively used

for vaccine production (de Abin *et al*., 2009), finally, PAM seem to be better than MARC-145 for diagnostic isolation of field PRRSV strains..

The PRRSV cell tropism *in vivo*, and the end the virus replication, is determined by specific cells surface markers. The first contact between PRRSV virions is with the HS on the susceptible cells surface (Vanderheijden *et al*., 2001). This is followed by the M/GP5 proteins heterodimer binding with the CD169 (sialoadhesin) on the cells (Delputte *et al*., 2005).This virus-receptor complex induces the virus internalization through the clathrin-dependent endocytosis (Vanderheijden *et al*., 2003). Inside the early endosomes, the virions interact with the scavenger receptor CD163, this induces the PRRSV genome release into the cell cytoplasm, in this process the endosome acidification and certain swine proteases are important (van Gorp *et al*., 2010). It has been suggested that GP4 protein mediates the interglycoprotein interactions, and that GP2a protein attached to the CD163 facilitates the virus entry in the host cell (Das *et al*., 2010).

When the PRRSV genome is in the cell cytoplasm, the ORF1 is translated to produce both ppt1a and ppt1ab controlled by RFS (den Boon *et al*., 1991). Ppts proteolysis results in the 14 nsp involved in the virus replication (genome amplification) and transcription (subgenomic mRNA synthesis) (Dokland *et al*., 2010; Johnson *et al*., 2011). The subgenomic mRNA translation results in the structural proteins generation, the multiple copies of N proteins bind to the genomic RNA, and this nucleocapsid complex buds into the lumen of the smooth endoplasmic reticulum and Golgi complex, where through the organelles networks the virions acquire their envelope and its corresponding proteins (Review: Yun and Lee, 2013). It has been suggested that the virus realease occur through exocytosis (Wissink *et al*., 2005). Recently, it has been reported that GP2 protein seems to play a important role in the apoptotic inhibition of

infected cells during the PRRSV replication (Pujhari *et al*., 2014); which is in line with the observation that indicates that PRRSV induces cell apoptosis at the end of the replication cycle (Costers *et al*., 2008).

MARC-145 cells express CD163, but not CD169 (Calvert *et al*., 2007), which suggests that the PRRSV entry process in these cells could be different respect to the *in vivo* model. The CD151 expression in these cells has been suggested as cooperating mechanism for the virus entry (Shanmukhappa *et al*., 2007); it is more, in these cells the simian vimenctin could act like CD169 of swine cells (Kim *et al*., 2006).

These studies showed that some PRRSV strains should to adapt to monkey's cells lines before to gets an efficient viral growth (Kim *et al*., 2006). Then, the results from *in vitro* experiments that includes the use cell lines should be carefully interpreted.

Epidemiology

The horizontal transmission of PRRSV from infected to naïve animals can occur through oro-nasal contact, semen (copula or artificial insemination), contaminated needles, fomites and farmers manipulations (Yaeger *et al*., 1993; Swenson et al., 1994; Magar *et al*., 1995). PRRSV can persist in infected animals for long periods of time. For instance in serum up to 210 dpi, oropharyngeal scrapings up to 156 dpi, semen up to 92 dpi, lymph nodes up 86 dpi, saliva up to 42 dpi or feces and urine up to 38 or 28 dpi, respectively (Christopher-Hennings *et al*., 1995; Benfield *et al*., 1997; Wills *et al*., 1997a; Wills *et al*., 1997b; Han *et al*., 2009; Metwall *et al*., 2010); all of them represent a potential sources for PRRSV transmission in the herd. PRRSV cannot support dry environmental conditions, and high pH variations (Pirtle and Beran, 1996). Under -20° or -70° C the PRRSV infectivity can remain during long-term whilst the virus is totally inactivated at 37°C after 48h or 45 min at 56°C (Benfield *et al*., 1992). PRRSV is very

sensible to pH variations, with the optimum pH range between 5.5 and 6.5 (Bloemraad *et al*., 1994). For these reasons, PRRSV cannot resist the environmental conditions during long-term, so the contact between PRRSV and the susceptible animals should occur in a short time. It is important to note, that under specific conditions (low sunlight intensity, high humidity and wind velocity) the PRRSV virions can be transport into aerosols over 4 km from the infected farm (Dee *et al*., 2009). The vertical transmission of PRRSV occurs during late gestation. It seems that endometrium/placenta development determines the passage of PRRSV to the fetuses, among the increasing number of endometrial $DC163^{+}$ and $CD169^{+}$ MØ that carry the virus to the fetuses (Karnyichuck *et al*., 2012).

Pathogenesis

Lung and mucosal surfaces (nasal, oro-pharyngeal or genital) are the normal entry site for PRRSV in the host. As other viruses, PRRSV has developed different strategies to reach the connective tissue under the epithelium (Steukers *et al*., 2012). It has been described varied mechanisms of invasion leading to different PRRSV virulence strains. PRRSV-1 subtype 1 susceptibility is restricted to the $CD163^{+}/169^{+}$ cells in the lamina propia; whereas PRRSV-1 subtype 3 access through both $CD163^{+}/169^{+}$ lamina propia cells and $CD163^{+}/169^{-}$ cells in the epithelium of nasal mucosa increasing its virulence (Frydas *et al*., 2013). Once the virus entry is produced, PRRSV replication cycle is completed in 12 hpi in local susceptible cells; it is followed by the viraemia, which spreads the virus to tissular MØ, especially to PAM (PRRSV main target cells), or MØ in secondary lymphoid tissues and in other organs as testes or utero (Lawson *et al*., 1997). The viraemia is typically prolonged during 4 to 6 weeks after infection, but the

virus can persist within the host for months in lymphoid tissues; inducing a PRRSV sub-clinical persistence (Wensvoort *et al*., 1991; Wills *et al*., 2003).

PRRSV infected animal in the herd can show a variety of clinical signs such as anorexia, lethargy, dyspnea, hyperthermia, periocular edema, cyanotic skin patches, reduction in the average daily gain, reproductive disorders, and increase in mortality rates (Halbur *et al*., 1995b; Hirose *et al*., 1995; Shibata *et al*., 2003). This clinical chart of PRRSV shows symptoms varying in severity depending of the strain virulence, presence of other pathogens, age of animals, environment conditions and farm proceedings (Brockmeier *et al*., 2002). Younger pigs between 6-8 weeks old are more susceptible to PRRSV infection compared with older animals, showing higher levels of viraemia, and excreting more quantity of virions (van der Linden *et al*., 2003).

PRRSV induces apoptosis in infected tissues (lung, testes, placenta, lymph nodes and thymus) both *in vivo* and in cell lines *in vitro* (Sur *et al*., 1997; Sur *et al*., 1998; Kim *et al*., 2002; Labarque *et al*., 2003; Karniychuck *et al*., 2011). Nowadays, it is not clear how PRRSV induces apoptosis directly on infected cells and indirectly on bystander cells (Sirinarumitr *et al*., 1998; Labarque *et al*., 2003). Recently it has been suggested that GP2 protein could play an anti-apoptotic role during the PRRSV replication in infected cells, which died by apoptosis after the virus release (Pujhari *et al*., 2014).

PRRSV replication in lung results in a PAM and pulmonary intravascular MØ lysis and apoptosis of infected and bystander MØ (Sirinarumitr *et al*., 1998) which induces the development of interstitial pneumonia. This is followed by a dramatic functional and quantitative decrease of these cells during the first week of infection (Molitor *et al*., 1996); which results in the weakening of the main lung cell defense barrier. This process is aggravated by the damage on the mucociliary apparatus, increased vascular

permeability, changes in the T-cell populations, decreased fuction of APC such as DC and MØ and cytokines imbalanced secretion produced by PRRSV (Molitor *et al*., 1997; Thanawongnuwech *et al*., 1998; Van Reeth *et al*., 1999; Xiao *et al*., 2010; Gómez-Laguna *et al*., 2013a). These alterations induced by PRRSV infection increase the susceptibility to secondary infections of others pathogens such us…, then PRRSV is the most common virus isolated from cases of porcine respiratory disease complex (Van Alstine, 2012). Co-infection of PRRSV with other pathogens such as *Mycoplasma hyopneumoniae* or swine influenza virus have been shown to increase the duration and severity of clinical chart than PRRSV single infection (Van Reeth *et al*., 1996; Thacker *et al*., 1999; Johnson *et al*., 2004)

Reproductive failure in gilts and sows normally occurs at the last third of gestation and is characterized by an increased number of abortions, mummified foetuses, stillbirth and weak-born piglets (Hopper *et al*., 1992; Done and Paton, 1995; Rossow, 1998). It has been shown PRRSV infection resistance in preimplantation embryos, which could be explained by the absence of CD169 expression. It can be concluded that the PRRSV infection susceptibility is age-dependent for embryos (Mateusen *et al*., 2007). intraamniotic and intrafetal PRRSV inoculation has demonstrated that during mid-gestation this virus is not able to cross ither from the mother to the foetuses or inversely; although PRRSV can replicate in the foetus (Christianson *et al*., 1993). These findings suggest that uterine-placental structures can act as barrier for the transplacental infection of PRRSV during the two first third of gestation. The fetuses transplacental infection by PRRSV mostly occur during the late-gestation (Kranker *et al*., 1998) when PRRSV can replicate in CD169+/163+ cells in the endometrial/placental tissue; which finally leads tothe virus transmission to the foetuses (Karniychuk and Nauwynck, 2009). The

endometrial and placental PRRSV replication induces to cell apoptosis that leads to the abortions and mummified fetuses during PRRS.

PRRSV immunity

Innate Immune Response

IIR is the primary immune barrier to pathogens and it assembles an effective initiation of AIR. The pathogens infection of host activates a battery of immune responses through interaction between pathogens structures and the immune surveillance mechanism of the host. Normally, the host recognizes conserved molecular structures known as PAMPs (Janeway and Medzhitov, 2002). These PAMPs are recognized by PRRs expressed on dencritic cells, MØ and neutrophils (Takeuchi and Akira, 2005). The effective PPRs-PAMPs interaction immediately induces the activation of complex signaling pathways to stimulate inflammatory responses via several cytokines and chemokines, which finally leads to the pathogens elimination (Kumar *et al*., 2009). The TLRs are considered one of the main PRRs in the recognition of pathogens, TLR1, 2, 4, 5 and 6 are located on the cell surface; whereas TLR3, 7, 8 (only described in humans) and 9 are expressed inside endocytic compartments and recognize nucleic acid of intra-cellular pathogens as virus and bacteria. *In vitro* studies with TLR7 demonstrated that this receptor is responsible for the PRRSV ssRNA recognition (Kumar *et al*., 2009; Baumann *et al*., 2013).

MØ immunity

MØ, although all of monocyte origin, represent a heterogenicfamily of cells as a result of cellular differentiation in specific tissues and local microenvironments including cytokine milieu (Gordon, 2003). MØ subset populations are differentiated based on

distinct phenotype, functional properties and the released cytokines (Mantovani *et al*., 2004). By analogy to Th1/Th2 nomenclature, the polarized MØ are referred to M1 and M2 (with different phenotypes M2a, M2b and M2c) (Mantovani *et al*., 2004). The polarization into classically (M1) or alternatively (M2) activated MØ is essential to understand MØ functions. M1 are activated through two signals, (1) the synthesis of IFN-γ which induces MØ differentiation and (2) the production of TNF-α as second signal. The later is produced by MØ after TLR ligation (Mosser, 2003); which is the main cytokine secretion inductor for Mo (Mosser and Zang, 2008). M1 generates high levels of nitric oxide, and secrete inflammatory cytokines as IL-1β, IL-6, and TNF-α; besides M1 are inducer and effector cells in Th1 inflammatory response (Mantovani *et al*., 2005). During the IIR the M1 acts as APC through the MHC II (Mitchel and Kumar, 2004). IL-4 and IL-13 induce the alternative MØ activation (M2). This M2 cannot produce nitric oxide, and are inefficient in killing of intracellular pathogens. M2 express major MHC II but are inefficient as antigen presenting cell, produce more IL-10, and can even polarize Th2 reactions (Mosser, 2003). M2 are involved in the process of tissue repair and regeneration (Mills, 2013). IL-10 can inhibit the macrophages functions; as well it is considered a immunosupressor cytokine that reduces the release of several cytokines such as IL-1α, TNF-α, IL-6, IL-10 (auto-regulation), IL-12 e IFN-γ (Biron and Sen, 2001).

DC Immunity

DC are a heterogeneous population of hematopoietic APC; which act as central cells in the onset of IIR and have alsoa main role in the activation of AIR (Ueno *et al*., 2010). The DC population are divided in mDC and pDC; and these have a common hematopoietic precursor (stem cells in the bone marrow), also monocytes that migrates to the inflammatory places derived into mDC (Siegal *et al*., 1999). The mDC can be

localized in circulating blood, peripheral tissues and in secondary lymphoid organs (Steinman and Inaba, 1999); whereas pDC are circulating in the blood, bone marrow and lymphoid organs. Unlike mDC, pDC express TLR7 and TLR9 ligands, which means that they are the main source of IFN-α in the organism. pDC can secrete 100 fold more IFN-α than another cellular type (Siegal *et al*., 1999; Akira *et al*., 2006). The most common *in vitro* model to study mDC is to use moDC. In secondary lymphatic organs, specialized fDC are found, these derived from mDC, and they function as APC to the B cells (Kapasi *et al*., 1994).

PRRSV infected pigs develop a poor innate and adaptive immune responses (Albina *et al*., 1998; Van Reeth 1999), including cytokine production modulation (Flores-Mendoza *et al*., 2008) which leads to co-infections and to the prolonged virus persistence in the host (Thanawongnuwech *et al*., 2004). PRRSV replicates in monocyte derived DC *In vitro*, although this replication has never been elucidated *in vivo* (Silva-Campa *et al*., 2009; Gimeno *et al*., 2011). Because MØ and DC are the central cells in the IIR, it is foreseeable that their infection by PRRSv leads to several impairment of IIR and AIR . PRRSV delays the induction of protective cellular and humoral immune response in the host, which leads to the virus replication, shed and infection of other naïve animals in the herd (López and Osorio, 2004). It should be taken into account that different PRRSV isolates can induce different patterns of secreted cytokines and immunologically relevant molecules; as well as the PRRSV replication can be limited by the pig breed (Ait-Ali *et al*., 2007; Gimeno *et al*., 2011).

IFN-I (IFN-α/β) modulation

The first cytokines were discovered in 1957 as secreted factors that induced a virus-resistant state. Because their capacity to “interfere” the viral replication, they were

named “interferon”.It had been discovered the IFN-I (Isaac and Lindenmann, 1957). The IFN-I is a heterogeneous family with multiple IFN-α subtypes, single IFN-β, IFN-ε, IFN-κ, IFN-δ (found in pig) and IFN-τ (found in ovine) (Theofilopoulos *et al*., 2005). IFN-I induces antiviral responses by binding a common receptor type I IFN-α/β receptor. These IFNs induce apoptosis of virus-infected cells, make naïve cells resistant to viral infection, and collaborate in the efficient induction of AIR (Theofilopoulos *et al*., 2005). It seems that all viral pathogens have developed strategies to avoid the IFN-I defense in the IIR (Haller *et al*., 2006).

It has been showed in *in vitro* studies that IFN-α significantly reduces PRRSV replication (Loving *et al*., 2007; Luo *et al*., 2011). As well as, *in vivo* administration of exogenous IFN-α at the same time that PRRSV challenge has demonstrated to reduce the PRRSV disease severity and the virus quantity in the host tissues (Brockmeier *et al*., 2012). On the other hand, PRRSV appear to induce weak IFN-α production in infected pigs (Van Reeth *et al*., 1999); although it has been demonstrated that several PRRSV strains promote systemic IFN-α secretion in infected pigs (Calzada-Nova *et al*., 2010; Guo *et al*., 2013). Also *in vitro* PRRSV infection of moDC or PAM showed a weak or null synthesis of IFN-I (Lee *et al*., 2004; Zhang *et al*., 2012); whereas the direct stimulation with several PRRSV strains over pDC render in the IFN-α secretion through TLR7 activation (Baumann *et al*., 2013). These findings suggest that some cells can drive the immune response against PRRSV depending on the virus isolate (Lee *et al*., 2004).

PRRSV has developed several mechanisms in order to inhibit the IFN-I production. PRRSV nsp, especially nsp1α, nsp1β, nsp2, nsp4 and nsp11, are involved in the virus modulation of IFN-I through the inhibition of IRF3, NF-κB, CBP, and STAT-1 (Beura *et al*., 2010; Kim *et al*., 2010a). PRRSV nsp1α and nsp1β play a key role in the IFN-I

inhibition. Nsp1β strongly inhibit the IFN-β promoter activity and nsp1β inhibits both the JAK-STAT pathway and IRF3 phosphorilation, avoiding its translocation into the nucleus (Beura *et al*., 2010; Chen *et al*. 2010); These mechanism finally lead to the inhibition of the IFN-I production. This IFN-I inhibition by nsp1 is partly due to the degradation of CBP in the nucleus in order to avoid the IFN gene expression (Li *et al*., 2010). PRRSV nsp inhibition of NF-κB, more important at the beginning of the infection, helps to the virus to subvert the host IIR in addition to produce high number of virions before the release from the infected cells (Costers *et al*., 2008).

TNF-α modulation

TNF-α is mainly produced by M1 MØ, although it is also secreted by monocytes, DC, endothelial cells, fibroblast, T and B cells (Pennica *et al*., 1984; Olszewski *et al*., 2007). TNF-α, belonging to the TNF superfamily, is a cytokine with potent proinflammatory effects in the establishment of the IIR; in which it is the first spread cytokine, followed by IL-1 and IL-6 (Biron and Sen, 2001). During the IIR initiation, TNF-α induces the production of cytokines and chemokines, as well as the adhesion, migration, attraction and activation of lymphocytes into the inflammation site. During the virus infection, the TNF-α induces apoptosis of infected cells (Wong and Goeddel, 1986). TNF-α improves the neutrophils and MØ endothelial adhesion and their phagocytic ability. During the inflammation process, the TNF-α high concentration cause the classical inflammation signs: heat, swelling, redness, pain and loss of function (Pennica *et al*., 1984).

The TNF-α modulation by PRRSV showed different results. On the one hand it has been described a TNF-α production in PRRSV infected pigs tissues (Choi *et al*., 2002; Barranco *et al*., 2012a); on the contrary both *in vivo* and *in vitro* studies reported PRRSV ability to induce a weak or suppress the TNF-α production (Van Reeth *et al*.,

1999; Lopez-Fuertes *et al*., 2000; Thanawongnuwech *et al*., 2004). These differences in the TNF-α secretion profiles have been related to variances depending in both cell type and PRRSV isolate (Gimeno *et al*., 2011). As described above, PRRSV nsp1α can suppress the NF-κB activation and nsp1β avoid the Sp1 translocation; which results in the TNF-α suppression in MØ and DC (Kim *et al*., 2010a; Subramaniam *et al*., 2010). These findings suggest that variations in the nsp1α sequenece along the PRRSV strains can induce variations in the TNF-α modulation. It has been shown that some epitopes of nsp2 are not essential for the PRRSV replication, but they act in the modulation of the host immune response, regulating the TNF-α production (Chen *et al*., 2010).

IFN-II (IFN-γ) modulation

IFN-II, or IFN-γ, is induced by both immunologic and mitogenic stimulation; and it works a key role in the IIR and AIR against viral and intracellular bacterial pathogens and cancer control (Baron *et al*., 1991; Biron and Sen, 2001; Samuel, 2001). IFN-γ is a potent MØ activator (Zlotnik *et al*., 1982), which differentiates into M1 after its stimulation (Zlotnik *et al*., 1982). Early in the IIR the stimulated NK induces the IFN-γ secretion, which primes mononuclear cells for the production of cytokines such as TNF-α and IL-12; later, when the antigen-specific immune response is developed, IL-12 drives the Th1 response to generates more IFN-γ by both activated CD4+ Th1 and CD8+ Tc cells (Senik *et al*., 1980; Fong and Mosmann, 1990; Chan *et al*., 1991; Harrington *et al*., 2005). IL-12 is produced by MØ, monocytes, DC and B cells (Trinchieri, 1994). IFN-γ induces the augmented expression of MHC-I on several cells and the MHC-II on APC in order to improve their ability to eliminate intracellular pathogens (Farrar and Schreiber, 1993; Bach *et al*., 1997). The stimulation of T cells subsets by IFN-γ induces Th1 development Th2 inhibition. (Gajewski and Fitch, 1988). Th1 cell cytokine profile includes IL-2 and TNF-β, but it is characterized by the

secretion of IFN-γ (which help MØ activation). On the contrary, Th2 cells produces IL-4 (it leads to M2 differentiation) and IL-5. Finally, Th17 lymphocytes secrete IL-10 (Chakraborty *et al*., 1999).

It has been reported that IFN-γ can reduces the number of PRRSV infected cells, virus yield, and the amount of viral RNA content in single cell; so these suggest that IFN-γ inhibits the PRRSV replication(Rowland *et al*., 2001) NK cells in the lung of PRRSV infected pigs are associated with a powerful IIR that decreases the number of PRRSV infected macrophages. Interestingly, PRRSV induces a NK depletion during the first days of infection; (Lohse *et al*., 2004); It is more, PRRSV infected PAM develop a low susceptibility to NK cytotoxicity (Cae *et al*; 2013); which finally leads to delayed IIR and the consequent virus replication.

Anti-inflammatory cytokine modulation

IL-10 develops several anti-inflammatory effects, such as the suppression of synthesis of proinflammatory cytokines, chemokines and adhesion molecules. Moreover, IL-10 can inhibit antigen-presenting and costimulatory molecules in APC, neutrophils, and T cells (Moore *et al*., 2001). The ability for IL-10 secretion has been described in several cells like T cells (Th2, Treg, Th1, and Th17), monocytes, MØ (major source *in vivo*), DC, fibroblast, epithelial cells, and tumor cells (Moore, 2001; Williams *et al*., 2004). IL-10 exerts its anti-inflammatory actions through the NF-κB inhibition by, (1) NF-κBp65/p50 nuclear translocation blocking through annulation of IKK, and (2) intra-nuclear inhibition of the binding between NF-κB and DNA (Johnson *et al*., 1999; Tergaonkar *et al*., 2005). These NF-κB signals are blocked in part by the IL-10 selective induction of NF-κB p50 homodimers, that does not need activation to be translocated into the nucleus and induces cytokines gene expression such us IL-10 (Li and Verma,

2002; Cao *et al.*, 2006). APC and lymphocytes are the major target cells for IL-10; in which have a high impact in the suppression of antigen presenting capacity and proinflammatory cytokines release (IL-1, IL-6, IL-12, TNF, and IFN-γ); and in the regulation of Th1/Th2 balance (priming Th2 differentiation) (Mosser and Zhang, 2008). Consequently, the cellular immune response is clearly downregulated by IL-10.

IL-10 is secreted in both *in vitro* and *in vivo* experiments of PRRSV infection (Charerntantanakul *et al*., 2006; Silva-Campa *et al*., 2009; Gómez-Laguna *et al*., 2010a; Barranco *et al*., 2012b). It has been showed on PRRSV-infected PBMC that different PRRSV strains induce different secreted IL-10 profiles (Gimeno *et al*., 2011). Then, it seems that IL-10 secretion in order to avoid an effective immune response in the host is not a common strategy for all PRRS strains.

TGF-β is the other anti-inflammatory cytokine (apart from Il10) that downregulates the host immune response during PRRSV infection (Letterio and Roberts, 1998). Actually, controversial information exists in the literature about the TGF-β induction by PRRSV. Previous reports indicate that PRRSV-2 strains induce TGF-β induction both at mRNA and protein levels in blood and tissues of infected animals (Silva-Campa *et al*., 2009; Renukaradhya *et al*., 2010; Dwivedi *et al*., 2011; Silva-Campa *et al*., 2012; Han *et al*., 2013a). Whereas for PRRSV-1 isolates, most of the reports did not show a TGF-β induction by PRRSV-1 (Díaz *et al*., 2006; Silva-Campa *et al*., 2009), although one paper showed TGF-β expression in PRRSV-1 infected tissues (Gómez-Laguna *et al*., 2012).

APCs cell-surface molecules modulation

PRRSV has the ability to change the cell-surface molecules on APC; which is another viral strategy to avoid the host immune response. This phenomenon has been reviewed

by our research group (Rodríguez-Gómez *et al*., 2013). Briefly, CD80/86 are strictly necessaries for the T cell activation the MHC-II and the costimulatory molecule (Banchereau and Steinman, 1998; Kindt *et al*., 2006). During the PRRSV infection of different subsets population of DC it has been described the downregulation of MHC-I, MHC-II, CD11b/c and CD14 (Flores-Mendoza *et al*., 2008; Park *et al*., 2008; Wang *et al*., 2007) or none changes for the case of MHC-II (Peng *et al.,* 2009). For CD80/86 expression it has been described both downrregulation (Flores-Mendoza *et al*., 2008) and overregulation (Park et al., 2008) in PRRSV-infected cells. Interestingly, it has been shown that PRRSV ability to downregulate these molecules is correlated with the virus IL-10 induced profile (Gimeno *et al*., 2011). In these studies, PRRSV infection downregulates at least one of these surface molecules in order to impair the T cell activation by APC.

Adaptive Immune Response

the host can induce an effective humoral immune response against the same PRRSV strains, with memory cells; but with absent or low cross-protection among different PRRSV strains (Osorio *et al*., 1998; Labarque *et al*., 2004). The most of the studies about pig AIR against PRRSV showed a delayed onset of both the cell mediated immunity and the NAb production.

Cell mediated immunity

Concerning to the AIR immunity, the IFN-γ expression by MØ and lymphocytes has been reported in the lung of PRRSV infected animals (Thanawongnuwech *et al*., 2003; Gómez-Laguna *et al*., 2010a), but this detected IFN-γ secretion was delayed in a range between 7 to 28 dpi. These findings are supported by several reports that point that the induction of IFN-γ specific cells is delayed at least 2-3 weeks after the infection; later

these cells showed a slowly increasing until reaches normal values after months post infection; it is more, the number of IFN-γ specific cells are lower compared with quantities induced by other viruses (Meier *et al*., 2003; Díaz *et al*., 2005). It has been reported that IFN-γ is induced by both structural (M, N, GP4 and GP5) and nsp (nsp2). On the contrary nsp5 and nsp11 have been suggested as IFN-γ suppressors (Díaz *et al*., 2009; Jeong *et al*., 2010; Burgara-Estrella *et al*., 2013). Sp1 is involved in the positive genetic expression of IL-12 (Andersen *et al*., 2007). As previously mentioned, PRRSV nsp1β can inhibit the Sp1, and then it could suppress the IL-12 secretion during the PRRSV infection, contributing to the delayed IFN-γ induction in PRRSV infected animals.

The IL-10 production due to PRRSV modulation of host immune system has been related with the switching to a Th2 response. Moreover PRRSV can induce CD8 Tc; although these cells fail in the cytolitic processing of PRRSV infected MØ (Costers *et al*., 2009); which suggests that these CD8 Tc are not able to eliminate virus infected cells

In the host, $CD4^+/CD25^+/Foxp3^+$ Treg are necessary for the auto-tolerance, inflammatory activity control and homeostasis management (Sakaguchi, 2004). Treg act as a defense mechanism in the control of excessive inflammatory response against pathogens. Activated Treg produces antiinflammmatory cytokines (IL-10 and TGF-β) and are able to reduce or inhibit the proliferative and effective ability of other T cells populations (Käser et al., 2008). Interestingly, several viruses induce Treg proliferation in order to avoid the host immune response, and favour its replication; which can finally lesd to chronic infections (Vahlenkamp *et al*., 2005; Belkaid, 2007). *In vitro* studies have suggested that PRRSV can induce Treg (Silva-Campa et al., 2009; Wongyanin *et al*., 2010); which was correlated with: 1) the TGF-β induction by PRRSV-2 strains

(Silva-Campa *et al*., 2009); and 2) the viral N immunomodulatory properties and the IL-10 production (Wongyanin *et al*., 2012). Nowadays it has not been demonstrated the relationship between Treg and the PRRS pathogenesis in infected pigs.

Humoral immunity

The PRRSV infection is characterized by an earlier specific not neutralizing Ab production after one week of infection (Loemba *et al*., 1996; Plageman *et al.*, 2006).On the contrary, the NAb production is low and delayed until 2 to 6weeks after PRRSV infection (Loemba *et al*, 1996; López and Osorio, 2004). The early humoral immune response produces non-NAb against N, M and GP5 (Nelson *et al*., 1994); but also it has been reported non-NAb against nsp1 and nsp2 (de Lima *et al*., 2006; Johnson *et al*., 2007). Several PRRSV proteins have shown to be targets for NAb, among which are GP3, GP4, GP5 and M (Kim and Yoon, 2008; Costers *et al*., 2010). The delayed production of NAb has been associated with the APC impairment induced by PRRSV and by the presence of a decoy epitope close to the main NAb inductor epitope present in the GP5 (Otrowski *et al*., 2002). Nowadays, attenuated or MLV vaccines (generated through several cell culture passages), and KV vaccines (chemically or physically killed virus) are available for PRRSV vaccination. MLV vaccines induce lower titters of NAb than KV vaccines; which are not able to induce NAb before virus challenge, then KV vaccines cannot protect against PRRSV infection (Zuckermann *et al*., 2007; Geldhof *et al*., 2012).

Material & Methods

To reach the proposed objectives we performed three studies: an *in vitro* study to establish the PRRSV ability to infect different cytokine-modulated MØ; and two *in vivo* studies first to determine how PRRSV-1 2982 modulate the immune response in swine tissues during the infection, and second we studied in different lymph node compartments the cytokines regulation induced by PRRSV-1 strains varying in virulence.

In vitro study

Monocyte-derived MØ

Monocyte-derived MØ were generated as previously described (Carrasco *et al*., 2001). Briefly, peripheral blood mononuclear cells were isolated from 3 to 12 months old SPF pigs using ficoll-paque density centrifugation (1.077 g/l; Amersham Pharmacia Biotech). Monocytes were then enriched by CD172a (clone 74-22-15A, hybridomas kindly given by Dr. A. Saalmüller, Veterinary University of Vienna, Austria) positive selection using the MACS with LS columns (Miltenyi Biotec GmbH, Germany), and seeded in 24-well culture plates at a density of 5×10^5 cells per well in DMEM with GlutaMax (Life Technologies, Switzerland) and 10% heat-inactivated porcine serum (Sigma Chemicals, Switzerland). The cells were cultured at 39 °C with 5% CO_2 for 72 h and then stimulated by using either IFN-γ (10 ng/ml, R&D Systems, UK), IL-4 (100 U/ml, own production, (Carrasco *et al*., 2001), IFN-β (100 U/ml, own production, (Husser *et al*., 2012) or were left untreated for another 24 h. These MØ were termed "IFN-γ MØ", "IL-4 MØ", "IFN-β MØ" or MØ, respectively.

