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CICLO XXXI

*In vitro* and *in vivo* infection of porcine monocyte-macrophages-lineage cells with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) isolates with different pathogenicity

Coordinatore:

Chiar.mo Prof. PAOLO MARTELLI

Tutore:

Chiar.mo Prof. PAOLO BORGHETTI

Dottorando: Dr. GIULIA OGNO

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## Abstract

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) represents one of the most important viruses affecting the swine population with a huge impact on pig production in many countries. Moreover, the recent emergence of some highly pathogenic isolates (HP-PRRSV-1) has caused more severe economic losses.

The effects of PRRSV on the immune system is poorly understood, indeed, the infection and persistence of the virus is related to a complex interaction with the immune system that determines a dysregulation on the mechanisms and the secretion of important cytokines of the host.

The virus shows a restricted tropism for cells from the monocyte-macrophage-lineage, the main *in vivo* target cells is represented by porcine alveolar macrophages (PAM). The aim of the present thesis is to analysing the response of the macrophages population to the infection of different PRRSV isolates, both *in vitro* and *in vivo*.

In the first *in vitro* study, we used as a model, monocytes derived macrophages (MDMs) pre-treated with different cytokines as IFN $\gamma$ , IL-4 and IFN $\beta$ . Nine PRRSV-1 isolates were analyzed: two Italian strains, five Eastern European strains, and Lena and Lelystad as reference-strains for HP and low pathogenic PRRSV, respectively. The different strains were able to infect MDMs, with the best efficiency in unpolarized M $\emptyset$  and IL-4 treated MDMs, while the IFN-treatment determined an antiviral state, this evidence was most pronounced with IFN $\beta$ . In general, the high pathogenicity isolates infected a higher percentage of MDMs and replicated to higher titers. Regarding the cytokines measurement, IFN $\alpha$  and IL-10 were not detected in the supernatant of infected MDMs; moreover, the Italian PR40 strain was the only one that induced a significant release of TNF- $\alpha$  and IL-1- $\beta$ .

The second study was performed by using as a model the polarized-porcine alveolar macrophages (PAMs) pre-treated with IFN $\gamma$ , IL-4 and IFN $\beta$ , in order to compare two PRRSV-1.1 Italian strains, the normal pathogenic PR11 strain and the HP-PRRSV PR40. Interesting, we did not see difference between the two isolates, and any difference in the infectivity among Mock, IL-4 and IFN $\gamma$

treatments, that evidence could found explanation in a re-polarization from the M1 to M2 phenotype after the infection.

While, the infection of both viruses was completely blocked by IFN $\beta$ -treatment, highlighting the antiviral state induced by Interferon. Moreover, both strains did not induce the production of the cytokines tested: IFN $\alpha$ , IL-10 and TNF-  $\alpha$ .

The in vivo study consisted in two different experiments, the aim of the first one was to analyse the impact of two Italian PRRSV-1 subtype 1 strains (PR11 and PR40), with different in vivo pathogenicity, on the macrophages population of the thymus, as target cell for both PRRSV replication and response of the host immune system. The second experiment aimed at evaluating the effect of a heterologous vaccination on macrophages population of the thymus after a challenge infection with a HP-PRRSV.

In order to define the macrophages population, immunohistochemistry for the N protein of the virus, TUNEL labelling and immunolabeling for CD163, CD172a, CD107a and BA4D5 was performed. The thymus of the animals infected with PRRSV strains, PR11 and PR40 dead at 10-14 dpi showed more severe lesions and the higher number of macrophages in all the compartments of the thymus gland compared with the others groups. This last result suggests a strong and early inflammatory process, without any difference between the normal and highly pathogenic strain.

## Riassunto

Il virus della sindrome respiratoria e riproduttiva suina (PRRSV) è considerato uno dei più importanti virus del suino, con un grave impatto economico sulla produzione suinicola mondiale. Recentemente, sono emersi nuovi isolati del virus, caratterizzati da maggiore patogenicità e mortalità, e quindi definiti ceppi ad alta patogenicità (HP-PRRSV).

Gli effetti dell'infezione da PRRSV sul sistema immunitario del suino sono ancora poco conosciuti, in quanto l'infezione e la persistenza del virus sono associate ad una complessa interazione con l'ospite, che determina una dis-regolazione dei meccanismi immunitari.

Il virus presenta un tropismo selettivo per le cellule della linea monocitaria, e il principale target *in vivo* è rappresentato dai macrofagi alveolari.

Lo scopo di questa tesi è stato quello di analizzare come viene influenzata la popolazione macrofagica in seguito all'infezione con differenti isolati di PRRSV, sia *in vitro* che *in vivo*.

Nel primo studio *in vitro*, il modello cellulare utilizzato è rappresentato dai "macrofagi derivanti da monociti" (monocytes derived macrophages (MDMs)), stimolati con diverse citochine quali IFN $\gamma$ , IL-4 and IFN $\beta$ . Sono stati utilizzati nove diversi isolati: due Italiani, due dell'Est Europa, mentre i ceppi Lena e Lelystad sono stati utilizzati come isolati di riferimento per i ceppi ad alta e bassa patogenicità, rispettivamente. Tutti i virus utilizzati sono stati in grado di infettare il modello; una maggiore efficienza è stata riscontrata in MDMs non trattati e in quelli trattati con IL-4. Il trattamento con IFN ha determinato invece uno stato antivirale, particolarmente evidente con IFN $\beta$ . In generale, i ceppi ad alta patogenicità hanno presentato una maggiore capacità infettiva e hanno replicato a un titolo maggiore. Riguardo la produzione di citochine, IFN $\alpha$  and IL-10 non sono state rilevate nel surnatante delle cellule infette, mentre, HP-PRRSV PR40 è stato l'unico isolato ad indurre un significativo rilascio di TNF- $\alpha$  and IL1- $\beta$ .

Il secondo studio *in vitro*, è stato invece condotto utilizzando come modello cellulare i macrofagi alveolari trattati con IFN $\gamma$ , IL-4 o IFN $\beta$ , per valutare la risposta all'infezione con due ceppi Italiani,

un ceppo a normale patogenicità PR11 e un ceppo ad alta patogenicità PR40. Inaspettatamente, non è stata rilevata alcuna differenza tra i due isolati, indipendentemente dal trattamento dei macrofagi. Questo risultato potrebbe trovare spiegazione in una ri-polarizzazione dei macrofagi dal fenotipo M1 al fenotipo M2 determinato dall'infezione. al contrario, l'infezione di entrambi gli isolati è stata completamente bloccata dal pre-trattamento con IFN $\beta$ , sottolineando lo stato antivirale indotto dall'interferone. Inoltre, entrambi gli isolati non hanno indotto la produzione di IFN $\alpha$ , IL-10 and TNF- $\alpha$ .

Lo studio *in vivo* si è articolato in due diversi esperimenti, il primo ha avuto lo scopo di analizzare l'effetto indotto dall'infezione con due isolati italiani (PR11 e PR40) a diversa patogenicità, sulla popolazione macrofagica del timo, target per la replicazione del virus e per valutare la risposta immunitaria. Il secondo esperimento ha invece valutato l'efficacia di un vaccino commerciale in seguito al challenge con un ceppo ad alta patogenicità (HP-PRRSV PR40).

Per valutare la risposta macrofagica all'infezione, è stata condotta un'analisi in immunohistochimica valutando la presenza del virus, TUNEL per valutare la morte cellulare e, la modulazione di diversi marker specifici per i macrofagi: CD163, CD172a, CD107a e BA4D5.

Il timo degli animali infetti con entrambi i virus, PR11 e PR40 morti prima della conclusione dell'esperimento, tra i dieci e i quattordici giorni post-infezione (10-14 dpi), mostrava gravi lesioni, una maggiore presenza del virus a livello cellulare, morte cellulare ed un aumento del numero di macrofagi in tutti i compartimenti. Questi risultati suggeriscono un forte e precoce processo infiammatorio, senza alcuna particolare differenza tra gli isolati utilizzati.

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# *Introduction*

## **Porcine Reproductive and Respiratory Syndrome (PRRS)**

### **Isolation**

Porcine Reproductive and Respiratory Syndrome (PRRS) is a viral disease with a huge impact on pig production in many countries all over the world (Holtkamp et al.,2013), emerged almost simultaneously in North America and Western Europe (Keffaberet, 1989).

Initially, it was defined as a “Mystery Swine Disease (MSD)” or “Blue Ear Disease” because the typical signs of the disease were the “blue ears”.

The causative agent was discovered independently in Europe and in USA in 1991. Particularly, in Netherlands it was isolated from herds with severe reproductive disorders, characterized and designated as Lelystad virus (LV) (Wensvoort et al.,1991). In the USA, researchers (Collins et al. 1992) isolated VR-2332 strains from herds undergoing severe respiratory and reproductive problems. These viruses represent prototype viruses of the two different genotypes: type 1 or European-like (prototype Lelystad) and type 2 or North American-like (prototype VR-2332).

The impact of PRRSV in the farm is drastically complex. The clinical signs were characterized by severe reproductive failure with a high rate of late abortion and early farrowing in sows, and by respiratory disease in growers and mortality in piglets. PRRSV infection impairs performance at several stages of production: it negatively influences the number of piglets weaned, determining lower numbers of piglets born alive or a higher pre-weaning mortality; furthermore, it leads to increased mortality rates and a reduced growth rate (Nieuwenhuis et al.2012). A considerable variation in the clinical responses was observed depending on the genetic and virulence differences among the involved isolates.

Despite various strategies had been developed and applied, PRRS is still endemic and causes significant economic losses to the pig industry worldwide.

## **Taxonomy**

PRRSV is a member of the family *Arteriviridae* of *Nidovirales* order. The *Arteriviridae* is composed by the genus *Arterivirus*, that also include Equine Arteritis Virus (EAV), Simian Hemorrhagic Fever Virus (SHFV) and Lactate Dehydrogenase-elevating Virus (LDV) in mice (Gorbalenya *et al.*, 2006). PRRSV is classified in two genotypes: type 1 or PRRSV-1 (European) and type 2 or PRRSV-2 (North American) (Nelsen *et al.* 1999). The genetic homology is of 60% in the entire genomes, with a variability of 55%-63% for the non-structural proteins, and 61%-81% in the structural proteins (Murtaugh *et al.*, 2010; Shi *et al.*, 2010).

The diversities are not only between the two recognized genotypes, but also within the same genotype, leading to the sub-classification into subtypes which differ in virulence and genetic makeup. PRRSV-1 is divided into 3 subtypes, the pan-European subtype 1 and East European subtypes 2, 3, and a fourth subtype has also been suggested (Stadejek, *et al.* 2008), whereas PRRSV-2 has been divided into at least nine lineages.

Stadejek *et al.* (2008) have suggested that this high variability in PRRSV-1 ORF5 and ORF7 determines the subtypes formation.

Recently, “highly pathogenic” (HP) variants of the virus have emerged within both PRRSV-1 and PRRSV-2, and they were characterized by differences in the clinical signs and virulence, determining an uncommon severe disease in conventional pig herds. The infection caused by a highly virulent strain results in more severe clinical signs, long-lasting viremia, higher virus level in blood and tissues, and higher frequency of mortality (Lunney *et al.*, 2010). Additionally, neurological signs as well as an erythematous rash have been associated with HP-PRRSV infection. HP-PRRSV infection causes clinical disease and death in all ages, including adult pigs and pregnant sows.

Regarding the epidemiology of HP-strains, in 2006 in Europe, a HP-PRRSV-1 subtype 3 designated as “Lena” was isolated from an outbreak of severe reproductive and respiratory disorders. The HP-PRRSV-2 strains emerged in USA (Brockmeier *et al.*, 2013), in China and Vietnam, giving rise to outbreaks with severe symptoms of hemorrhagic fever (Tian K *et al.*, 2007). Canelli *et al.* (2017)

described the infection by an Italian HP-PRRSV 1.1 isolate designated as PR40/2014, characterized by severe respiratory and systemic disease, associated with a mortality rate of more than 50%. Since 2006, HP-PRRSV variants have continued to emerge, and they all share the presence of discontinuous deletions in the nsp2-coding region, considered as a genetic marker.

## **Structural biology**

The virion is a spherical-shaped particle of 50-60 nm in diameter constituted by surface glycoproteins (GPs) and membrane proteins inserted into the lipidic-bilayer envelope.

The genome, approximately 15 kb in length of positive-strain of RNA, is packed by nucleocapsid proteins and contains 11 open reading frame (ORFs) organized similarly to the ORFs of coronavirus (Nelsen et al.,1999).

The ORF1a and 1b are the precursors of two large polyproteins pp1a and pp1ab, and cleaved into 14 non-structural proteins (nsp) which are involved in genome replication and sub-genomic mRNA transcription.

ORF1a is a variable region, because of the hypervariability in the central region (nsp2), whereas ORF1b is more conserved.

The structural proteins are coded by ORF2a to ORF7 in the 3'-end viral genome. Particularly, eight relatively overlapping small genes encode four membrane associated glycoproteins (GP2a, GP3, GP4 and GP5); three un-glycosylated membrane proteins (E, ORF5a, and M), and a nucleocapsid protein (N) (Joan et al.,2007).

GP5 and M protein constitute a heterodimer, that represent the major component of the PRRSV envelope, which also comprehend minor proteins such a GP2a, GP3 and GP4.

The nucleocapsid (N) protein with the GP5, assembled on the virus surface as a dimer, are the most abundant and immunogenic components of the virion. The N protein, encoded by ORF7, is the most abundant protein expressed in the infected cells (Dea et al.,2000). Through the N-terminal domain, the protein interacts with the RNA to form the viral nucleocapside (Spilman et al.,2009).

The occurrence of insertions and deletions in the genome regions coding for the structural and non-structural proteins has been well described for PRRSV and it represent a further evidence that diversification from the wild-type strain is an important mechanism for the generation of the genetic diversity of PRRSV (Murtaugh et al.,2010).

### **Cell tropism and viral replication**

The virus has a limited cell tropism and infects porcine alveolar macrophages or other tissue macrophages (MØ) (Van Breedam, et al.,2010).

It is also able to infect and replicate in monocyte-derived cells as MØ and DC generated *in vitro* (Wang et al.,2007).

To obtain a high number of PAMs for isolation and *in vitro* studies, it is necessary to sacrifice animals, as a consequence, alternative *in vitro* models have been developed.

The African monkey kidney cells line MA-104, and their derivate CL2621, CRL-11171, and MARC-145 are permissive to infection by PRRSV (Kim et al.,1993).

PAMs and MARC-145, are the fully permissive cell for PRRSV growth (Kim et al.,1993) and are commonly used for *in vitro* studies. Recently, an alternative model to PAMs has been developed using monocyte-derived macrophages (MDMs) (García-Nicolás et al.,2014), MDMs can be modulated and polarized *in vitro* by using cytokines to mime the functions of tissue MØ during the activation of inflammatory/immune responses. During the infection, a scavenger receptor member of cysteine-rich family, CD163 was identified to be the major receptor that mediates viral internalization (Lunney et al.,2010). GP2a and GP4 were determined as viral proteins that mediate and facilitate the entry into the host cell by interacting with CD163 (Das et al., 2010).

The sialoadhesin CD169 was also identified as receptor mediating the viral internalization by interacting with the ectodomains of GP5/M; instead, a recent study demonstrated that this protein is not needed for the attachment of the virus at the host cells (Prather et al.,2013).

PRRSV enters in the host cells through endocytosis, and the viral genome is released into the cytosol following endosome acidification and membrane fusion (Van Breedam, et al.,2010).

At this step both ppt1a and ppt1ab are encoding by ORF1, and consequently processed into 14 nsp, which are assembled into a replication and transcription complex (RTC). The RTC determine the production of both single-strand full-length and subgenomic-length-strand RNSs, the translation of this last results in the structural proteins generation. The new RNA genomes are packaged into nucleocapsids and successively the new virions are released through exocytosis (Lunney et al.,2010).

## **Pathogenesis**

The entry site for the PRRSV is represented by mucosal surface of the respiratory tract, in which is essential the interaction with susceptible cells such as macrophages and dendritic cells.

Besides the respiratory infection, the virus can also spread by ingestion of infected saliva and fomites, blood, mammary secretions or damaged skin.

Clinical signs of PRRS are extremely variable and influenced by several factors such as the virus strain, the immune status of the infected pig and management factors (Blaha et al., 2000).

PRRS infection can be divided into distinct stages: acute infection, persistence and extinction.

In the acute stage of infection, the primarily target of virus is represented by alveolar macrophages, in which the virus completes the replication cycle in 6-12 hpi (Lunney et al.,2010), and the viremia may be present for 4 to 6 weeks' post-infection. The acute infection is associated with an acute illness and the major symptom lie from respiratory distress, indeed virus replication in the lung induces the release of inflammatory cytokines (Chand et al.,2012), development of interstitial pneumonia, followed by a quantitative and functional decrease of PAM and pulmonary intravascular MØ (Chand et al.,2012; Halbur et al., 1995a; Molitor et al.,1996). During the persistence of infection, the virus is no longer detected in blood and lungs and the viral replication is primarily localized in the lymphoid

organs, particularly tonsils, sternal, tracheobronchial lymph nodes and in the thymus (Lunney et al.,2010).

In the thymus the infection results in size decrease, associated to a great reduction number of cells number, especially in the cortex; recently study, has been demonstrated how viruses with different virulence determine different changes in histopathology and histomorphometry (Amarilla et al.,2016).

However, it has been described that the high pathogenic PRRSV predispose piglets to weak cellular immunity with thymus atrophy, T cell depletion and affecting the development of naïve T cell (Han et al.,2017).

During the viremic period, infected pigs can transmit the virus to naïve pigs by direct or indirect contact (Wills et al.,1997; Christopher-Hennings et al.,2008). The reproductive failure is determined by the transmission of the virus from dams to piglets either in utero through placenta or post-partum; the infections before farrowing are often associated with weak piglets and death before weaning, or virus persistence.

Together, the clinical alterations induced by PRRS infection increase the susceptibility to secondary infections of others pathogens. The stage of the extinction of the virus represents the gradually decline of the virus replication, but the spontaneous elimination has been rarely reported, indeed the replication can be maintained for as long as 250 days after infection (Wills et al.,2003).

## **PRRSV Immunity**

### **The immune response to viral infection**

The immunity against infectious agents is a complex network of physical and humoral signals, which interconnects the cells of innate and specific immunity (humoral and cell-mediated immunity). In fact, innate and specific immune responses are closely related each other through the cooperation between their cellular components.

The innate immunity is the first efficient line of the defense against the pathogens and has an essential role in activating the specific immunity. The recognition of invading pathogen and the activation of innate immune cells represent the fundamental steps for both controlling and eliminating pathogens at their entry site, and also promoting the differentiation of dendritic cell (DCs) for providing an efficient antigen presentation to the cells of specific immunity (Mair et al.,2014)

Pattern Recognition Receptors (PRRs), expressed by the innate immune cells (neutrophils, dendritic cells, macrophages) and by other tissue cells, are able to recognize conserved molecular structures known as PAMPs (Pathogen-Associated Molecular Patterns) only presents in the pathogens. Among PRRs, some are expressed on the cell surface, able to recognize bacterial products outside the cell, while other located in the intracellular compartments, can recognize viral nucleic acid inside the cell (Figure 1). The PRRs-PAMPs interaction induces the activation of complex signaling pathways to stimulate inflammatory response.

Toll-like receptors (TLR) are the most studied PRRs in the recognition of pathogens, particularly, ten members have been identified in pigs (Kumar et al.,2011).

The natural pathogen recognition triggers a production of cytokines (IL-1, TNF- $\alpha$ , IFN, IL-8, IL-6, chemokines) that sustains the recruitment and activation of inflammatory and innate cells at the sites of infection, and also the recruitment of lymphocytes and DC in lymphoid tissues and tributary lymph nodes. During viral infection one of the most effective innate immune response is the production of Type I Interferon (Theofilopoulos et al.,2005).

The TLR-mediated immune cells activation and cytokines production has a major role in the connection between the innate and specific immunity.

Natural Killer (NK) cells are activated by IL-12, INF, TNF- $\alpha$ , IL-15, IL-18 secreted by infected cells and inflammatory macrophages. NK cells are able to kill infected cells and represents the major sources of IFN $\gamma$  in the early stages of viral infection; in turn, NKs stimulate macrophages and dendritic cells to secrete other cytokines as IL-12 driving a subsequent Th1 response of Lymphocytes (Biron et al.,1999; Pintaric et al. 2008; Toka et al.,2009; Gerner et al.,2009).

This bi-univocal cooperation between NKs and macrophages has a critical role to sustain the defensive function of innate immunity (Figure 2 and 4) and promote the development of cell-mediated immunity (CMI) (Hoebe et al.,2004; Della Chiesa et al.,2005)

NK cells kill infected cells by inducing apoptosis and by the production of cytotoxic (TNF- $\alpha$ , TNF- $\beta$ ) and cytostatic (IFN $\gamma$ ) cytokines (Loeden & Lanier,2006).

Inflammatory activated Macrophages (M1) act as professional phagocytes towards opsonized pathogens and apoptotic infected cells; they produce cytokines and growth factors that regulate and modulate innate immune mechanisms and can act as antigen-presenting cells in inflamed tissues and as effector cells cell-mediated immunity (Murthaug et al.,2002).

Therefore, they are fundamental for the innate immunity and play an essential role in defense mechanisms, tissue development, and homeostasis. T Lymphocytes represent a first line of defense against biological pathogens, being able to act as APC, to perform cytotoxic activity towards different target cells and, above all, to secrete IFN (Reddehase et al.,1991; Takamatsu et al.,2006). The activation of specific immunity sustains a more efficient pathogen elimination, both at systemic and mucosal level, definitively providing the clearance of the pathogen from the tissues.

Specific immunity is triggered when naïve B and T lymphocytes encounter their specific antigen in the secondary lymphoid tissues where the antigen arrives as it is or is presented by mature dendritic cells.

Moreover, some cytokines from the innate cells induce the maturation of DCs for antigen presentation and for driving the differentiation of T helper Lymphocytes and establishing specific immunity (Murthaug & Foss,2002)

After specific antigen recognition, B and T lymphocytes clonally proliferate, triggering the effector immune response (activation of the primary response), with production of antibodies, cytotoxicity and phagocytosis of infected cells; in parallel, a circulating pool of long-living specific immune cells (T and B memory cells) is established for responding a subsequent exposure to the same antigen.



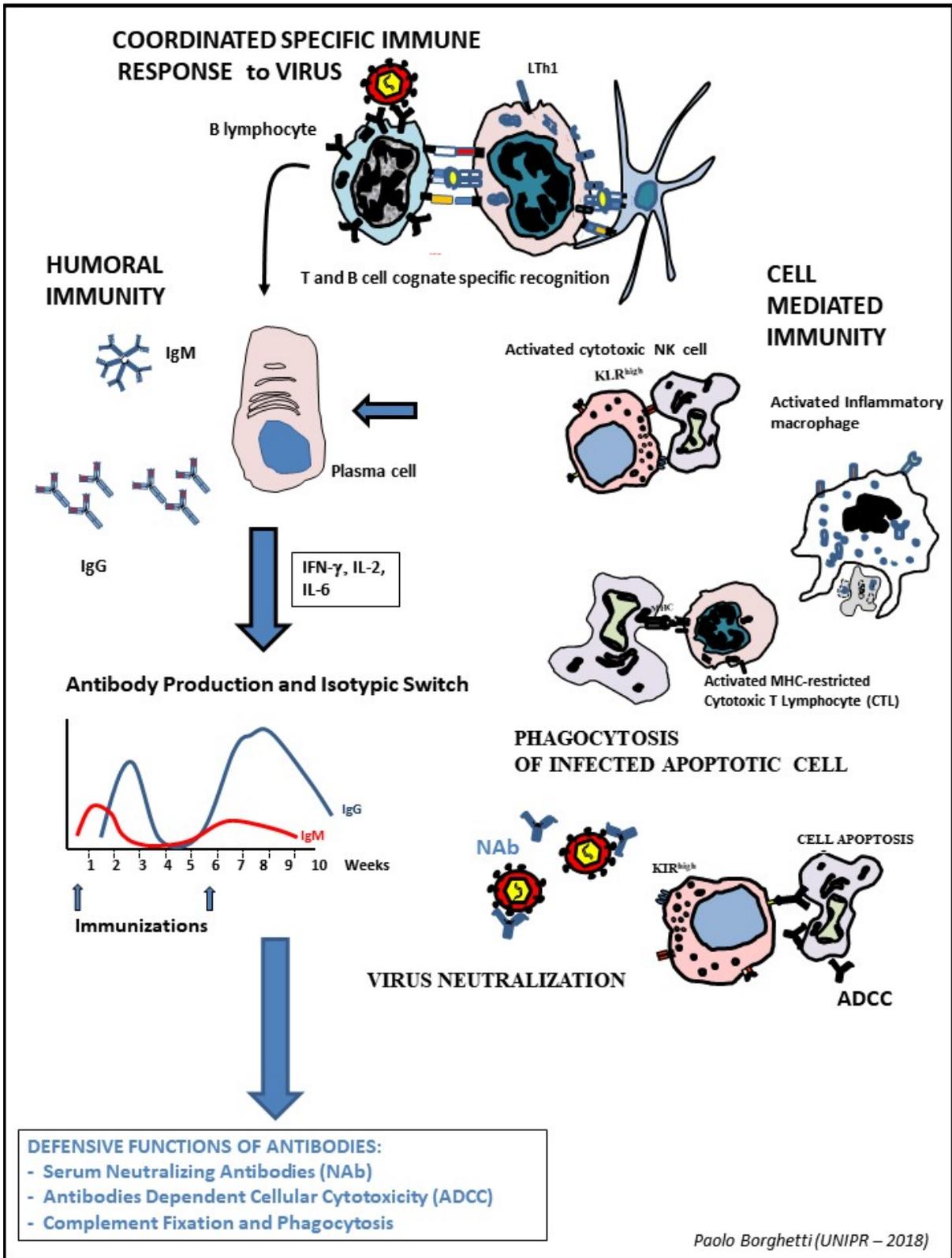
The specific immunity is regulated by T helper lymphocytes (Th). The mature DCs, activated by signal from innate cells, capture and process the antigen, migrate into the lymph node and present it with MHC to naïve helper T cells (CD4+). Antigen specific-activated T helper cells modulate the response through cytokine secretion (Saalmuller et al.,1999).

Different subsets of T helper lymphocytes have been defined on the basis on the different secreted cytokine pattern and may differently involve in different sites of immune responses and/or against different types of pathogen. Particularly, Th1 response plays a pivotal protective role in the defense against intracellular microorganisms (viruses, intracellular bacteria and protozoa).

During viral infection, IFN $\gamma$ , IL-12, IL-18 produced by immune cells and by activated mDC presenting antigen, drive the differentiation of specific T helper naïve lymphocyte towards Th1 differentiation (Figure 1).

Activated Th1 lymphocytes reach the sites of infection and secrete cytokines (IL-2, IFN $\gamma$ ), which mainly regulate cell-mediated response by activating specific cytotoxic T cells and NK cells as effectors of cytotoxicity and macrophages for killing and phagocytosis of infected apoptotic cells (Figure 2).

The effectors of acquired immunity are the cytotoxic T lymphocytes (CTL), in cooperation with NK cells and macrophages (Cell Mediated Immunity), and antibodies [Immunoglobulins (Ig)] produced by the activated B lymphocytes, that proliferate and differentiate in Antibody Secreting Cells (Plasma Cell) (Antibody Mediated Immunity) ([https://www.pig333.com/articles/the-immune-system-and-the-immunity-in-swine-the-immune-cells\\_12522/](https://www.pig333.com/articles/the-immune-system-and-the-immunity-in-swine-the-immune-cells_12522/)).



**Figure 2.** Cellular and molecular effector mechanisms of the specific immune response against virus (by permission of Prof. Paolo Borghetti, Dip. Veterinary Sciences – University of Parma).

## **PRRSV immunopathogenesis**

PRRSV infection and persistence are related to a dysregulation of the immune response to the virus (Loving et al., 2015), involving an altered activation of innate immunity and consequently, a negative effect of the development of protective adaptive immunity.

PRRSV stimulates an antibody response by 7-9 post-infection (pi), but without evidence of protection (Labarque et al.,2000; Lopez et al.,2007)

Moreover, the development of PRRSV-specific cell-mediated immunity is also delayed and altered (Meier et al.,2000 and 2003; Martelli et al.,2009; Borghetti et al.,2011). This complex interaction of PRRSV with specific immunity finds a key role in the negative effect of the infection on the innate immune response efficiency and early inflammatory cytokine production.

## **Effect of PRRSV on innate immunity**

Van Reeth (1999,2000) demonstrated that, during the infection, the secretion of some important pro-inflammatory cytokines is very lower if compared with other viral infections.