Viruses

As a genotype 1 PRRSV we used Lelystad virus adapted to grow in MARC-145 (LVP23; kindly obtained from Dr. Barbara Thür, IVI, Switzerland), two Spanish field

isolates 2982, 3267 (kindly provided by Dr. Enric Mateu, Centre de Recerca en Sanitat Animal-CReSA, Barcelona, Spain) (Gimeno *et al*., 2011) and Olot/91 (passaged several times, kindly obtained from the PoRRSCon Con-sortium through Dr. Luis Enjuanes, Universidad Autónoma, Madrid, Spain). The HP-PRRSV Lena strain (kindly provided by Prof. Hans Nauwynk, Ghent University, Belgium) which belongs to Eastern European subtype 3 (Karniychuk *et al*., 2010) was also included. As a genotype 2 PRRSV we used VR-2332 (Collins *et al*., 1992) (ATCC, LGC Standards, Molsheim, France), JA-1262, MN184, SS144 (kindly obtained from Dr. Michael Murtaugh, University of Minnesota, St. Paul, MN, USA) and RVB-581 (kindly obtained from Dr. Martin Beer, Friedrich-Loeffler-Institut, Riems, Germany) (Wernike *et a*l., 2012) representing a highly pathogenic field isolated in China. The PRRSV isolate SS144 was isolated in 2010 from a severe PRRS outbreak with high mortality rates in Missouri, USA. The MN184 isolate was obtained in 2001 from a swine farm experiencing severe reproductive disorder and sow mortality in Minnesota, USA. JA-1262 represents a 2009 Midwestern US isolate from a sow herd enduring abortions and holding infected piglets. Lena, 2982, 3267, MN184 and SS144 were propagated in alveolar MØ isolated and cultured as described (Basta *et al*., 2000). Strains of Olot/91, LVP23, RVB-581, JA-1262 and VR-2332 were propagated in the MARC-145 cell line (ATCC, LGC Standards, Molsheim, France) cultured in DMEM GlutaMax supplemented with 10% of foetal bovine serum (Biowest, France). Viral stock was obtained from cells lysed by freezing when 50% CPE was reached, clarified by 2500 × g centrifugation at 4 °C for 15 min, and stored at -70 °C. Lysates from uninfected MØ or MARC-145 cells were used as mock-infected controls. All strains were titrated in their corresponding propagating cell type by CPE evaluation or by using the IPMA with PRRSV anti-nucleocapsid mAb SDOW17-A (Rural Technology Inc., South Dakota, USA). Titres

were calculated and expressed as 50% tissue culture infective dose per ml (TCID50/ml). All inocula were tested for PCV2 by qPCR as described by Olvera *et al*. and found to be negative (Olvera *et al*., 2004).

PRRSV infection of MØ

MØ were infected at a MOI of 0.1 TCID50/cell in medium containing 10% SPF pig serum, free of anti-bodies against PRRSV. Mock-treated MØ were included as controls. Virus adsorption was allowed for 1 h before the cells were washed three times with PBS, new cell culture medium was added and the cells were cultured at 39 °C. Culture supernatants were collected at 0, 6, 12, 16, 20 and 24 hpi for virus titrations, PRRSV N expression or cytokine measurements. All experiments were performed in triplicates and repeated at least two times with different blood donors.

Cytokines measurements in MØ supernatants

Supernatants of MØ were harvested at 20 hpi. IFN-α production was determined by in-house ELISA (Guzylack-Piriou *et al*., 2004). TNF-α and IL-10 expression was measured using commercial kits (R&D Systems, UK). The detection limits are given at 30 and 60 pg/ml, respectively.

Monoclonal antibodies and flow cytometry

The following mouse anti-pig cell surface antibodies were used: anti-CD172a (clone 74-22-15A), anti-CD163 (clone 2A10/11; AbD Serotec, Puchheim, Germany), anti-α-MHC I and anti-MHC II (clone74-22-15A, 74-11-10 and MSA3, respectively, hybridomas kindly given by Dr. A. Saalmüller, Veterinary University of Vienna, Austria), anti-CD86 (clone HA5.2B7, Beckman Coulter, Nyon Switzerland), anti-CD16 (clone G7; AbD Serotec) and anti-CD14 (clone CAM36A; VMRD, Inc., Pullman, WA, USA).

Specific goat anti-mouse IgG conjugated with RPE (Dako, Zug, Switzerland) was used as secondary antibody. At each time point post infection the cells were seeded and fixed with 4% paraformaldehyde, washed and permeabilized with 0.3% (wt/vol) saponin in PBS. For detection of the PRRSV N protein the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed and then permeabilized with 0.3% (wt/vol) saponin, during which staining with the monoclonal antibody SDOW17-A (Rural Technologies Inc., South Dakota, USA) was performed for 15 min on ice. Acquisition was done on a FACSCalibur (Becton Dickinson, Mountain View, CA, USA). Electronic gating based on the forward/side scatter plots was applied to identify living cells using the FlowJo V.7.2.5 software (Tree stars Inc., Ashland, OR, USA).

In vivo studies

Virus, animals and experimental designs

For the first *in vivo* study, thirty-two specific pathogen-free, five-week-old pigs from a PRRSV seronegative farm were used in this study. These animals were randomly allocated in eight groups of four animals per group, seven groups were inoculated by the intramuscular route with 1 ml of third passage of PRRSV field isolate 2982 (kindly provide by Dr. E. Mateu, CReSA, Barcelona, Spain) at $10^{3.0}$ $TCID_{50}$. The virus was initially isolated from the serum of naturally infected piglets during a respiratory outbreak of PRRS affecting a Spanish farm. The remaining control group was inoculated intramuscularly with 1 ml of sterile RPMI 1640 medium. The virus-inoculated pigs were humanely euthanized at 3, 7, 10, 14, 17, 21 and 24 dpi and the controls at 24 dpi. All animals were sedated with tiletamine-zolazepam (Zoletil, Virbac, France) followed by intravenous injection of a lethal dose of 5% sodium thiopental (Thiovet, Vet Limited, UK), the animals were perfectly bled. This study was carried out at the Animal Health Research Centre (CISA-INIA, Valdeolmos, Madrid, Spain),

according to European Union guidelines (Directive 86/609/EEC) and following the approval of the institute's Ethics Committee.

In the second *in vivo* study, three PRRSV-1 strains were used. The LV was selected as the prototype subtype 1 strain. The 215-06 strain (subtype I) was isolated in 2006 from the serum of a post-weaning piglet showing signs of wasting and poor condition on a farm in England, and isolated at the Animal Health and Veterinary Laboratories Agency (AHVLA).The eastern European PRRSV SU1-bel (subtype 3), isolated at the AHVLA from infected lungs from pigs from Belarus (kindly provided by Dr. Tomasz Stadejek, Warsaw University of Life Sciences, Poland) and belonging to the Eastern European subtype 3 was also included. Virus propagation was carried out as previously described by Morgan *et al*. (2012).

Seventy-six SPF five week old male piglets from a PRRSV and porcine circovirus type 2 (PCV2) seronegative farm in the Netherlands were used in this *in vivo* experiment. These animals were matched by weight and randomly allocated in four groups; for the control group was allocated 16 animals, whereas for each infected group 20 animals were used. Each group was housed in separate pens of a containment building at AHVLA, which allowed the free airflow from the outside; the animals were let to acclimatise for 14 days prior to the beginning for the experiment. To prevent viral cross-infections between groups the staff uses specific clothes and tools for each room. At seven weeks old, 3 groups of piglets were inoculated intranasally with 1.5 ml of cRPMI with 10^5 $TCID_{50}$ of each PRRSV strain (LV, 215-06 and SU1-bel, respectively). The remaining control group was inoculated intranasally with 1.5 ml of PAM cryolysate diluted in cRPMI. Four animals in the control group and five for each virus-inoculated group were humanely euthanized at 3 and 7 dpi. At 7 dpi five of the remaining animals in each group were vaccinated intramuscularly with an Aujeszky's Disease (ADV)

vaccine (Suvaxyn Aujeszky I.N./I.M., Fort Dodge, The Netherlands), following the manufacturer's instructions; a booster vaccination at 21 dpi was included. All remaining animals were humanely euthanized at 35 dpi. This experiment was performed in accordance to the Animals (Scientific Procedures) Act, 1986, UK; following the approval of the AHVLA ethical review committee.

Clinical Signs, Gross Pathology, Histopathology and Viraemia

In both *in vivo* studies pigs were monitored daily for clinical signs, including rectal temperature and a clinical signs were scored as previously described (Halbur *et al*., 1995a). Lungs, tonsils and Tb-LN, Rf-LN, and Med-LN were examined at post-mortem and lung lesions were evaluated by visual inspection and each lobe was scored to reflect the approximate volume percentage of the lung tissue affected, according to the previously described method by Halbur *et al*. (1995a). In the second *in vivo* study a clinical respiratory score was performed, as previously described (Morgan *et al*., 2012). Med-LN is the main apical and medial lung lobes draining lymph node. PRRSV is most frequently detected in these lung lobes (Halbur *et al*., 1996). For this reason, Med-LN was selected in this study. For first gene expression analysis, tissue samples were immediately frozen in liquid nitrogen after euthanasia, and stored at -80 °C until laboratory processing. For the analysis of transcript levels in the second *in vivo* study, a piece of Med-LN was embedded and cryopreserved in OCT^{TM} as previously described (Irving *et al*., 1996).

For microscopical examination samples were fixed in 10% buffered formalin, routinely processed and embedded in paraffin-wax and tissue sections (4 µm) were stained with haematoxylin and eosin. Virus titration was carried out on blood samples at the different time-points using an immunoperoxidase monolayer assay (IPMA) or RT-qPCR in

previous studies from our research group (Gómez-Laguna *et al*., 2010b; Morgan et al., 2012).

Laser capture microdissection

Frozen Med-LN in OCT were prepared to obtain 10 µm thick sections using a cryostat. Tissue sections were placed on membrane-coated slides (PEN-Membrane 2.0 µm; Leica Microsystems, Germany). The cryostat was treated with DNAZap Solution (Life Technologies, Germany) between each sample in order to avoid acid nucleic samples cross-contamination. Sections were then air-dried and decontaminated/fixed in 70% ethanol for 5 minutes, and stained with RNase free haematoxylin for 1 minute. Two consecutive Med-LN tissue sections were used per animal. All observed F and peripheral IF areas were dissected and captured separately in different RNase-free PCR tubes (Greiner bio-one, UK) using a laser microdissector (Leica LMD6500, Leica, Germany). The sample tubes were sealed and immediately frozen and stored at -80 °C until laboratory processing.

Reverse Transcription quantitative Polymerase Chain Reaction

In the first *in vivo* study, tissue samples from lung, tonsil, Tb-LN and Rf-LN were dissected to obtain 20 mg of tissue in order to extract total RNA with the RNeasy Plus Mini kit (Qiagen, USA) following the manufacturer's guidelines. RNA was treated with DNase by using the Turbo DNA-free™ kit (Ambion, USA) following the manufacturer's instructions. The concentration and the purity of mRNA samples were assessed with the ratio of absorbance at 260/280 and 260/230 nm using a Nanodrop ND 1000 spectrophotometer (Thermo Scientific, USA). mRNA integrity was assessed by agarose-TAE electrophoresis and mRNA was stored at -80 ºC until using.

In this experiment cDNA was synthesized from total mRNA obtained by using OligodT/random hexamers and the GeneAmp RNA PCR Core kit (Applied Biosystems, USA) with MuLV Reverse Transcriptase and RNase Inhibitor in a 20 µl volume reaction containing 20 U enzyme, 1 µg total RNA, 100 ng/µl random hexamers, 10mM DNTP mix and DEPC treated water. The reaction was carried out for 15 min at 42 ºC followed by inactivation at 99 ºC for 5 min. cDNA was stored at -80 °C until laboratory processing.

For the first *in vivo* experiment all primers were synthesized by TIB Molbiol (Germany). Primers for each proinflammatory (IFN-α, IFN-γ, IIL-12p35, IL-12p40 and TNF-α) and immunomodulatory (IL-10 and TGF-β) cytokine were selected from previously published reports (integrated into the Porcine Immunology Nutrition Database) (Royaee *et al*., 2004; Gabler *et al*., 2006; Moue *et al*., 2007; Kim *et al*., 2010b). All primer pairs produced amplicons smaller than 160 bp. A primer set for the highly conserved ORF7 that encodes information to virus N protein was selected (Lurchachaiwong *et al*., 2007). In order to generate the standard curves, the transcripts of each cytokine of interest and PRRSV ORF7 were encoded in a plasmid (pGEM-T easy plasmid, Promega, Germany) following the manufacturer's instructions. The plasmids were cut using restriction enzymes Pst I or Xmn I (Promega, Germany), depending on the nucleotide sequence. Synthesized cDNA was amplified using the ABI 7300 Real Time PCR system (Applied Biosystems, USA). Absolute quantification real time qPCR was performed using SYBR green chemistry (Power SYBR Green, Applied Biosystems, USA) for a total volume of 25 µl. Thermal cycle conditions were 10 min at 95 ºC, 40 cycles of 95 ºC for 15 s, and annealing-extension at 60 ºC for one minute for all tested primers. Reverse transcription negative controls and non template controls were included. Finally, a dissociation curve was performed with 110 cycles of

denaturation at 90 ºC for 15 s and renaturation at 60 ºC for one minute with a temperature decrease of 0.3 ºC for each cycle, in order to ensure that a single product had been amplified. All RT-PCR reactions were performed in duplicate in 96 well reaction plates (Applied Biosystems, USA). PRRSV infection kinetics in each tissue were studied by measuring the viral load contents in 200 µg of tissue is rand represented by changes in the Ct as previously described (Kuzemtseva *et al*., 2014). Cytokines gene expression was calculated as target/reference ratio of each sample normalized by target/reference ratio of endogenous calibrators (β-actin, cyclophilin and GAPDH) by geometric averaging of multiple endogenous control genes method (Vandesompele *et al*., 2002). The Fold Change was calculated for each cytokine to compare the cytokine gene expression in the infected animals groups with the control group. To ease their interpretation, these results are expressed in base 2 logarithmic scale.

Table 1. Primers sequences, amplicon size, locus and references for each qPCR assay

Cytokine	Primer Forward	Primer Reverse	Amplicon Length	Reference
PRRSV ORF7	5´-GGGGAATGGCCAGTCAGTCAA-3´	5´-AGGGGAAAATGTGGCTTCTC-3´	**123 bp**	Lurchachaiwong et al., 2007
IFN-α	5'-CCCCTGTGCCTGGGAGAT-3'	5'-AGGTTTCTGGAGGAAGAGAAGGA-3'	**63 bp**	Moue *et al*., 2007
IFN-γ	5'-TGGTAGCTCTGGGAAACTGAATG-3'	5'-GGCTTTGCGCTGGATCTG-3'	**79 bp**	Royaee *et al.* , 2004
TNF-α	5'-ACTCGGAACCTCATGGACAG-3'	5'-AGGGGTGAGTCAGTGTGACC-3'	**134 bp**	Gabler *et al.* ,2008
IL-12p35	5'-AGTTCCAGGCCATGAATGCA-3'	5'-TGGCACAGTCTCACTGTTGA-3'	**84 bp**	Moue *et al*., 2007
IL-12p40	5'-TTTCAGACCCGACGAACTCT-3'	5'-CATTGGGGTACCAGTCCAAC-3'	**160 bp**	Kim *et al.* ,2010b
IL-10	5'-TGAGAACAGCTGCATCCACTTC-3'	5'-TCTGGTCCTTCGTTTGAAAGAAA-3'	**109 bp**	Royaee *et al.* , 2004
TGF-β	5'-CACGTGGAGCTATACCAGAA-3'	5'-TCCGGTGACATCAAAGGACA-3'	**108 bp**	Moue *et al*., 2007
B-Actine	5´- CTACGTCGCCCTGGACTTC-3´	5´- GATGCCGCAGGATTCCAT-3´	**172 bp**	Skovgaard et al., 2009
Cyclophilin	5´- TGCTTTCACAGAATAATTCCAGGATTTA-3´	5´- GACTTGCCACCAGTGCCATTA-3´	**77 bp**	Duvigneau et al., 2005
GAPDH	5´- ACATGGCCTCCAAGGAGTAAGA-3´	5´- GATCGAGTTGGGGCTGTGACT-3´	**106 bp**	Duvigneau et al., 2005

For each LMC sample a total RNA was extracted using the RNAqueous-Micro Kit (Ambion Ltd, UK) following the manufacturer's instructions, including the DNase I treatment and DNase inactivation step. The total RNA was quantified using Qubit 2.0

Fluorometer (Invitrogen, Life Technologies, UK) samples adjusted to the same RNA concentration (2 ng/µl).

In these samples, PRRSV RT-qPCR was done as previously described by Frossard *et al*. (2012). Briefly, 2 µl of samples and standard RNA dilutions were added as template to the QuantiTec Probe RT-PCR Kit (Qiagen, Germany) following the manufacturer's instructions for a total volume of 25 µl. The thermal cycling condition included a retrotranscription phase in two steps 30 min at 50 °C and 15 min at 95 °C; following by a touchdown phase of 10 cycles of denaturation (20 s at 94 °C) and annealing/extension (45 s at 72 °C), with the annealing temperature in these cycles reduced by 1 °C after each cycle. Finally, 38 cycles of denaturation (20 s at 94 °C) and annealing/extension (45 s at 55 °C) were performed.

For this second *in vivo* experiment, cytokine primers and TaqMan probes sets were synthesized by Sigma-Aldrich (UK) (summarized in Table2). All cytokines primer pairs produced amplicons smaller than 150 bp. All primers and probes sets were optimized for our laboratory conditions. In order to obtain complementary DNA (cDNA) a RT-PCR was done using the SuperScript VILO^{TM} cDNA Synthesis Kit (Applied Biosystems, UK) following the guidelines of the manufacturer. cDNA was stored at -80 °C until laboratory processing. 2 µl of diluted cDNA (100x) were used as template for cytokines qPCR using EXPRESS qPCR Supermix (Invitrogen) with ROX reference dye and TaqMan probes designed and validated in silico for each specific cytokine primer set in a total volume of 20 µl. Thermal cycle conditions were two minutes at 95 ºC, 45 cycles of denaturation at 95 ºC for 15 seconds and annealing-extension at 60 ºC for one minute. Reverse transcription negative controls and non-template controls were included. The plates were centrifuged before being run on Stratagene MX-3000P platform (Stratagene, UK). The determination of PRRSV replication was done

measuring the viral load content in each Med-LN compartment; which is represented by changes in the Ct as previously described (Kuzemtseva *et al*., 2014). Cytokines fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and, the β-actine was used as endogenous calibrator. Relative gene expression results are expressed in

Table 2. Primers and probes sequencies and concentration of use for each qPCR assay.

Gene	Primer Forward nM	Primer Reverse nM	Probe nM
β-Actin	5´-cactcctaacgctgtggatcag-3´ 300	5´-ccacttaactatcttgggcttatcg-3´ 300	5´-[6FAM]-cacgtgcttcacgcggcagc-[TAM]-3´ 50
TNF-α	5´-tggccccttgagcatca-3´ 900	5´-cgggcttatctgaggtttgaga-3´ 600	5´-[6FAM]-ccctctggcccaaggactcagatca-[TAM]-3´ 50
IFN-α	5´-tcagctgcaatgccatctg-3´ 150	5´-agggagagattctcctcatttgtg-3´ 600	5´-[6FAM]-tgacctgcctcagacccacagcc-[TAM]-3´ 50
IFN-γ	5´-gaaaagctgattaaattccggtag-3´ 300	5´-aggttagatcttggtgacagatc-3´ 900	5´-[6FAM]-tctgcagatccagcgcaaagccatcag-[TAM]-3´ 50
SOCS1	5´-ttcttcgccctcagtgtgaa-3´ 300	5´-ggcctggaagtgcacgc-3´ 300	5´-[6FAM]-ttcgggccccacaagcatcc-[TAM]-3´ 50
IL-10	5´-tgagaacagctgcatccacttc-3´ 300	5´-tctggtccttcgtttgaaagaaa-3´ 300	5´-[6FAM]-caaccagcctgccccacatgc-[TAM]-3´ 150
TGF-β	5´-agggctaccatgccaattt-3´ 600	5´-ccgggttgtgctggttgt-3´ 600	5´-[6FAM]-cactcagtacagcaaggtcctggctctgta-[TAM]-3´ 50
IL-23	5´-agaagagggagatgatgagac-3´ 900	5´-agcaggactgactgccgtcc-3´ 300	5´-[6FAM]-ctgaggatcacagccatccccgc-[TAM]-3´ 50

base 2 logarithmic scale.

Evaluation of RT-qPCRs based in SYBR Green chemistry

RT-qPCR amplification conditions were carried out as described above for the first *in vivo* experiment. qPCR was optimized for target specificity (ratio cDNA:water 1:1, 1:5, and 1:25) and primer concentration (50, 200, 300 and 900 nM) and these were run in duplicate. The amplification specificity was evaluated by gel electrophoresis in 2% (wt/vol) agarose gel in 1 x TAE (Tris Acetate-EDTA buffer; Sigma-Aldrich, USA), and RedSafe™ (Intron Biotechnology, South Korea) staining (Fig. 2). The qPCR conditions were based on: (i) absence of primer dimers; and, (ii) minimum Cq value at same

amount of cDNA. Cq is defined as the cycle number at which the SYBR-Green-DNA interaction fluorescent signal attained a threshold level avoiding the background fluorescence (equivalent to the Ct) (Trichopad *et al*., 2010).

Standar curves for each primer set were prepared in DNase and RNase free MiliQ water such that the final copy number in the qPCR assay ranged from 10^8-10^1 copies/µl for 10-fold dilutions and from 10^7-305 copies/µl for 2-fold dilutions. Replicates for 10-fold and 2-fold serial dilutions were completed in quintuplicate which indicates the repeatability of qPCR assays; includin g non-template controls.

Efficiency: $E = 10^{(-1/slope)}$. The %E = (E x 100) / 2. An E value of 2 implies that two copies of DNA are generated from every template during the amplification phase of PCR reaction in each cycle. The slope was provided by the ABI 7300 Real Time PCR system (Applied Biosystems, USA).

Coefficient of Variation: CV = (SD/Cq average) x 100; where SD is the standard deviation of replicates of the same amount of template, and Cq average is the arithmetic mean of the Cq values in the replicates of the same amount of template. The CV shows the extent of variability in relation to mean of the Cq.

Factor of Discrimination: $FD = E^2xSD$. FD determines the assay ability to distinguish different amounts of DNA along the fold dynamic range. This estimates the amplitude between the maximum and minimum Cq values in the replicates of the same amount of cDNA template.

The analytical sensitivity of these qPCR assays was characterized by the LOD, taken as the last concentration in which all qPCR replicates were detected with a CV% lower than 2%. All parameters were calculated using Microsoft Office Excel 2007 (Microsoft Co., USA).

Immunohistochemistry

The Med-LN cell markers immunolabelling was done by the avidin-biotin complex method (ABC Vector Elite, Vector laboratories, USA) as described previously (Gómez-Laguna *et al*., 2010a; Barranco *et al*., 2011). Briefly, 4 µm thick sections were dewaxed and rehydrated followed by endogenous peroxidase inhibition through H_2O_2 3% in methanol during 30 min. Depending on the epitope of interest, antigen retrieval in the tissues section was performed by enzymatic trypsin/alpha-chymotrypsin (CD3 and MAC-387) or by microwaving the sections in citric acid pH6 (CD79a) or pH6.0 citrate buffer (PRRSV nucleocapsid N protein). The slides were mounted in a Sequenza Immunostaining Centre (Shandon Scientific, Runcorn, UK) and washed with Tris buffered saline (TBS; pH 7.6, 0.005 M) (Sigma–Aldrich, USA) and incubated for 30 min at room temperature with 100 µl per slide of blocking solution. The primary antibodies used were monoclonal anti-human CD3 (1:1000; Dako), monoclonal anti-human CD79a (2.5:1000; Dako), monoclonal anti-human MAC-387 (1:100; Serotec). All these antibodies were applied for 1 h at room temperature. In each case, the corresponding biotinylated secondary antibody (Vector Laboratories) was incubated for 30 min at room temperature. After this step, slides were incubated during 30 min with avidin-biotin complex and the labelling carried out using 3,30-diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Positive and negative controls, as well as isotype controls, were included in each IHC run.

The immunolabelled Med-LN sections were examined by light microscopy. The immunolabelling measurements were recorded using a score ranking from -3 to 0 (cellular depletion) and from 0 to 3 (cellular increment) comparing to the control group.

Positive scale: 0 = absence (<1 positive cell/structure); 1 = scarce (1-10 positive cells/structure); 2 mild-moderate (11-30 positive cells/structure); 3 abundant (>31 positive cells/structure). Negative scale: 0 = absence (no different to control group); -1 = scarce (5% less positive cells compares with control group); -2 mild-moderate (6-10% less positive cells compares with control group); -3 abundant (more than10% less positive cells compares with control group).

Statistical analysis

Statistical analyses were done with GraphPad PrismV.5 software; and SPSS 15.0 software. Significant effects on polarized MØ, compared to unpolarized MØ were assessed by repeated measures ANOVA and Dunnett's Multiple Comparison tests. For cell surface markers and cytokines expression the differences were assigned by Kruskal-Wallis test followed by the Mann-Whitney-U non parametric test. The correlations between PRRSV and cytokines were determined by the Spearman's Rho analysis. $P < 0.05$ was at least considered statistically significant. Significance variations were indicated by asterisk or different letters based on statistical analysis.

Results

In vitro study

MØ phenotype

In order to determine the impact of the selected cytokines on MØ differentiation, CD163, CD14, MHC I, MHC II, CD16 and CD86were measured by flow cytometry (Fig. 2A). The PRRSV receptor CD163 was significantly down-regulated by IL-4 as shown in Fig. 2B. The expression of CD14 was down-regulated by each cytokine. As expected, MHC I was up-regulated by IFN-β and IFN-γ but not by IL-4. MHC II was induced by IFN-γ whereas IFN-β and IL-4 did not significantly influence MHC II expression. Similarly, CD16 was up-regulated by IFN-γ only. Finally, CD80/86 remained at low level even after cytokine stimulation. In conclusion, while these results demonstrate that the cytokines used modulate MØ, the main effect of the IFN's was found to be an up-regulation of MHCI and MHCII, whereas the main effect of IL-4 was found to be a down-regulation of CD163.

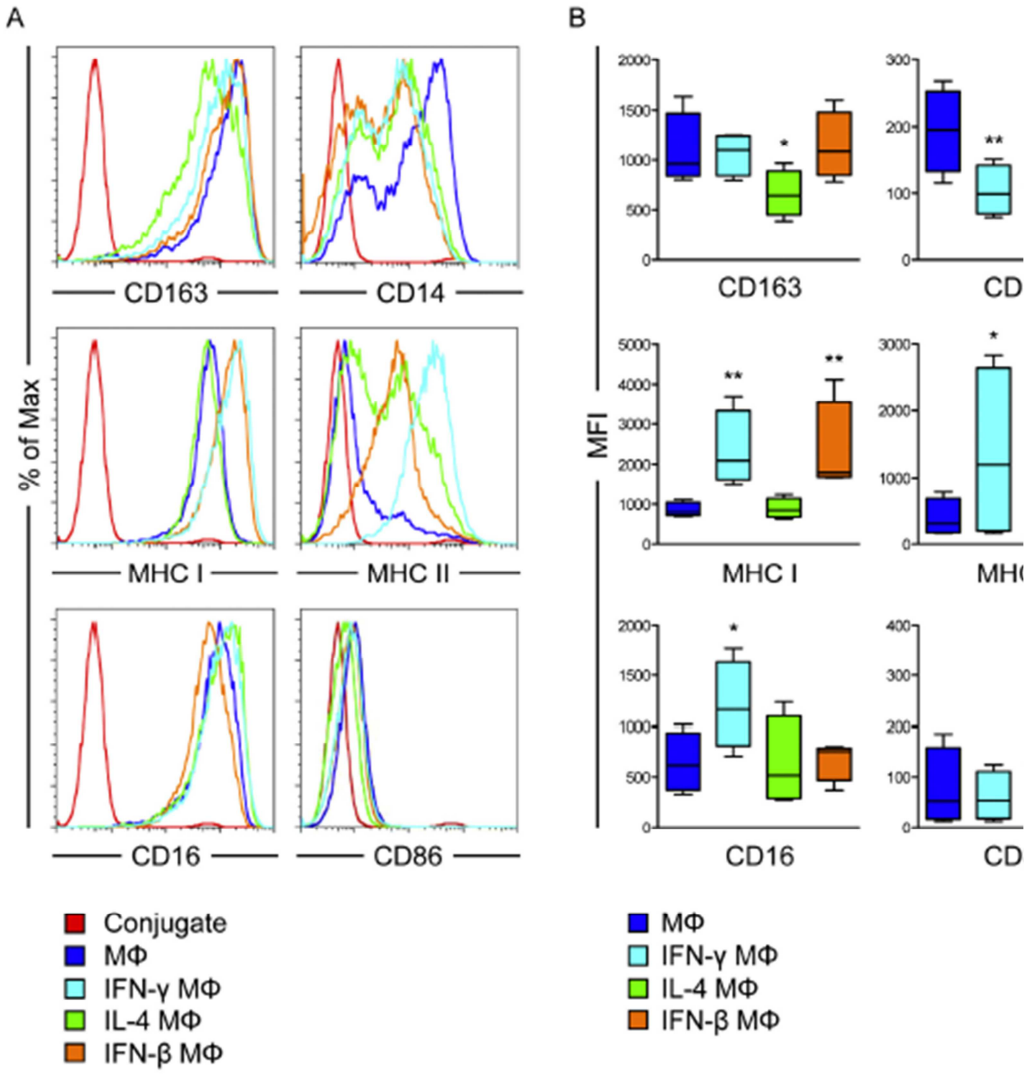

Fig. 2. Differential expression of cellular markers after MØ polarization. (A) MØ were incubated with IFN- γ (IFN-γ MØ), IL-4 (IL-4 MØ), IFN-β (IFN-β MØ) or with medium only (MØ) for 24 h, and CD163, CD14, MHC I, MHC II, CD16 and CD86 were measured by flow cytometry. The data are representative of four independent experiments performed in culture triplicates. (B) Mean fluorescence intensity (MFI) of unpolarized MØ, IFN-γ MØ, IL-4 MØ and IFN-β MØ shown in A. Boxplots represent four independent experiments performed in culture triplicates. Significant differences compared to unpolarized MØ were denoted by * and ** where $P < 0.05$ and $P < 0.01$ respectively.

MØ viability

To measure MØ, we evaluated the cultures by microscopy but did not employ a cell viability stain such as propidium iodide, as MØ are highly phagocytic and rapidly remove necrotic and apoptotic cells. The viability of MØ varied depending on the MØ

activation and the PRRSV strain used (Fig. 3A). As determined by the disappearance of adherent MØ and presence of detached shrunken cells with highly granular cytoplasm vesicles and cell debris, undifferentiated MØ and "IL-4 MØ" were sensitive to all PRRSV strains. Infection with VR-2332 and RVB-581 strain particularly destroyed the MØ. In contrast, IFN-γ protected the MØ against cytopathogenic effects induced by LVP23 and VR-2332 but not against RVB-581. "IFN-β MØ" were the most resistant against all 3 viruses, although the density of MØ was reduced as well after infection with VR-2332 and RVB-581

To quantify MØ viability we employed the FSC/SSC profiles obtained by flow cytometry. To this end, an electronic gate was defined in the mock-treated MØ. After infection, many MØ had strongly reduced FSC/SSC and remained outside of this gate implying a shrinking process or cellular disintegration (Fig. 3B). As shown in Fig. 3C, the cytokine treatments did not influence the percentage of MØ in the FSC/SSC gate.

Table 3. MØ polarization with IFN- γ and IFN-β impacts on PRRSV infection and viral titre.

		MØ	IFN-γ MØ	IL-4 MØ	IFN-β MØ
PRRSV N* cells (%)[a]	**LVP23**	55.73 ± 9.07	7.67 ± 7.59*	43.10 ± 4.42	17.81 ± 15.95*
	VR-2332	65.68 ± 4.89	18.30 ± 7.48*	57.23 ± 1.24	32.83 ± 24.44*
	RVB-581	74.9 ± 10.49	52.48 ± 15.03	75.45 ± 9.29	59.98 ± 24.13
Titre log10 ($TCID_{50}$/ml)[b]	**LVP23**	5.31 ± 0.12	3.81 ± 0.24*	6.00 ± 0.35	4.00 ± 0.58*
	VR-2332	6.12 ± 0.14	4.94 ± 0.37*	5.87 ± 0.25	4.81 ± 0.37*
	RVB-581	5.00 ± 0.41	4.25 ± 0.46	5.62 ± 0.32	4.56 ± 0.51

[a]Percentage of PRRSV N+ cells at 16 hpi (MOI of 0.1 TCID50/cell).

[b]Viral titre at 16 hpi ± standard deviation of two independent experiments performed in culture duplicates are shown.

*Significant difference compared to unpolarized M_ using ANOVA and Dunnett's multiple comparison test ($P < 0.05$). The data are from the kinetic experiment (Fig. 5).

This analysis confirmed that all viruses reduced MØ viability of undifferentiated MØ. IFN-γ induced good protection against LVP23 and VR-2332 but not RVB-581. IFN-β

induced complete protection against LVP23 and VR-2332 but only partial protection against RVB-581. IL-4 appeared to induce a partial resistance against all viruses.

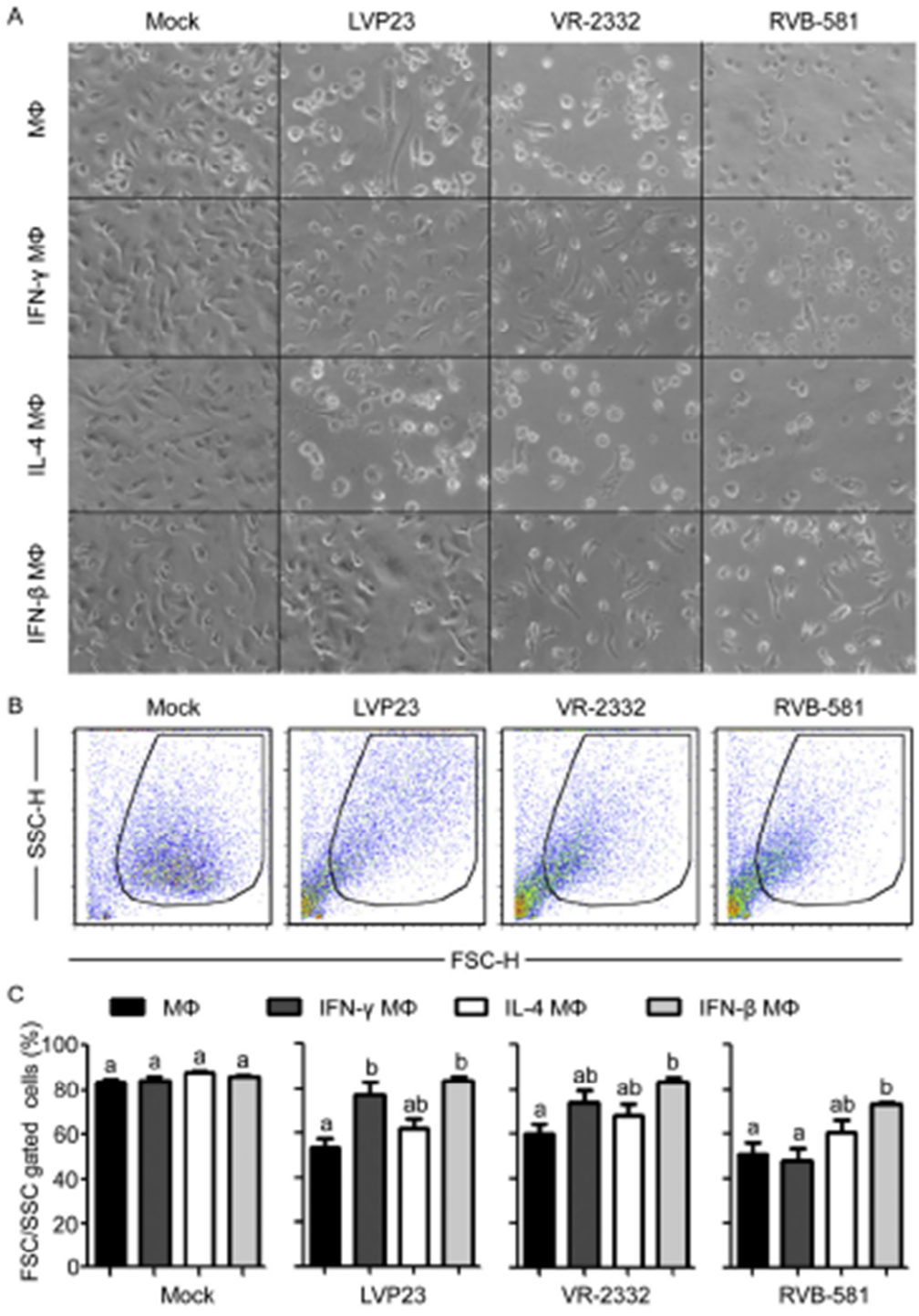

Fig. 3. Cell morphology and FSC/SSC of M_ after PRRSV exposure. (A) Photography (400×) of MØ, IFN-γ MØ, IL-4 MØ and IFN-β MØ infected at an MOI of 0.1 $TCID_{50}$/cell with LVP23, VR-2332, RVB-581 or treated with mock. Phase-contrast microscopy analysis was performed at 20 hpi. (B and C) FSC/SSC data of PRRSV infected M_. B. FSC/SSC plot of unpolarized MØ. FSC/SSC plot with gating to exclude shrunken (dead) cells and debris at 20 hpi with LVP23, VR-2332, RVB-581 (MOI of 0.1 $TCID_{50}$/cell) or mock treatment. (C) Percentage of FSC/SSC-gated MØ defined in B with bars show the mean ± standard deviations of three independent experiments performed in culture triplicates. Different letters indicate statistically significant differences ($P < 0.05$).

MØ susceptibility to PRRSV infection

Considering these results, we tested MØ infection by quantification of the nucleocapsid (N) expression by flow cytometry. Undifferentiated MØ and "IL-4 MØ" were highly susceptible to all PRRSV tested with higher infection rates obtained with the PRRSV-2 isolates when compared to LVP23, although this was not statistically significant (Fig. 4A and C). In contrast, IFN-γ and IFN-β almost completely prevented MØ infection by LVP23 but only partially against VR-2332 and RVB-581 (Fig. 4B and D). RVB-581 was significantly more resistant to the antiviral effects of both IFN's when compared to LVP23.

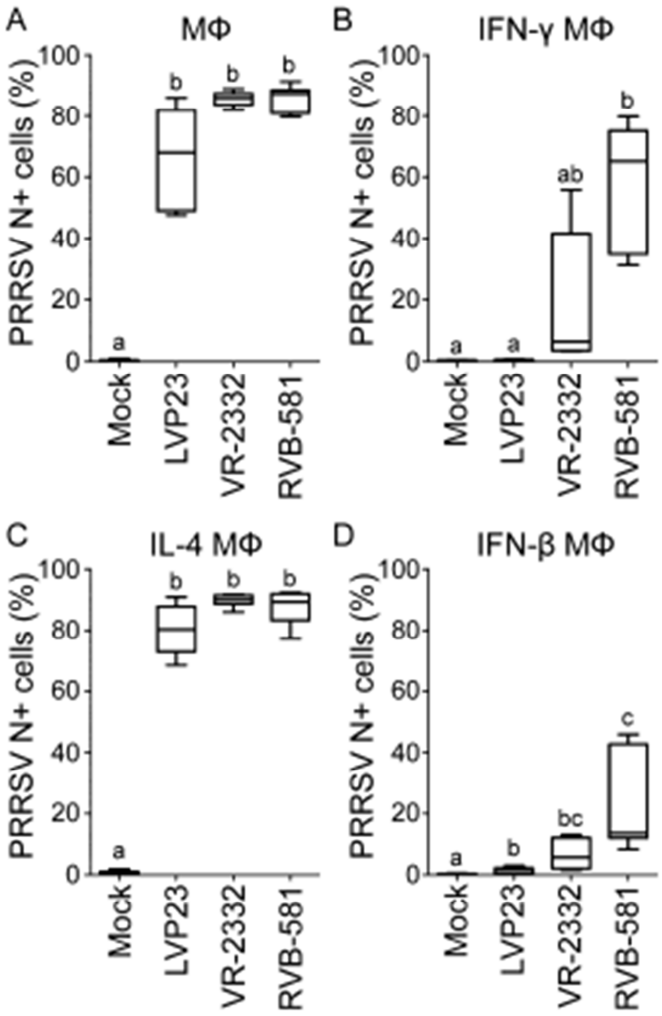

Fig. 4. Effect of cytokine polarization on M_ susceptibility to infection with LVP23, VR-2332 and RVB-581. Unpolarized MØ (A), IFN-γ MØ (B), IL-4 MØ (C) and IFN-β MØ (D) were infected with LVP23, VR-2332, RVB-581 or mock control for 20 h. Percentage of PRRSV nucleocapsid (N) expression was measured by flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N^+) cells of three independent experiments performed in culture triplicates. The different letters indicate significant differences between viruses ($P < 0.05$).

Taking this into account, we performed a kinetic analysis of PRRSV infection and replication in MØ. As for LVP23 infection, the results confirmed that both IFN prevented the expression of N protein in MØ, but did not completely stop virus replication (Fig. 5A-D, left panels). Nevertheless, the highest titres found at16 hpi were reduced by 1.5 logs by IFN-γ and 1.3 logs by IFN-β (Table 2). As for VR-2332, again a

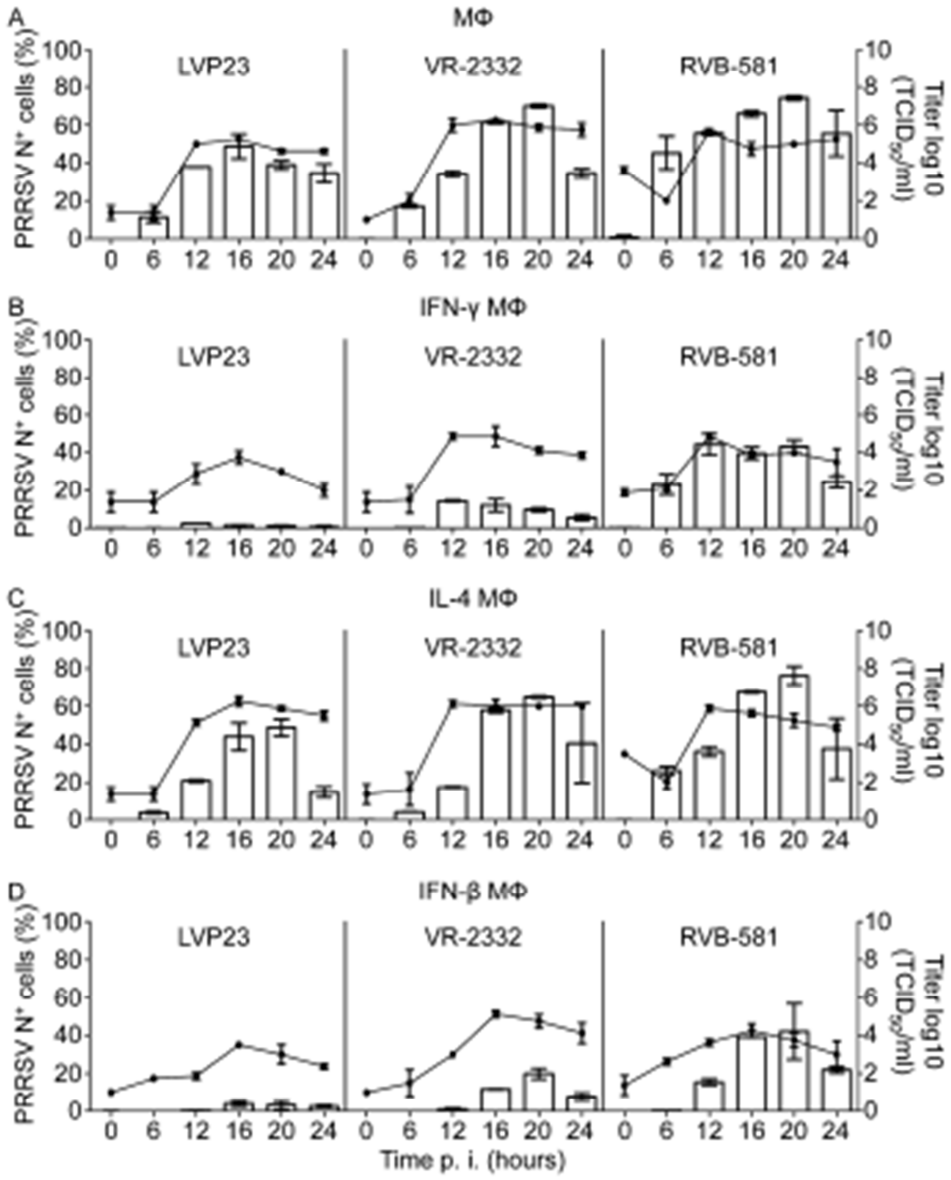

Fig. 5. Kinetic of N expression and virus titre in polarized MØ infected with LVP23, VR-2332 and RVB-581. Unpolarized MØ (A), IFN-γ MØ (B), IL-4 MØ (C) and IFN-β MØ (D) were infected with LVP23, VR-2332 and RVB-581 (MOI of 0.1 $TCID_{50}$/cell) for the indicated time on the x-axis. Bars indicate the mean ± standard deviation of PRRSV nucleocapsid expression (%N^+ cells, left y-axis) measured by flow cytometry. The dotted shows viral titre ($TCID_{50}$/ml, right y-axis) measured in the supernatants. One out of two representative experiments in duplicate is shown.

clear antiviral effect was found with the IFN-γ and IFN-β both at the level of N expression and viral titres (Fig. 5A–D, middle panels, Table 2). Nevertheless, the reduction in N^+ MØ and viral titres was lower when compared to LVP23 infected MØ (Table 2). As for RVB-581, the IFN's only had a minor effect on the peak of N^+ MØ and on viral titres (Fig. 5A–D, right panels and Table 2). These findings confirm that PRRSV isolates differ in their IFN sensitivity, with LVP23 being the most sensitive isolate and the HP isolate RVB-581 the most resistant.

We consequently tested more PRRSV-1 and PRRSV-2 isolates of different virulence to confirm these findings. Statistical analysis of at least two independent experiments demonstrated that all PRRSV-2 isolates had a significantly higher resistance to IFN-γ when compared to LVP23, while this discrimination was not possible in undifferentiated MØ (Fig. 6 A and B).

Furthermore, the Chinese HP PRRSV isolate RVB-581 was significantly more resistant than all PRRSV-1 isolates. In undifferentiated MØ, the infection rates by the Olot/91 and the Spanish field isolate 2982 were lower than with the other viruses

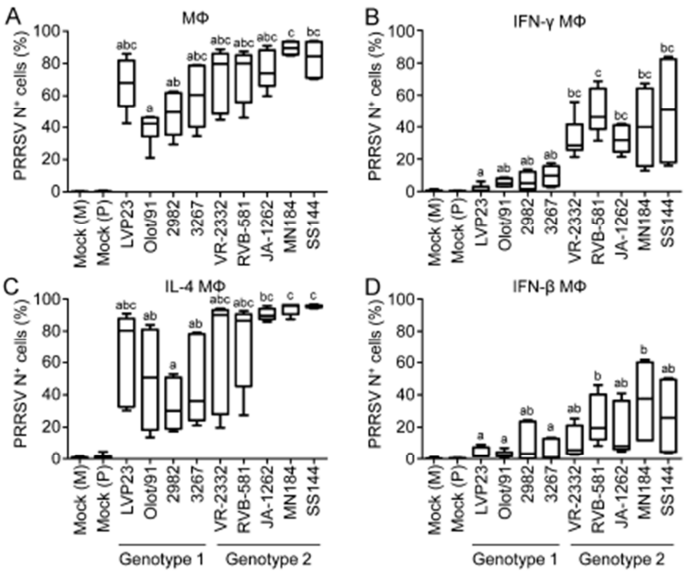

Fig. 6. Differential infection of IFN-γ MØ by PRRSV-1 and PRRSV-2 strains. Unpolarized MØ (A), IFN-γ MØ (B), IL-4 MØ (C) and IFN-β MØ (D) were infected with various genotype 1 PRRSV (LVP23, Olot/91, 2982 and 3267) or various genotype 2 PRRSV (VR-2332, RVB-581, JA-1262, SS144 and MN184) at an MOI of 0.1 TCID50/cell or treated with mock for 20 h. Mock (M) and mock (P) stands for MARC-145 cells and PAM lysates respectively. The percentage of PRRSV N expression was measured by flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells of a pooled data from triplicate culture of at least two independent experiments. The different letters indicate significance between viruses ($P < 0.05$).

tested but this was not observed in the IFN-γ- and IFN-β-treated MØ. Differences in infection rates were observed in the "IL-4 MØ" as well but these did neither relate to genotype nor virulence (Fig. 6C). It appeared also that differences relating to virulence and genotypes could be found using "IFN-β MØ" (Fig. 6D). PRRSV-2 isolates were more resistant to the effect of IFN-β but the PRRSV-1 isolate 2982 did not differ from this group. A statistical analysis demonstrated that both IFN-γ and IFN-β significantly reduced the N expression for all isolates compared to MØ treated with medium only.

These findings pointed on a genotype-dependent resistance in IFN-treated MØ. Considering that PRRSV-2 is generally more virulent than PRRSV-1, an alternative interpretation would be an inverse relationship of resistance to virulence independent of the genotype. We therefore tested HP-PRRSV isolate Lena, a PRRSV-1 belonging to subtype III in MØ polarized by IFN-γ and IFN-β. The levels of N expression in MØ infected with Lena were clearly and significantly higher than LVP23, and at least as high as those reached by RVB-581 (Fig. 7A and B). From these data, we propose that IFN-treated MØ may allow to discriminate PRRSV isolate also relating to their virulence.

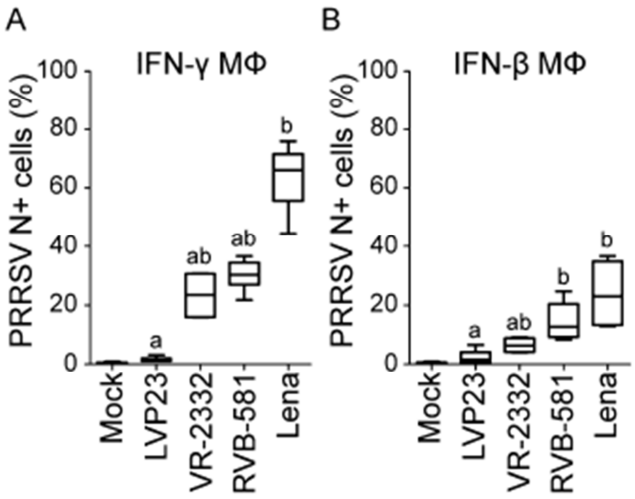

Fig. 7. Genotype-independent IFN-resistance of HP-PRRSV in MØ. IFN-γ (A) and IFN-β MØ were infected with LVP23, VR-2332, RVB-581 and Lena (MOI of 0.1 $TCID_{50}$/cell) or mock control. Percentage of PRRSV nucleocapsid (N) expression was measured by flow cytometry. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N^+ cells of two independent experiments performed in culture triplicates. The different letters indicate significant differences between viruses ($P < 0.05$).

Production of cytokines and IFN-α in infected MØ

We did not find TNF-α or IL-10 production in any of the supernatants harvested from the infected MØ. Whereas TNF-α was induced by LPS (10 µg/ml) in undifferentiated MØ (2.3 ng/ml), IFN-γ MØ (4.3 ng/ml), IL-4 MØ (4.1 ng/ml) and IFN-β MØ (3.3 ng/ml), no detectable amounts of IL-10 were found in any types of LPS-stimulated MØ. Furthermore, IFN-α was not detected with one exception. LVP23 induced IFN-α in "IFN-γ MØ" at 20 hpi (82.65 U/ml, $P < 0.05$; data not shown).

In vivo studies

Clinical Signs, Gross Pathology, Histopathology and Viraemia

Neither clinical sings nor gross lesions were observed in control animals throughout both *in vivo* studies. In infected pigs, rectal temperature remained within the normal physiological range. In animals of the first study a mild increase at 3 and 10 dpi was detected (39.78 ± 0.44 ºC, and 39.34 ± 0.38 ºC, respectively), whereas in the second *in vivo* experiment only SU1-bel infected animals showed hyperthermia at 3, 8-10 dpi (over 40ºC). In the last experiment Su1-Belstrain infected animals developed higher mean score of clinical signs (mean clinical score from 5 to 10) compared with the rest of animals of this study (mean clinical score up to 3).

Inoculated animals showed non-collapsed, mottled tan and rubbery lung parenchyma from 3 dpi onward. A mild to moderate enlargement of retropharyngeal Tb-LN and Rf-LN was observed from 7 dpi until the end of the first *in vivo* study, at 35 dpi Med-LN no damage was observed during the second study. For the first *in vivo* experiment, lung microscopic lesions were characterized by interstitial pneumonia, infiltration of mononuclear cells within the alveolar septa and type-2 pneumocyte hyperplasia from 7 dpi onward. Histopathological examination of lymphoid organs revealed, from 7 dpi

until the end of this study, a mild hypertrophy of germinal centers and the presence of apoptotic bodies in the lymphoid follicles of the lymph nodes, and in a lesser extent in the tonsil, with cell pyknosis, mitosis and necrosis. From the second *in vivo* experiment virus-infected groups showed no significant histological lesions at 35 dpi.

PRRSV was detected in blood samples from inoculated animals from 3 dpi until the end of the first study (24 dpi), with the maximum level at 10 dpi (3.280 ± 0.657, expressed as log 10). In blood from the second experiment the virus was detected until 28 dpi, these animals has not circulating PRRSV at 35 dpi. No virus was detected in control animals throughout both studies. More details on the clinical signs, viraemia and gross and microscopic lesions have been previoulsy described by our group (Gómez-Laguna *et al*., 2010b; Barranco *et al*., 2012a; Morgan et al., 2012). As previously described by Morgan *et al*. (2012), in the second *in vivo* experiment, the vaccination against ADV did not affect the progression of PRRSV infection, and vice versa, the infection with PRRSV did not affect the immune response to ADV vaccination.

Evaluation of RT-qPCRs based in SYBR Green chemistry

BLAST in silico tool was used for all primers and resulted in 100% homology to target genes. Primer concentration for each cytokine was selected based on the lowest Ct value

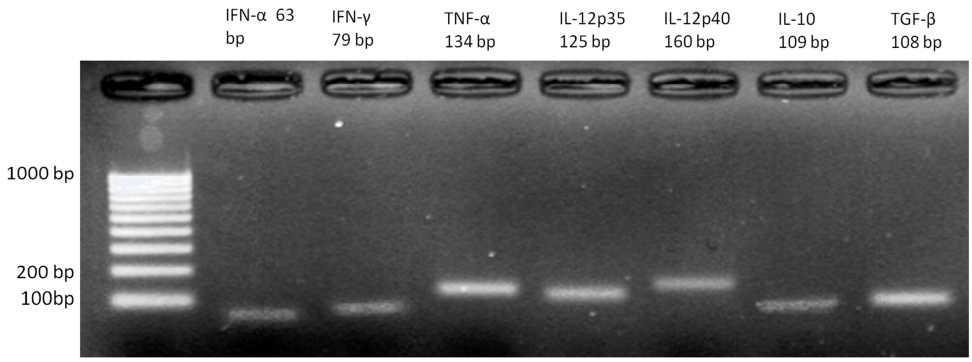

Fig. 8. Agarose gel electrophoresis of the real time qPCR product of each cytokine.

obtained with the lowest amount of cDNA: 200 nM for IFN-γ, 300nM for IFN-α, TNF-α, IL-10 and TGF-β, and 900 nM for IL-12p35 and IL-12p40. Specific single bands with the expected amplicon sizes were obtained by agarose electrophoresis and confirmed by DNA sequencing of the PCR products (Fig. 8).

These qPCRs showed dissociation curves with single peaks for IFN-γ, TNF-α and IL-12p35. A small peak preceding the main peak was observed for IFN-α, IL-12p40, IL-10 and TGF-β (these peaks were identified as artefact). No primer dimer formation was observed in no-template or negative RT controls for any cytokine.

All qPCR amplification plots displayed amplification curves with an exponential phase followed by a non-exponential phase, ending with a plateau. The LOD was 100 copies for the qPCR assays of IFN-α, IFN-γ, IL-12p35, IL-12p40 and IL-10. In the case of TGF-β and TNF-α qPCR assays, the LOD was 305 copies. These assays are sensitive enough to detect even low levels of cytokine gene expression in porcine tissues.

Table 4. Correlacion coefficient of standard curve (R^2), Efficeincy (E) and percentage of Efficiency (%E) of each qPCR assay.

	R^2		E		%E	
Cytokine	**1:2**	**1:10**	**1:2**	**1:10**	**1:2**	**1:10**
IFN-α	0.99	0.99	1.90	1.83	95.2	91.7
IFN-γ	0.99	0.99	1.92	1.90	96.25	95.25
TNF-α	0.99	0.99	2.00	1.68	100.45	84.2
IL-12 p35	0.97	0.99	1.91	1.97	95.55	98.75
IL-12 p40	0.95	0.99	1.82	1.97	91.1	98.9
IL-10	0.75	0.99	2.38	1.92	119.35	96.45
TGF-β	0.99	0.97	1.79	1.97	89.53	98.45

All qPCR assays rendered correlation coefficients of $R^2 > 0.95$ (Table 1) except for IL-10 2-fold dilution (R^2 = 0.75). E values ranged between 1.68 (TNF-α) and 2.38 (IL-10) using a plasmid DNA template. The E and percentage of E of each 2-fold and 10-fold serial dilution qPCR for each cytokine are summarized in Table 4.

The CV results indicated that the qPCR assay variability was lower than 3% along the 2 and 10-fold dynamic range for all cytokines, except for IL-12p40 and TGF-β 10-fold dilutions which CV was higher than 5% just in some dilutions.

The mean FD in the 2-fold dilutions qPCR assays was lower than 1.29 for all cytokines except for IL-10 (FD = 2.59), which showed an irregular FD along the 2-fold and 10-fold dilution dynamic range. The mean FD in the 10-fold dilutions qPCR assays was lower than 1.97 for all the cytokines. The mean FD was higher in the 10-fold dilution than in the 2-fold dilutions qPCRs assays. The FD increased in two-fold and ten-fold dilutions qPCR when less than 1000 template copies were used.

PRRSV kinetics of replication in lung, tonsil and lymph nodes

During the first *in vivo* experiment the replication of PRRSV-1 2982 strain was significantly increased in all examined tissues from inoculated pigs from 3 dpi onwards (Fig. 9). PRRSV-1 2982 strain replication showed a two peak curve in the lung of inoculated animals with a first significant increase of expression at 7 dpi (Ct 17.61 ± 0.96) and a maximum peak of virus replication at 21 dpi (Ct 15.11 ± 1.90). PRRSV kinetics of replication in tonsil showed a progressive increasing with the maximum at 21 dpi (Ct 13.06 ± 0.75). In Tb-LN, the PRRSV associated to this tissue increased at 3 dpi remaining at similar levels until 10 dpi (Cts from 15.73 ± 1.48 to 15.65 ± 1.75, respectively); following it decreased to reach again similar values at the end of the study (24 dpi) (Ct 16.43 ± 0.64). In Rf-LN, PRRSV kinetics of replication showed a high levels of virus associated to Rf-LN at 3 dpi (Ct 12.89 ± 1.74) followed by an intense decreased at 7 dpi (Ct 12.89 ± 0.83) describing then a progressive increase from 10 to 24 dpi (Cts from 16.52 ± 1.64 to 15.86 ± 0.73, respectively).