Despite *in vivo* studies shown some production of IFN $\alpha$  during PRRSV infection (Barranco et al.,2012; García-Nicolàs et al.,2014), others have demonstrated that PRRSV infection impairs immune responses (Darwich et al.,2010), both at respiratory level and in alveolar macrophages. The reduced production of type 1 IFNs from infected cell has been explained by an inhibition of the RIG-I pathway, specifically (Albina et al.,1998; Genini et al.,2008; Jung et al.,2009, Dobrescu et al.,2014). It has been showed that the largest proteins in PRRSV play a role in the reduction of IL-1 IL-1 $\beta$  and TNF- $\alpha$  and, in addition, negatively affect type1 IFN by NF-kB signalling pathway in different stages of viral replication (by post-transcriptional mechanisms) (Lee et al.,2004).

Nsp1, nsp2, nsp11, and N are antagonists in the IFN induction. Particularly, it has been demonstrated that nsp1b can inhibit the IRF3 association with CREB-binding protein in the nucleus (Kim et al.,2010), and can also inhibit double-strain RNA-induced IRF3 phosphorylation and nuclear

translocation (Beura et al., 2010). Indeed, nsp2 blocks IRF3 activation, and the antiviral function of ISG15; the N protein inhibits IFN $\beta$  by interfering with double-strain RNA-induced IRF3 activation (Luo et al., 2008). PRRSV interferes also in the IFN-activated signaling through the inhibition of IRF3, NF- $\kappa$ B, CBP, and STAT-1. It has been demonstrated how the presence of IFN $\alpha$  before or during the infection induces significant differences in viral clearance and adaptive immune responses (Brockmeier et al., 2009, 2012).

Moreover, different PRRSV isolates have different capacities to affect IFN $\alpha$  production and this is correlated with a significant inhibition of NK cell activity. (Renukaradhya et al., 2010; Dwivedi et al., 2011; Lee et al., 2014; Lunney et al., 2016)

The IFN $\gamma$  response to the virus is also poor appearing dampened and delayed (Murtaugh et al., 2002; Xiao et al., 2004)

This cytokine is necessary for the macrophage activation determining the differentiation into M1, and also for the production of other cytokines such as IL-12 and TNF $\alpha$  by the mononuclear cells (Zlotnik et al., 1982). When the immune response is developed, CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes secrete IFN $\gamma$ . Moreover, IFN $\gamma$  increases the expression of MHC-II on APC and MHC-I on several cells. It is known that IFN $\gamma$  can inhibit the PRRSV replication with an increase of infected cells and the amount of viral RNA content in single cell (Rowland et al., 2001).

Despite a normal or increased secretion of IL-12 in PRRSV-infected pigs, the secretion of IFN $\gamma$  remains low even after 3 weeks' post-infection. Some evidences could involve an altered production of IL-12 p40 homodimer which is a potent antagonist of immune responses or, alternatively, the upregulation of IL-12 might be involved in the differentiation of macrophages which aids viral infection (Dwivedi et al., 2012).

The CD163, a member of the SRCR (Scavenger receptor cysteine-rich) family, is strongly associated with the development of the innate response to infection, its expression on macrophages increases

after maturation and activation, and is also regulated by the TLR signal and resulting proinflammatory or anti-inflammatory signals such as IL-6 and IL-10 (Fabriek et al.,2009).

CD163 is a receptor for PRRSV and a correlation between its expression and PRRSV infectivity has been reported. Interestingly, the expression of CD163 during PRRSV infection can be upregulated by IL-10 (Patton et al.,2009).

The tumor necrosis factor (TNF) is a pleiotropic pro-inflammatory cytokine mainly secreted by neutrophils and macrophages, but it is also produced by tissue cells (epithelial and endothelial cells) infected cells NK cells, during the early phase (pathogen recognition) of innate immune response (Tizard,2013). TNF- $\alpha$  promotes inflammation at the site of infection promoting a further secretion of cytokines and chemokines. TNF- $\alpha$  also induces adhesion molecules expression on leukocytes and endothelial cells improving the adhesion, migration, recruitment and activation of innate cells and lymphocytes. TNF- $\alpha$  increases neutrophils and macrophages phagocytic ability and NK cytotoxicity. (Toews et al.,2001). TNF- $\alpha$  improves the macrophages ability to deal intracellular pathogens and induces apoptosis in infected cells, both effects are increased by the presence of IFN $\gamma$  (Wong et al.,1986; Lopez-Fuertes et al.,2000).

Data regarding the modulation of the TNF- $\alpha$  production by the PRRSV infection are controversial, strains belong to different genotypes, subtypes and also with different pathogenicity might have distinct ability of TNF- $\alpha$  induction. Li et al. (2016) showed that a large amount of soluble TNF- $\alpha$  produced by PAMs upon PRRSV-2 infection, but the increase overtime of TNF- $\alpha$  was documented also *in vivo* (Borghetti et al.,2011; Darwich et al.,2010).

Other study showed that PRRSV is able to suppress the *in vivo* and *in vitro* production of this cytokine (Lopez-Fuertes et al.,2000; Van Reeth et al.,1999; Choi and Chae 2002). The non-structural protein of PRRSV, Nsp1 is involved in the TNF- $\alpha$  suppression. Particularly, this protein inhibits TNF- $\alpha$  promoter blocking NF- $\kappa$ B activation and Sp1 transactivation (Subramaniam et al.,2010). In addition to Nsp1, also Nsp11 plays an important role in the TNF- $\alpha$  suppression (He et al.,2015) and, it has also

been suggested that TNF- $\alpha$  production could be regulating by the non-structural protein Nsp2 (Chen et al., 2010).

Interleukin-1 $\beta$  is one of the classical pro-inflammatory cytokines produced and secreted by a variety of innate immune cells and by different stimuli, and during the inflammation, is able to activate neutrophils and macrophages (Lopez-Castejon et al.,2011). An inactive precursor called pro-IL-1 $\beta$ , represents the first step for the production of the cytokine. In response to PAMPs, the immune cells make the 31-kDa precursor, after at least two separate signaling cascades there are the production and secretion of biologically active IL-1 $\beta$  (Bi et al.,2014). Many viruses are able to induce the production of IL-1 $\beta$ , other viruses indeed induce the expression of the inactive precursor and process it through different signaling molecules and pathways. The role of IL-1 $\beta$  during PRRSV infection is not completely known, but several studies consider that during systemic disease, massive mononuclear cell infiltration in lungs, fever and anorexia can be attributed to IL-1 $\beta$  activity (Van Reeth et al.,1999; Shibata et al., 1997; Hill et al.,1997). Additionally, severe lung inflammation and disease result when IL-1 $\beta$  and TNF- $\alpha$  occur at high levels or in combination (Van Reeth et al.,1999). PRRSV induces, both *in vitro* and *in vivo*, IL-1 $\beta$  expression (Thanawongnuwech et al.,2004; Qiao et al.,2011; Lunney et al.,2010; Borghetti et al.,2011), but it has been also reported a low IL-1 $\beta$ -gene expression in the early phase of PRRSV infection (Darwich et al., 2010).

The reduction of early INF-alfa production is one of the key mechanisms for viral persistence, but another important mechanism could be related to the increased production of IL-10 due to the ability of the virus to interact with the dendritic cell, directly interfering with the induction of acquired immunity. This mechanism was recently correlated to the role of viral proteins N. IL-10 is a pro-inflammatory cytokine produced by lymphocytes, monocytes and macrophages, dendritic cells and PAMs (Díaz et al.,2005; Gimeno et al.,2011). This cytokine is crucial in controlling the extent of immune-mediated inflammatory responses and in preventing immune-mediated tissue damage. IL-10 strongly inhibits the production of several chemokines, adhesion molecules and cytokines (IL-1,

IL-6, IL-12, TNF- $\alpha$ , and IFN $\gamma$ ), particularly, its effects on IL-1 and TNF- $\alpha$  production are essential for the anti-inflammatory activity (Moore *et al.*, 2001), and it also inhibits the proliferation of Th1. The mechanism through which IL-10 employs its anti-inflammatory action is the NF- $\kappa$ B inhibition (Johnson *et al.*, 1999). It is known that PRRSV may interfere with the immune response cells through the induction of immunosuppressive factors, like IL-10. Many studies have been reported that PRRSV infection induce IL-10 production in dendritic cells, PAMs or CD172a<sup>+</sup> (Gimeno *et al.*, 2011; Suradhat *et al.*, 2003), and also *in vivo* (Gómez-Laguna *et al.*, 2010; Barranco *et al.*, 2012; Amarilla *et al.*, 2015).

Highly Pathogenic isolates appear to stimulate a higher secretion of cytokines. Particularly, *in vitro* infection of PAM, it induces an overexpression of IL-8 (Xiao *et al.*, 2015), while some *in vivo* studies showed the production of IFN $\alpha$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 (Fan B *et al.*, 2016). (García-Nicolàs *et al.*, 2016; Amarilla *et al.*, 2016). Moreover, compared to normal strains, HP-PRRSV are also stronger inducers of TLR3, 7, 8 expressions (Zhang *et al.*, 2013).

### **Effect of PRRSV infection on the acquired immunity**

PRRSV infection suppresses inflammatory and antiviral cytokines (type 1 INF, IL-1, IL-8, IFN $\gamma$ ) production and this negatively influences the development of an efficient specific immunity.

Serum antibodies with PRRSV-neutralizing activity (NAbs) appear only at 4 weeks PI or more (Meier *et al.*, 2000 and 2003) and are not associated with viral clearance (Yoon *et al.*, 1994; Loving *et al.*, 2015), so they result detectable also in presence of viremia and viral persistence at the tissue level. Moreover, either natural exposure or vaccination provides only limited protection against secondary challenge; protective levels of NAbs usually requires multiple vaccinations or repeated infections and cross-protection (heterologous) can be limited (Vu HL *et al.*, 2011).

Conversely, a production of ELISA antibodies promptly occurs after infection; these antibodies are not involved in the neutralization of virus and in the protection, but that may enhance the internalization of the virus in macrophages as a result of a opsonization phenomenon [antibody-

dependent enhancement (ADE)]. (Lopez OJ, Osorio FA.,2004). Overall, considering some contrast results from *in vitro* and *in vivo* experiments, the contribution of ADE to PRRS pathogenesis remains controversial (Murtaugh et al.,2011).

During the early phases of the infection the neutralizing Ab (Nab) against N-protein, GP5 and nsps appear, particularly against nsp1 (a/b), nsp2 and nsp7 (a/b) (Lunney et al.,2015), while these serum neutralizing antibodies appear later, after 28-day post-infection (Loving et al.,2015).

Different potential mechanisms may be responsible for the inhibition and/or the delay of NAb production (Lunney et al.,2016)

Cell-mediated Immunity (CMI) is also delayed: an antigen-specific lymphocyte proliferation may be detected only four weeks' post-infection (Bautista E.M., Molitor T.W.,1997).

Moreover, PRRSV-specific T cells able to produce IFN $\gamma$  in blood were observed as early as 2 weeks' post-infection with substantial variation over time and among animals with no apparent correlation to the level of virus in lymphoid tissues, indicating that the PRRSV infection persistence can be related to a weak cell-mediated immune response unable to recognize permissive infected macrophages (Xiao et al.,2004)

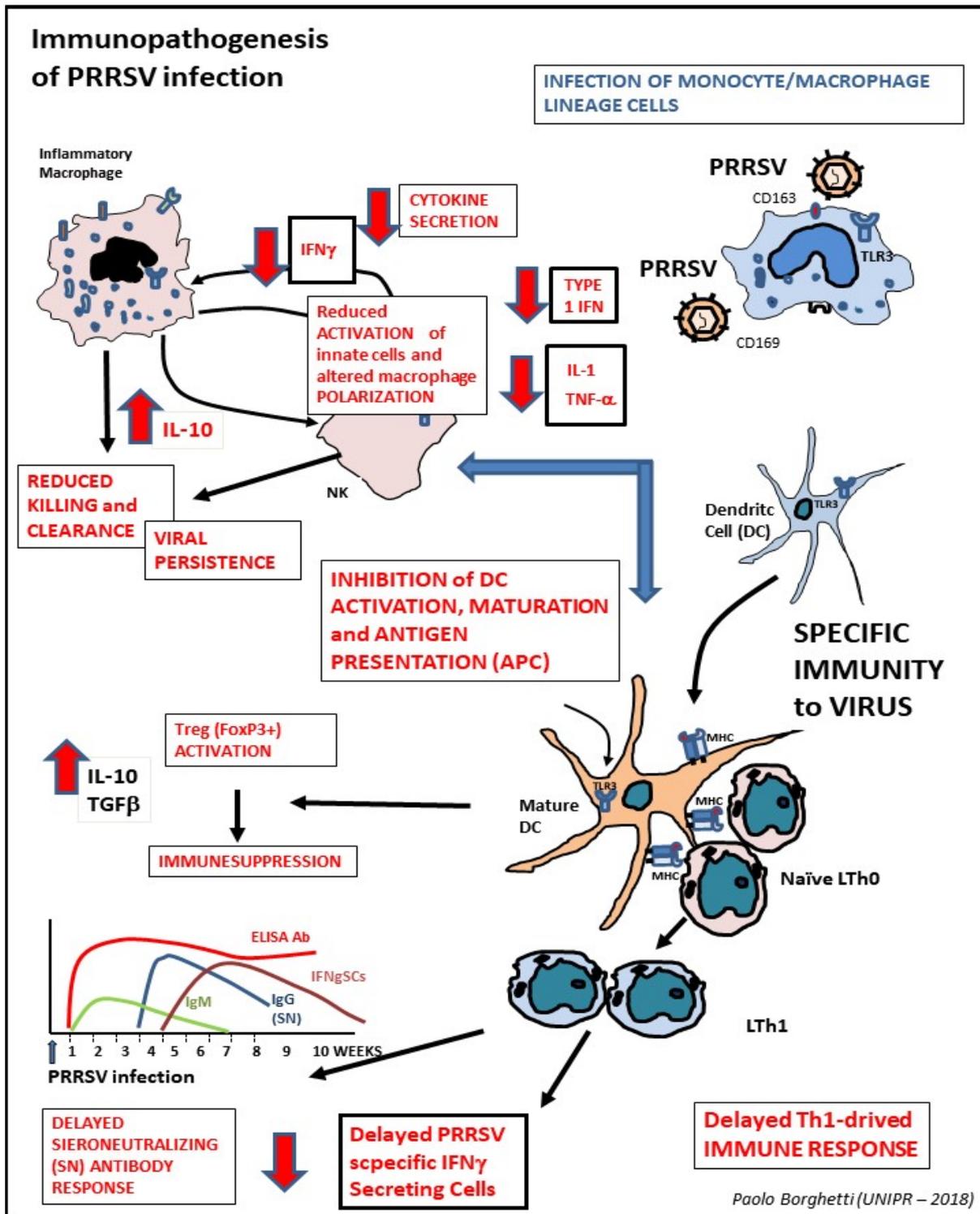
CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes as well as Double Positive (DP: CD4<sup>+</sup>CD8<sup>+</sup>) are important in viral clearance by secreting IFN $\gamma$  and mediating pathogen specific cytotoxicity. When the IFN $\gamma$ -secreting CD8<sup>+</sup>T-cell (Cytotoxic T-Lymphocytes - CTL) response was evaluated, a late and low virus-specific response was observed (Ferrari L. et al.,2013)

No exhaustive studies were performed on the role of CD8<sup>+</sup> cytotoxic T cells (CTLs) in controlling primary PRRSV infection; anti-PRRSV-targeted CTLs have been detected only after clearance of the virus from blood (Costers et al.,2009). Also, the role of memory T cells in anti-PRRSV immunity has not been studied extensively.