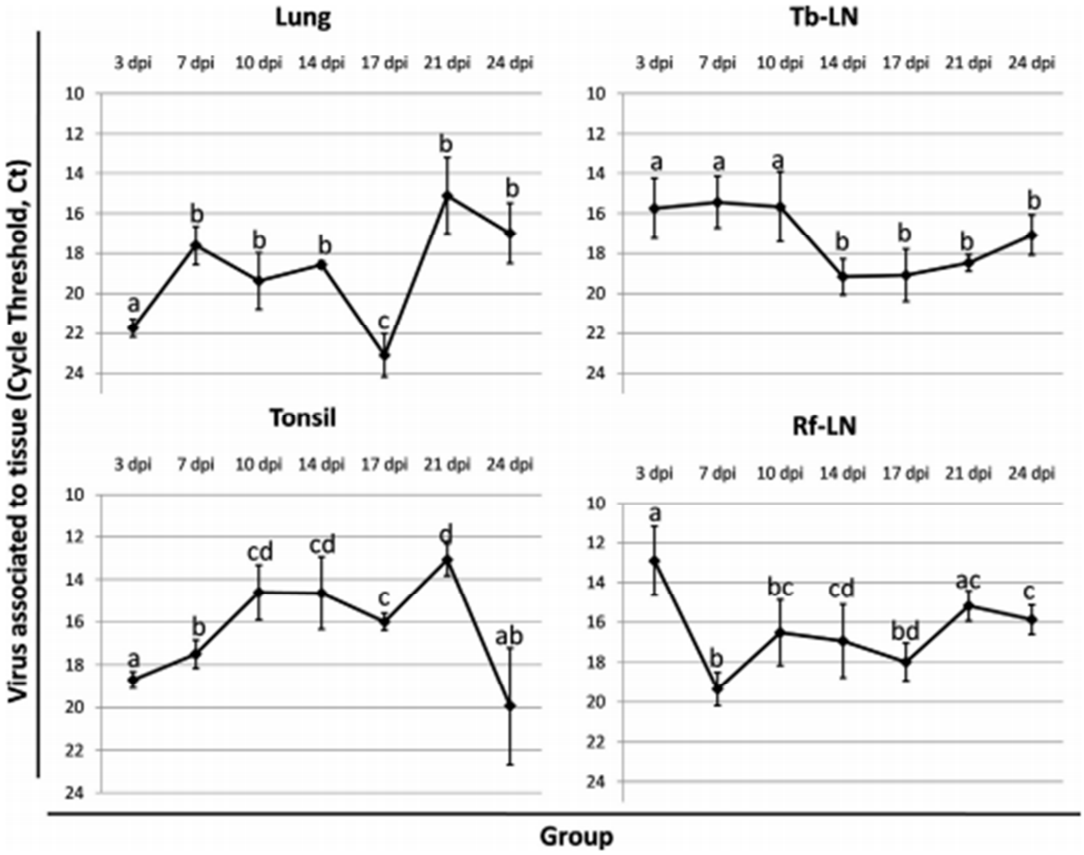

Fig. 9. PRRSV-1 2982 kinetics of replication in 200 µg of lung, tonsil, Tb-LN and Rf-LN. Viral load is represented by changes in the cycle threshold, Ct. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

The PRRSV-1 2982 strain kinetics of replication was compared between tissues at each time point of this study (Fig. 10). PRRSV was not detected in control animals. Viral replication was higher in lymphoid organs than in the lung of PRRSV-inoculated animals throughout the study. At 3 dpi, the highest PRRSV replication was detected in both lymph nodes, especially in the Rf-LN and in a lesser extent in the Tb-LN, and remained elevated at 7 dpi in Tb-LN (Table 5). From 10 to 21 dpi the maximum PRRSV viral load was detected in tonsil (Table 5). At 17 dpi PRRSV replication decreased more markedly in the lung to increase at 21dpi (Table 5). At 24 dpi the PRRSV kinetics of replication was similar in all examined organs, showing the highest

viral replication in Rf-LN, with the lowest PRRSV replication being detected in the tonsil (Table 5).

Table 5. PRRSV kinetics of replication in each tissue at each time-point.

	Lung	**Tonsil**	**Tb-LN**	**Rf-LN**
	Ct±SD	**Ct**±SD	**Ct**±SD	**Ct**±SD
Control	No detected	No detected	No detected	No detected
3dpi	21.73±0.45	18.70±0.39	15.74±1.49	12.90±1.74
7 dpi	17.61±0.96	17.50±0.66	15.43±1.28	19.34±0.84
10 dpi	19.38±1.41	14.62±1.29	15.65±1.75	16.52±1.65
14 dpi	18.58±0.18	14.64±1.70	19.17±0.90	16.93±1.84
17 dpi	23.10±1.07	16.00±0.39	19.09±1.31	17.98±0.94
21 dpi	15.11±1.90	13.07±0.75	18.47±0.41	15.19±0.75
24 dpi	16.99±1.51	19.93±2.71	17.08±1.00	15.87±0.73

Tb-LN: TracheoBronchial Lymph Node; Rf-LN: Retropahryngeal Lymph Node.

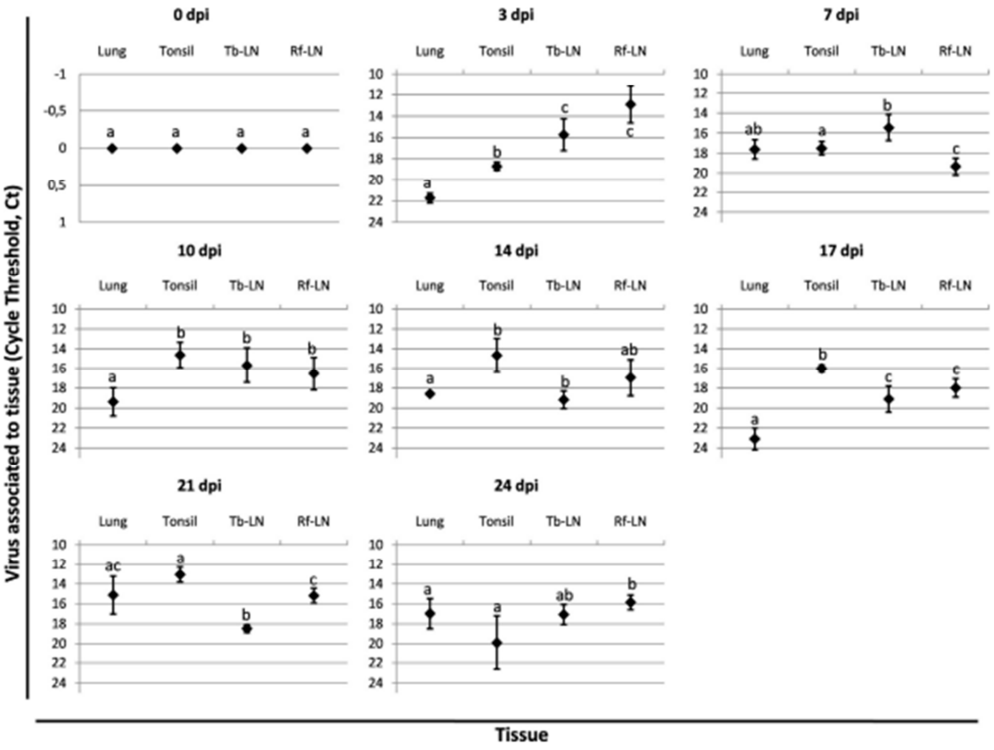

Fig. 10. PRRSV-1 2982 kinetics of replication in 200 µg in lung, tonsil, Tb-LN and Rf-LN were compared between tissues in each time-point of this study. Viral load is represented by changes in the cycle threshold, Ct. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

For the second *in vivo* study all PRRSV RNA measurements results are reflected in figure 11. There was no expression of PRRSV RNA in the Med-LN of control animals (Fig. 11). All PRRSV infected animals showed a higher PRRSV replication at 3-7 dpi than at 35 dpi ($P < 0.05$). The LV group showed at 3 dpi a maximum replication in IF area of Med-LN (Ct = 19.11 ± 1.27 SD). At 7 dpi the PRRSV replication was very similar between Med-LN compartments (Ct = 19.52 ± 1.90 SD; Ct = 18.92 ± 0.58 SD; F and IF area respectively).

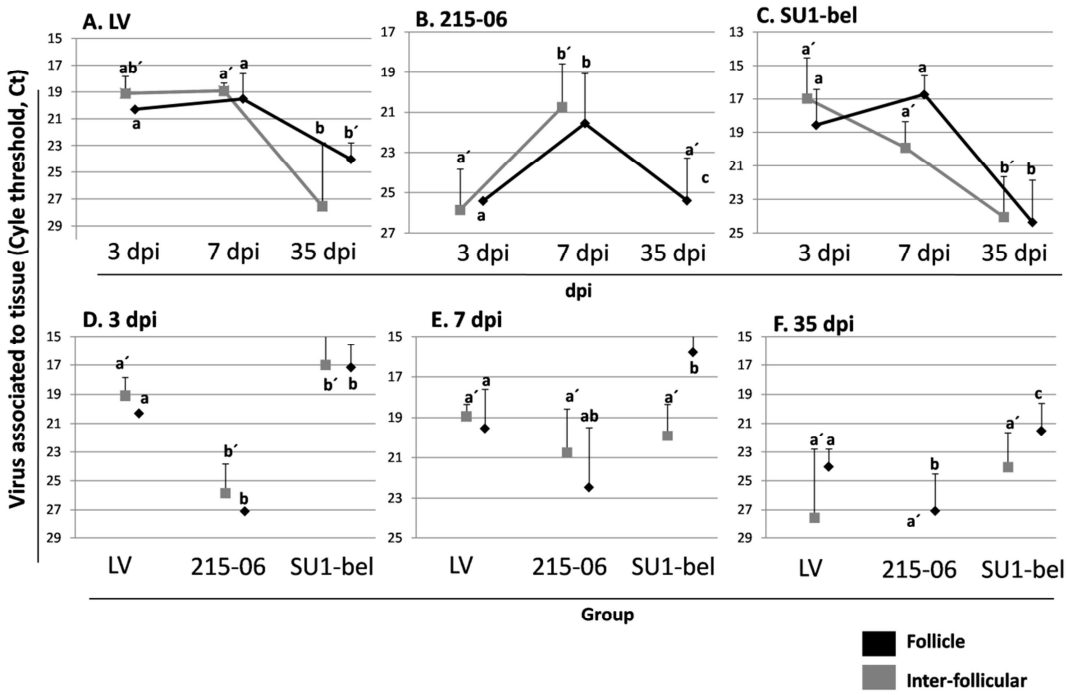

Fig 11. PRRSV-1 strains kinetic of replication in Med-LN structures. Viral load is represented by changes in the cycle threshold, Ct. The virus detection was negative in the control animals group. PRRSV detection in follicle and inerfollicular areas of mediastinal lymph nodes of LV (A), 215-06 (B) and SU1-bel (C) infected animals during this study. Differences between distinct PRRSV-1 infected pigs are showed for 3 (D), 7 (E), and 35 (F) days post-infection. Statistically significant differences ($P < 0.05$) between groups are indicated by different letters (a, b, and c for follicle; a´, b´, and c´ for interfollicular area) on the bars.

At 35 dpi the LV RNA was significantly reduced in Med-LN compartments (Ct 24.02 ± 1.23; Ct 27.54 ± 4.76; F and IF area respectively) (Fig. 11A). The 215-06 RNA was detected at 3 dpi mainly in IF area of Med-LN (Ct 25.84 ± 2.02). At 7 dpi the 215-06 RNA increased in the Med-LN and the virus presence was higher in IF area than in F (Ct 20.73 ± 2.16; Ct 22.45 ± 2.96; respectively). Interestingly the 215-06 was only detected in F of Med-LN at 35 dpi (Ct 27.06 ± 2.55) (Fig. 11B). SU1-bel group showed the higher virus replication in Med-LN at each time post infection in this study. At 3 dpi the SU1-bel was mostly detected in IF (Ct 16.96 ± 2.43). On other hand, at 7 dpi the SU1-bel was mostly replicated in F (Ct 15.85 ± 0.85). At 35 dpi the SU1-bel was mainly detected in F of Med-LN (Ct 21.50 ± 1.88) (Fig. 11C).

Along this study, SU1-bel was the PRRSV strain that express higher replication in Med-LN compartments both F and IF area (Fig. 11D-F; $P < 0.05$). For all infected animals at 3 dpi, PRRSV replication was mostly in IF area (Fig. 11D). At 7 dpi the virus replication was similar in both Med-LN compartments for LV and 215-06 PRRSV strains, whereas SU1-bel replication was higher in F (Fig. 11E). In F the virus replication was different for each PRRSV strain, the minimum was detected for 215-06 animals, followed by LV group, and the maximum was detected in F of SU1-bel inoculated animals (Fig. 11E). At 35 dpi the virus replication was focused in the F of Med-LN for all tested PRRSV-1 strains (Fig. 11F).

Cytokine gene expression in the first *in vivo* experiment

No significant increase in IFN-α subtype 1 (IFN-α 1) gene expression was detected in any examined tissue along the study (Fig. 12). Conversely, IFN-α 1 gene expression was down-regulated in the lung of inoculated animals at 21-24 dpi (-1.619 and -1.873

log 2 Fold Change, respectively) and in Tb-LN at 10 and 24 dpi (-2.075 and -1.822 log

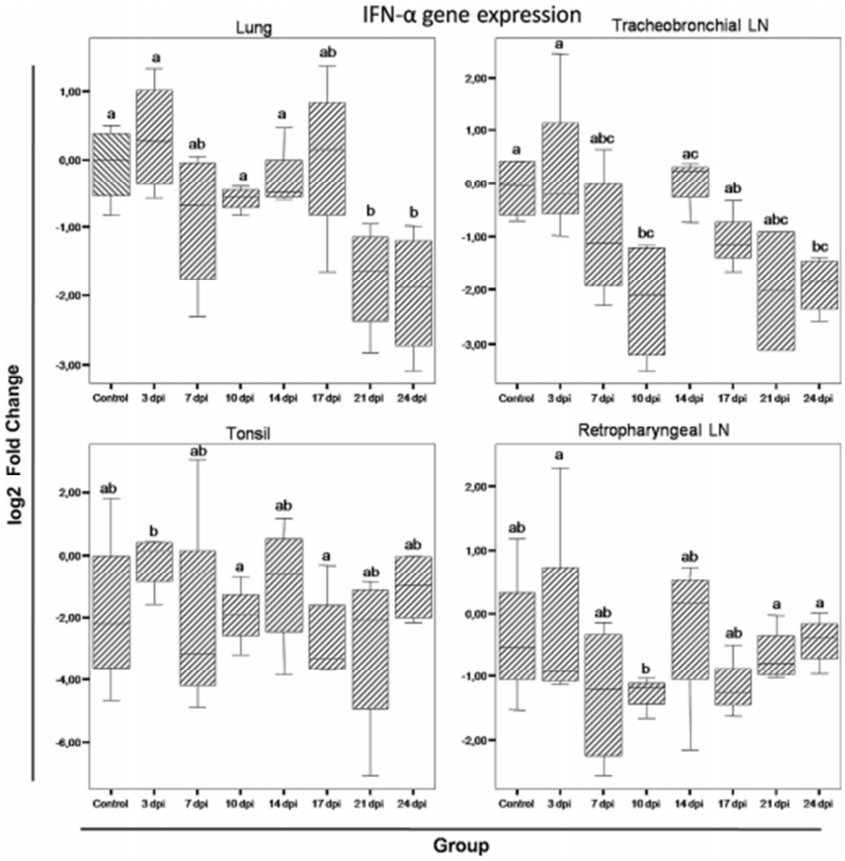

Fig. 12. IFN-α 1gene expression was not induced in lung, tonsil, Tb-LN and Rf-LN during this study. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

2 Fold Change, respectively).

IFN-γ mRNA expression increased in the lung and Tb-LN, whereas it remained without significant changes in the tonsil and Rf-LN (Fig. 13). In the lung the IFN-γ transcripts were higher at 10 dpi (1.837 log 2 Fold Change), and from 17 to 24 dpi (from 2.213 to 1.132 log 2 Fold Change), with the maximum increase at 21 dpi (2.787 Log 2 Fold Change). Additionally, the IFN-γ gene expression described a two peak curve in Tb-LN of PRRSV-inoculated animals with maximum expression levels at 7 and 24 dpi (3.141 and 3.602 log 2 Fold Change, respectively).

No significant changes were detected in TNF-α mRNA expression in the infected group with respect to the control group throughout the study, but a down-regulation was

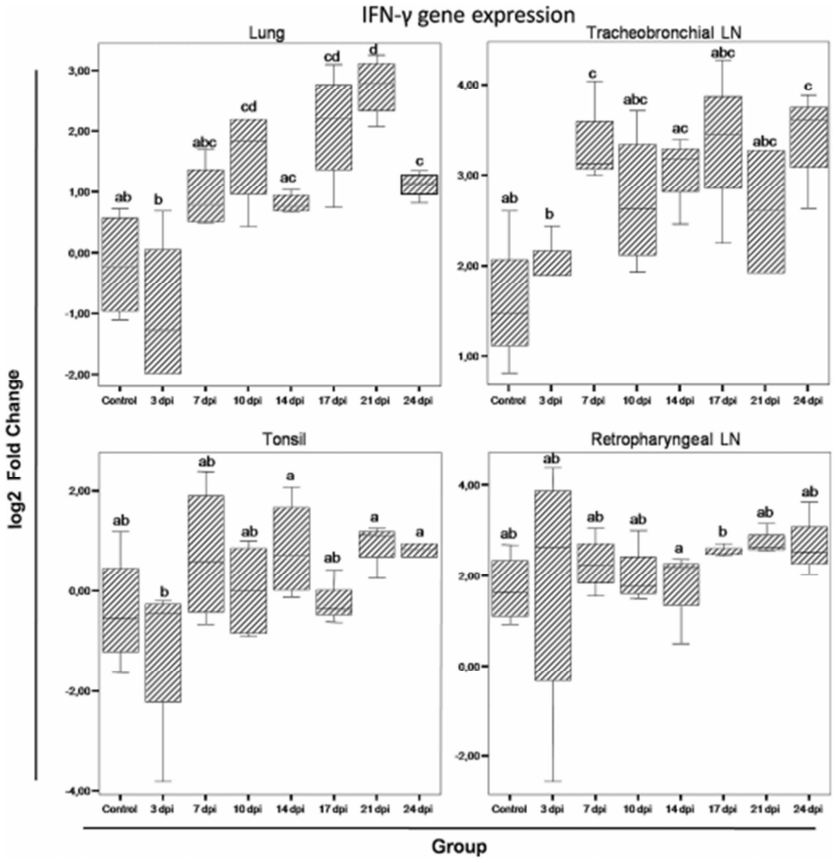

Fig. 13. IFN-γ gene expression was over-expressed in lung and Tb-LN, whereas it was not regulated in tonsil and Rf-LN. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars

observed at 14 and 24 dpi in the lung (-2.197 and -2.641 log 2 Fold Change, respectively) and at 21 dpi in the tonsil (-1.105 log 2 Fold Change) (Fig. 14). A high inter-animal gene expression variance was observed for this cytokine in control animals (up to 2.395 in lung).

IL-12p35 gene expression was down-regulated in the lung at 24dpi (-1.858 log 2 Fold Change) and up-regulated in the Rf-LN at 21 and 24 dpi (0.845 and 1.163 log 2 Fold Change, respectively), whilst no significant changes were detected in the tonsil and Tb-Ln along the study (Fig. 15). In contrast to the IL-12p35, the IL-12p40 mRNA expression was only statistically up-regulated in the tonsil of PRRSV-inoculated

animals at 14, 21 and 24 dpi (2.389, 4.655 and 2.184 log 2 Fold Change, respectively) (Figs. 15 and 16).

An up-regulation in the IL-10 gene expression was detected in the lung of inoculated animals at 10 and 21 dpi (1.216 and 2.042 log 2 Fold Change, respectively), and in Tb-LN at 3 and 17 dpi (1.478 and 1.06 log 2 Fold Change, respectively) (Fig. 17). The gene expression of IL-10 did not present significant differences in the tonsil and Rf-LN of PRRSV-inoculated animals with respect to control group.

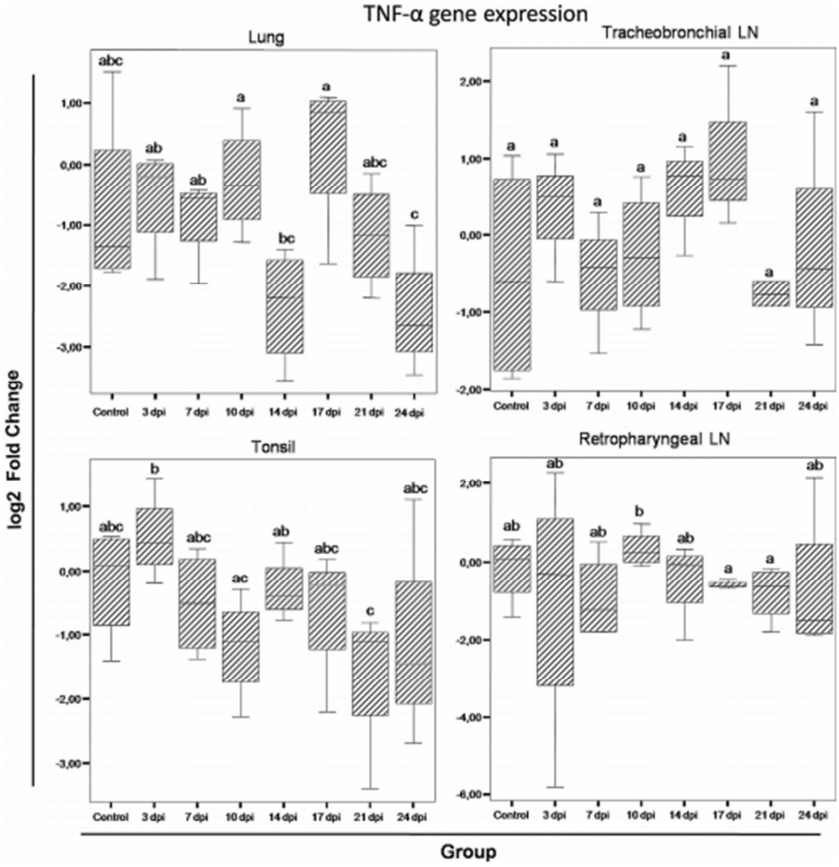

Fig. 14. TNF-α gene expression was not induced in lung, tonsil, Tb-LN and Rf-LN during this study. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

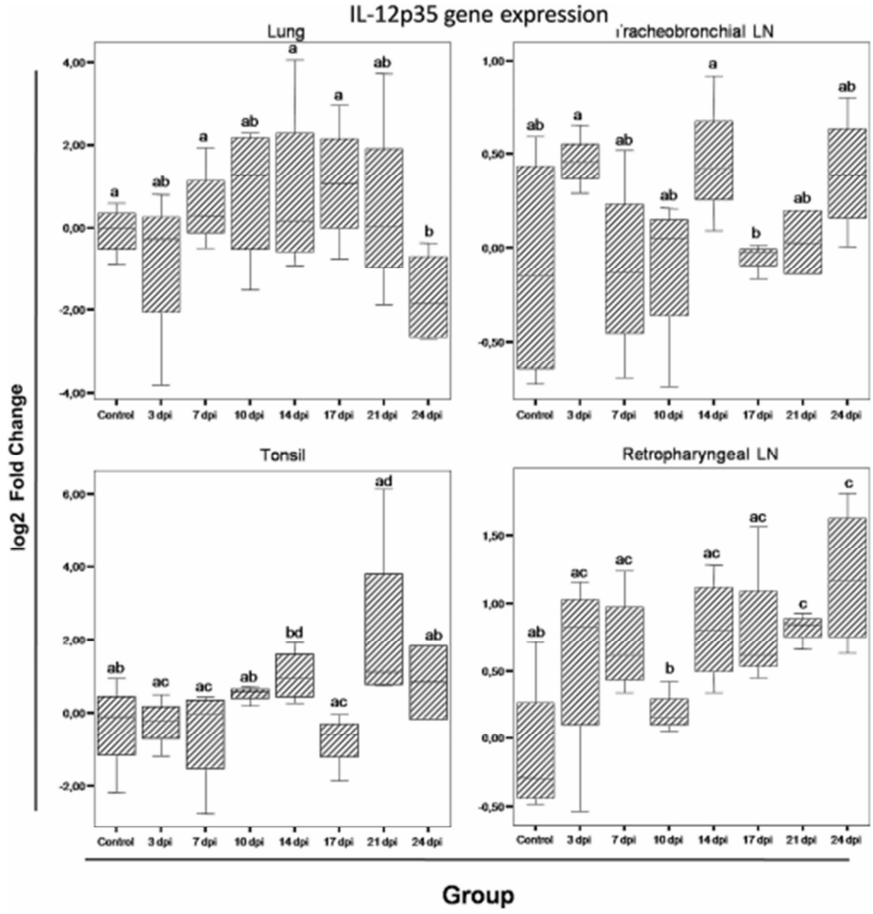

Fig. 15. IL-12p35 gene expression was down-regulated in lung, it was not induced in Tb-LN, but it was over-regulated in tonsil and Rf-LN during this study.The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

The TGF-β mRNA expression did not show statistically significant differences in any evaluated tissue between control and infected groups throughout the study (data not shown).

The correlations between PRRSV-1 2982, IFN-α 1, IFN-γ, TNF-α, IL-12p35, IL-12p40, IL-10, and TGF-β in lung, tonsil, Tb-LN and Rf-LN of PRRSV-infected pigs are shown in Tables 6, 7, 8 and 9, respectively.

In the lung of inoculated animals, a negative correlation was detected between PRRSV replication and the gene expression of IFN-α 1 (-0.857, $P < 0.01$). A positive correlation

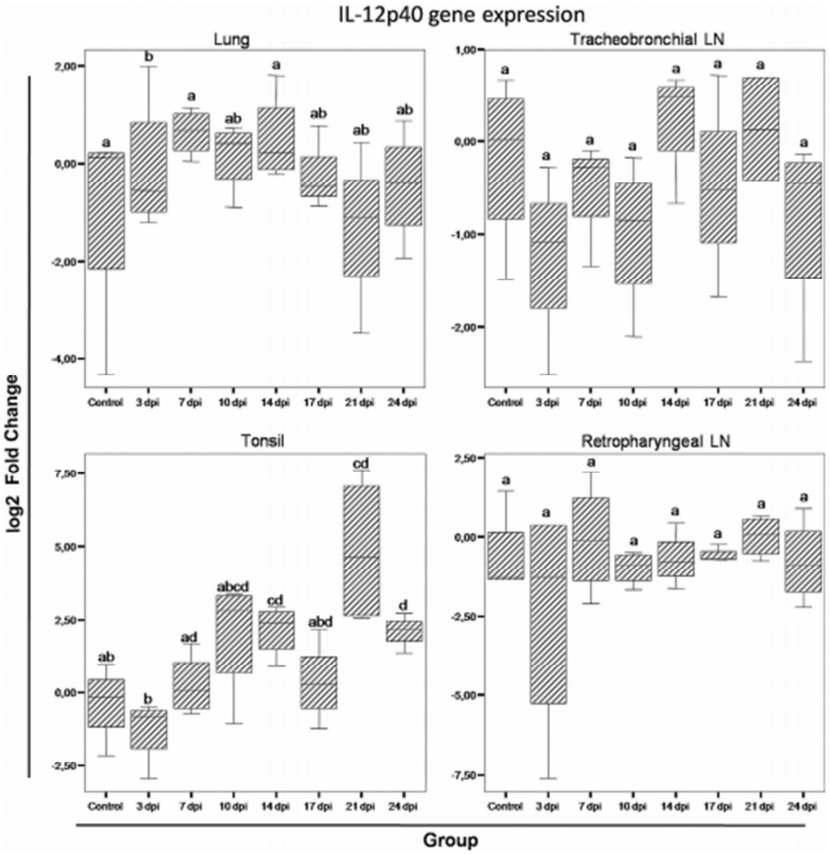

Fig. 16. IL-12p40 gene expression was not induced in lung, Tb-LN and Rf-LN during this study, it was over-expressed in tonsil. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

was detected between IFN-α 1 and TNF-α in lung and tonsil when both cytokines gene expression were down-regulated (0.690, $P < 0.05$; in both organs).

In the tonsil of PRRSV-1 2982 infected animals a positive correlation was observed between both subunits of IL-12 among them (0.762, $P < 0.05$) and between each one of these subunits (IL12p35 and IL12p40) with PRRSV replication and IFN-γ gene expression (0.857, and 0.714, $P < 0.05$, respectively for the IL12p35; and, 0.762 and 0.928, $P < 0.05$, respectively for the IL12-p40). Furthermore, the IL-12p40 and IFN-γ over-regulation in tonsil was negatively correlated with TNF-α down-regulation (-0.738 and -0.642, $P < 0.05$, respectively).

The IFN-γ over-regulation in the Rf-LN was correlated with the IL-12p35 regulation (0.857, $P < 0.05$). In addition, a positive correlation was detected between TNF-α and TGF-β in the Rf-LN (0.833, $P < 0.01$).

A positive correlation between IL-10 and IFN-γ were detected in lung (0.643, $P < 0.05$). IL-10 gene expression was negatively correlated with the IL-12p40 detected in Tb-LN (-0.690; $P < 0.01$).

Table 6. Correlation between PRRSV RNA quantities, IFN- α 1, IFN-γ, TNF- α, IL-12p35, IL-12p40, IL-10, and TGF-β in the Lung of PRRSV-1 infected animals.

	IFN-α 1	**IFN-γ**	**TNF-α**	**IL-12p35**	**IL-12p40**	**IL-10**	**TGF-β**
PRRSV	-0,857**	0,571	-0,524	-0,214	-0,119	0,357	0,214
IFN-α 1	-	-0,500	0,690*	0,261	-0,142	-0,023	-0,071
IFN-γ		-	0,071	0,380	-0,071	0,642*	0,762*
TNF-α			-	0,428	-0,047	0,380	0,380
IL-12p35				-	0,547	0,047	0,690*
IL-12p40					-	-0,619	0,142
IL-10						-	0,523

** $P < 0.01$
* $P < 0.05$

Table 7. Correlation between PRRSV RNA quantities, IFN- α 1, IFN-γ, TNF- α, IL-12p35, IL-12p40, IL-10, and TGF-β in the tonsil of PRRSV-1 infected animals.

	IFN-α 1	**IFN-γ**	**TNF-α**	**IL-12p35**	**IL-12p40**	**IL-10**	**TGF-β**
PRRSV	0,119	0,738*	-0,547	0,857**	0,762*	0,023	0,047
IFN-α 1	-	-0,333	0,690*	0,142	-0,333	-0,547	-0,238
IFN-γ		-	-0,642*	0,714*	0,928**	0,071	0,476
TNF-α			-	-0,404	-0,738*	-0,476	-0,047
IL-12p35				-	0,762*	0,119	0,285
IL-12p40					-	0,285	0,214
IL-10						-	-0,333

** $P < 0.01$
* $P < 0.05$

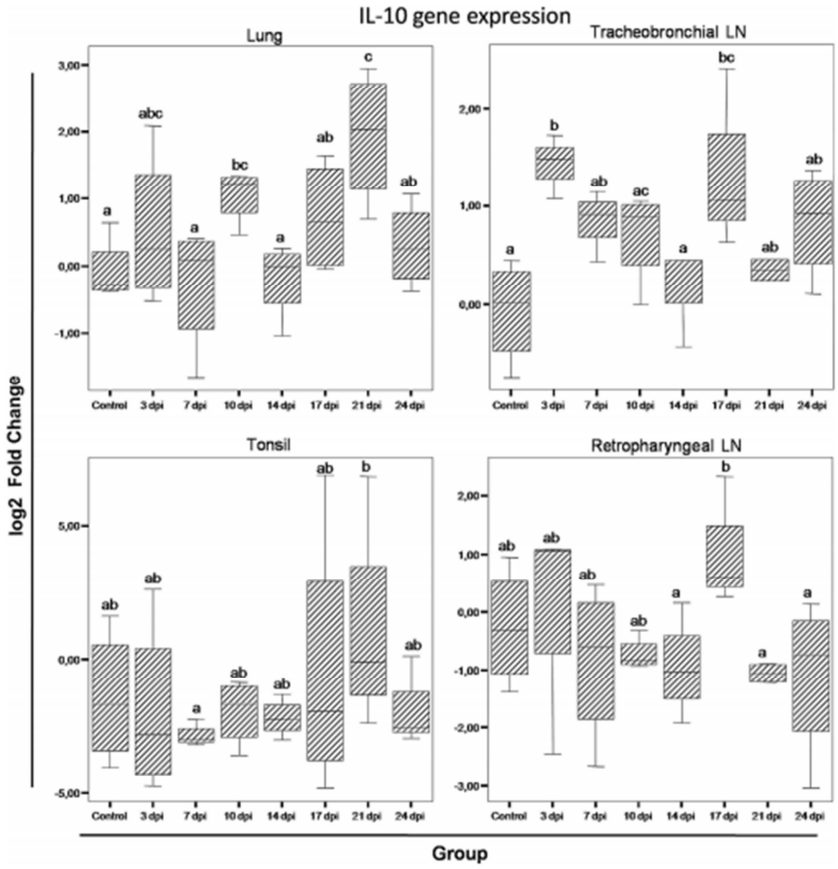

Fig. 17. IL-10 gene expression was induced in lung and Tb-LN. The statistically significant differences ($P < 0.05$) between groups are indicated by letters on the bars.

Table 8. Correlation between PRRSV RNA quantities, IFN- α 1, IFN-γ, TNF- α, IL-12p35, IL-12p40, IL-10, and TGF-β in the Tb-LN of PRRSV-1 infected animals.

	IFN-α 1	**IFN-γ**	**TNF-α**	**IL-12p35**	**IL-12p40**	**IL-10**	**TGF-β**
PRRSV	0,047	0,333	0,071	0,333	-0,738*	0,619	0,142
IFN-α 1	-	-0,404	0,285	0,309	0,071	0,000	0,905**
IFN-γ		-	0,214	0,000	-0,119	0,357	-0,357
TNF-α			-	0,547	-0,238	0,428	0,428
IL-12p35				-	0,023	0,190	0,214
IL-12p40					-	-0,690*	-0,214
IL-10						-	0,047

** $P < 0.01$
* $P < 0.05$

Table 9. Correlation between PRRSV RNA quantities, IFN- α 1, IFN-γ, TNF- α, IL-12p35, IL-12p40, IL-10, and TGF-β in the Rf-LN of PRRSV-1 infected animals.

	IFN-α 1	IFN-γ	TNF-α	IL-12p35	IL-12p40	IL-10	TGF-β
PRRSV	0,357	0,285	-0,452	0,2853	-0,404	-0,2619	-0,500
IFN-α 1	-	-0,476	-0,119	-0,095	-0,476	0,047	-0,261
IFN-γ		-	-0,238	0,857*	0,380	-0,547	-0,404
TNF-α			-	-0,309	-0,142	0,071	0,833**
IL-12p35				-	0,285	-0,428	-0,523
IL-12p40					-	-0,190	0,071
IL-10						-	0,428

** $P < 0.01$
* $P < 0.05$

PRRSV-1 Cytokine gene expression in the second *in vivo* experiment

At 3 dpi all PRRSV groups shown lower IFN-α transcript levels, and it was statistically significant for the total IFN-α transcription of SU1-bel group (Fig. 18). This generalized lower IFN-α transcripts level continued at 7 dpi, and it was statistically significant different in IF areas of LV animals and in both Med-LN compartment of 215-06 infected pigs (Fig.18) in which SU1-bel showed the lower

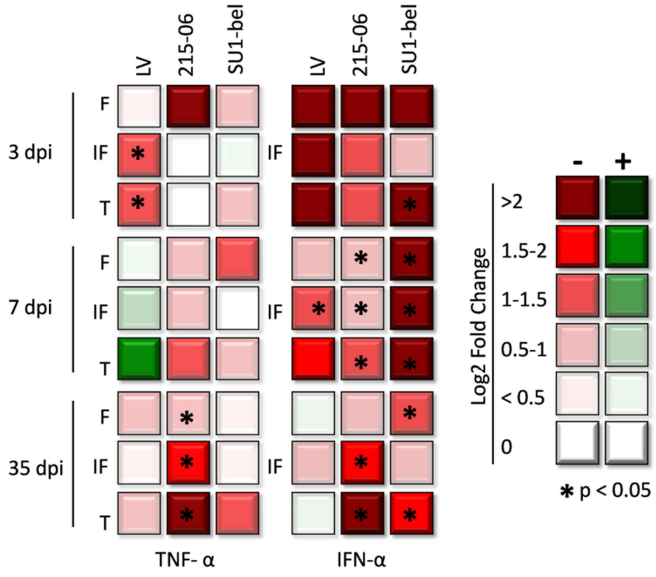

Fig 18. RT-PCR analysis of TNF-α and IFN-α. Log 2 Fold change in transcript level of the indicated genes relative to the control gene β-Actin; which was determinated by $2^{-\Delta\Delta Ct}$ method. The statistically significant differences ($P < 0.05$) with control group are indicated by asterisks.

IFN-α transcript levels. At 35 dpi, LV inoculated animals did not show any IFN-α transcript, 215-06 pigs showed lower IFN-α transcripts at IF area level, whereas SU1-bel reduced transcripts levels of IFN-α in the F of Med-LN (Fig. 18).

In this study a TNF-α non-regulation or lower transcript levels was observed at 3 dpi in all PRRSV infected animals. Only in IF area of Med-LN of LV group a statistically significant lower TNF-α transcripts levels (Fig. 18) was detected. At 7 dpi the TNF-α transcription trended to be high in LV group, whereas for PRRSV field strains the TNF-α transcript were lower (Fig. 18). At the end of this study the TNF-α was not statistically significant regulated in both LV and SU1-bel animals, but this cytokine was statistically significantly lower in Med-LN IF area of 215-06 group (Fig. 18).

IFN-γ transcription was not statistically significant regulated at 3 dpi for any PRRSV inoculated group (Fig. 19) at this time post-infection the lower gene expression were detected in LV group. At 7 dpi, the IFN-γ transcripts were higher than control animals for all PRRSV groups at Med-LN follicular compartment; and this was statistically significant for LV

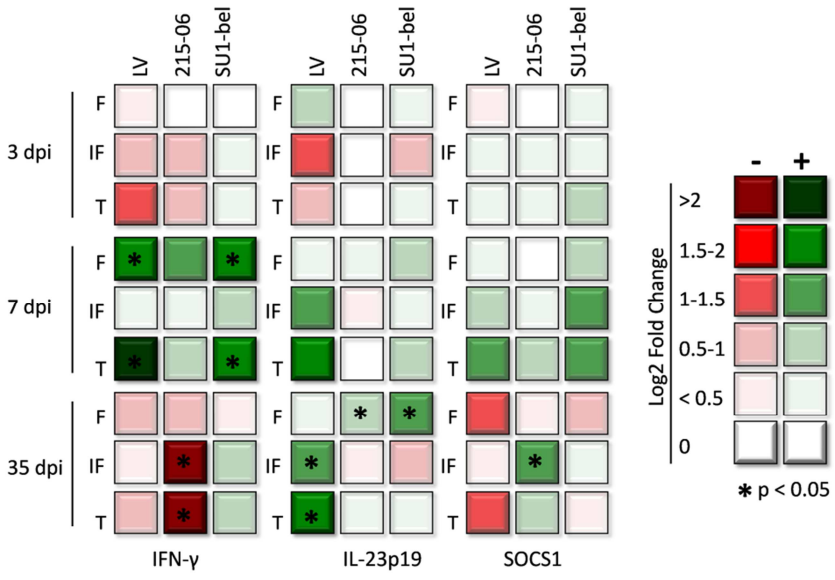

Fig 19. RT-PCR analysis of IFN-γ, IL-23p19, and SOCS1. Log 2 Fold change in transcript level of the indicated genes relative to the control gene β-Actin; which was determinated by $2^{-\Delta\Delta Ct}$ method. The statistically significant differences ($P < 0.05$) with control group are indicated by asterisks.

and SU1-bel groups (Fig. 19). Finally, at 35 dpi the IFN-γ transcript levels were not difference compared with the control animals (LV and SU1-bel animals) or was statistically significant lower in IF area of Med-LN 215-06 infected pigs (Fig. 19).

Neither at 3 dpi nor 7 dpi the IL-23p19 was statistically significant transcribed for any PRRSV group. At 35 dpi, IL-23p19 transcription was higher for all PRRSV field strain groups in F of Med-LN and IF area of LV group (Fig. 19). The detection of IL-12 mRNA was not statistically significant detected in this study (data not shown).

SOCS1transcription was not statistically significant regulated for any PRRSV group during the first week of this experiment. However at 35 dpi, SOCS1 transcription was increased in IF area or Med-LN of 215-06 group (Fig. 19).

The IL-10 gene expression was not statistically significant transcribed at 3 dpi for any PRRSV group. At 7 dpi, LV infected animals showed higher IL-10 transcript levels in IF area, whereas the PRRSV field strains showed a lower IL-10 transcription in F (both 215-06 and SU1-bel groups) and in IF area (only 215-06 group) of Med-LN. Although at 35dpi the IL-10 transcription was similar than 7dpi, the IL-10 transcription modulation was not

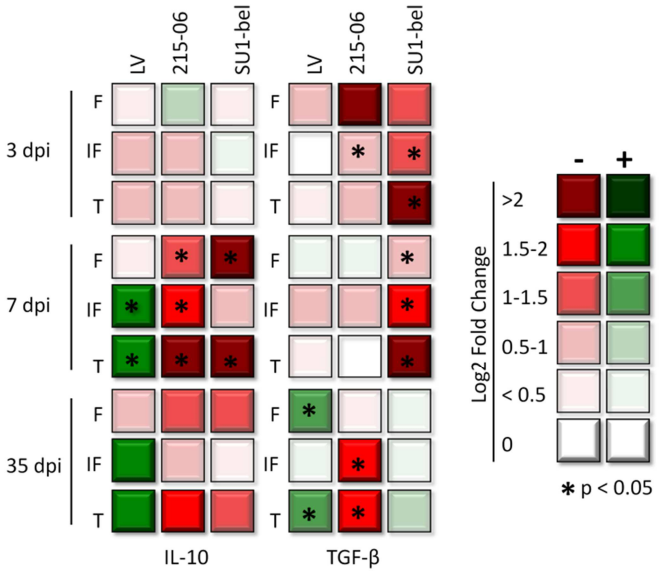

Fig 20. RT-PCR analysis of IL-10 and TGF-β. Log 2 Fold change in transcript level of the indicated genes relative to the control gene β-Actin; which was determinated by $2^{-\Delta\Delta Ct}$ method. The statistically significant differences ($P < 0.05$) with control group are indicated by asterisks.

statistically significant (Fig. 20) in any group.

The TGF-β transcription was statistically downregulated in IF area of Med-LN of 215-06 and SU1-bel infected pigs. At 7 dpi this lower TGF-β transcription continued in all Med-LN compartments of SU1-bel group. TGF-β transcription was higher in F of LV group and lower in IF area of Med-LN 215-06 inoculated group at 35 dpi (Fig. 20).

In this experiment the statistically significant correlations between PRRSV-1 strains and RNA quantification and gene expression of immune genes in Med-LN compartments are summarized below.

In LV infected animals, a negative correlation was detected between the virus and IFN-α both in F and IF (-0.663, and -0.499, $P < 0.05$; respectively); whereas this virus correlation was positive with IFN-γ for a total Med-LN (0.527, $P < 0.05$), and with SOCS1 in F and for a total Med-LN (0.527, and 0.569, $P < 0.05$; respectively). At the same time, the IFN-γ and SOCS1 were positively correlated in all Med-LN compartments (0.607 in F, and 0.680 in IF, $P < 0.05$). For these animals, TNF-α was positively correlated both IFNs, IFN-α and IFN-γ (0.857, and 0.630, $P < 0.05$, respectively).

The most noteworthy correlations detected in Med-LN of 215-06 infected group were between PRRSV and IFN-γ (both in F and IF, 0.612 and 0.661, $P < 0.05$, respectively); and between TNF-α and IFN-γ (in F, 0.490, $P < 0.05$).

For SU1-bel infected animals, the most significantly correlations were displayed related to the virus, with TNF-α (-0.683, $P < 0.05$), IFN-α (-0.772, $P < 0.05$), and SOCS1 (0.515, $P < 0.05$).

Immunohistochemistry

CD3 immunolabelling defined the T cell population in Med-LN (Jones et al., 1993). During the second *in vivo* study the number of T cells was found to be increased in F of Med-LN at 7 dpi in all infected animals compared with the control animals (2 s-qic for LV and 215-06 groups and 3 s-qic for SU1-bel group). At 35 dpi this follicular T cell population remained statically increased in LV infected animals (2 s-qic for LV; Fig. 21A). Contrarily, the T cell immunolabelling decreased in the Med-LN IF from 3 dpi in LV (-1 s-qic) and specially for SU1-bel inoculated animals (-2 s-qic), remaining lower than control animals at 7 and 35 dpi for all infected animals (-2 s-qic for LV and 215-06, and from -2.5 to -1 s-qic for SU1-bel;Fig. 18A).

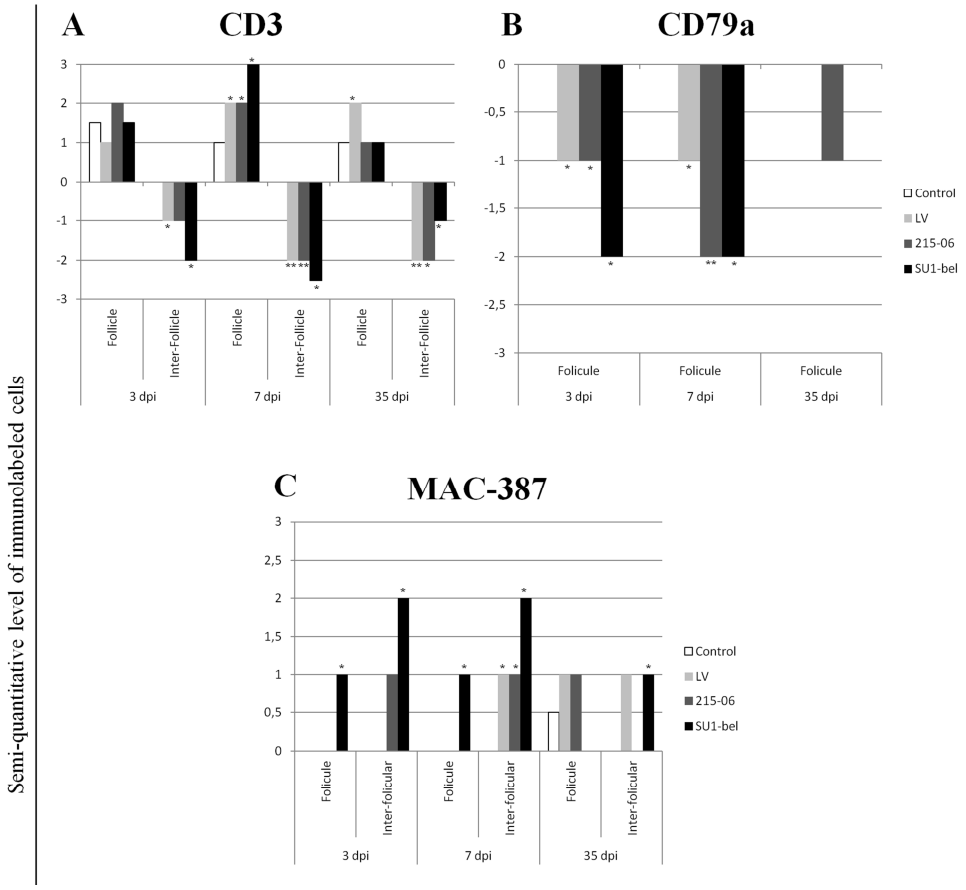

Fig 21. Med-LN IHC results. **A.** CD3 staining for T cells detection. **B.** CD79 staining for B cell staining in F. **C.** MØ detection with MAC-387 staining. The bars represent s-qic mean values; the statistically significant differences ($P < 0.05$) with control group are indicated by asterisks.

The B cell immunolabelling of CD79a surface protein (Mason *et al*., 1995) showed cells only in the F of Med-LN of animals from the second *in vivo* experiment. In all infected animals these cells decreased in immunolabelling intensity compared with the control at 3 dpi (-1s-qic for LV and 215-06 and especially for SU1-bel group, -2 s-qic) and 7 dpi (especially for 215-06 and SU1-bel groups, -2 and -1 s-qic for LV and 215-06 respectively). At 35 dpi the B cell population did not shown a statistically significant change for any inoculated group (Fig. 21B).

MØ were identified through MAC-387 imunolabelling of Med-LN sections (Gómez-Laguna et al., 2010a; Soulas *et al*., 2011). In the F, MØ immunolabelling was found to be statistically increased in SU1-bel infected animals at 3 and 7 dpi (1 s-qic, respectively). In the IF areas, the macrophage population was increased from 3 dpi until the end of the study in SU1-bel infected animals (from 2 to 1 s-qic), showing the maximum levels at 3 and 7 dpi in this Med-LN area. However the animals inoculated with LV and 215-06 PRRSV strains showed a macrophage immunolabelling increase in IF area only at 7 dpi (1 s-qic) lower than SU1-bel group (Fig. 21C).

Representative images of CD3, CD79a and MAC387 immunhistochemical staining in all the groups are shown in Figure 22.

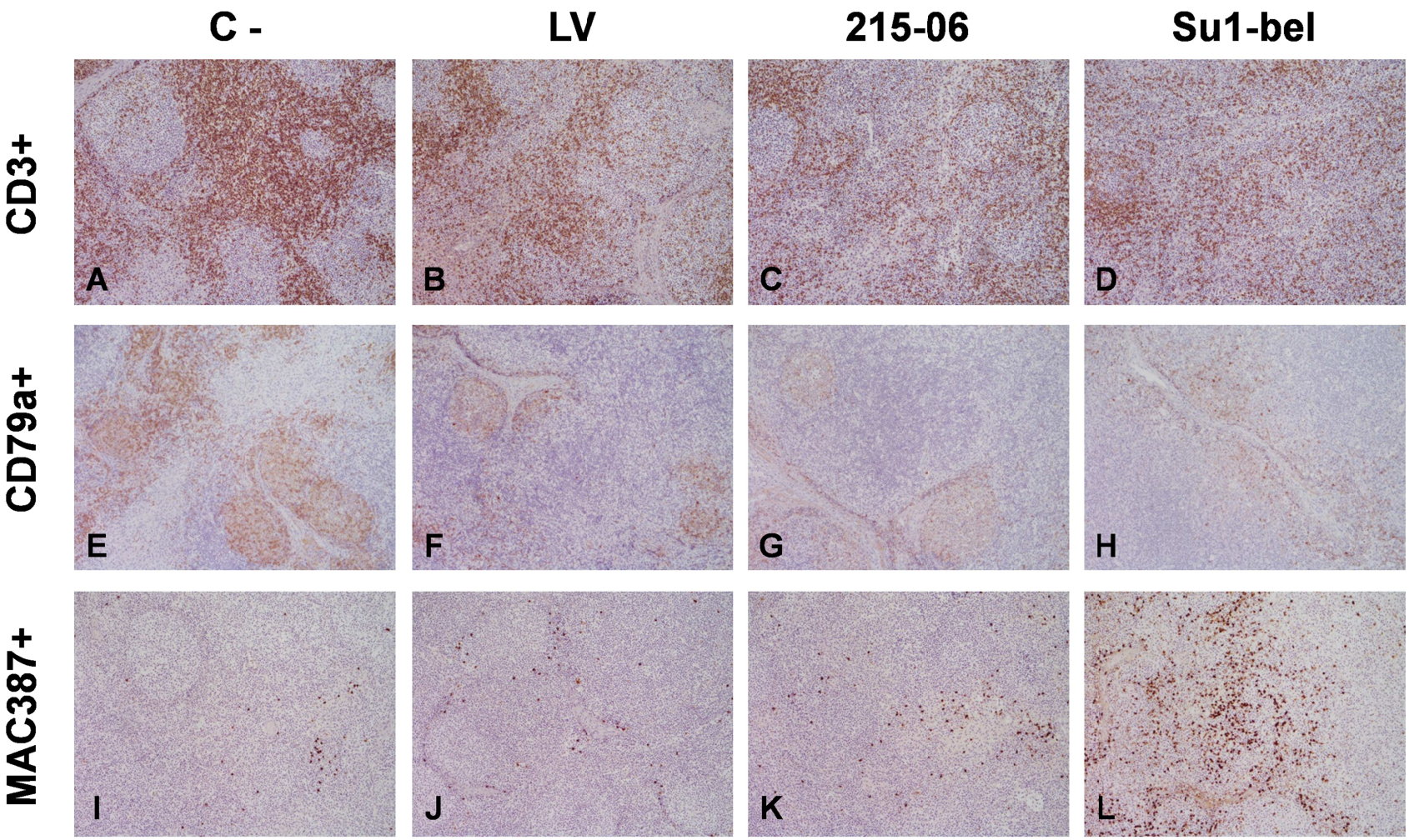

Fig. 22. Immuohistochemical detection of CD3 (A, B, C, D), CD79a (E, F, G, H) and MAC387 (I, J, K, L) in mediastinal lymph nodes of control pigs (A, E, I) and infected with LV (B, F, J), 215-06 (C, G, K) and SU1-bel (D, H, J) strains, at 7 dpi. An increased in the number of CD3+ cells is observed in the lymphoid follicles from all infected groups together with a depletion of these cells in the interfollicular areas. A decrease in the number of CD79 cells in the follicles is also observed in all the infected groups. A substantial increase in the number of MAC387 is observed in the follicles and interfollicular areas from SU1-bel infected animals (L) together with a mild increase in the LV (J) and 215-06 (K) groups. Original magnification: 20x.

Discussion

PRRSV shows a restricted cell tropism for cells from the monocyte/macrophage lineage, the main *in vivo* target cells for replication being PAM, and other tissue MØ (Van Breedam *et al*., 2010). Consequently, the interaction of the virus with this cell type is of major importance to understand the pathogenesis. PRRSV has numerous pathways to prevent antiviral responses in MØ as well as in cell lines used for experimental *in vitro* studies (Chand *et al*., 2012), but is sensitive to the action of many IFN-I (Sang *et al*., 2010) as well as IFN-γ (Rowland *et al*., 2001). Despite the ability to suppress antiviral responses in MØ *in vivo*, IFN-α is found both locally and systemically at early time points post infection (Albina *et al*., 1998; Dwivedi *et al*., 2012; Liu *et al*., 2010; van Reeth *et al*., 2002). This response is weak with low pathogenic PRRSV isolates but can reach high levels with HP-PRRSV isolates (Guo *et al*., 2013). *In vitro* studies indicate that plasmacytoid dendritic cells are likely to represent a source of IFN-α since they can be activated by PRRSV (Baumann *et al*., 2013). In addition, IFN-γ is found locally and systemically during PRRSV infection of pigs, although this is found at later time points, most likely reflecting adaptive T cell responses (Darwich *et al*., 2010).

MØ, although all of monocyte origin, represent a heterogenic family of cells as a result of cellular differentiation in specific tissues and local microenvironments including cytokine milieu (Gordon, 2003). Differentiated MØ exhibit marked phenotypic and functional heterogeneity due to polarization depending on the imprinting by the environment and the phase of the immune response (Gordon and Taylor, 2005; Mosser and Edwards, 2008). At least three distinct functional states have been proposed, classically activated M1, alternatively activated M2 and regulatory MØ, also called M2b or M2-like. In mouse and man, the differentiation of these states is typically but not exclusively promoted by IFN-γ, IL-4/IL-13 and IL-10, respectively. M1 differentiation is typically induced by IFN-γ and results in an inflammatory phenotype.

These MØ secrete high levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α in response to TLR ligands. Furthermore, they have a reduced phagocytic activity but enhanced antigen-presenting functions. IL-4 and IL-13 induce the alternative MØ activation (M2), which have more anti-inflammatory and tissuere-modelling functions. Typically, M2 MØ produce more IL-10, are highly phagocytic, but inefficient at antigen presentation (Mosser, 2003).

Nowadays, the functional and phenotypic characteristics of M1 and M2 MØ have not yet been described in pigs. As this was not the objective of the present study, we cannot give details on phenotypic and functional markers of this system in the pig. Nevertheless, we can state that as expected M1 MØ have a higher MHCI and MHCII expression than unpolarized and M2 MØ. Compared to unpolarized MØ, both M1 and M2 have reduced CD14, CD16 is up-regulated in M1, while CD163 is down-regulated in M2. Considering that CD163 represents one of the PRRSV receptors also involved in virus internalization (Welch and Calvert, 2010), a reduced infection rate could be expected in M2 MØ. Indeed, the levels of N expressions of LVP23, VR-2332 and RVB-581 in IL-4 MØ were significantly reduced compared to those observed in unpolarized MØ at 6 and 12 hpi ($P < 0.05$), but not at later time points (20 and 20 hpi). Furthermore, the end point titres reached in the "IL-4 MØ" were not statistically different from those in unstimulated MØ, indicating that M2 polarization does not interfere with virus replication. In contrast to IL-4, IFN-γ and IFN-β treatment of MØ induced a clear antiviral state in MØ with reductions in virus infection and replication. Importantly, these effects were significantly stronger against low virulent PRRSV-1 strains when compared to the PRRSV-2 and HP-PRRSV-1 Lena isolates. This was particularly evident for IFN-γ. Furthermore, it appeared that the HP-PRRSV isolate from China and Eastern Europe were particularly resistant to the effects of IFN-γ. Consequently, we

propose that such MØ cultures could help to better understand the immunological and genetic basis of PRRSV virulence. It could be speculated that most of the PRRSV-2 and the HP isolates possess enhanced abilities to interfere with antiviral genes induced by both IFN-γ and IFN-β, and this could be one of the mechanisms of their increased virulence. In the same idea, the reduced replication yield of low virulent PRRSV-1 isolates in IFN-γ and IFN-β MØ should be more deeply investigated to identify which cellular elements could be of particular relevance for MØ resistance to PRRSV. Another important aspect which might reflect the fact that HP-PRRSV isolates differentially replicate in IFN-γ MØ, could be the use of alternative receptors than CD163 for cell binding and entry. In this study, CD16 upregulation was observed in M1 MØ. Accordingly, it can be suggested that other potential receptors for PRRSV binding would be upregulated. It would lead to a more efficient viral uptake, increasing the antigen expression per cell. Such phenomena would explain why HP-PRRSV have higher N expression compare to low virulent isolates.

Interestingly, none of the PRRSV isolates tested induced pro-inflammatory TNF-α or anti-inflammatory IL-10, at least at the protein level detectable by ELISA. This challenges a direct role of MØ-derived cytokines during the pathogenesis of PRRSV. Nevertheless, both pro- and anti-inflammatory cytokines are found in the lung and lymphoid organs of PRRSV infected pigs and have been associated to MØ and other accessory cells (Barranco *et al*., 2012a, b; Gómez-Laguna *et al*., 2010a, 2013). We therefore conclude that the currently available cell culture models cannot fully reflect the complex events induced in the cytokine network of a multitude of cell types found *in vivo*. On the other hand, PRRSV infection of MØ and cell lines inhibits IFN-α and IFN-β production both at them RNA and protein levels through interactions of several viral non-structural proteins with various cellular products including IRF-3, IRF-7, NF-

κB and STAT1/2/IRF-9 (Sang *et al*., 2011). These pathways are likely to also affect cytokine responses in MØ, which would fit our *in vitro* data.

Considering that lung MØ are the main PRRSV target cells *in vivo*, they represent an appropriate model for *in vitro* studies to identify viral and cellular factors of importance for virus replication and host responses at the cellular level. Nevertheless, it is necessary to sacrifice pigs to collect sufficient MØ from lung lavages. Our data support an alternative model using monocyte-derived MØ which can be obtained without slaughtering animals. In addition, these MØ can be modulated and polarized by cytokines to mimic certain functions of tissue MØ during steady state conditions or during the course of immune responses. In fact, it can be expected that during early phases of PRRSV infection the virus would encounter unprimed as well as IFN type I-primed MØ, followed by M1 "IFN-γ MØ", M2 "IL-4 MØ" and finally M2b "IL-10 MØ". M2 and M2b functional states would be important in restoring tissue integrity and counteracting damaging inflammatory responses, and thereby protect lung functions. Future studies are still required to address the interaction of PRRSV with such regulatory MØ.