Researches focusing on the role of T Regulatory Lymphocytes (Treg) gave some contrasting results. Some studies suggest that Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> cells (T-regulatory cells: Tregs) with

immunosuppressive properties can be involved in PRRSV immunopathogenesis and that PRRSV infection can increase the frequency of these lymphocytes producing TGF- $\beta$  (Wongyanin et al.,2010; Silva-Campa E. et al.,2012). The secretion of TGF- $\beta$  and/or IL-10, could may explain an immunosuppressive response, resulting in the delayed onset of a Th1 immune response (Suradhat et al.,2003; Wongyanin et al.,2010; Dwivedi et al.,2011; Wongyanin et al.,2012; Renukaradhya et al.,2010)

However, it has also been shown that IL-10 increase can be dependent on the different strains of the PRRSV and also that *in vivo* IL-10 increase could be dependent on the age of infected animals was not induced in adult infected pigs (Diaz et al.,2006; Klinge et al.,2009).



**Figure 3.** General molecular mechanisms of the specific immune response on response to PRRSV infection (by permission of Prof. Paolo Borghetti, Dip. Veterinary Sciences – University of Parma).

## **Macrophages**

Macrophages are essential components of the innate immunity. When recruited, inflammatory macrophages are activated in response to signals of different nature (viral, bacterial and parasitic antigens, immune complexes, apoptotic or necrotic cells) they become a fundamental weapon to fight pathogens and tissue damage.

Recently, it has been demonstrated that, in relation to different micro-environmental stimuli and to the type of secreted cytokine, macrophages could undergo phenotypically and functionally dynamic switch, showing specific capacities and a different status of differentiation (Murray et al.,2017).

Indeed, inside the innate response against pathogens, macrophages act not only like activators but they are the main regulators of the inflammation, being implicated also in its resolution and in the triggering of the reparative processes.

Therefore, recently, it has been possible to identify different macrophage subpopulations characterized by a particular polarization state; a macrophages model has been established considering two main programs of polarization/differentiation, “classical” activated macrophages (M1) and alternatively activated macrophages (M2) (Fantino, 2015).

Classical M1 activation is triggered by danger signals (TLR-mediated activation) such as intracellular infection, bacterial components, pro-inflammatory cytokines (IFN $\gamma$ , TNF- $\alpha$ ); M1 macrophages have a high level of phagocytic activity, are able to produce inflammatory cytokines (IL-1, TNF- $\alpha$ , IL-8, IL-6, chemokines), nitric oxide (NO) promoting an efficient killing and clearance of the pathogen and driving Th1 polarization of CD4 cells (Mantovani et al.,2004; Zhang and Mosser 2008).

Alternatively, M2 activation is induced by apoptotic and damage cells, Th2 mediated responses (parasitic and fungal infections), immune complexes, cytokines such as interleukin-4 (IL-4), IL-13, IL-10 and anti-inflammatory inducers (glucocorticoids and TGF- $\beta$ ).

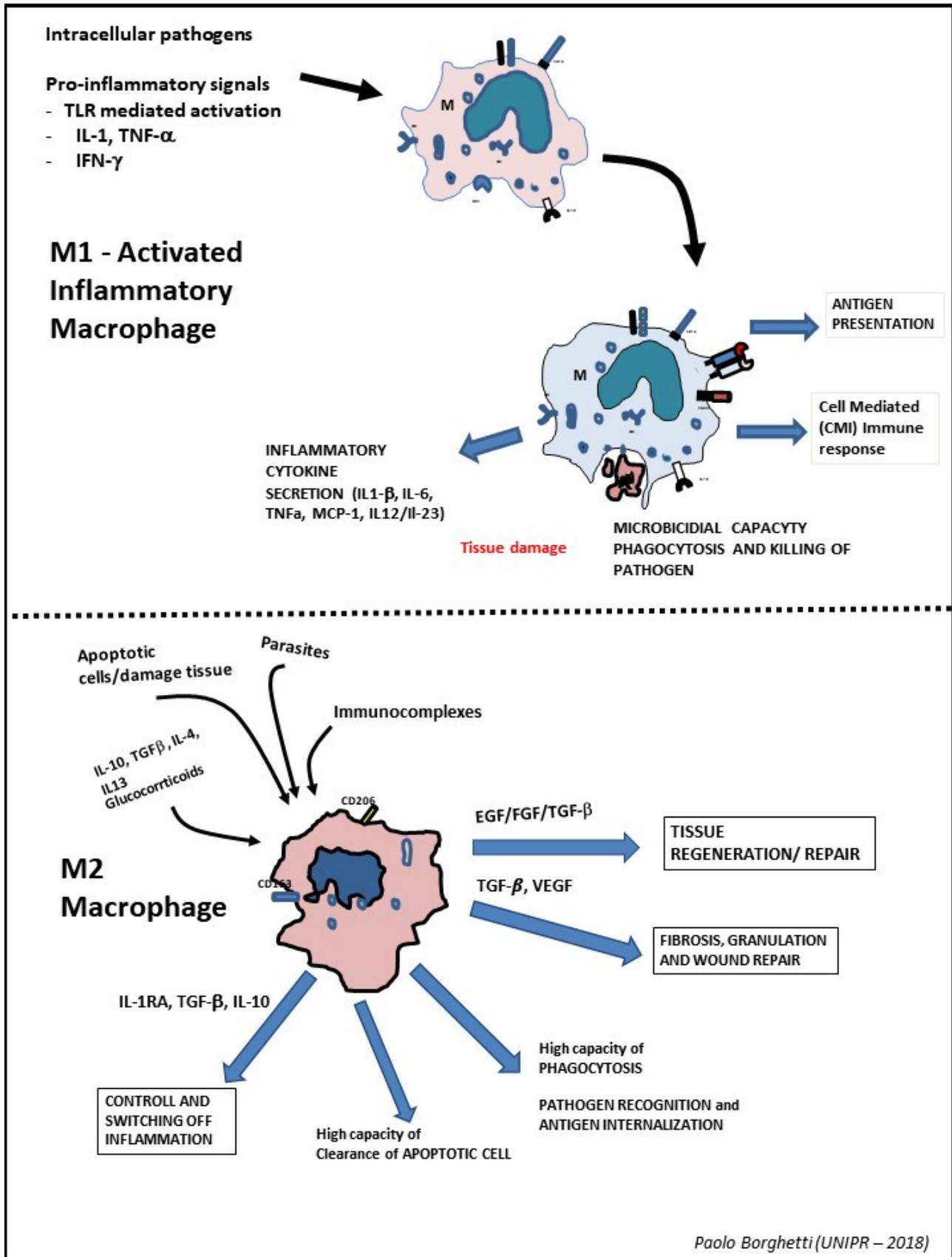
M2 macrophages are in fact involved in the elimination of apoptotic cells, in controlling the intensity and duration of inflammatory response. M2 show high phagocytosis, secrete IL-10 and are able to produce growth factors (such as TGF- $\beta$ , angiogenic factor) involving in the tissue repairing. In parallel, the role of M2 macrophages has been studied also in pathology, in the context of allergic reactions, in the development of tumors and as cellular reservoirs of various pathogens (Herbert et al., 2004; Pollard 2009; Duffield et al., 2013; Martinez and Gordon 2014; Novak and Koh 2013).

Polarization of macrophages occurs also during viral infections. Typically, virus recognition and macrophage internalization of virus, induces a M1 polarization (Figure 4) with a pro-inflammatory response focusing to control the infection and viral clearance while a subsequent M2-polarization occurs to control a potential over-inflammation preventing a tissue damage. Recent studies performed on this model of infection have demonstrated that, depending of the type of virus, the macrophage polarization could be modulated and variable across time influencing the pathogen clearance.

Indeed, viral infection may affect macrophage polarization and thereby induce immunosuppression which are generally associated with co-infections by other pathogens (Mc Cullers 2014).

Moreover, the different status of polarization can be differently modulated by the susceptibility to the virus; in fact, this model of study can help to explain some mechanism of resistance, viral spreading or persistence (Zanoni and Gandhi 2014; Poglitsch et al., 2012; Cassol et al., 2009).

Recently, some studies investigated the effects of PRRSV infection in porcine alveolar macrophage and macrophage polarization after cytokine treatment (García-Nicolàs et al., 2014; Whang et al., 2017; Singleton et al., 2016).



**Figure 4.** General aspects of M1-M2 model of macrophage polarization (by permission of Prof. Paolo Borghetti, Dip. Veterinary Sciences – University of Parma).

# *Materials and Methods*

## **In vitro study 1**

### **Monocyte-derived macrophages (MDMs)**

MDMs were generated from blood monocytes as previously described (Garcia et al.,2014). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from blood of specific-pathogen-free (SPF) pigs (Swiss White Landrace) 5 to 10 months old, using Ficoll-paque density centrifugation (Ficoll-Paque Plus, GE Healthcare, Chicago, IL, USA). Cells were enriched by positive selection for CD172a (mAb, clone 74-22-15A, hybridomas kindly provided by Prof. A. Saalmüller, Veterinary University of Vienna, Austria) using the magnetic cell sorting system (MACS) with LS columns (Miltenyi Biotec GmbH, Germany). Cells were then seeded onto 48-well culture plates at a density of  $0.5 \times 10^6$  cells/ml in Dulbecco's modified Eagle's medium without phenol red (DMEM, Gibco, 21063-029, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany) and recombinant porcine M-CSF (20 U/ml) (Sautter et al.,2018), and cultured at 39 °C with 5% CO<sub>2</sub> for 72 h for differentiation to MDMs.

MDMs were then stimulated for further 24h with one of the following treatment regimens: IFN $\gamma$  (10 ng/ml, R&D Systems, UK), IL-4 (100 U/ml) (Carrasco et al.,2001), IFN $\beta$  (100 U/ml) (Husser et al., 2011) and untreated, to obtain “IFN $\gamma$  MØ”, “IL-4 MØ”, “IFN $\beta$  MØ” and untreated “MØ”, respectively.

### **Viruses**

Six different PRRSV-1 isolates were analysed (two prototype/reference strains and four field strains). These isolates belong to different subtypes and they were selected based on the different *in vivo* virulence patterns. Lelystad virus (LVP, cell-culture-adapted to MARC-145 cells in 28 passages (P28), working stock was propagated once on MDMs to have a similar background as the other viruses) was chosen as the prototype strain of a classical European PRRSV-1 subtype 1. Lena strain (5<sup>th</sup> passage on MDMs, virus kindly provided by Prof. Hans Nauwynk, Ghent University, Belgium), isolated in Eastern Europe, was used as prototype strain of a highly pathogenic PRRSV-1. It belongs

to the subtype 3 (Karniychuk et al.,2010) and differs from the Lelystad prototype virus at both a genetic and antigenic level. Moreover, two Italian field isolates belonging to the subtype 1, Parma11/2014 (PR11) and Parma40/2014 (PR40), both passaged four times on MDMs, were included in the study. PR11 was isolated from a grower during a classical PRRSV outbreak. PR40 is an HP-PRRSV strain, isolated from a grower during an uncommon and severe PRRSV outbreak (Canelli et al.,2017). Finally, two Eastern European field PRRSV-1 strains, belonging to subtype 2, and passed four times on MDMs, were also tested: 17008/ILI 6 (ILI6) and 9/9A-BOR59 (BOR59). BOR59 is a HP strain isolated in Belarus in 2009 from the lungs of a pig died with respiratory symptoms; the intermediate pathogenic Russian ILI6 was isolated in 2009 from the lungs of a weaner pig (Stadejek et al.,2017). The different isolates were divided as highly pathogenic or not, on the basis of the *in vivo* pathogenic behaviour (Canelli et al.,2017; Stadejek et al.,2017; Karniychuk et al.,2010). In particular, Lena, PR40 and BOR59 can be classified as highly pathogenic strains, while LVP, PR11 and ILI6 showed lower pathogenicity and virulence (Tab1).

### **Virus propagation**

All strains were propagated *in vitro* on MDMs cultured at  $1 \times 10^6$  cells/ml in T150 flasks. Three-days-cultured MDMs were infected with 0.1 MOI. When the cytopathic effect (CPE) reached 50%, cells were lysed by freezing at  $-70^\circ\text{C}$  overnight. The thawed lysate was clarified by  $2000 \times g$  centrifugation at  $4^\circ\text{C}$  for 20 min, and stored at  $-70^\circ\text{C}$ .

The titer of the different viruses was determined in quintuplicate serial dilutions on 3-days-old MDMs, plated as  $1 \times 10^6$  cells/ml in 96-wells plates. After 48 h of incubation, the virus was inactivated with 80% acetone for 10 min at room temperature. Then, an immunoperoxidase monolayer assay (IPMA) was performed to detect PRRSV N protein by using the monoclonal antibody (mAb) SR30-A (RTI, Brookings, SD, USA). The titer was calculated with the Reed and Muench method (Reed L.J. and Muench H.,1938), and expressed as 50% tissue culture infective dose per ml (TCID<sub>50</sub>/ml).

Virus	Species	Subtype	Pathogenicity	Origin	Passage
Lelystad (LVP)*	PRRSV-1	1	Low	The Netherlands	29
Lena	PRRSV-1	3	High	Belgium	5
PR11/2014	PRRSV-1	1	Low	Italy	4
PR40/2014	PRRSV-1	1	High	Italy	4
17008/ILI 6	PRRSV-1	2	Intermediate <sup>1</sup>	Russia	4
9/9A-BOR59	PRRSV-1	2	High <sup>1</sup>	Belarus	4

**Tab. 1:** Viruses used for the *in vitro* analyses, divided by genotype, subtypes, *in vivo* pathogenicity, origin and cell culture passages. \*cell culture-adapted; <sup>1</sup>Stadejek et al., 2017; <sup>2</sup>Canelli et al., 2017; <sup>3</sup>Karniychuk et al., 2010; <sup>4</sup>Weesendorp et al., 2013 (Weesendorp et al. analysed Lelystad virus that had been propagated 7 times on PAMs (Weesendorp et al., 2013). Arguably, the MARC-145-adapted Lelystad “LVP” would be even less pathogenic *in vivo*).

## PRRSV infection of MDMs

MDMs were infected with a MOI of 0.1. Purified cell-lysate of MDMs served as a mock-control. After virus adsorption of 1 hour at 39°C and 5% CO<sub>2</sub>, MDMs were washed two times with 37°C PBS with calcium and magnesium (DPBS (1x), Gibco, 14040-019, Thermo Fisher Scientific), and cultured at 39°C with fresh culture medium. Sixteen h post-infection, the isolate infectivity and shedding were assessed by flow cytometry of harvested cells and viral titration of supernatants, respectively; moreover, the cytokine production in the supernatants was evaluated by using ELISA kits.

## Flow cytometry

The PRRSV N protein expression was evaluated by flow cytometry in order to assess the percentage of infected MDMs. For PRRSV nucleocapsid expression analysis, MDMs were harvested by 20 min incubation at room temperature (RT) with Accutase (Innovative Cell Technologies, San Diego, CA, USA), washed with 1 ml cold PBS/5xEDTA and fixed with 4% paraformaldehyde (PFA; Polysciences, Warrington, PA, USA), dissolved in PBS, for 10 min at RT, washed and permeabilized with 0.1% saponin (Applichem, Darmstadt, Germany) in PBS. The mAb SR30-A was diluted in PBS containing 0.3% of saponin (w/v) and added for 20 minutes to the cells on ice; after washing with

PBS containing 0.1% saponin (w/v), cells were incubated with the secondary antibody, Alexa Fluor 647, (A21240, Molecular Probes) diluted in 0.3% saponin (w/v) in PBS, for 15 minutes on ice. After a final wash, the cells were resuspended in the cell wash (BD Biosciences) for acquisition with the FACS Canto (Becton Dickinson, Eysins, Switzerland). After doublet exclusion, electronic gating based on the forward/side scatter plots was applied to identify living cells, using the FlowJo V.7.2.6 software (Tree stars Inc., Ashland, OR, USA).

### **Titration of supernatant**

Supernatant was titrated for viral growth assessment, as described above, in triplicate.

### **Cytokine measurement**

Cytokine production was evaluated after harvesting the culture supernatants at 16 hpi. IFN $\alpha$  production was determined by using an in-house ELISA (Guzylack-Piriou et al.,2004), with a detection limit of 30 pg/ml; IL-1 $\beta$ , IL-10 and TNF- $\alpha$  production was measured using commercial kits (R&D Systems, UK), with a detection limit of 60 pg/ml.

### **Statistical analysis**

All experiments were repeated at least five times and performed in triplicate. Statistical analysis was performed by using GraphPad Prism V.7 software (GraphPad Software, San Diego, California, USA) and FlowJo V.9.6.

Data were analysed with the ONE-way ANOVA test, followed by the Bonferroni's multiple comparisons test. A *p* value of < 0.05 was considered as statistically significant.

## **In vitro study 2**

### **PAM isolation and polarization**

PAMs were isolated from 4–6-week-old conventional pigs from a farm serologically free of PRRSV, as described in the OIE Terrestrial Manual 2015, with minor modifications. Briefly, piglet was humanely euthanized, then three lungs lavages were performed with PBS. The three lavages were centrifuged at  $1,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min twice, and the obtained cells resuspended and counted. Cells were then seeded into 48-well culture plates at a density of  $0.5 \times 10^6$  cells/ml in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with l-glutamine, non-essential amino-acids, penicillin G, streptomycin and amphotericin B and cultured at  $39^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 2 h for differentiation to make it stick. PAMs were then stimulated for further 24h with one of the following treatments: IFN $\gamma$  (10ng/ml), IL-4 (25ng/ml), IFN $\beta$  (25ng/ml) and untreated.

### **Viruses**

Two Italian field isolates belonging to the subtype 1, Parma11/2014 (PR11) and Parma40/2014 (PR40), both passaged four times on PAMs, were included in the study. PR11 was isolated from a grower during a classical PRRSV outbreak. PR40 is an HP-PRRSV strain, isolated from a grower during an uncommon and severe PRRSV outbreak (Canelli et al.,2017).

### **Virus propagation**

All strains were propagated *in vitro* on PAMs cultured at  $0.25 \times 10^6$  cells/ml in 96 wells/plate. One-day-cultured PAM were infected with 0.1 MOI. When the cytopathic effect (CPE) had reached 50%, the cells were lysed by freezing at  $-70^{\circ}\text{C}$  overnight. The thawed lysate was clarified by  $2000 \times g$  centrifugation at  $4^{\circ}\text{C}$  for 20 min, and stored at  $-80^{\circ}\text{C}$ .

## **PRRSV infection of PAMs**

PAMs were infected with a MOI of 0.1. Purified cell-lysate of PAM served as a mock-control. After virus adsorption of 1 hour at 39°C and 5% CO<sub>2</sub>, PAMs were washed two times with 37°C PBS with calcium and magnesium (DPBS (1x), Gibco, 14040-019, Thermo Fisher Scientific), and cultured at 37°C with fresh culture medium. 16 h post-infection, the isolate infectivity and shedding were assessed by flow cytometry of harvested cells and ELISA of supernatants.

## **Flow cytometry**

The PRRSV N protein expression was evaluated by flow cytometry in order to assess the percentage of infected PAM, as described above, in duplicate.

## **Cytokines measurement**

Cytokine production was evaluated after harvesting the culture supernatants at 16 hpi. Cytokine concentration was quantified by using commercial species-specific solid-phase sandwich ELISA kits (porcine TNF- $\alpha$ , swine IFN- $\gamma$  and IL-10 ELISA Kits, Thermo Fisher Scientific – Life Technologies, Carlsbad, CA, USA; according to the manufacturer's instructions and a Victor-3<sup>™</sup> 1420 Multilabel Counter (450 nm filter, PerkinElmer, Waltham, MA, USA).

## **Statistical analysis**

All experiments were repeated at least three times and performed in duplicate. Statistical analysis was performed by using GraphPad Prism V.7 software (GraphPad Software, San Diego, California, USA) and FlowJo V.9.6.

Data were analysed with ONE-way ANOVA test, followed by the Bonferroni's multiple comparisons test. A *p* value of < 0.05 was considered as statistically significant.

## **In vivo study**

### **Animals and experimental infection**

The in vivo study is part of a large project carried out to investigate the pathogenesis and control of PRRSV-1 strains of differing virulence (Canelli et al.,2017; Canelli et al.,2018). Two different experiments were performed. In the first experiment, a total of twelve 4-week-old conventional pigs from seventeen different litters of a PRRSV-free herd were housed in a biosafety level 2 (BSL-2) facility and randomly allocated in three separate rooms six days prior to inoculation and assigned to three different experimental groups: (i) PR40 group (PR40), with 5 pigs inoculated intra-nasally (IN) with 2 ml, 1 ml per nostril, of  $10^5$  TCD<sub>50</sub>/pig of PRRSV1\_PR40/2014 at 0 days post-inoculation (dpi); (ii) PR11 group (PR11), with 4 piglets inoculated IN with 2 ml, 1 ml per nostril, of  $10^5$  TCD<sub>50</sub>/pig of PRRSV1 PR11/2014 at 0 dpi; and, (iii) Control group (C), with 3 animals inoculated IN with sterile medium (MOCK/negative control)(Tab2). In the second one four different groups were considered: (iv) VAC-C: 2 pigs were IM-vaccinated against PRRSV at 4 weeks of age (35 days from inoculation) and left uninfected; (v) VAC-PR40: 6 pigs were IM-vaccinated against PRRSV at -35 days and intra-nasally (IN) infected with PR40 at 35-day post-vaccination (dpv)/0 days post-inoculation (dpi) (Tab2).

The following parts were common for both experiments. No relevant pathogens (PRRSV, SIV, PCV2) were detected in the animals before the beginning of the study. Animals suffering from severe clinical signs with a fatal prognosis were humanely euthanized according to standard protocols. All the survivors were humanely euthanized at 35 dpi (end of the experiment). At necropsy gross pathology was recorded and thymus samples were collected and stored at  $-70$  °C and/or fixed in buffered-formalin pH 7.4 for histopathology, immunohistochemical. The experimental design and all the procedures were fully in agreement and approved by the Ethical Committee, and by the Ministry of Health in Italy according to European and National rules on experimental infection studies and animal welfare.

## **Histopathology and grading of thymus**

Four  $\mu\text{m}$  tissue sections were stained with haematoxylin and eosin (H&E). The severity of the lesions in thymus was scored as follows (adapted and modified from Amarilla et al., 2016): (i) Grade 0, the cortex:medulla ratio (C/M) is about 2:1 with typical histological characteristics of the thymus; (ii) Grade I, diffuse cortical reduction with focal cortical disappearance, 5–9 tingible body macrophages/ $\text{mm}^2$  within the thymic cortex, typical medulla and stroma; (iii) Grade II, focal or multifocal decrease of C/M (<2:1), decrease of cortical layer with slight proportional increase of the stroma and 10–15 tingible body macrophages/ $\text{mm}^2$  within the thymic cortex; (iv) Grade III, focal to multifocal blurring of normal corticomedullary demarcation, increase of the stroma, occasional increase in the number of lymphocytes, mast and plasma cells and  $\geq 16$  tingible body macrophages/ $\text{mm}^2$ , with a “starry sky” appearance of the tissue; and, (v) Grade IV, extensive cell death of cortical thymocytes with complete disappearance of corticomedullary boundary demarcation and increase of the stroma.

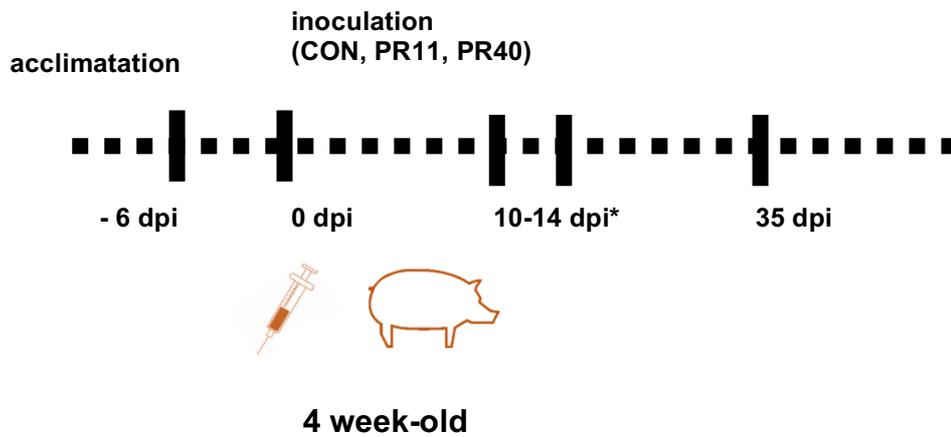
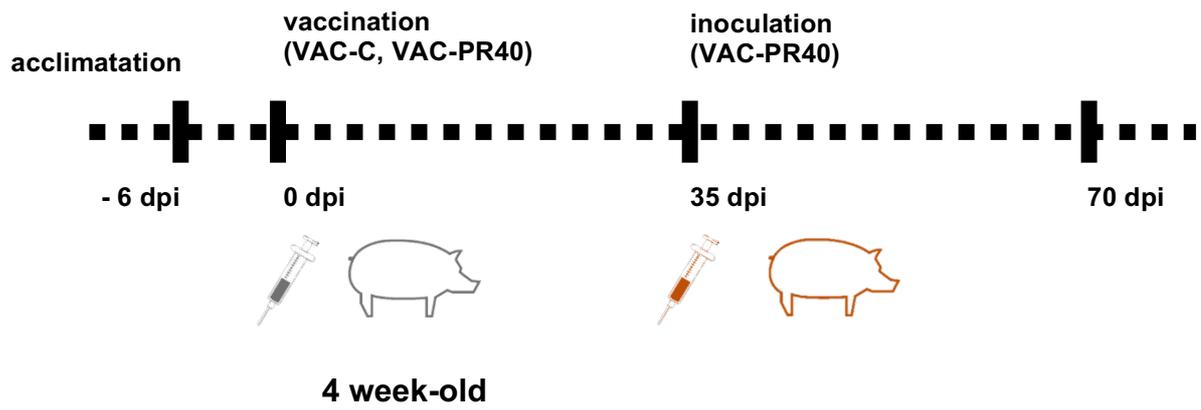
Manual quantification of tingible body macrophages in thymic cortex was assessed in 25 non-overlapping, consecutively selected high magnification fields of  $0.2 \text{ mm}^2$ . Results were expressed as number of cells per  $\text{mm}^2$ .

## **Immunohistochemistry**

The Avidin–Biotin–Peroxidase complex technique (ABC Vector Elite, Vector laboratories, USA) was used for the immunolabelling of PRRSV antigen and the different macrophages markers. Terminal dUTP Nick End-Labeling (TUNEL) was carried out by using a commercial kit (In Situ Cell Death Detection Kit, POD, Roche, Germany) following manufacturer’s instructions. Briefly,  $4 \mu\text{m}$  tissue sections were dewaxed and rehydrated in a gradient of ethanol, followed by endogenous peroxidase inhibition with 3 %  $\text{H}_2\text{O}_2$  solution in methanol for 30 minutes (min). After treatment with different antigen retrieval methods (Tab3), the slides were washed with PBS (pH 7.4, 0.01 M) and

incubated for 30 min at room temperature with 100  $\mu$ l of blocking solution in a humid chamber. Primary antibodies were incubated overnight at 4 °C in a humid chamber (see dilutions in Tab2 for each antibody), while for the negative controls the primary antibody was replaced by either an isotype control or by blocking solution. Biotinylated secondary antibody was incubated for 30 min at room temperature. An avidin-biotin-peroxidase complex (Vector Laboratories) was applied for 1 hour at room temperature in the darkness. Labelling was visualized by application of the NovaRED™ substrate kit (Vector Laboratories). Sections were counterstained with Harris's haematoxylin, dehydrated and mounted.