This *in vitro* model has identified important strain differences using cytokine-primed MØ cultures, which will be very valuable to understand the virological and cellular elements important for PRRSV–cell interactions and relating to virulence.

Further understanding of the role of cytokines in the pathobiological mechanisms of respiratory swine diseases holds the key to the development of effective prophylactic and therapeutic strategies (Coussens *et al*., 2004; Gómez-Laguna *et al*., 2013b). RT-qPCR is a sensitive, specific, rapid, reproducible and reliable method for mRNA quantification (Ficko and Cernelc, 2005) and it has been used frequently to measure the

immune responses gaining popularity in vaccine immunology. It is more, the analysis of transcript levels represents an alternative to immunoassays for the detection of cytokines that sometimes are difficult to detect due to their low amounts (Quereda *et al*., 2013).

Many qPCR assays are poorly described and validation is lacking in published papers. Poor choice of RT primers and optimization of their concentrations leads to inefficient assay performance (Bustin *et al*., 2009). In this evaluation it has been demonstrated each primer set are gene specific as produced unique PCR products. The results suggested that these qPCR assays were specific, sensitive, robust and reproducible when performed simultaneously with the same qPCR amplifying conditions. E is an important parameter to perform proper gene expression analysis (Bustin *et al*., 2009). Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal reference transcript (Pfaffl, 2004); it does not require standards with known concentrations. In the present study, we observed that the different qPCR assays tested yielded different E values; all of them within the acceptable range between 1.9 and 2.1 at least in one of the two dilutions series tested (10-fold or 2-fold) as previously recommended (Nolan *et al*., 2006). This finding implies that methods that correct for different E should be applied in the relative gene quantification of these cytokines (Pfaffl, 2004).

Although no studies of validation of qPCR assays for porcine cytokines have been reported in the literature, the results obtained in the current study were comparable to the previously qPCR parameters published for human virus qPCR assays in which CV values were between 0.7 and 5.6% (Ruelle *et al*., 2004; Champagain *et al*., 2006). Therefore, the present qPCR assays are satisfactory for monitoring proinflammatory and immunomodulatory swine responses, with robust results with low template inputs.

Nowadays, it has no published a reference study about FD in qPCR regarding swine gene expression. The mean FD in 2-fold and 10-fold serial dilutions was always lower than 3, which could be considered optimal results because of their power to detect small differences in gene expression.

We show that all these qPCR assays can be run simultaneously in one plate in separate wells under the same amplification conditions. We provide here evidence that the development of the tested qPCR assays is sensitive, specific, robust and highly useful in studying proinflammatory and immunomodulatory swine respiratory responses.

MØ act as antigen presenting cells by expressing MHC II and induce cytokine production that modulates the immune response against pathogens (Mitchell and Kumar, 2004). IL-12, TNF-α, IFN-α and IFN-γ act as potent MØ activators, whereas IL-10 and TGF-β down-regulate the phagocytic activation of these cells (Mitchell and Kumar, 2004). We showed that PRRSV-1 cannot proliferate in MØ belonging an effective host immune response against the virus. Otherwise, the M2 MØ are susceptible to be infected by PRRSV-1 strains. Complex virus-host cell interactions occur during PRRSV infection, however; a little is still known about the exact mechanisms triggered off during PRRSV infection in the host. As described above, the currently available cell culture models cannot fully reflect the cytokine production complexity that involved different cells networks in the tissues. Therefore, the study of the cytokine dynamics in infected pigs can be of help to understand the interactions between different PRRSV strains and PRRSV-infected host tissues (Gómez-Laguna *et al*., 2013b).

In the first *in vivo* experiment, despite inoculated animals showed only mild clinical signs, the detection of gross and histopathological lesions as well as PRRSV replication in lung and lymphoid organs, support the efficient infection of the animals. PRRSV was

inoculated by intramuscular route to ensure an efficient and homogeneous infection of all inoculated animals, as it has been previously described in many other viral infection models of swine with oral-nasal natural route of infection (Gómez-Laguna *et al*., 2010b; Barranco *et al*., 2012a; Graham *et al*., 2012; Kittawornrat *et al*., 2013). This inoculation route resulted in the highest PRRSV replication in lymph nodes at 3dpi, which might be filtering the virus from blood probably reflecting the filtration of the lower number of PRRSV copies quantified in the lung at 3 dpi.

A bimodal PRRSV replication was observed in this study, accordingly with previous immunohistochemical studies carried out in the same tissues by our research group (Gómez-Laguna *et al*., 2010a; Barranco *et al*., 2012a). The present study confirms the ability of PRRSV to replicate during long periods in infected tissues, which may allow the depression of the immune system in PRRSV-infected pigs.

It is important to note that the basal cytokine expression is somewhat variable among pigs (Suradhat *et al*., 2003), for example it has been described a considerable variation for IFN-α levels between porcine alveolar macrophages of different pigs (du Manoir *et al*., 2002).The mRNA expression of IFN-α 1was not regulated or down-regulated at transcriptional level in either lungs or lymphoid organs of inoculated animals. Interestingly, the gene expression of IFN-α 1 was negatively correlated with PRRSV replication in the lung of inoculated animals, and coincided with both IL-10 over-expression and with the PRRSV replication increase in this organ at 21 dpi. These results are in agreement with the previously reported PRRSV ability to suppress IFN-α production in the site of infection (Murtaugh *et al*., 2002; Baumann *et al*., 2013). On the other hand, it has been reported the IFN type I secretion by cells infected with the A2MC2 PRRSV strain both *in vivo* and *in vitro* models (Nan *et al*., 2012; Wang *et al*., 2013). Besides plasmacytoid dendritic cells may produce IFN-α in presence of PRRSV

(Baumann *et al*., 2013). Additionally, an *in vivo* study showed IFN-α production after pigs infection with a Chinese highly-pathogenic PRRS strain (Guo *et al*., 2013), nonetheless, other studies have suggested an inefficient PRRSV clearance in association with a higher expression of IFN-α (Gómez-Laguna *et al*., 2009).

In our study we did not detect significant TNF-α transcriptional induction in examined tissues but a down-regulation was observed with the second increase of PRRSV replication in the lung of infected animals. This is in agreement with previous studies that have shown that PRRSV is able to suppress or induce a weak TNF-α response during infection (Lopez-Fuertes *et al*., 2000; Thanawongnuwech *et al*., 2004). Furthermore, a TNF-α transcriptional induction without effective protein secretion after *in vitro* PRRSV infection of pig monocyte derived macrophages has been described (Subramaniam *et al*., 2010). Our transcriptional findings are in agreement with the protein expression detected in tonsil and Rf-LN of PRRSV-1 infected animals in parallel studies of our research group (Barranco *et al*., 2012a), but contrast with the TNF-α protein expression observed in the lung of infected animals (Gómez-Laguna *et al*., 2010a), which points to different post-transcriptional regulation mechanism in the lung and lymphoid organs. IFN-α 1 and TNF-α down-regulation was correlated in lung and tonsil. These results indicate that PRRSV isolate 2982 suppresses *in vivo* the IFN-α 1 and TNF-α gene expression in immune cells, such as macrophages and dendritic cells, whatever it prevents the effective onset of innate immune response during the virus infection (Beura *et al*., 2009), and interfere with the induction of adaptive immunity (Kimman *et al*., 2009).

The mRNA expression of IFN-γ was up-regulated in the lung and Tb-LN of inoculated animals, whereas it did not show changes neither in tonsil nor in Rf-LN. The up-regulation of IFN-γ coincided with an enhancement of the PRRSV replication in these

tissues. It has been suggested that the impairment of the host immune response to remove PRRSV infection could be linked to the lower IFN-γ levels observed in PRRSV infected pigs (Suradhat *et al*., 2003; Meier *et al*.,2003; Xiao *et al*., 2004; Zuckermann *et al*., 2007; Molina *et al*., 2008; Jung *et al*., 2009). Recently, it has been demonstrated that M1 macrophages (macrophages stimulated with IFN-γ) resist to Pan-European PRRSV-1 infection, together with an increased expression of IFN-α, pointing to an effective innate immune response onset in this M1 macrophages (see in this thesis). The gene expression of IFN-γ both in lung and lymphoid organs, coincided with the protein expression of this cytokine previously reported in parallel studies from our research group (Gómez-Laguna *et al*., 2010a; Barranco *et al*., 2012b), which rule out a post-transcriptional regulation of this cytokine. The suppressor of cytokines signaling 1 (SOCS-1) is a key physiological negative regulator of IFN-γ signalling (Brysha *et al*., 2001). An enhancement in the expression of SOCS-1 has been reported in PRRSV-infected PAMs (Zhou *et al*., 2011), which could explain why the IFN-γ up-regulation observed in the lung and Tb-LN of inoculated animals in our study was not effective to avoid PRRSV replication in these tissues, since PRRSV replication was still detected at high levels at 21-24 dpi.

IL-12 induces IFN-γ secretion by T cells (Trinchieri, 1994). Only the IL-12p70 heterodimer has biological activity, as well the simultaneous expression of IL-12p35 and IL-12p40 is necessary to an effective IL-12p70 production (Wolf *et al*., 1991). Moreover, the production of large amounts of IL-12p40 homodimer (in excess to the IL-12p70 heterodimer), has shown antagonistic activity against IL-12p70 heterodimer (Trinchieri *et al*., 2003). In our study, a positive correlation between IL-12p35 and IL-12p40 was detected in tonsil; and both IL-12 subunits were positively correlated with PRRSV replication and IFN-γ. These results suggest that in tonsil PRRSV induces the

secretion of IFN-γ through IL-12 signaling, but it was not strong enough or efficient to clear PRRSV from the tonsil. IL-12p70 has been reported to suppress efficiently PRRSV proliferation both *in vitro* and *in vivo* statements (Carter and Curiel, 2005). Therefore, our results suggest that PRRSV infection may modulate the host IL-12 response to suppress its biological effects in lung and LNs.

The IL-10 induction by PRRSV is controversial, and it has been associated with high PRRSV strains variability (Gimeno *et al*., 2011). IL-10 mostly acts on dendritic cells and macrophages inhibiting the antigen presentation by decreasing the MHC-II expression and increasing the CD80/86 expression (Ding *et al*., 1993). The IL-10 exposition of PAMs increases the CD-163 expression in these cells, which leads to increase the virus infection (Patton *et al*., 2009). This fact could explain the increased PRRSV expression observed in the lung of inoculated animals followed after IL-10 up-regulation in the present study. The mRNA expression of IL-10 is in agreement and supports the IL-10 protein expression detected in lung and lymphoid organs in other studies from our group (Gómez-Laguna *et al*., 2010a; Barranco *et al*., 2012b).In addition, the up-regulation of IL-10 mRNA was correlated with the gene expression of IFN-γ in the lung and Rf-LN of PRRSV-infected pigs, contrary to previous findings which suggest that PRRSV induces IL-10 as an strategy to proliferate avoiding an efficient IFN-γ secretionin infected tissues (Díaz *et al*., 2006). This fact points out that the modulatory role of IL-10 on the immune response may vary among PRRSV strains.

During this study, TGF-β was not induced in any analysed tissue. This finding is in agreement with previous reports that indicate that TGF-β gene expression is induced by PRRSV-2, whereas PRRSV-1 does not induce this cytokine (Silva-Campa *et al*. 2009).

Viruses have developed efficient strategies to evade host immune response; likewise, the host employs a number of responses to generate an antiviral state (Emmott et al.,

2010). Upon virus recognition, a signaling to induce type I IFN (IFN-α/β) is promptly initiated to promote viral clearance or induce apoptosis of the infected cells, with the establishment of an antiviral state in non-infected cells (Randall and Goodbourn, 2008).

The analysis of the expression of cytokines during PRRS infection is crucial to understand PRRS pathogenesis and to identify new approaches that allow the control of this disease. In this study, we have observed a different PRRSV kinetic of replication in the main virus target organ, the lung compared to lymphoid organs where the virus may persist or may be drained from circulation. At difference of previously *in vitro* study, during the *in vivo* infection with PRRSV strain 2982 evaded the onset of an effective innate immune response, leading to an impaired expression of IFN-α 1 and TNF-α gene expression, which finally induced a weak and delayed adaptive immune response trough an inefficient IL-12 and IFN-γ expression, and PRRSV replication favoured the expression of the anti-inflammatory IL-10 cytokine in infected pigs. Further studies are necessary to determine the exact mechanisms developed by PRRSV to modulate the host immune response, such as determining the role of SOCS-1 in the modulation of IFN-γ during PRRSV infection.

The high genetic and pathogenic diversity within PRRSV-1 strains make more comparative studies about the pathogenesis of European PRRSV genotype strains more necessary. PRRS replication has been reported in lymphoid organs (Xiao *et al*., 2004), as a lack of protein homogeneity in proinflammatory cytokines response in lymph nodes of infected animals (Barranco *et al*., 2012a). These suggest a role of this tissue in the pathogenesis of PRRS, so the study of lymphoid organs in PRRSV infected animals is crucial to understand the host-pathogen interaction.

Porcine lymph nodes are structurally inverted, presenting an unusual route for lymphocyte circulation compared to other domestic species (Binns *et al*., 1992).

Lymphatic vessels enter the node through the hilum, and the lymph passes through the node to leave it through the periphery (Binns *et al*., 1992). The lymph node has a dense medulla, where T cells are predominant lacking sinuses and cords. The follicles are the B cell area with fDC and Th cells that collaborate to the antigen presentation to B cells and it antibodies production, respectively. Circulation of B cells, T cells, macrophages and DC through lymph nodes is also important for facilitating cellular interactions needed for the antigen recognition and the induction of the immune response (Binns *et al*., 1994; Rezk *et al*., 2013). PRRSV can induce a delayed NAb secretion in infected animals (López and Osorio, 2004). It has been described that the immunopathogenesis of PCV2 infection is associated to follicular changes in lymph nodes (Lin et al., 2011) and it is suggested that it could also be possible in PRRSV infections.

Transcriptional expression profiling studies can help to understand the biology and the molecular basis of disease (Mendrick, 2011), as we showed above. Several authors have published results on the transcriptional profiles during PRRSV infection in different organs from small pieces of tissue (Lunney *et al*., 2010; Miller *et al*., 2012; Wysocki *et al*., 2012), but neither of them have shown results about transcriptional profiling in different tissues structures. LCM is a powerful tool for the acquisition of homogeneous cells populations or specific tissue structures for the downstream application of various molecular biology techniques (Okuducu *et al*., 2005; Aranday-Cortes *et al*., 2012); for instance, LMC has provided a wide knowledge on tumor biology (Cheng *et al*., 2013).

During this experiment, the SU1-bel PRRSV strain infected pigs showed hyperthermia and higher Med-LN gross pathology compared with strains of pan-European subtype I (LV and 215-06 PRRSV strains). This was in correlation with the previous observation in lung gross pathology (Morgan *et al*., 2012), that together with Med-LN inflammation could explain the observed hyperthermia in SU1-bel group. These results indicate that

SU1-bel East European subtype 3 PRRSV differs from pan-European subtype I strains in virulence.

It has been reported that animals infected with strains differing in pathogenicity did not differ between groups in viral loads at serum level (Martínez-Lobo *et al*., 2001). However, during this experiment, differences in virulence among the PRRSV-1 strains in Med-LN compartments have been observed. For instance, the PRRSV-1subtype 3 SU1-bel strain showed the higher capacity to replicate in Med-LN compared with the PRRSV-1 pan-European strains. PRRSV is mostly detected in the main apical and medial lung lobes drained principally by Med-LN (Halbur *et al*., 1996), as at 3 dpi the higher PRRS RNA level was detected in IF area of Med-LN, suggesting the virus lymphatic circulation. In addition, viral load was shown to be delayed for 215-06 strain at 7 dpi, in agreement with its lower virulence observed in field infected pigs.

The PRRSV N protein was detected by IHC mainly in the cytoplasm of macrophages and dendritic cells (data not shown). PRRSV antigen was detected in the Med-LN of infected pigs from 3 dpi until the end of the present study, with high immunolabelling found in SU1-bel infected animals and no detection of PRRSV in Med-LN of control animals (data not shown). The serum blood samples for virus titration was carried out on at the different time-points, for all PRRSV infected groups the viraemia persisted until 28 dpi where the lower virus trite was measured in SU1-bel group (Morgan *et al*., 2012). The lower virus titration and higher tissue presence of SU1-bel strain suggest that PRRSV-1 subtype 3 virus can link and enter into its target cells more efficiently that PRRSV-1 subtype I strains.

PRRSV remains infective in lymphoid tissues, lasting for several months (Batista *et al*., 2004). Our results showed that PRRSV replication in Med-LN until 35 dpi. In the present study, the presence of different strains of PRRSV-1 within different Med-LN

compartments has been measured for the first time; showing the presence of PRRSV in F of Med-LN at 35 dpi. We hypothesized that PRRSV may infect fDC in order to remain replicating in secondary lymph organs, such as the Med-LN, therefore escaping from the host immune response. fDC may present PRRSV antigens to B cells in order to produce antibodies (Ab) against the virus, but these animals did not show neutralizing antibodies at this time post infection (Morgan *et al*., 2012). This could suggest that PRRSV may interact with fDC of Med-LN. It has been reported $DC163^{+}$ dendritic cells in swine (Marquet *et al*., 2011); as well the expression of CD151 in fDC from human tonsil has been previously described (Sincock *et al*., 1997) and also it has been recently reported that cells expressing CD151 are susceptible to PRRSV infection (Provost *et al*., 2012; Han *et al*., 2013b). Moreover, it has been reported that $CD163^{-}$ and $CD169^{-}$ cells can be infected by a highly virulent PRRSV-I subtype 3 (Lena) strain (Frydas *et al*., 2013). In this sense, the determination of which swine DC population express CD163 and CD151 and its susceptibility to PRRSV infection could help to understand the pathology induced by PRRSV and to design effectively vaccines against this disease.

PRRSV infection has been associated with B and T cell depletion in cortical areas of both prymary and secondary lymph organs as thymus and lymph nodes, respectively (He et al., 2012; Gómez-Laguna *et al*., 2013a). In these studies, the immune cell depletion was related with the apoptotic cell death induced by PRRSV infection. This phenomenon could explain the B and T cell depletion observed in this study. As PRRSV indirectly induce apoptosis on bystander cells (Sirinarumitr *et al*., 1998; Labarque *et al*., 2003); we hypothesize that PRRSV replication in cells like as fDC could induces the apoptosis of neighbor cells such us B cell and in consequence its depletion. In consequence, the Med-LN compromised functions that delay the production of non-neutralizing PRRSV-specific Ab production in PRRSV infected

animals from 7 and 9 dpi (Su1-bel and LV, respectively; Morgan *et al*., 2012) and a weak onset of cell-mediated immune response (Xiao et al., 2004). The $CD3^+$ cell increase detected in F, mostly at 7 dpi, could be related to the Th cell migration to F area in order to assist to B cell Ab production, in this case non-neutralazing Ab. The macrophage population increase in Med-LN at earlier time points for the SU1-bel group, and at 7 dpi for the other infected groups, as it has been detected by IHC labeling in the IF area, which was prolonged until 35 dpi for PRRSV-1 subtype 3 SU1-bel group. This indicates that SU1-bel strain induces a higher inflammatory response in the lung that other PRRS-1 tested strains according with previous reports (Karnychuk *et al*., 2010; Weesendorp *et al*., 2013). This fact supports the idea that SU1-bel strain is more pathogenic than PRRSV-1 subtype 1 strains.

Production of type I IFNs (IFN-α/β) is critical to activate the innate IIR against viral infection as well as regulation of effective induction of AIR (Overend *et al*., 2007; Sadler and Williams, 2008). As it is supported by previous reports, in this study low or non-transcription changes have been detected for IFN-α transcript levels in all PRRSV inoculated groups during this experiment, more noticeably at 7 dpi. This fact was more persistent for PRRSV field strains, playing a role in delaying the onset of an effective IIR (Albina *et al*, 1998; Labarque *et al*., 2000). Interestingly, the LV and SU1-bel RNA were negatively correlated with the IFN-α gene expression, this indicates that PRRSV replication directly down-regulates the IFN-α gene induction in the site of infection, in agreement with previous studies (Murtaugh *et al*., 2002). Recently, it has been published that PRRSV induces IFN-α production by pDC but neither PRRSV-1 nor PRRSV-2 strains infect pDC (Baumann *et al*., 2013). These results suggested that PRRSV could modulate the pDC activation avoiding the IFN-α secretion in infected

pigs. It is important to note that LCM coupled with RT-qPCR lead to show differences in the transcriptomic profile among Med-LN F and IF compartments.

As it has been previously described in *in vitro* and *in vivo* studies (Labarque *et al*., 2003; Subramaniam *et al*., 2010), we showed that PRRSV can reduces the TNF-α transcription during infection to avoid the host IIR and to delay the effective AIR in Med-LN. SU1-bel replication was negatively correlated with the TNF-α transcription in the F of Med-LN, this could suggest that PRRSV-1 subtype 3 directly affect the TNF-α production in the site of replication, especially in IF area at 3 dpi and in F at 35 dpi. These results support the idea that PRRSV-1 subtype 3 strains have developed the most efficient strategies to avoid the host immune responses among the PRRSV-1 genotype.

The IFN-γ up-regulation at 7 dpi is an evidence of delayed adaptive immune response in all PRRSV inoculated groups as supported several reports (Zuckermann *et al*., 2007; Jung *et al*., 2009). The positive correlation between IFN-γ gene expression and PRRSV LV ad 215-06 strains can suggest that PRRSV-1 subtype 1 induction of IFN-γ expression depends directly of the virus presence. SOCS proteins are a pivotal regulator in both IIR and AIR (Dalpke *et al*., 2008). SOCS1 act as negative regulator of IFN-γ signaling inhibiting the STAT1 activation and thereby the expression of IFN-γ mediated genes (Alexander, 2002). Recently, it has been described a SOCS1 induction in PRRSV vaccinated/infected pigs (Royaee et al., 2004; Zhou *et al*., 2011). This suggests that PRRSV could induce SOCS1 expression in infected cells to evade the host immune response. In this study the SOCS1 gene expression was not statistically significant regulated for any PRRSV infected group. On the other hand, the SOCS1 and IFN-γ gene expression were positively correlated in LV group. This could indicate that the IFN-γ up-regulation at 7 dpi in this group induce the SOCS1 expression in its negative

feedback control system. Additional *in vitro* experiments may be used to elucidate the suggested SOCS1 induction by PRRSV at the initial phase of virus infection.

IL-12p40 transcription among the different PRRSV infected groups throughout this study was not detected. On the other hand, the IL-23 was up-regulated from 7 dpi, and it was statistically significant at 35 dpi in F of all PRRSV groups. IL-12 is secreted by activated DCs and macrophages and it is necessary to induce Th1 differentiation (Shimizu *et al*., 2013). IL-23 protein is an IL-12p40-IL-23p19 hetero-dimer that is secreted by antigen presenting cells as MØ and DCs, and it is necessary for the Th17 differentiation and surviving, these cells can induce an anti-inmflamatory reaction and the TGF-β secretion (Vignali and Kuchroo, 2012). In this study we did not detect IL-12p40 transcripts, it could be explained by the transcription kinetics that express earlier or later the mRNA of this cytokine compared with the tissue sample collection. The IL-23p19 is a limiting factor to produce a biologically active IL-23 protein, as IL-23p19 is not secreted in absence of the IL-12p40 chain (Cooper and Khader, 2007) and the IL-23p19 induction is usually lower than IL-12p40 induction (Shimizu *et al*., 2013). For this reason, the higher transcription of IL-23p19 in Med-LN suggests a delayed Th17 differentiation in PRRSV infected pigs at 35 dpi. These results suggest that PRRSV replication can modulate the transcription mechanism in infected cells in order to avoid the adequate host immune response.

The IL-10 transcription detected in this study was generally lower for all PRRSV infected groups, except for LV at IF area of Med-LN from 7 dpi, in which it was higher. This fact points out that the induction of IL-10 varies among PRRSV strains in infected pigs as previously described (Gimeno *et al*., 2011). Therefore, it seems that the induction of IL-10 by PRRSV and the consequent delay of the host immune response is not a common strategy among the PRRSV-1 genotype. The TGF-β gene expression was

not induced or was downregulated for PRRSV-1 field strains in agreement with Silva-Campa *et al*. (2009). The TGF-β up-regulation induces by LV at 35 dpi was negatively correlated with the virus quantification. These results suggest that neither subtype 1 nor 3 of PRRSV-1 genotype induce TGF-β to evade the host immune response to prolong the infection. These differences between LV and the field PRRSV-1 strains suggest that LV strain may not represent the PRRSV-1 strains circulating in the field at this moment. It is possible that the PRRSV evolution has been different for field strains along the "commercial animals passages" compared with the LV since it was isolated for first time (Wensvoort *et al*., 1991), mostly propagated and adapted to grow in cells line culture as MARC-145 cells.

The classical technique to take samples for mRNA extraction cannot select different organic structures as LMC can do it. For instance, at 35 dpi IFN-α gene expression is up-regulated in Med-LN for 215-06 and SU1-bel groups, but this up-regulation is happening in IF or F, respectively. In this study we showed that LMC coupled to RT-qPCR is a powerful tool that leads to differentiate the transcriptomic profile among different structures from the same tissue. In this study we show several evidences that demonstrate how the immunopathogenesis of PRRSV-1 infection is associated to the site of infection. SU1-bel belongs to PRRSV-1 subtype 3 showed a higher tissue pathogenesis and virulence compared with PRRSV-1 subtype 1 strains. We have also demonstrated that PRRSV-1 strains avoid the IIR in infected pigs through the IFN-α and TNF-α down-regulation in F and IF area of Med-LN. The AIR was delayed avoiding the early IFN-γ expression. Also, the virus presence in follicles of Med-LN and the non-neutralizing Ab secretion at 35 dpi suggest an important role of fDC in the PRRSV immunopathogenesis. Both induction of IL-10 and TGF-β gene expression by PRRSV in order to delay the host immune response is not a common strategy among the

PRRSV-1 genotype. The transcriptomic cytokines profiles showed in this study in PRRSV-1 infected animals, with the T and B cell depletion, could explain the PRRSV-1 allocation in F of Med-LN of infected animals at the end of this study, and it could explain the high lymphatic persistence in infected animals.

The knowledge presented in this thesis is crucial for the rational design and development of more effective vaccines against PRRSV.

General Conclusions

1. Monocyte-derived MØ cultures can help to better understand the immunological and genetic basis of PRRSV virulence. This Monocyte-derived MØ represent a flexible *in vitro* model alternative against: 1) monkey cell lines; which present limitations in results interpretation because of their physiological differences with pig cells; and 2) PAM, which should be obtained with slaughtering animals, and present technical limitations regarding to polarization.
2. Cytokine-primed MØ cultures can reveal important PRRSV strain differences; whereas undifferentiated MØ and M2 (IL-4) are sensitive to all PRRSV strains, IFN-γ and IFN-β treatment of MØ induced a clear antiviral state in MØ with reductions in virus infection and replication. Hence, differences related to virulence and genotypes could be revealed using IFNs treated MØ. Despite this, the HP-PRRSV isolates from China and Eastern Europe were particularly resistant to the effects of IFN-γ.
3. Regardless the absence of technical information in the literature about cytokines qPCR based in SYBR Green chemistry, the evaluation of these optimized qPCR assays are satisfactory for monitoring proinflammatory and immunomodulatory swine responses, with robust results with low template inputs.
4. PRRSV strain 2982 is able to evade the onset of an effective innate immune response, leading to an impaired expression of IFN-α 1 and TNF-α gene expression, which finally induces a weak and delayed acquired immune response trough an inefficient IL-12 and IFN-γ expression. Additionally, PRRSV strain 2982 replication favoured the expression of the anti-inflammatory IL-10 cytokine in infected pigs.

5. Laser Microdissection acquisition coupled to RT-qPCR is a powerful tool that allows differentiating the transcriptomic profile among different structures o the same tissue.
6. PRRSV remains infecting lymphoid tissues allocated in the follicle which shows differences in the virus replication level among various viral strains. SU1-bel strain that belongs to subtype 3 of PRRSV-1 has higher capacity to replicate in Med-LN compared with the PRRSV-1 pan-European strains.
7. PRRSV-1 strains avoid the correct innate immune response in infected pigs through lymphatic cell depletion, IFN-α and TNF-α down-regulation in follicle and interfollicular area of Med-LN. Additionally, PRRSV-1 subtype 3 SU1-bel strain has developed the most efficiently strategies to avoid the host immune responses among the PRRSV-1 genotype.
8. The IL-10 induction by PRRSV is not a common strategy among the PRRSV-1 to delay of the host immune response.

Summary

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is characterized by respiratory disease in neonatal and growing pigs and reproductive failure in gilts and sows (increased number of abortions, mummified foetuses, stillbirth and weak-born piglets) (Hopper *et al*., 1992; Done and Paton, 1995; Rossow, 1998). PRRS is considered one of the most economically important swine infectious diseases around the world. It has been calculated that PRRS generates $664 million annually in losses only in USA (Zimmerman *et al*., 2006; Holtkamp *et al*., 2013).

PRRS virus (PRRSV) is classified in two genotypes, type 1 (European genotypes or PRRSV-1), represented by Lelystad virus (LV) prototype, and type 2 (American genotypes or PRRSV-2), represented by VR-2332 strain prototype (Nelsen *et al*., 1999). PRRSV-1 is divided into 3 subtypes: pan-European subtype 1 and East European subtypes 2 and 3 (Stadejek *et al*., 2008), and it has been suggested the possibility of a fourth subtype (Stadejek *et al*., 2013); the highly pathogenic (HP) PRRS-1 strains belonging to subtype 3 (Karniychuk *et al*., 2010). PRRSV-2 has been divided into at least nine lineages, including the HP-PRRSV which emerged in 2006 in China; during this outbreak the virus killed more than 2.000.000 of pigs (Shi *et al*., 2010). Despite these significant genetic differences between both PRRSV genotypes and also intra-genotype, are considered the same virus (Murtaugh *et al*., 2010).