Labelled cells were analysed in 25 non-overlapping and consecutive high magnification fields of 0.2 mm<sup>2</sup>. The expression of all markers was manually counted and the results were expressed as the number of cells per mm<sup>2</sup>.



**\*humanely killing of animals due to animal welfare issues**

**Tab 2.** Experimental designs (for an easier interpretation the two study were described in a unique picture)

<b>Specificity (clone)</b>	<b>Type of antibody</b>	<b>Commercial origin</b>	<b>Fixative</b>	<b>Blocking solution</b>	<b>Dilution</b>	<b>Antigen retrieval</b>
<b>Anti-PRRSV (clone SDOW17)</b>	mAb	Rural Technologies Inc., Brookings, SD, USA	Formalin	BSA 1%	1:500	Protease Type XIV <sup>a</sup>
<b>TUNEL</b>	N.A.	Roche Diagnostics, Indianapolis, USA	Formalin	N.A.	N.A.	Proteinase K <sup>b</sup>
<b>Anti-CD172a (BA1C11)</b>	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
<b>Anti-CD163 (2A10/11)</b>	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
<b>Anti-CD107a (4E9/11)</b>	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2
<b>Anti-BA4D5 (BA4D5)</b>	mAb	In house, INIA	Formalin	BSA 1%	Neat	Citrate pH 3.2

**Tab 3.** Clones, sources and dilutions of the primary antibodies used for the immunohistochemical detection of macrophages markers.

N.A.: Not applicable; <sup>a</sup>Protease Type XIV (Sigma-Aldrich): 7 min at 37 °C in water bath ; <sup>b</sup>Proteinase K (Roche): 15 min at 37 °C in heat incubator.

# *Results*

## **In vitro study 1**

### **Infectivity on MDMs of different PRRSV strains**

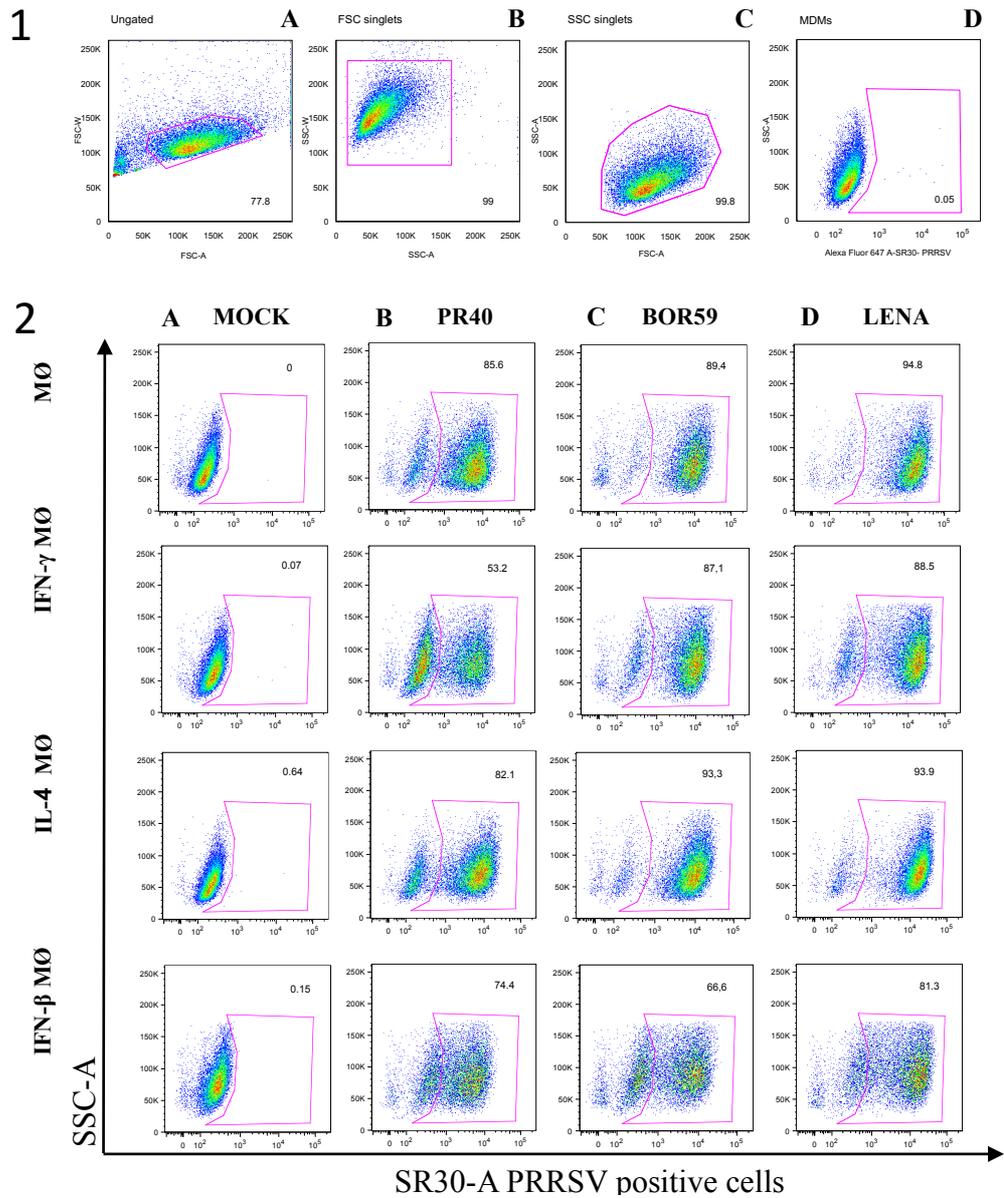
The infectivity on MDMs in terms of was determined as nucleocapsid protein expression after 16h post infection (Fig. 1). In untreated MDMs, the highest levels of infectivity were found for the HP strains Lena, Bor59, PR40 as well as for the ILI6 PRRSV, classified as intermediate virulent isolates (Fig. 2A). In IFN $\gamma$  MDMs, Lena and BOR59 showed the highest infectivity, followed by ILI6, PR40 and PR11 strains. LVP induced the lowest levels of nucleocapsid expression, which was statistically different from all other strains (Fig. 2B). In IL-4 MDMs, the levels of infection and the differences between strains were similar to untreated MDMs (Fig. 2C). In IFN $\beta$  MDMs, the highest levels of infectivity were found for the HP strains Lena, PR40 and BOR59, followed by ILI6 and PR11. PR11 was less effective than the aforementioned four strains, in a statistically significant manner ( $p < 0.05$ ). IFN $\beta$  almost completely suppressed nucleocapsid expression of LVP (Fig. 2D). Of note, within the PRRSV type 1.1 strains, PR40 infectivity was higher than that of PR11 in all MDM-conditions ( $p < 0.05$ ), except for IFN $\gamma$  MDMs.

### **Virus replication in MDMs after infection with different PRRSV strains**

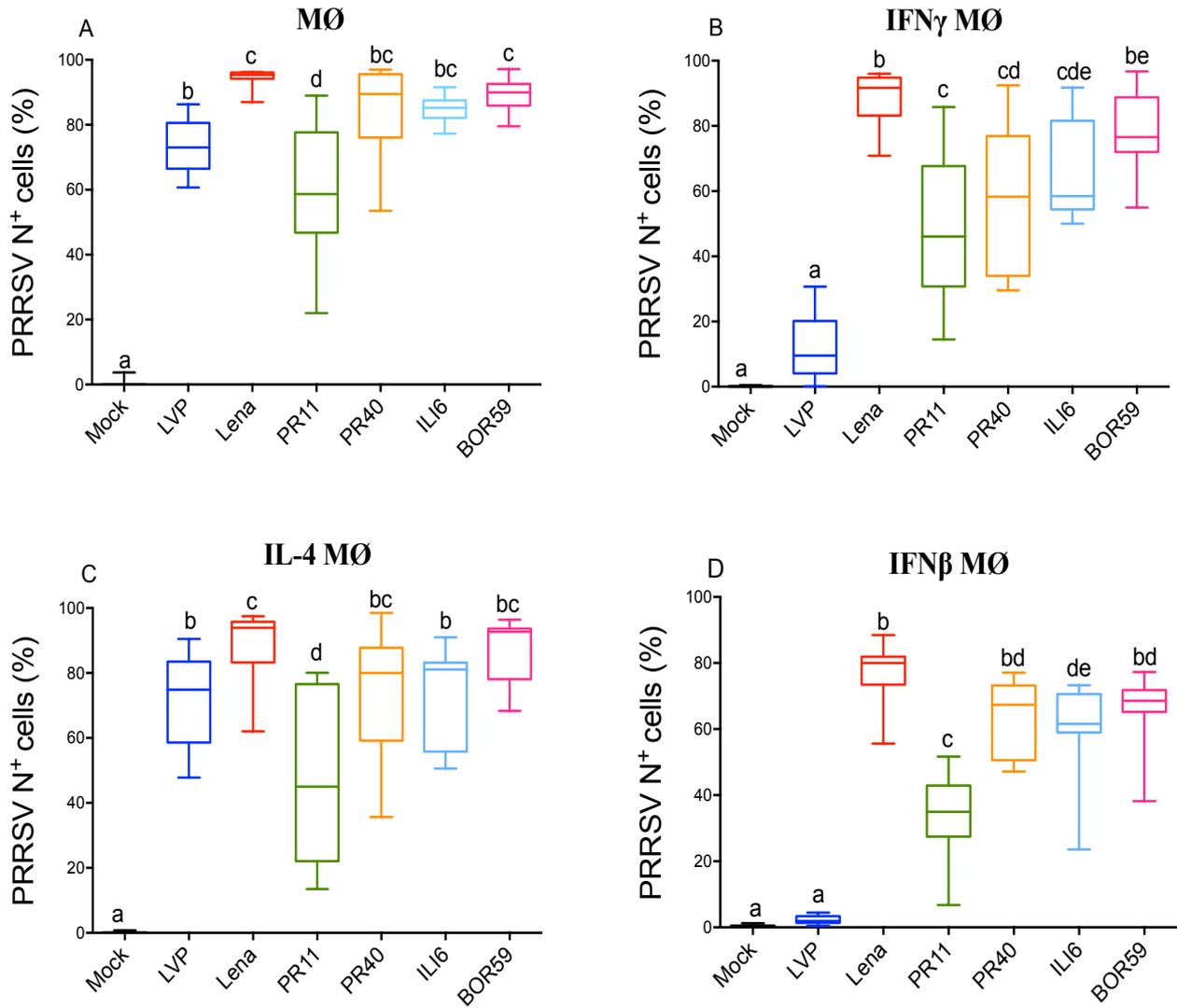
Virus replication was determined by measuring virus shedding into the supernatant of infected MDMs. As shown in Fig. 3A and 3C, no virulence-dependent differences in viral titers were found when untreated MDMs or IL-4 MDMs were used. In contrast, when using IFN $\gamma$  MDMs, the HP strains PR40, BOR59, Lena and the intermediate strain ILI6 grew at higher titers than LVP and PR11 (Fig. 3B), but, except for Lena, they were not statistically distinguishable. On IL-4 MDMs, no strain-dependent differences were found. In IFN $\beta$  MDMs, Lena and PR40 stood out as the most replicative strains, followed by BOR59, which was significantly less replicative than Lena, but not than PR40 (Fig. 3D). A deeper analyse of how the viruses are compared to each other, considering their *in vivo* pathogenicity. Therefore, data in Fig. 2 were used to divide isolated into two groups, then these

isolates were analysed for their relative infectivity, compared to a reference strain. HP-PRRSV strains PR40, BOR59 and Lena (subtypes 1, 2 and 3, respectively) were compared among each other, using the Lena as the reference strain for the HP-PRRSV. The same data analysis was also performed among the “less pathogenic” strains (PR11, ILI6, and LVP) in comparison to LVP, chosen as low pathogenic reference strain.

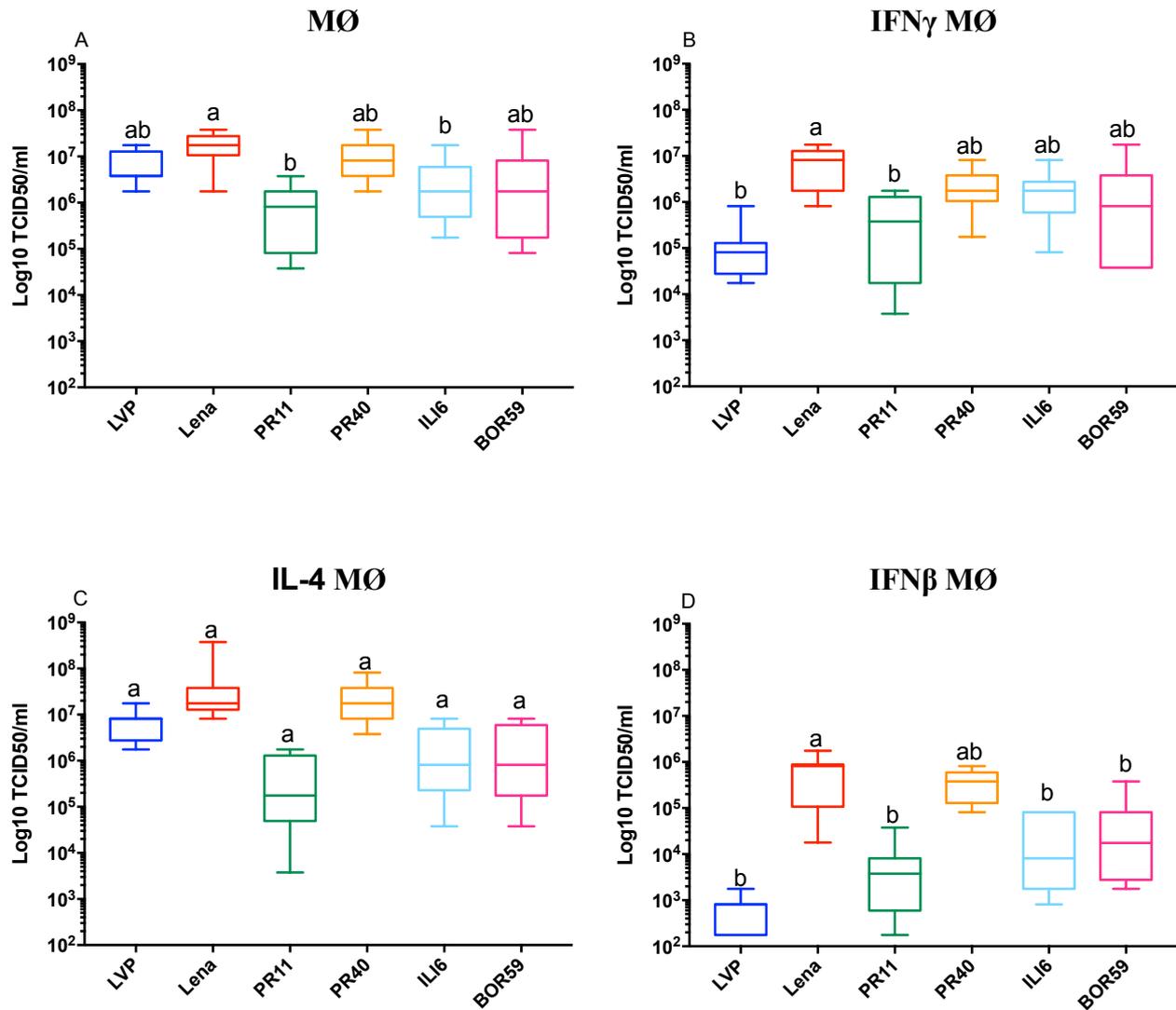
BOR59 was statistically as infective as Lena, with the only exception of the IFN $\beta$ -primed MDMs. PR40 had reduced infectivity for M $\emptyset$ s under all conditions when compared to Lena, and also when compared to BOR59 for IFN $\gamma$  and IL-4-primed M $\emptyset$ s. When we analysed the less pathogenic isolates (LVP, PR11, and ILI6), ILI6 was the most efficient at infecting M $\emptyset$  in different conditions. The Italian strain PR11 was statistically less able than ILI6 to infect any of the treated M $\emptyset$ , and also performed worse than LVP on untreated and IL-4-primed M $\emptyset$  ( $p < 0.05$ ). Curiously though, PR11 had a higher efficiency than LVP in infecting the MDMs pre-treated with either of the IFNs, and LVP infected IL-4 M $\emptyset$  as successfully as ILI6.



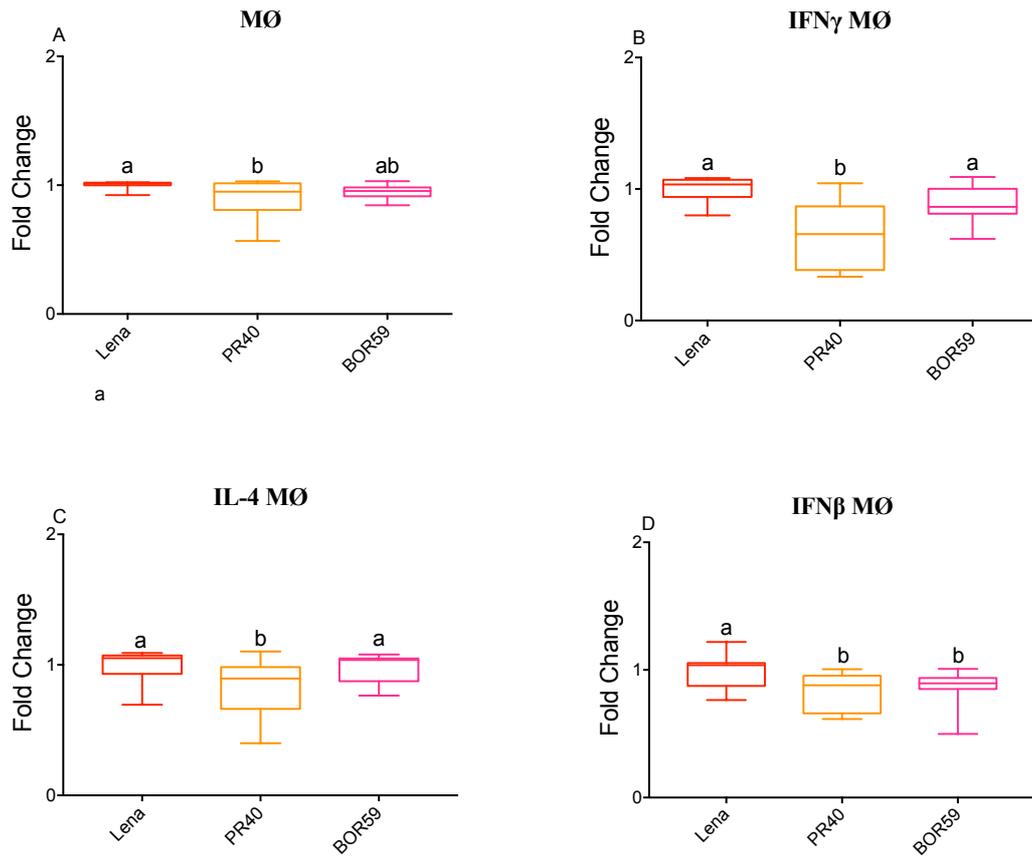
**Fig. 1:** 1) Gating strategy of porcine MDMs. Cell suspensions were gated for singlets by doublet discrimination (A, B) and then gated for MDMs (C). PRRSV positive cells were detected by using the monoclonal antibody SR30A (D). 2) Untreated MDMs, IFN $\gamma$  MØ, IL-4 MØ and IFN $\beta$  MØ infected with PR40 (B), BOR59 (C) and Lena (D) or Mock (A). After 16 hpi flow cytometry with mAb SR30-A was performed for PRRSV nucleocapsid protein detection.



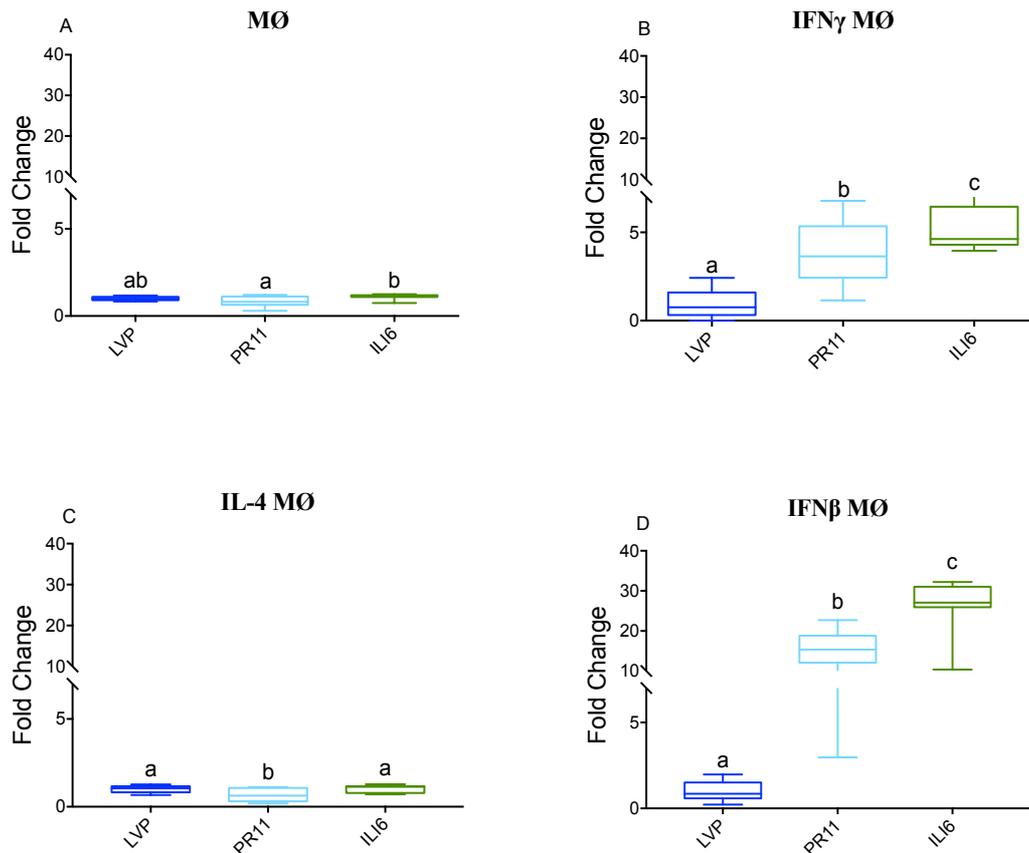
**Fig. 2: Infectivity of different PRRSV strains.** Percentage of PRRSV nucleocapsid (N) expression measured by flow cytometry. Untreated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C) and IFN $\beta$  MØ (D) were infected at MOI 0.1 with: LVP, Lena, PR11, PR40, ILI6, BOR59 or Mock-treated for 16 h. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N<sup>+</sup>) cells; data from triplicate culture of at least five independent experiments. Different letters indicate significance between viruses (p < 0.05).



**Fig. 3: Viral shedding of different PRRSV strains.** Supernatant was collected from untreated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C) and IFN $\beta$  MØ (D), which were infected at MOI 0.1 with LVP, Lena, PR11, PR40, ILI6 or BOR59 for 16 h. The viral titer was determined in quintuplicate serial dilutions on 3-day-old MØ after 48 h of incubation. The titer was calculated, in at least three independent experiments, with the Reed and Muench method and expressed as log<sub>10</sub> TCID<sub>50</sub>/ml. Different letters indicate significance between viruses (p<0.05).



**Fig. 4: Relative infectivity of HP-strains.** Untreated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C), and IFN $\beta$  MØ (D), were infected with MOI 0.1 of Lena, PR40 or BOR59 for 16 h. PRRSV nucleocapsid (N) expression was measured by flow cytometry, and Fold Change was calculated in reference to Lena. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells. Data from triplicate culture of at least five independent experiments. The different letters indicate significance between viruses ( $p < 0.05$ ). This is a further analysis of the data seen in Fig.1.



**Fig. 5: Relative infectivity of “not highly pathogenic” PRRSV strains.** Untreated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C), and IFN $\beta$  MØ (D), were infected with MOI 0.1 of LVP, PR11 or ILI6 for 16 h. PRRSV nucleocapsid (N) expression was measured by flow cytometry, and Fold Change was calculated in reference to Lena. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N+) cells. Data from triplicate culture of at least five independent experiments. The different letters indicate significance between viruses ( $p < 0.05$ ). This is a further analysis of the data seen in Fig. 1.

## Cytokine production

Since several cytokines are differentially regulated by the individual PRRSV strains, the secretion of IFN $\alpha$ , IL-1 $\beta$ , IL-10 and TNF into the supernatant of infected cells at 16 hours post infection (hpi) was evaluated.

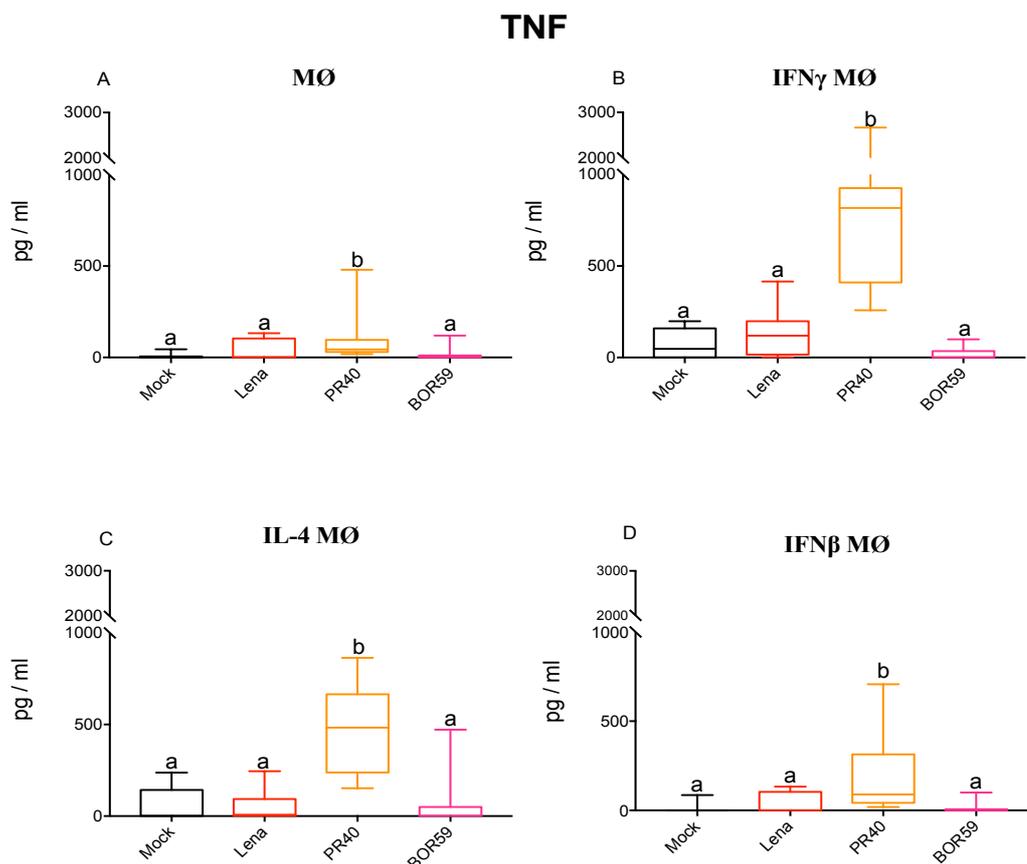
No IFN $\alpha$  or IL-10 were detected in the supernatants of untreated MØ, IFN $\gamma$  MØ, IL-4 MØ, or IFN $\beta$  MØ infected with the different PRRSV strains (data not shown).