PRRSV shows a restricted tropism for cells from the monocyte/macrophage lineage, the main *in vivo* target cells for replication being porcine alveolar macrophages (PAM), and other tissue macrophages (MØ) (Van Breedam *et al*., 2010). The virus is also able to infect and/or replicate in monocyte-derived cells as MØ and dendritic cells (DC) generated *in vitro* (Wang *et al*., 2007). It has been proposed that PRRSV can infect and

replicate in CD163+ and CD169+ endometric and placental cells of infected sows (Karniychuck *et al*., 2011). PRRSV is able to infect and/or replicate in monocyte-derived cells as MØ and DC generated *in vitro* (Wang *et al*., 2007). Beyond to MØ, it has been described the PRRSV presence in other swine cells during the *in vivo* infection, such as testicular germ cells, heart endothelial cells, thymus interdigitating cells, and DC in secondary lymphatic organs (Halbur *et al*., 1995a; Sur *et al*., 1997).

Lung and mucosal surfaces (nasal, oro-pharyngeal or genital) are the normal entry site; then PRRSV replication cycle is completed in 12 hours post-infection (hpi) in local susceptible cells. This is followed by the viraemia, which spreads the virus to tissular MØ, especially to PAM or MØ in secondary lymphoid tissues and in other organs as testes or utero (Lawson *et al*., 1997). The viraemia is typically prolonged during 4 to 6 weeks after infection, but the virus can persist within the host for months in lymphoid tissues, inducing a PRRSV sub-clinical persistence (Wensvoort *et al*., 1991; Wills *et al*., 2003).

PRRSV infected pigs develop a poor innate and acquired immune responses (Albina *et al*., 1998; Van Reeth 1999), including cytokine production modulation (Flores-Mendoza *et al*., 2008) which leads to co-infections and to the prolonged virus persistence in the host (Thanawongnuwech *et al*., 2004). A lack or weak IFN-α secretion (Albina*et al*., 1998), and a consequent weak and delayed cell-mediated immune response with low levels of IFN-γ, has been described in PRRSV infections (Mateu and Díaz, 2008; Meier *et al*., 2003). Infected animals also show a delayed production of neutralizing antibodies (NAb). The high genetic and pathogenic diversity within PRRSV-1 strains make more necessary to do comparative studies about the pathogenesis of PRRSV type 1 strains.

Objectives

The general aim of this thesis will be the evaluation of the swine immune response against PRRSV infection in the swine target cells and organs, paying special attention to the PRRSV ability to modulate the innate immune response.

Considering the central importance of MØ for PRRS pathogenesis and the role of IFNs and cytokines in regulating MØ during different types of immune response, we hypothesized that PRRSV varying in virulence should differ in their interaction with cytokine-modulated MØ which represented distinct swine immune scenarios. For this reason, the **first** objective was to evaluate the IFN-γ-(M1), IL-4-(M2) and IFN-β-activated monocyte derived MØ susceptibility to different PRRSV-1 and PRRSV-2 strains as well their ability to secrete cytokines after infection.

The **second** objective was to determine how the swine immune response is modulated against PRRSV 2982 field strain; belonging to the predominant PRRSV-1 subtype 1 in Western Europe. Once the gene expression technique was validated, we determined the differences in the transcripts levels of proinflammatory and immunomodulatory cytokines in order to get more insights into the pathogenesis and the immune response in the main lung, tonsil, tracheobronchial lymph node (Tb-LN) and retropharyngeal LN (Rf-LN) of infected pigs.

PRRSV 2982 regulates the host immune response in the site of infection, both in lung and secondary lymph organs. Swine PRRSV infection has been associated with B and T cell depletion in cortical areas of secondary lymph organs as lymph nodes; which suggest an important role of these tissues in the host immunity modulation by PRRSV. For this reason, the **third** objective was to evaluate the immune response against PRRSV in different compartments of mediastinal LN (Med-LN) [lymphoid follicle (F)

and inter-follicular area (IF)] of pigs infected with three different PRRSV-1 strains varying in virulence.

Material and Methods

To reach the proposed objectives we performed three studies: an *in vitro* study to establish the PRRSV ability to infect different cytokine-modulated MØ, and two *in vivo* studies, the first one to determine how PRRSV-1 2982 modulate the immune response in swine tissues during the infection, and the second one to study the cytokines regulation induced by PRRSV-1 strains varying in virulence in different lymph node compartments.

In vitro study

Monocyte-derived MØ

PBMCs were isolated from specific pathogen free (SPF) pigs using ficoll-paque density centrifugation (1.077 g/l). Monocytes were then enriched by CD172a positive selection using the MACS with LS columns, and seeded in 24-well culture plates at a density of 5 × 10^5 cells per well in DMEM with GlutaMax and 10% heat-inactivated porcine serumat 39°C with 5% CO_2 for 72 h, and then stimulated by using either IFN-γ (10 ng/ml; "IFN-γ MØ"), IL-4 (100 U/ml; "IL-4 MØ"), IFN-β (100 U/ml; IFN-β MØ") or were left untreated for another 24 h (MØ).

Viruses and experimental design

For the MØ infection we used as PRRSV-1, the Lelystad virus adapted to grow in MARC-145 (LVP23), two Spanish field isolates (2982 and 3267), Olot/91, and HP-PRRSV Lena strain. As a PRRSV-2, VR-2332, JA-1262, MN184, SS144 and HP-China isolate RVB-581 were used. MØ were infected at a MOI of 0.1 TCID50/cell in medium containing 10% SPF pig serum. Mock-treated MØ were included as controls.

Cytokines measurements

In the supernatant IFN-α, TNF-α, and IL-10 expression production were determined by ELISA.

Monoclonal antibodies and flow cytometry

The following mouse anti-pig cell surface antibodies were used: anti-CD172a, anti-CD16, anti-α-MHC I and anti-MHC II, anti-CD86, anti-CD16 and anti-CD14. Specific goat anti-mouse IgG conjugated with RPE was used as secondary antibody. For detection of PRRSV nucleocapsid (N) protein the cells were stained with the monoclonal antibody SDOW17-A. Acquisition was done on a FACSCalibur.

***In vivo* studies**

Viruses and experimental designs

For the *in vivo* study of PRRSV-1 2982 stain infection, thirty-two SPF pigs were randomly allocated in eight groups of four animals per group; seven groups were inoculated by the intramuscular route with 1 ml of third passage of PRRSV field isolate 2982 at $1^{3.0}$ $TCID_{50}$, and with 1 ml of sterile RPMI 1640 medium the control group. The virus-inoculated pigs were humanely euthanized at 3, 7, 10, 14, 17, 21 and 24 days post-infection (dpi) and the controls at 24 dpi.

In the second *in vivo* study three PRRSV-1 strains were used; LV as the prototype subtype 1 strain, 215-06 strain (subtype 1) and SU1-bel (subtype 3). Seventy-six SPF piglets were matched by weight and randomly allocated in four groups; control group (16 animals), and three infected groups (20 animals/group). At seven weeks old, 3 groups were inoculated intranasally with 1.5 ml of cRPMI with 10^5 $TCID_{50}$ of each PRRSV strain (LV, 215-06 and SU1-bel, respectively). Four animals in the control

group and five for each virus-inoculated group were humanely euthanized at 3 and 7 dpi. At 7 dpi five of the remaining animals in each group were vaccinated intramuscularly with an Aujeszky disease virus vaccine. All remaining animals were humanely euthanized at 35 dpi.

Clinical signs, gross pathology, histopathology and viraemia

In both *in vivo* studies pigs were monitored daily for clinical signs, including rectal temperature. Lungs, tonsils, Tb-LN, Rf-LN, and Med-LN were examined at post-mortem and lung lesions were scored as previously described (Halbur *et al.*, 1995). Tissues were microscopically examined. Virus titration was carried out on blood samples using an immunoperoxidase monolayer assay and RT-qPCR (for the first and second *in vivo* experiments, respectively). For the analysis of transcript levels the half Med-LN was cryopreserved in OCT.

Laser capture microdissection (LCM)

In the second *in vivo* study, two consecutive Med-LN cryostat tissue sections were placed on membrane-coated slides, and all observed F and peripheral IF area were dissected and captured separately using a laser microdissector.

Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

For the PRRSV-1 2982 infected animals a tisular RNA was extracted and treated with DNase; then cDNA was synthesized by mRNA retrotranscription. For the samples of LMC, RNA was extracted using the RNAqueous-Micro Kit, including the DNase I treatment and DNase inactivation step. Primers for each proinflammatory (IFN-α, IFN-γ, IIL-12p35, IL-12p40 and TNF-α) and immunomodulatory (IL-10 and TGF-β)

cytokine were selected from previously published reports (Royaee*et al*., 2004; Gabler*et al*., 2006; Moue *et al*., 2007; Kim *et al*., 2010). cDNA was amplified using SYBR green chemistry, these qPCR assays were optimized for target specificity (ratio cDNA:water 1:1, 1:5, and 1:25) and primer concentration (50, 200, 300 and 900 nM). To generate the standard curves, the transcripts of each cytokine of interest and PRRSV ORF7 were encoded in a plasmid. Standard curves for each primer set were prepared at the final copy number in the qPCR assay ranged from 10^8 in 10-fold and 2-fold serial dilutions. For each qPCR was calculated efficiency, coefficient of variation, factor of discrimination and limit of detection. Cytokines gene expression was calculated as target/reference ratio of each sample normalized by target/reference ratio of endogenous calibrators (β-actin, cyclophilin and GAPDH) by geometric averaging of multiple endogenous control genes method (Vandesompele *et al*., 2002).

In the second study, for the samples of LMC, cDNA were used as template for cytokines qPCR using TaqMan probes. Cytokines fold change was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), the β-actin was used as endogenous calibrator. PRRSV RT-qPCR was done as previously described Lurchachaiwong *et al*., (2007) for SYBR green chemistry, and Frossard et al. (2012) for LMC samples. PRRSV replication was represented by changes in the Ct as previously described (Kuzemtseva *et al*., 2014).

Immunohistochemistry (IHC)

For the second in vivo experiment cell markers immunolabelling were done by the ABC method as previously described (Gómez-Laguna et al., 2010; Barranco et al., 2011). The primary monoclonal antibodies used were anti-CD3, anti-CD79a and anti- MAC-387; in each case the corresponding biotinylated secondary antibody; finally, the labelling was carried out using DAB. The immunolabelled Med-LN sections were examined by light

microscopy, and the measurements were recorded using a score ranking from -3 to 0 (cellular depletion) and from 0 to 3 (cellular increment) comparing to control group.

Statistical analysis

Statistical analyses were done with GraphPad PrismV.5 software and SPSS 15.0 software. Significant effects on polarized MØ, compared to unpolarized MØ were assessed by repeated measures ANOVA and Dunnett's Multiple Comparison tests. For cell surface markers and cytokines expression the differences were assigned by Kruskal-Wallis test followed by the Mann-Whitney-U non parametric test. The correlations between PRRSV and cytokines were determined by the Spearman's Rho analysis. $P < 0.05$ was at least considered statistically significant. Significance variations were indicated by asterisk or different letters based on statistical analysis.

Results

In vitro study

MØ phenotype

The *in vitro* experiment showed that stimulated MØ developed different phenotype compared with non polarized MØ. In general, IFNs-treated MØ expressed more MHC I, whereas only M1 MØ produces more MHC II and CD16. In contrast, the CD14 was down-regulated by each cytokine treatment. Interestingly, M2 MØ produced the lower CD163 expression.

MØ viability and susceptibility to PRRSV infection

Undifferentiated and M2 MØ were susceptible to be infected by all PRRSV strains tested in the present studies; in these cells PRRSV-2 strains and LENA developed the higher cytopatic effect and infections rates. On the other hand, IFNs-treated MØ were resistant to the infection and the cytopathogenic actions of the PRRSV-1 subtype 1

strains, but these cells were only partially resistant against PRRSV-2 strains. Finally, it is important to remark that the IFN-β stimulated MØ were the most resistant cells against PRRSV. Interestingly, HP-PRRSV isolates of both genotypes showed particularly high levels of infection compared with the prototype viruses in both M1 and IFN-β treated MØ.

Production of cytokines and IFN-α in infected MØ

All tested MØ did not secrete TNF-α or IL-10; anywise the IFN-α production was detected in the supernatant of M1 MØ infected with LVP23 PRRSV-1 strain.

As the currently cell culture systems cannot totally exhibit the complex relations between cells during the immune response, we had carried out two *in vivo* experiments in order to evaluate the swine immune response against PRRSV-1.

***In vivo* studies**

Clinical Signs, Gross Pathology, Histopathology and Viraemia and virus replication

During the first *in vivo* experiment we evaluated the immune response in lung, tonsil and lymph nodes of 2982 PRRSV-1 strain infected pigs. In this study only the inoculated pigs showed clinical signs and lesions in lung and lymphatic organs; in these animals the viraemia was detected from 3 dpi onward (24 dpi). Generally, viral replication was higher in lymphoid organs than in the lung of PRRSV-inoculated animals throughout the study.

We did a second *in vivo* study in which pigs were infected with PRRSV-1 strains varying in virulence in order to study the induced immune response in different Med-LN compartments. We selected LV as prototype PRRSV-1 strain, 215-06 UK field isolate as low virulent PRRSV-1 subtype 1, and SU1-bel Belarus field strain as virulent PRRSV-1 subtype 3. The viral inoculated animals showed clinical signs and lesions in Med-LN at 3 and 7 dpi; which were higher for SU1-bel infected pigs. Generally, during this study PRRSV was detected at 3 dpi in the IF area of Med-LN. Later the virus

increased in the F area, were PRRSV was mainly detected at the end of the study. At each time post-infection, the higher virus replication was observed in the animals infected with SU1-bel PRRSV-1 strain.

Cytokines RT-qPCRs evaluation for swine tissues using SYBR green chemistry

The SYBR green chemistry qPCR assays were evaluated prior to measure the cytokine production in swine PRRSV infected tissues. All qPCR assays rendered a correlation coefficients of R^2>0.95 and efficiency values ranged between 1.68 (TNF-α) and 2.38 (IL-10). The coefficient of variation results indicated that the qPCR assays variability was lower than 3% along the 2 and 10-fold dynamic range for the most of cytokines. Finally, the mean factor of discrimination was always lower than 3 in both 2-fold and 10-fold serial dilutions. This qPCR assays evaluation showed robust results with low template inputs.

Cytokine gene expression

PRRSV strain 2982 avoided the onset of an effective innate immune response, especially in PRRSV main target (lung) and reservoir (tonsil) organs. PRRSV lead to an impaired expression of IFN-α and TNF-α gene expression, later a weak and delayed IL-12 and IFN-γ expression. Finally, PRRSV replication favored the expression of the anti-inflammatory IL-10 cytokine in infected pigs.

Field PRRSV-1 strains delayed the onset of an effective innate immune response in Med-LN compartments; these strains induced a down-regulation of both IFN-α and TNF-α pro-inflammatory cytokines in the site of infection. This was followed by a delayed induction of IFN-γ at 7 dpi. The gene expression of anti-inflammatory cytokines IL-10 and TGF-β were detected only in LV infected animals; whereas the field PRRSV-1 strains did not induce the expression of these cytokines in inoculated pigs.

Immunohistochemistry

During the second in vivo experiment T cell population was increased in the F of infected animals at 7 dpi; whereas in IF from 3 dpi onward T cell depletion was detected in LV and SU1-bel PRRS-1 strains infected animals. The B cell population decreased in all PRRSV infected animals from 3 dpi onward. Finally, the MØ detection was higher for SU1-bel infected animals compared with other groups along this study.

Discussion

Considering the central importance of MØ for PRRS pathogenesis and the role of IFNs and cytokines in regulating MØ (Cancel-Tirado *et al*., 2004; Zlotnik *et al*., 1982), we showed that PRRSV strains differing in virulence were distinct in their interaction with cytokine-modulated MØ. IFN-γ-(M1), IL-4-(M2) and IFN-β-activated MØ showed distinct susceptibility to different PRRSV-1 and PRRSV-2 strains as well their ability to secrete cytokines after infection. Therefore, IFN-treated MØ may allow discrimination between PRRSV isolates varying in virulence.

As polarized MØ has shown distinct susceptibility to be infected by different PRRSV strains; MØ cultures could help to better understand the immunological and genetic basis of PRRSV virulence. In general, PRRSV-1 subtype 1 strains were not able to proliferate in M1 and IFN-β MØ, which represent an effective swine immune response against viral infection (Shahangian *et al*., 2009). On the other hand, PRRSV-1 subtype 3 LENA strain and PRRSV-2 showed higher ability to replicate in these cells compared with PRRSV-1 subtype 1 strains, which supports results reported by other authors (Karniychuk *et al*., 2010; Shi *et al*., 2010). M2 MØ represent the anti-inflammatory immune response (Mosser, 2003), these cells were susceptible to be infected by all PRRSV genotypes

Our data support an alternative model to PAM using monocyte-derived MØ, which can be obtained without slaughtering animals (Wensvoort *et al*., 1991). In addition, this MØ can be modulated and polarized by cytokines to mimic certain functions of tissue MØ during steady state conditions or during the course of immune responses (Carrasco *et al*., 2001). This *in vitro* study has identified important strain differences using cytokine-primed MØ cultures, which will be very valuable to understand the virological and cellular elements important for PRRSV–cell interactions and relating to virulence.

PRRSV has developed several strategies in order to avoid the proper onset of the host innate immune response as well to delay the acquired immunity (Albina *et al*., 1998; Van Reeth 1999; Thanawongnuwech *et al*., 2004; Flores-Mendoza *et al*., 2008). Our results showed that M2 MØ are susceptible for PRRSV like non-polarized MØ; then if PRRSV can modulate the host immune response it could avoid the M1 MØ differentiation in order to induce more susceptible cells to the infection.

Nowadays, the currently available cell culture models cannot fully reflect the complex events induced in the cytokine network of a multitude of cell types found *in vivo*. Transcriptional expression profiling studies can help to understand the biology and the molecular basis of disease (Mendrick, 2011). Therefore, the analysis of cytokines transcript levels in PRRSV infected swine tissues represents a useful tool to evaluate the virus immune modulation in the host during the infection.

Intramuscular inoculation route resulted in the highest PRRSV-1 2982 replication in lymph nodes at 3dpi, which might be the result of the viral filtration process from the lymph. This inoculation route could explain the lower number of PRRSV copies quantified in the lung at 3 dpi.

During the PRRSV-1 2982 isolate pig infection, our results confirmed the ability of PRRSV to replicate during long periods in infected tissues, which may allow the

depression of the immune system in PRRSV infected pigs. Intramuscular inoculation route resulted in the highest PRRSV-1 2982 replication in lymph nodes at 3dpi, which might be the result of the viral filtration process from the lymph. This inoculation route could explain the lower number of PRRSV copies quantified in the lung at 3 dpi.

PRRSV isolate 2982 *in vivo* suppressed the IFN-α 1 and TNF-α gene expression in immune cells, such as MØ and DC, consequently the host effective onset of innate immune response was impaired during the virus infection (Beura *et al*., 2009), then the induction of adaptive immunity was interfered (Kimman *et al*., 2009).

The observed IFN-γ up-regulation in the lung and Tb-LN of inoculated animals was not effective to avoid PRRSV replication in these tissues. Probably, in tonsil PRRSV induces the secretion of IFN-γ through IL-12 signalling (Trinchieri, 1994). Therefore, our results suggest that PRRSV infection may modulate the host IL-12 response to suppress its biological effects in lung and LNs.

The IL-10 up-regulation was correlated with the gene expression of IFN-γ in the lung and Rf-LN of PRRSV-infected pigs, contrary to previous findings which suggest that PRRSV-1 induces IL-10 as a strategy to proliferate avoiding an efficient IFN-γ secretion in infected tissues (Díaz *et al*., 2006). This fact points out that the modulatory role of IL-10 on the immune response may vary among PRRSV-1 strains.

PRRSV 2982 strain avoided the onset of an effective innate immune response, through the impairment of IFN-α 1 and TNF-α gene expression; which finally induces a weak and delayed cellular acquired immune response with an inefficient IL-12 and IFN-γ expression, and PRRSV replication favoured the expression of the anti-inflammatory IL-10 cytokine in infected pigs.

As we showed above, the PRRSV-1 2982 strain can modulate the host immune response in the infected tissues. Swine PRRSV infection has been associated with B and

T cell depletion in cortical areas of secondary lymph organs as lymph nodes; which suggest an important role of these tissues in the host immunity modulation by PRRSV. Several authors have published results on the transcriptional profiles during PRRSV infection in different organs by taking small pieces of tissue (Lunney *et al*., 2010; Miller *et al*., 2012; Wysocki *et al*., 2012), but neither of them have shown results about transcriptional profiling in different tissues structures. The use of LCM is a powerful tool to the acquisition of homogeneous cells populations or specific tissues structures for the application of molecular biology techniques (Okuducu *et al*., 2005; Aranday-Cortes *et al*., 2012).

SU1-bel East European subtype 3 PRRSV differs from pan-European strains in virulence. In addition, SU1-bel strain showed the higher capacity of replication in Med-LN compared with the PRRSV-1 subtype 1; which can remain in the Med-LN until 35 dpi.

The immune cell depletion has been related with the apoptotic cell death induced by PRRSV infection in these tissues; which could explain the B and T cell depletion observed in this study (He *et al*., 2012; Gómez-Laguna *et al*., 2013).

LV and SU1-bel RNA were negatively correlated with the IFN-α gene expression, this indicates that PRRSV replication directly down-regulates the IFN-α gene induction in the site of infection. It is important to note that LCM coupled with RT-qPCR showed differences in the transcriptomic profile among Med-LN F and IF compartments.

We showed that PRRSV reduces the TNF-α transcription during infection, and a delay of the host innate andacquired immune response. PRRSV-1 subtype 3 directly affected the TNF-α production in the site of replication, especially in IF area at 3 dpi and in F at 35 dpi. These results support the idea that PRRSV-1 subtype 3 strains has developed the

most efficiently strategies to avoid the host immune responses among the PRRSV-1 genotype (Frydas *et al*., 2013).

The IFN-γ up-regulation at 7 dpi is an evidence of a delayed acquired immune response in all PRRSV inoculated groups as supported several reports (Zuckermann *et al*., 2007; Jung *et al*., 2009). The positive correlation between IFN-γ gene expression and PRRSV LV ad 215-06 strains suggested that PRRSV-1 subtype 1 induction of IFN-γ expression depends directly on the virus presence (Murtaugh *et al*., 2002). In this study the SOCS1 gene expression was not statistically significant regulated for any PRRSV infected group. On the other hand, the SOCS1 and IFN-γ gene expression were positively correlated in LV group. This could indicate that the IFN-γ up-regulation at 7 dpi in this group induce the SOCS1 expression in its negative feedback control system (Brysha *et al*., 2001).

IL-23 is secreted by antigen presenting cells as MØ and DCs, and it is necessary for the Th17 differentiation and surviving, these cells can induce anti-inflammatory reaction and the TGF-β secretion (Vignali and Kuchroo, 2012). The IL-23p19 higher transcription detected in the present work suggests a delay in the Th17 differentiation in Med-LN of PRRSV infected pigs at 35 dpi.

The induction of IL-10 varies among PRRSV strains in infected pigs (Gimeno *et al*., 2011), suggesting that the induction of IL-10 by PRRSV is not a common strategy among the PRRSV-1 genotype to delay the host immune response. The TGF-β gene expression was not induced or lower transcript levels were measured for PRRSV-1 field strains in agreement with Silva-Campa *et al*. (2009).

In this study we showed several evidences that immunopathogenesis of PRRSV-1 infection is associated to the site of infection. SU1-bel that belongs to PRRSV-1

subtype 3 showed a higher tissue pathogenesis and virulence compared with PRRSV-1 subtype 1 strains. Our results demonstrated that PRRSV-1 strains avoid the host immune response, first the virus prevent the correct onset of an effective innate immune response in infected pigs through the IFN-α and TNF-α down-regulation in F and IF area of Med-LN, and finally the cellular acquire immune response was delayed avoiding the early IFN-γ expression.

General conclusions

1. Monocyte-derived MØ cultures can help to better understand the immunological and genetic basis of PRRSV virulence. This Monocyte-derived MØ represent a flexible *in vitro* model alternative against: 1) monkey cell lines; which present limitations in results interpretation because of their physiological differences with pig cells; and 2) PAM, which should be obtained with slaughtering animals, and present technical limitations regarding to polarization.
2. Cytokine-primed MØ cultures can reveal important PRRSV strain differences; whereas undifferentiated MØ and M2 (IL-4) are sensitive to all PRRSV strains, IFN-γ and IFN-β treatment of MØ induced a clear antiviral state in MØ with reductions in virus infection and replication. Hence, differences related to virulence and genotypes could be revealed using IFNs treated MØ. Despite this, the HP-PRRSV isolates from China and Eastern Europe were particularly resistant to the effects of IFN-γ.
3. Regardless the absence of technical information in the literature about cytokines qPCR based in SYBR Green chemistry, the evaluation of these optimized qPCR assays are satisfactory for monitoring proinflammatory and immunomodulatory swine responses, with robust results with low template inputs.

4. PRRSV strain 2982 is able to evade the onset of an effective innate immune response, leading to an impaired expression of IFN-α 1 and TNF-α gene expression, which finally induces a weak and delayed acquired immune response trough an inefficient IL-12 and IFN-γ expression. Additionally, PRRSV strain 2982 replication favoured the expression of the anti-inflammatory IL-10 cytokine in infected pigs.
5. Laser Microdissection acquisition coupled to RT-qPCR is a powerful tool that allows differentiating the transcriptomic profile among different structures o the same tissue.
6. PRRSV remains infecting lymphoid tissues allocated in the follicle which shows differences in the virus replication level among various viral strains. SU1-bel strain that belongs to subtype 3 of PRRSV-1 has higher capacity to replicate in Med-LN compared with the PRRSV-1 pan-European strains.
7. PRRSV-1 strains avoid the correct innate immune response in infected pigs through lymphatic cell depletion, IFN-α and TNF-α down-regulation in follicle and interfollicular area of Med-LN. Additionally, PRRSV-1 subtype 3 SU1-bel strain has developed the most efficiently strategies to avoid the host immune responses among the PRRSV-1 genotype.
8. The IL-10 induction by PRRSV is not a common strategy among the PRRSV-1 to delay of the host immune response.

Resumen

Introducción

El síndrome respiratorio y reproductivo porcino (PRRS, del inglés “porcine reproductive and respiratory syndrome”) se caracteriza por causar enfermedad respiratoria en neonatos y cerdos en crecimiento y un fallo reproductivo en cerdas de reproductoras (incremento de los abortos, cerdos momificados, muerte de neonatos y lechones débiles al nacimiento) (Hopper *et al*., 1992; Done y Paton, 1995; Rossow, 1998). El PRRS se considera una de las enfermedades infecciosas con un mayor impacto económico a nivel mundial. Se ha estimado que PRRS causa pérdidas anuales a la industria porcina de EEUU por valor de 664 millones de dólares (Zimmerman *et al*., 2006; Holtkamp *et al*., 2013).

El virus PRRS (PRRSV) se clasifica en dos genotipos, el tipo 1 (genotipos europeos o PRRSV-1), representado por la cepa prototipo virus Lelystad (LV), y el tipo 2 (genotipos americanos o PRRSV-2), representado por la cepa prototipo VR-2332 (Nelsen *et al*., 1999). PRRSV-1 se divide en tres subtipos: subtipo 1 pan-Europeo, y subtipos 2 y 3 del Este de Europa (Stadejek *et al*., 2008), últimamente se ha sugerido la posibilidad de un cuarto subtipo (Stadejek *et al*., 2013); las cepas altamente patogénicas (HP, del inglés “*higly pathogenic*”) de PRRSV-1 pertenecen al subtipo 3 (Karniychuk *et al*., 2010). PRRSV-2 se a subdividido en al menos nueve linajes, incluyendo las cepas HP-PRRSV que emergieron en China en 2006, que durante ese brote llegaron a causar 2.000.000 de cerdos muertos (Shi *et al*., 2010). A pesar de estas significativas diferencias genéticas entre genotipos de PRRSV e incluso entre subtipos dentro de cada genotipo, se consideran como el mismo virus (Murtaugh *et al*., 2010).

PRRSV muestra un tropismo muy restringido hacia células de la línea monocito/macrófago, siendo la principal célula diana para la replicación los macrófagos

alveolares porcinos (MAP), y otros macrófagos (MØ) tisulares (Van Breedam *et al*., 2010). El virus también es capaz de infectar y/o replicar en células previamente generadas *in vitro* que han sido derivadas de monocitos como MØ y células dendríticas (DC, del inglés "*dendritic cells*") (Wang *et al*., 2007). Además de MØ, se ha descrito que la presencia de PRRSV en otras células porcinas durante la infección *in vivo*, cómo en células germinales testiculares, células endoteliales cardíacas, células interdigitantes de timo, y DC en órganos linfáticos secundarios (Halbur *et al*., 1995a; Sur *et al*., 1997).

El pulmón y las superficies mucosas (nasales, oro-faríngea o genitales) son los sitios normales de entrada del virus; por lo tanto el ciclo de replicación de PRRSV es completado en 12 horas post-infección (hpi) en las células locales susceptibles al virus. Este proceso es seguido por la viremia, que disemina el virus hacia los MØ tisulares, especialmente hacia MAP o MØ en tejidos linfoides secundarios y otros órganos como testículos o útero (Lawson *et al*., 1997). La viremia suele prolongarse durante 4 a 6 semanas después de la infección, pero el virus puede persistir en el hospedador durante meses en tejido linfoide, lo que induce una persistencia sub-clínica de PRRSV (Wensvoort *et al*., 1991; Wills *et al*., 2003).

Los cerdos infectados por PRRSV desarrollan unas respuestas innata y adquirida pobres (Albina *et al*., 1998; Van Reeth 1999), incluyendo la modulación de producción de citocinas (Flores-Mendoza *et al*., 2008) lo que permite la proliferación de co-infecciones y prolonga la persistencia del virus en el hospedador (Thanawongnuwech *et al*., 2004). Durante la infección por PRRSV se ha descrito la ausencia o una escasa secreción de IFN-α (Albina *et al*., 1998), y en consecuencia una débil y retrasada respuesta inmune celular con bajos niveles de IFN-γ (Mateu y Díaz, 2008; Meier *et al*., 2003). Además, lo animales infectados por PRRSV muestran una producción de anticuerpos neutralizantes muy retrasada en el tiempo (NAc). La gran variedad genética

y patogénica dentro de PRRSV-1 hace más necesario realizar estudios comparativos sobre la patogénesis de cepas de PRRSV-1.

Objetivos

El objetivo general de esta tesis será la evaluación de la respuesta inmune de cerdo contra la infección por PRRSV en los órganos diana del virus, poniendo especial atención en la habilidad del PRRSV para modular la respuesta innata inmune del cerdo.

Considerando la importancia central de los MØ para la patogénesis de PRRSV y el rol central de IFNs y citocinas en la regulación de MØ durante diferentes tipos de respuesta inmune, partimos de la hipótesis de que cepas de PRRSV que varíen en virulencia deben de diferir en sus interacciones con MØ modulados por citocinas que representen distintos escenarios de la respuesta inmune del cerdo. Por esa razón, el **primer** objetivo será la evaluación de la susceptibilidad a la infección por diferentes cepas de PRRSV-1 y PRRSV-2 que presentan MØ derivados de monocitos sin estimular o estimulados por IFN-γ (M1), IL-4 (M2) o IFN-β, así como se evaluará la capacidad de estas células de secretar diferentes citocinas después de la infección.

El **segundo** objetivo será la determinación de cómo la cepa 2982 de PRRSV modula la respuesta inmune en cerdos infectado, esta cepa pertenece al subtipo 1 del Oeste de Europa de PRRSV-1. Una vez que la técnica de detección de expresión génica de citocinas sea validada, determinaremos diferencias en los niveles de transcripción de citocinas proinflamatorias y antiinflamatorias con el propósito de obtener un mayor conocimiento en la patogénesis y en la respuesta inmune que se da en pulmón, tonsila, nódulos linfáticos traqueobronquial (Tb-LN) y retrofaríngeo (Rf-LN) de cerdos infectados.

La cepa 2982 de PRRSV regula la respuesta inmune del hospedador en el sitio de infección, tanto en pulmón como en órganos linfáticos secundarios. Además la infección en cerdos por PRRSV se ha asociado con una depleción de linfocitos B y T en las áreas corticales de órganos linfáticos secundarios como nódulos linfáticos, lo que sugiere in rol importante de estos tejidos en la modulación de la respuesta inmune del cerdo por parte del PRRSV. Por estos motivos, el tercer objetivo será la evaluación de la repuesta inmune contra PRRSV en diferentes compartimentos del nódulo linfático mediastínico (MEd-LN) [folículo linfoide (F) y área interfolicular (IF)] de cerdos infectados por diferentes cepas de PRRSV-1 que varían en virulencia.

Material y Métodos

Para alcanzar los objetivos propuestos realizamos tres estudios: un estudio *in vitro* para establecer la habilidad de PRRSV para infectar diferentes MØ derivados de monocitos estimulados por citocinas, y dos estudios *in vivo*, el primero para determinar como la cepa 2982 del PRRSV-1 modula la respuesta inmune en tejidos de cerdo durante la infección, y el segundo de ellos para estudiar la regulación de citocinas inducida por diferentes cepas de PRRSV-1 que varían en virulencia en los diferentes compartimentos de la corteza de nódulo linfático.

Estudio *in vitro*

MØ derivados de monocitos

Se aislaron PBMC procedentes de cerdos libres de patógenos específicos (SPF del inglés “specific pathogen free”) mediante el uso de centrifugación por densidad en ficoll-paque (1.077 g/l). Los monocitos fueron seleccionados mediante el marcaje de células positivas a CD172a utilizando tecnología magnética de MACS con columnas

LS, y estas células se sembraron en placas de cultivo de 24 pocillos a una densidad de 5 x 10^5 células por pocillo en medio DMEM conGlutaMAX y un 10% de suero porcino inactivado por calor e incubadas a 39 ºC con un 5% de CO_2 durante 72 h, después se estimularon con IFN-γ (10 ng/ml; "IFN-γ MØ"), IL-4 (100 U/ml; "IL-4 MØ"), IFN-β (100 U/ml; IFN-β MØ") o se dejaron sin estímulo durante otras 24 h (MØ).

Virus y diseño experimental

Para la infección de MØ empleamos cepas del PRRSV-1, Lelystad virus adaptada a crecer en MARC-145 (LVP23), dos cepas de campo españolas (2982 y 3267), Olot/91, y la cepa HP-PRRSV Lena. Del PRRSV-2 empleamos VR-2332, JA-1262, MN184, SS144 y la cepa HP-China RVB-581. Los MØ se infectaron a una MOI de 0.1 TCID50/pocillo en medio con un 10% de suero de cerdos SPF. Como controles se incluyeron MØ tratados con Mock.

Determinación de citocinas

En los sobrenadantes se determinaron mediante ELISA los niveles de expresión de IFN-α, TNF-α, e IL-10.

Anticuerpos monoclonales y citometría de flujo.

Utilizamos los siguientes anticuerpos monoclonales anti-marcadores de superficie celular de cerdo: anti-CD172a, anti-CD16, anti-α-MHC I and anti-MHC II, anti-CD86, anti-CD16 and anti-CD14. Como anticuerpos secundarios se emplearon anticuerpos específicos cabra anti-IgG de ratón conjugados con RPE. Para la detección de la proteína de nucleocápside de PRRSV (N) las células se marcaron con el anticuerpo monoclonal SDOW17-A. Para la cuantificación celular se empleó el FACSCalibur.

Estudios *in vivo*

Virus y diseños experimentales

Para el estudio *in vivo* de la infección con la cepa 2982 de PRRSV-1, se emplearon 32 cerdos SPF que se alojaron aleatoriamente en ocho grupos de cuatro animales por grupo; siete grupos se inocularon intramuscularmente con 1ml de un tercer pasaje de la cepa de campo de PRRSV 2982 a $1^{3.0}$ $TCID_{50}$, y el grupo control con 1 ml de medio estéril RPMI 1640. Los cerdos inoculados por PRRSV fueron eutanasiados humanitariamente a 3, 7, 10, 14, 17, 21 y 24 días post-infección (dpi) y el grupo control a 24 dpi.

En el segundo estudio *in vivo* se emplearon tres cepas de PRRSV-1: LV se empleó como cepa prototipo del PRRSV-1, cepa de campo 215-06 (del subtipo 1), y SU1-bel (del subtipo 3). 76 cerdos SPF se equilibraron por pesos y agrupados aleatoriamente en cuatro grupos, uno control (16 animales), y tres grupos de animales infectados (20animales por grupo). A las siete semanas de edad, tres grupos se inocularon intranasalmente con 1.5 ml de cRPMI con 10^5 $TCID_{50}$ de cada cepa de PRRSV (LV, 215-06 y SU1-bel, respectivamente). Cuatro animales del grupo control y cinco de cada grupo infectado se eutanasiaron humanitariamente a 3 y 7 dpi. A 7 dpi cinco de los restantes animales de cada grupo fueron vacunados intramuscularmente contra el virus de la enfermedad de Aujeszky. Todos los animales restantes fueron humanitariamente eutanasiados a los 35 dpi.

Signos clínicos, patología macroscópica, histopatología y viremia

En ambos estudios *in vivo* los signos clínicos fueron monitorizados diariamente, incluyendo temperatura rectal. Pulmón, tonsila, TB-LN, RF-LN y Med-LN fueron

examinados post-mortem y las lesiones pulmonares se evaluaron como previamente describieron Halbur et al., (1995a). Los tejidos se examinaron microscópicamente. La titulación del virus se realizó en muestras de sangre utilizando el ensayo de monocapa inmunoperoxidasa y por RT-qPCR (respectivamente para el primer y el segundo estudio *in vivo*). Para el análisis de niveles de transcripción las muestras de MEd-LN fueron criopreservadas en OCT.

Captura por microdisección laser (CML)

En el segundo estudio *in vivo*, dos secciones consecutivas de Med-LN crioperservado fueron colocadas en portas cubiertos con una membrana para corte por láser, todos los F e IF área circundante fueron diseccionados y capturados separadamente mediante un microdisector láser.

RT-qPCR

Para los animales infectados por la cepa 2982 del PRRSV-1 el RNA tisular se extrajo y trató con DNase; entonces el cDNA se sintetizó por retrotranscripción del mRNA. Para las muestras de CML, el RNA se extrajo con el kit RNAqueous-Micro, incluyendo el tratamiento con DNase I y un paso de inactivación de esta enzima. Para el primer estudio los cebadores para cada citocina proinflamatoria (IFN-α, IFN-γ, IIL-12p35, IL-12p40 y TNF-α) e inmunomoduladora (IL-10 y TGF-β) fueron seleccionados en previas publicaciones (Royaee *et al*., 2004; Gabler *et al*., 2006; Moue *et al*., 2007; Kim *et al*., 2010). El cDNA se amplificó utilizando química de SYBR Green, estos ensayos de qPCR se optimizaron para una cantidad específica de muestra (ratio cDNA:agua 1:1, 1:5 y 1:25) y para concentración de cebadores (50, 200, 300 y 900 nM). Para generar las curvas estándar los transcriptos para cada citocina y de ORF7 de PRRSV-1 se insertaron

en un plásmido. Las curvas estándar se prepararon a una concentración de 10^8 copias por µl y se hicieron curvas con diluciones seriadas decimales y medias. Para la evaluación de cada qPCR se calcularon la eficiencia, el coeficiente de variación, el factor de discriminación y el límite de detección. La expresión de citocinas se calculó como la ratio gen objetivo/gen de referencia normalizado con tres genes de referencia (β-actina, cyclofilina y GAPDH) por el método de media geométrica de múltiples genes endógenos de control (Vandesompele *et al*., 2002).

Para el segundo estudio, las muestras de CML la cuantificación de citocinas se empleó el cDNA como base tras la retrotranscripción, y se emplearon sondas TaqMan. La expresión relativa de citocinas se calculó con el método $2^{-\Delta\Delta Ct}$ (Livak y Schmittgen, 2001), la β-actina se utilizó como gen control. Las RT-qPCR para la cuantificación de PRRSV se realizaron como describió Lurchachaiwong *et al*. (2007) para las muestras que se empleó química basada en SYBR Green, y Frossard *et al*. (2012) paralas muestras de CML. La replicación de PRRSV se representa como variaciones en las Cts como previamente describió Kuzemtseva et al. (2014).

Inmunohistoquímica

En el segundo estudio *in vivo* se realizó inmunomarcaje de marcadores celulares con el método ABC como había sido previamente publicado (Gómez-Laguna et al., 2010; Barranco et al., 2011). Los anticuerpos monoclonales empleados fueron anti-CD3, anti CD79a y anti CD-387; en cada caso se empleó el correspondiente anticuerpo secundario biotinado; finalmente el marcaje se realizó utilizando DAB. Las secciones inmunomarcadas se examinaron mediante microscopía, y las marcadas se evaluaron de acuerdo con una escala desde -3 a 0 (para depleción celular) y de 0 a 3 (para incremento celular) comparando con el grupo control.

Análisis estadístico

Los análisis estadísticos se realizaron con el empleo de los programas informáticos GraphPad PrismV.5 y SPSS 15.0. Los efectos significativos sobre los MØ polarizados, comparados con los no polarizados, se realizaron mediante medidas repetidas en ANOCA y test de comparación múltiple de Dunnett. Para el análisis de marcadores celulares de superficie, expresión de virus y de citocinas se realizaron con los test no paramétricos de Kruskal-Wallis seguido por la U de Mann-Whitney. Las correlaciones entre PRRSV y las citocinas se determinaron por el análisis de la Rho de Spearman. Se consideró estadísticamente significativo una $P < 0.05$. Las variaciones significativas basadas en los análisis realizados se indicaron en las gráficas con asteriscos o letras.

Resultados

Estudio in vitro

Fenotipos de MØ

El experimento *in vitro* mostró que los MØ estimulados por citocinas desarrollan diferentes fenotipos comparados con los no polarizados. En general, los MØ tratados con IFNs expresan más CMH I, mientras que solo los M1 expresan más CMH II y CD16. En contraste, el CD14 se vio disminuido con cada tratamiento por citocinas. Interesantemente, M2 expresan la menor cantidad de CD163.

Viabilidad de MØ y susceptibilidad a la infección por PRRSV

Los MØ no polarizados y los M2 son susceptibles a la infección por todas las cepas probadas de PRRSV en el presente estudio. En estas células las cepas de PRRSV-2 y Lena provocaron los mayores ratios de infección y el mayor efecto citopático. Por etro

lado, los MØ tratados con IFNs fueron resistentes a la infección y efecto citopático de las cepas del subtipo 1 de PRRSV-1, pero esas células fueron parcialmente resistentes a las cepas del PRRSV-2. Finalmente, es importante remarcar que los MØ tratados con IFN-β fueron lo más resistentes contra PRRSV. Interesantemente, las cepas HP de PRRSV de ambos genotipos mostraron niveles particularmente altos de infección comparados con el correspondiente virus prototipo de genotipo in ambos tipos de MØ tratados con IFNs.

Producción de citocinas e IFN-α en MØ infectados

Todos los MØ testados no secretaron TNF-α o IL-10; mientras que la producción de IFN-α solo se detectó en el sobrenadante de M1 infectados con la cepa LVP23 de PRRSV-1.

Como los actuales sistemas de cultivo celular no pueden exhibir totalmente las complejas relaciones entre células durante la respuesta inmune, llevamos a cabo dos experimentos *in vivo* con el propósito de evaluar la respuesta inmune del cerdo contra el PRRSV-1.

Estudios in vivo

Signos clínicos, patología macroscópica, histopatología, viremia y replicación vírica

Durante el primer estudio *in vivo* evaluamos la respuesta inmune en pulmón, tonsila y nódulos linfáticos de cerdos infectados con la cepa 2982 del PRRSV-1. En este estudio solo los animales inoculados con virus mostraron signos clínicos y lesiones en pulmón y órganos linfoides; en estos animales la viremia fue detectada desde 3 dpi en adelante (24 dpi). Generalmente, la replicación vírica fue mayor en órganos linfoides que en pulmón durante el estudio.

En el segundo experimento *in vivo* que realizamos, en el que los animales fueron con cepas del PRRSV-1 que variaban en virulencia con el propósito de estudiar la respuesta inmune que se da en diferentes compartimentos de nódulo linfático. Como prototipo de PRRSV-1 seleccionamos la cepa LV, como cepa poco virulenta del subtipo 1 del PRRSV-1 escogimos la cepa de campo 215-06 del Reino Unido, y la cepa de Bielorrusia SU1-bel representa una cepa virulenta del subtipo 3 del PRRSV-1. Los animales inoculados mostraron signos clínicos y lesiones en Med-LN a los 3 y 7 dpi, en los que eran mayores en el caso de animales infectados con SU1-bel. Generalmente, durante el estudio PRRSV se detectó a 3 dpi en el área IF de Med-LN. Posteriormente, la replicación de virus incrementó en el F, dónde el virus fue detectado principalmente al final del estudio. En cada tiempo post-infección, la mayor tasa de replicación se observó en los animales infectados con la cepa SU1-bel.

Evaluación de RT-qPCR para citocinas de tejidos de cerdo utilizando química de SYBR Green

Las qPCRs basadas en química de SYBR Green se evaluaron con el objetivo de cuantificar citocinas en tejidos de cerdo infectados por PRRSV. Todos los ensayos de qPCR rindieron coeficientes de correlación con una $R^2 > 0.95$ y una eficiencia con valores entre 1.68 (TNF-α) y 2.38 (IL-10). Los resultados de los coeficientes de variación indicaron que la variabilidad de los ensayos de qPCR fueron menores del 3% para las curvas estándar de diluciones medias y decimales en la mayoría de ensayos. Finalmente, el factor de discriminación medio fue siempre inferior a 3 en todos los rangos de dilución seriada media o decimal. Esta evaluación de los ensayos de qPCR mostró resultados robustos con una baja cantidad de muestra.

Expresión Génica de citocinas

La cepa 2982 del PRRSV-1 evita el establecimiento efectivo de una respuesta inmune innata, especialmente en el principal órgano diana (pulmón) y reservorio (tonsila) de PRRSV. El virus hace que la expresión génica de IFN-α y TNF-α sean inadecuadas, posteriormente se produce una respuesta débil y tardía en la producción de IL-12 e IFN-γ. Finalmente la replicación de PRRSV favorece la expresión de la citocina antiinflamatoria IL-10.

En el segundo estudio *in vivo* se vio que las cepas de campo de PRRSV-1 retrasan el establecimiento de una respuesta inmune innata efectiva en los compartimentos de Med-LN. En el sitio de infección estas cepas indujeron una menor expresión de citocinas proinflamatorias tanto de IFN-α como de TNF-α. Esto fue seguido por un retraso en la producción de IFN-γ hasta los 7 dpi. La expresión génica de citocinas antiinflamatorias como la IL-10 y TGF-β solo se detectó en animales infectados por la cepa LV; mientras que las cepas de campo de PRRSV-1 no indujeron la expresión de estas citocinas en los cerdos infectados.

Inmunohistoquímica

Durante el segundo experimento *in vivo* la población de linfocitos T incrementó en el F de animales infectados a 7 dpi; mientras que en IF desde 3 dpi en adelante se detectó una depleción de linfocitos T en los animales infectados por las cepas LV y SU1-bel. La población de linfocitos B decreció en todos los animales infectados por cualquier cepa de PRRSV desde los 3 dpi hasta el final del estudio. Finalmente, los MØ detectados

fueron mayores en los animales infectados por SU1-belcomparado con otros grupos durante todo el estudio.

Discusión

Considerando la importancia central de los MØ para la patogénesis de PRRSV y el rol de los IFNs y citocinas en la regulación de MØ Cancel-Tirado *et al*., 2004; Zlotnik *et al*., 1982), nosotros mostramos que las cepas de PRRSV que varían en virulencia son diferentes en su interacción con MØ modulados por citocinas. M1, M2 y los MØ activados por IFN-β mostraron diferentes susceptibilidades la infección por cepas del PRRSV-1 y PRRSV-2 así como su habilidad a secretar citoquinas después de la infección. Por lo tanto, los macrófagos tratados con IFNs pueden permitir la discriminación entre aislados de PRRSV que difieran en virulencia.

Como los MØ polarizados han mostrado una susceptibilidad diferente a ser infectados por distintas cepas de PRRSV, los cultivos de MØ pueden ayudar a un mejor entendimiento de las bases inmunológicas de la virulencia de PRRSV. En general, las cepas del subtipo 1 de PRRSV-1 no son capaces de proliferar en MØ estimulados por IFNs, que representan una respuesta inmune efectiva en el hospedador contra la infección vírica (Shahangian *et al*., 2009). Por otro lado, la cepa Lena del subtipo 3 de PRRSV-1 y el PRRSV-2 muestran una mayor habilidad para replicar en esas células comparado con las cepas del subtipo 1 del PRRSV-1, lo que apoya los resultados presentados por otros autores (Karniychuk *et al*., 2010; Shi *et al*., 2010). Los M2, son células que representan una respuesta inmune antiinflamatoria (Mosser, 2003), son susceptibles a la infección por todas las cepas testadas de ambos genotipos de PRRSV.

Nuestros datos muestran que los MØ derivados de monocitos son una alternativa a la experimentación con MAP, evitándose el sacrificio de animales para la obtención de las células (Wensvoort *et al*., 1991). Además, estos MØ pueden ser modulados y polarizados por citocinas para imitar ciertas funciones de MØ tisulares durante el estado estacionario o durante el curso de una respuesta inmune (Carrasco *et al*., 2001). Este estudio *in vitro* ha identificado importantes diferencias de cepas de PRRSV utilizando cultivos de MØ estimulados, lo que lo puede hacer una técnica muy valiosa para entender elementos celulares y virológicos que son importantes para la interacción célula-PRRSV y relacionado a la virulencia.

El PRRSV ha desarrollado varias estrategias con el propósito de evadir un establecimiento adecuado de la respuesta inmune innata del hospedador así como retrasar la inmunidad adquirida (Albina *et al*., 1998; Van Reeth 1999; Thanawongnuwech *et al*., 2004; Flores-Mendoza *et al*., 2008). Nuestros resultados mostraron que M2 son susceptibles de ser infectados por PRRSV al igual que los MØ no polarizados; por ello si PRRSV puede modular la respuesta inmune del hospedador podría lograr bloquear la diferenciación de M1 con el propósito de mantener un mayor número de células susceptibles a la infección.

Actualmente, los modelos celulares disponibles no pueden reflejar completamente los complejos eventos que se inducen en las conexiones de citocinas entre múltiples tipos celulares que se dan *in vivo*. El estudio de perfiles de expresión transcripcional pueden ayudar al entendimiento de la biología y las bases moleculares de la enfermedad (Mendrick, 2011). Por lo tanto, el análisis de niveles transcripcionales de citocinas en tejidos de cerdo infectados por PRRSV representa una herramienta útil para evaluar la modulación inmune que causa el virus en el hospedador durante la infección.

En el primer estudio *in vivo* la inoculación intramuscular resultó en una mayor replicación de la cepa 2982 en los nódulos linfáticos a 3 dpi, lo que puede ser resultado de un proceso de filtración de virus desde la linfa. Esta ruta de inoculación podría explicar la menor cantidad de PRRSV cuantificada en pulmón a los 3 dpi.

Durante la infección en cerdos por 2982 del PRRSV-1, nuestros resultados confirmaron la habilidad de PRRSV a replicar durante largos periodos en tejidos infectados, lo que puede permitir la depresión del sistema inmune de cerdos infectados por PRRSV.

La cepa 2982 durante la infección suprime la expresión génica de IFN-α 1 y TNF-α en células inmunes, como MØ y DC, en consecuencia el establecimiento de una respuesta inmune innata efectiva se ve inhabilitada durante la infección vírica (Beura *et al*, 2009), por ello la inducción de la inmunida adquirida se ve alterada (Kimman *et al*., 2009).

El aumento de la expresión génica de IFN-γ que se observó en pulmón y Tb-LN de los animales infectados no fue suficiente como para evitar la replicación de PRRSV en esos tejidos. Probablemente, en tonsila PRRSV pudo inducir la expresión de IFN-γ a través de IL-12 (Trinchieri, 1994). Por lo tanto, nuestros resultados sugieren que la infección por PRRSV puede modular en el hospedador la respuesta de IL-12 para suprimir su efecto biológico en pulmón y LNs.

El aumento de expresión de IL-10 se correlacionó con la expresión génica de IFN-γ en el pulmón y Rf-LN de los cerdos infectados por la cepa 2982, al contrario que hallazgos previos que sugieren que el PRRSV-1 induce IL-10 como estrategia para proliferar evitando una eficiente secreción de IFN-γ en los tejidos infectados (Díaz *et al*., 2006). Este hecho pone de manifiesto que el rol inmunomodulador de la IL-10 en la respuesta inmune debe de variar a lo largo de las cepas del PRRSV-1.

Como se ha visto, la cepa 2982 del PRRSV-1 puede modular la respuesta inmune del hospedador en los tejidos infectados. La infección en cerdos por PRRSV se ha relacionado con una depleción de linfocitos B y T en las áreas corticales de órganos linfoides secundarios como nódulos linfáticos; lo que puede sugerir un rol importante de estos tejidos en la modulación de la inmunidad del hospedador mediada por el PRRSV.

Varios autores han publicado resultados sobre perfiles transcripcionales en diferentes órganos durante la infección de PRRSV, pero siempre tomando pequeñas muestras de tejido de manera macroscópica (Lunney *et al*., 2010; Miller *et al*., 2012; Wysocki *et al*., 2012), pero sin mostrar resultados de estructuras concretas de cada órgano. El uso de CML es una poderosa herramienta en la adquisición de poblaciones homogéneas de células o de estructuras tisulares específicas para la aplicación de técnicas moleculares (Okuducu *et al*., 2005; Aranday-Cortes *et al*., 2012).

La cepa SU1-bel de PRRSV del Este de Europa del subtipo 3 difiere en virulencia de las cepas pan-Europeas. Además, la cepa SU1-bel muestra la mayor capacidad de replicación en Med-LN comparada con las cepas del subtipo 1 del PRRSV-1; que además pueden permanecer en Med-LN hasta 35 dpi.

La depleción de células inmunes se ha relacionado con la muerte celular por apoptosis inducida por PRRSV en los tejidos, esto podría explicar la depleción de linfocitos B y T observadas en este estudio (He *et al*., 2012; Gómez-Laguna *et al*., 2013).

El RNA de las cepas LV y SU1-bel se correlacionó negativamente con la expresión génica de IFN-α, lo que indica que la replicación de PRRSV directamente está relacionada con la menor regulación génica de IFN-α en el sitio de infección. Es importante resaltar que MCL junto con RT-qPCR mostraron diferencias en el perfil de transcripción a lo largo de los compartimentos de Med-LN.

Hemos mostrado que PRRSV-1 reduce la transcripción de TNF-α durante la infección, y que la respuesta inmune innata se ve retrasada. El subtipo 3 del PRRSV-1 afecta directamente a la producción d eTNF-α en el sitio de replicación, especialmente en el área IF a 3 dpi y en el F a 35 dpi. Estos resultados apoyan la idea de que las cepas del subtipo 3 de PRRSV-1 han desarrollado las estrategias más efectivas en la evasión de la respuesta inmune del cerdo a lo largo del PRRSV-1 (Frydas *et al*., 2013).

La mayor expresión de IFN-γ a 7 dpi es una evidencia del retraso de la respuesta inmune adquirida en todos los grupos inoculados por PRRSV-1 como sugieren otros autores (Zuckermann *et al*., 2007; Jung *et al*., 2009). La correlación positiva entre la expresión génica de IFN-γ y las cepas LV y 215-06 sugieren que la expresión de IFN-γ por el subtipo-1 de PRRSV-1 depende directamente de la presencia del virus (Murtaugh *et al*., 2002). En este estudio la expresión génica de SOCS1no fue regulada estadísticamente significativa en ningún grupo infectado por PRRSV. Por otro lado, las expresiones génicas de SOCS1 y de IFN-γ se correlacionaron positivamente en el grupo infectado por LV. Esto puede sugerir que el aumento de expresión de IFN-γ en este grupo a 7 dpi puede inducir una mayor expresión de SOCS1 como un controlador negativo en un sistema de feedback (Brysha *et al*., 2001).

La IL-23 es secretada por células presentadoras de antígeno como MØ y DC, y es necesaria para la diferenciación y supervivencia de linfocitos Th17, estas células pueden inducir una reacción antiinflamatoria y la secreción de TGF-β (Vignali y Kuchroo, 2012). A los 35 dpi el aumento de la transcripción de IL-23p19 detectada en este estudio sugiere un retraso en la diferenciación de linfocitos Th17 en Med-LN de cerdos infectados por PRRSV.

La inducción de IL-10 varia a lo largo de los animales infectados por diferentes cepas de PRRSV-1 (Gimeno *et al*., 2011), lo que sugiere que la el retraso de la respuesta inmune en el hospedador mediada por la inducción de IL-10 por el virus no es una estrategia común en las cepas de PRRSV-1. La expresión génica de TGF-β no se indujo o se dio a bajos niveles en las cepas de campo de PRRSV-1, de acuerdo con Silva-Campa *et al*. (2009).

En este estudio hemos mostrado varias evidencias de que la inmunopatogénesis de la infección por PRRSV-1 está asociada al sitio de infección. SU1-bel que pertenece al subtipo 3 del PRRSV-1 mostró la mayor virulencia patogénesis tisular comparada con las cepas del subtipo 1 del PRRSV-1. Nuestros resultados demostraron que las cepas del PRRSV-q evitan una correcta respuesta inmune en el hospedador, en primer lugar el virus previene el establecimiento de una respuesta inmune innata efectiva en los cerdos infectados a través de una menor expresión de IFN-α y TNF-α tanto en F como en IF d eMed-LN, y finalmente la respuesta inmune adquirida celular es retrasada evitando la expresión temprana de IFN-γ.

Conclusiones Generales

1. Los cultivos de MØ derivados de monocitos pueden ayudar a una mejor comprensión de las bases inmunológicas y genéticas de la virulencia de PRRSV. Estas células representan una alternativa flexible frente a otros modelos *in vitro*: 1) líneas celulares derivadas de mono; que presentan limitaciones en la interpretación de resultados por sus diferencias de especie con células porcinas; y 2) con MAP, que requieren sacrificar a los cerdos donantes para su obtención, y presentan algunas limitaciones técnicas con respecto a su polarización por citocinas.

2. Los cultivos de MØ estimulados por citocinas pueden revelar importantes diferencias entre cepas de PRRSV. Mientras que los MØ no diferenciados y M2 son sensibles a todas las cepas de PRRSV, el tratamiento de MØ con IFNs induce un claro estado antivírico en dichas células lo que conduce a una reducción en la infección y replicación vírica. Por lo tanto, diferencias relacionadas con la virulencia y el genotipo de PRRSV pueden ser reveladas con el empleo de MØ estimulados con IFNs. A pesar de esto, las cepas HP de PRRSV procedentes de China y del Este de Europa son particularmente resistentes a los efectos del IFN-γ.
3. Independientemente de la ausencia de información técnica en la literatura sobre qPCR basadas en química de SYBR Green de citocinas, la evaluación de dichas pruebas optimizadas han demostrado ser satisfactorias para el monitorizado de respuestas proinflamatorias e immunomoduladoras en el cerdo, con unos resultados robustos con baja cantidad de muestra.
4. La cepa 2982 es capaz de evadir el establecimiento de una respuesta inmune innata efectiva, causando una inadecuada expresión génica de IFN-α 1 y de TNF-α, que finalmente induce una débil y retrasada respuesta inmune adquirida mediante la expresión ineficiente de IL-12 e IFN-γ.
5. La captura por microdisección láser junto con la RT-qPCR es una poderosa herramienta que permite diferenciar perfiles de transcripción de citocinas en estructuras diferentes pertenecientes a nódulos linfáticos de cerdo.
6. PRRSV-1 es capaz de permanecer infectando tejidos linfoides alojado en el folículo que presenta diferentes niveles de replicación entre distintas cepas del virus. La cepa SU1-bel del subtipo 3 del PRRSV-1 presenta la mayor capacidad de replicación en Med-LN comparado con cepas pertenecientes al subtipo 1.

7. Las cepas de PRRSV-1 evitan una correcta respuesta inmune innata en los cerdos infectados causando una depleción linfoide así como una menor expresión de IFN-α y TNF-α tanto en el folículo como en el área inter-folicular de Med-LN. Además, la cepa SU1-bel perteneciente al subtipo 3 ha desarrollado las estrategias más efectivas para evadir las respuestas inmunes del hospedador a lo largo del genotipo 1 de PRRSV.
8. La inducción de IL-10 por PRRSV para evadir o retrasar la respuesta inmune en el hospedador no parece ser una estrategia común en el genotipo 1 de PRRSV.

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