The production of the pro-inflammatory cytokines (TNF and IL-1 $\beta$ ) varied among the different HP-PRRSV strains (Fig 6 and 7). While BOR59 induced almost no TNF, Lena led to the production of

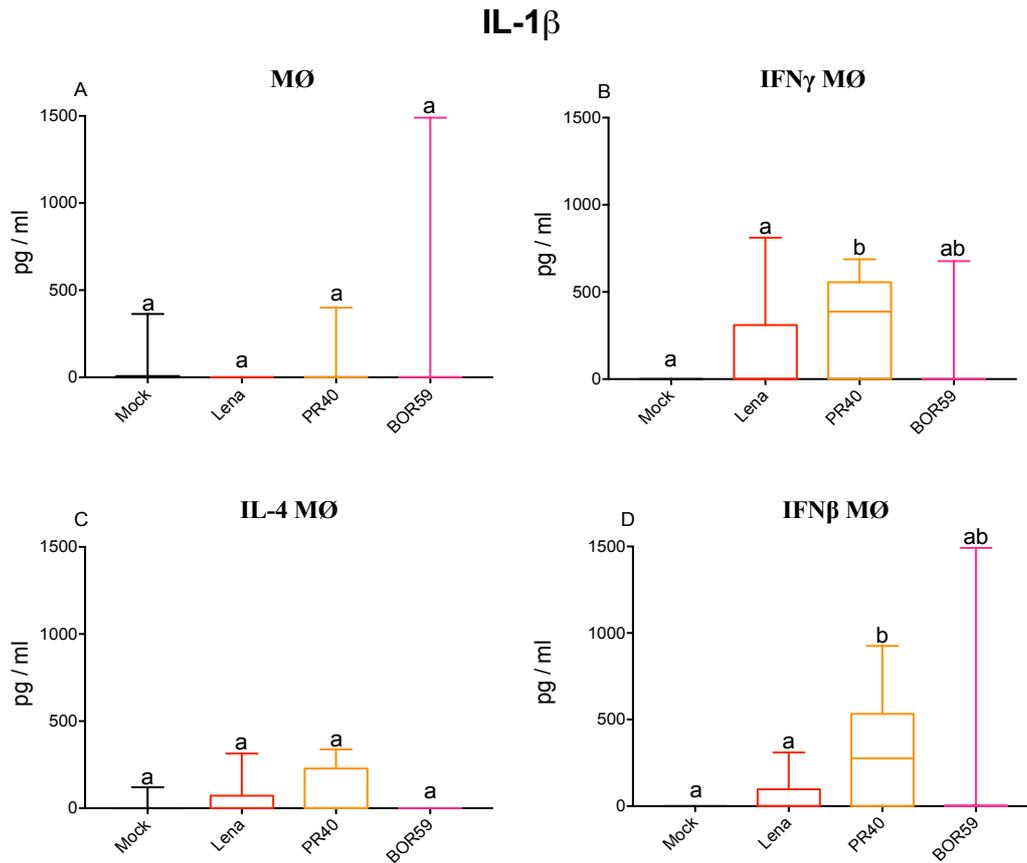
some TNF, and PR40 stimulated the release of quite a lot. The mean values of TNF production after infection with PR40 are: untreated MØ (mean value 212.3 pg/ml), IFN $\gamma$  MØ (982.3 pg/ml), IL-4 MØ (480.8 pg/ml) and IFN $\beta$  MØ (212.3 pg/ml) (Fig. 5 – cave, the middle line in the box plots indicates the median, not the mean).

Infection with “low pathogenic” PRRSV strains yielded no levels TNF above the spontaneous production. The increased production induced by PR40, was statistically significant in comparison to all other strains (both HP and not) under all MØ conditions ( $p < 0.05$ ).

The production of IL-1 $\beta$  varied greatly. A significant production could only be seen in IFN $\gamma$ - or IFN $\beta$ -primed MØ that were infected with PR40 (Fig. 7).



**Fig. 6: TNF production induced by HP-PRRSV.** Supernatants of cells infected with different HP-PRRSV strains: PR40, BOR59 and Lena (subtype 1, 2 and 3, respectively). Undifferentiated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C) and IFN $\beta$  MØ (D) were infected at MOI 0.1 or Mock-treated for 16 h. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of TNF (pg/ml) from triplicate culture of at least five independent experiments. The different letters indicate significance between viruses ( $p < 0.05$ ).



**Fig. 7: IL-1 $\beta$  production induced by HP-PRRSV.** Supernatants of cells infected with different HP-PRRSV strains: PR40, BOR59 and Lena (subtype 1, 2 and 3, respectively). Undifferentiated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C) and IFN $\beta$  MØ (D) were infected at MOI 0.1 or Mock-treated for 16 h. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of TNF (pg/ml) from triplicate culture of at least five independent experiments. The different letters indicate significance between viruses ( $p < 0.05$ ).

## **In vitro study 2**

### **Infectivity on PAM of different PRRSV strains**

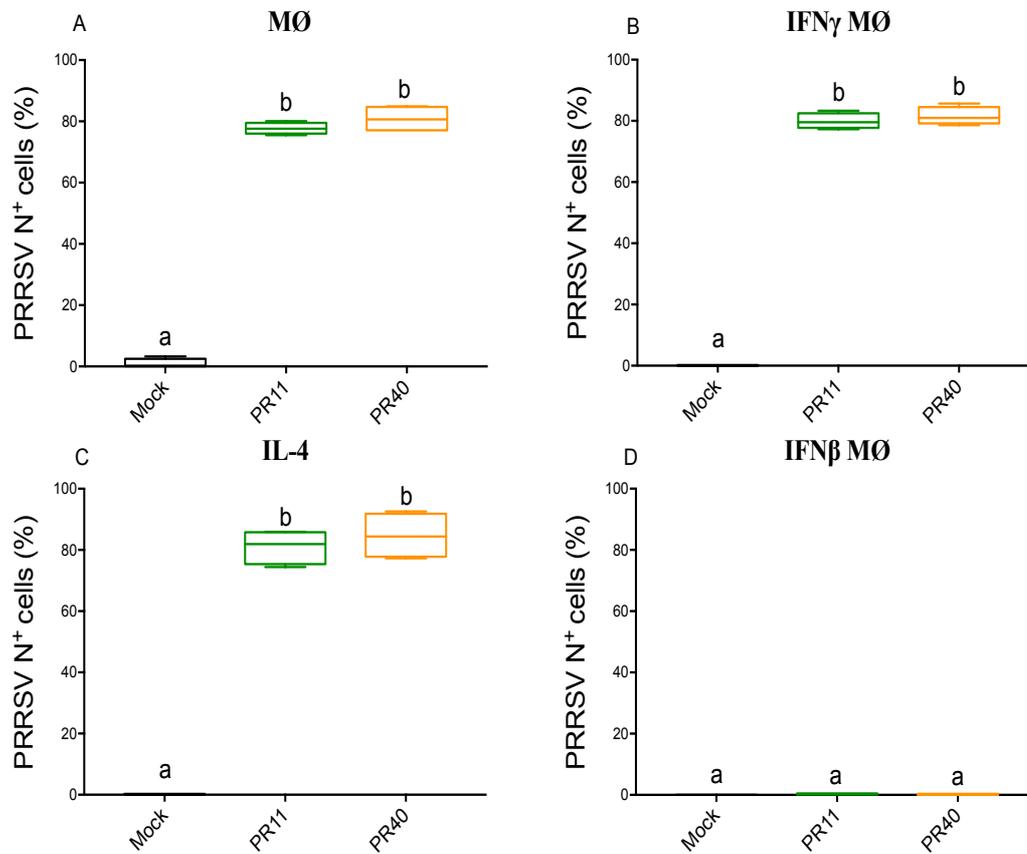
The infectivity on PAMs of different PRRSV isolates was tested by quantification of the nucleocapsid (N) expression after 16h of infection.

The results did not show significant differences between the two Italian strains tested. In the untreated and IL-4 treatments the highly pathogenic strain PR40 showed a higher capacity of infection compared with PR11, but this difference was not significant (Fig 8C).

Interesting, the treatment with IFN $\gamma$  did not determine an antiviral state, instead the levels of N protein detected were similar to untreated and IL-4 PAMs. On the contrary, IFN $\beta$  completely suppressed nucleocapsid expression after the infection with both viruses, like as in the Mock treatment, and also in this case, any difference between the two strains was found (Fig 8D).

### **Cytokine production**

The secretion of IFN $\alpha$ , IL-10 and TNF was evaluated in the supernatant of infected cells at 16 hpi. No cytokines production in any of the supernatants harvested from the infected PAM was found (data not shown).



**Fig. 8. Infectivity of different PRRSV strains.** Percentage of PRRSV nucleocapsid (N) expression measured by flow cytometry. Untreated MØ (A), IFN $\gamma$  MØ (B), IL-4 MØ (C) and IFN $\beta$  MØ (D) were infected at MOI 0.1 with: PR11 and PR40 or Mock-treated for 16 h. Boxplots indicate the median (middle line), 25th and 75th percentiles (boxes), maximum and minimum (whiskers) of N positive (N<sup>+</sup>) cells; data from triplicate culture of at least five independent experiments. Different letters indicate significance between viruses ( $p < 0.05$ ).

## **In vivo study**

### **Gross lesions, histopathology and grading of thymus**

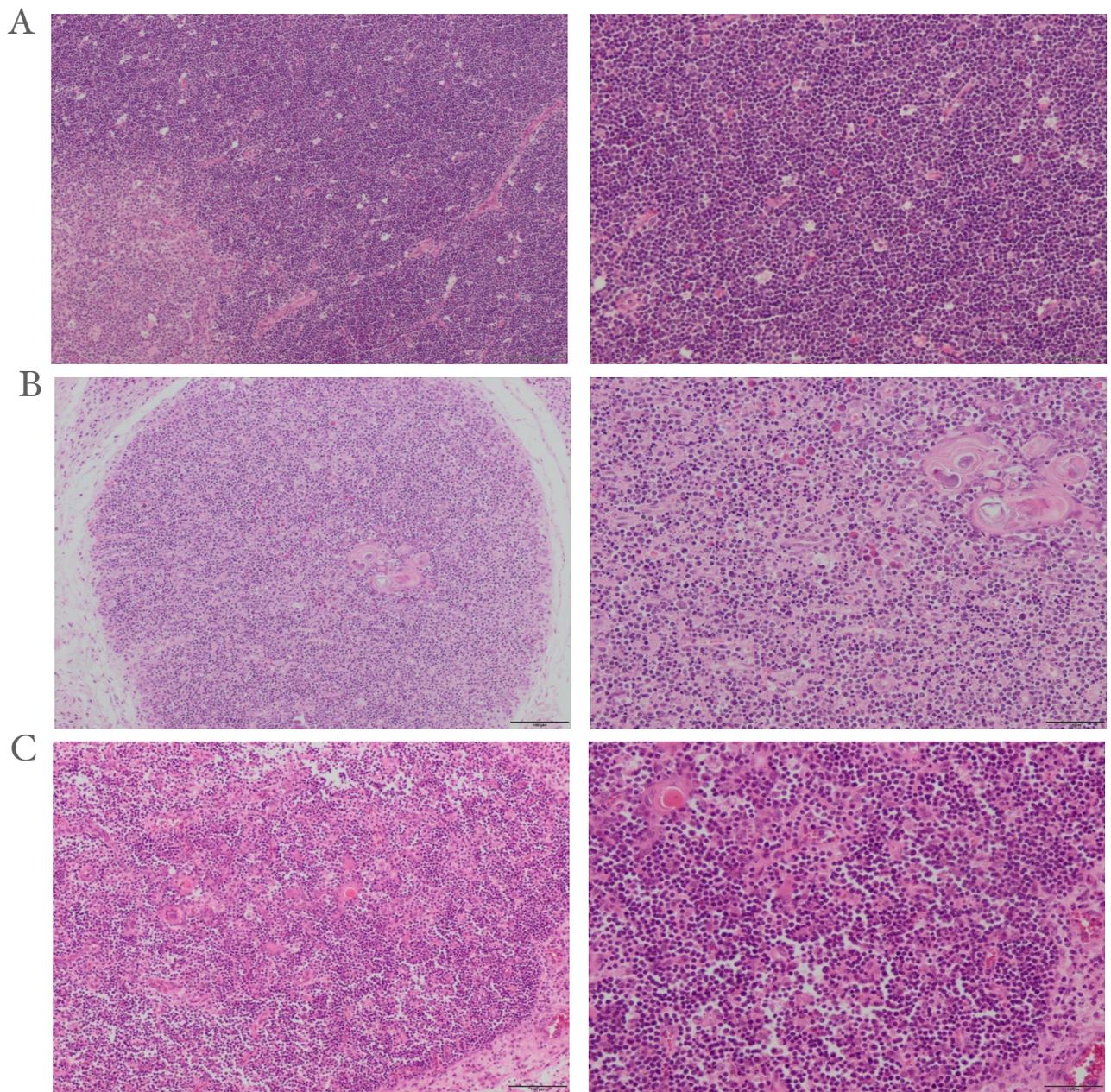
The clinical signs and gross lesions have been previously described (Canelli et al., 2017). Mortality rate was similar in the two infected groups, with two and three pigs euthanized due to welfare conditions in PR40 and PR11 groups, respectively, between 10 and 14 dpi. Lung lesions were more severe in the PR40 group compared to the PR11 group and consisted of interstitial pneumonia with multifocal, mottled, tanned appearance of the lungs accompanied, in some cases, by bronchopneumonia associated to secondary bacterial infections. Atrophy of the thymus was detected in both infected groups, with an almost complete atrophy of the cervical part of the thymus in the PR40 group. Control animals did not exhibit significant gross or microscopic lesions.

The thymus of the infected animals with both viruses was characterized by diffuse cortical reduction, disappearance of the corticomedullary boundary, and, in some cases, a consistent inflammation of the stroma. The most intense changes were observed in the thymus from PR11- and PR40-infected pigs that died at 10-14 dpi, which presented extensive cell death phenomena in the cortex with a strong disappearance of the corticomedullary boundary (Tab1)(Fig. 2A-2B). In most of these animals, a marked interstitial inflammatory infiltrate of the stroma by abundant neutrophils and mononuclear cells (macrophages, lymphocytes and plasma cells in a lesser extent) together with oedema of the connective tissue was also observed (Fig. 2C). This infiltrate was particularly intense at perivascular level as well as was the intravascular trafficking of these immune cells (Fig. 2D).

	<b>CON (35 dpi)</b>	<b>PR11 (35 dpi)</b>	<b>PR40 (35 dpi)</b>	<b>PR11 (10-14 dpi)</b>	<b>PR40 (10-14 dpi)</b>	<b>VAC-C</b>	<b>VAC-PR40</b>
<b>Grades</b>							
0	2/3		1/3				3/5
I							1/5
II	1/3	1/1	2/3			2/2	1/5
III							
IV				3/3	2/2		
<b>Tingible body macrophages</b>	9.87 ± 1.79	7	8.87 ± 6.82	ND*	ND*	13.1 ± 8.63	10.4 ± 1.79
<b>TUNEL positive cells</b>	54.97 ± 63.40	67.91	43.53 ± 31.07	ND*	ND*	53.82 ± 40.20	53.18 ± 34.59

ND\*: Not determined due to extensive cell death of thymocytes in the cortex.

**Tab 1.** Histopathology grading of the thymus of piglets from each experimental group and average number of tingible body macrophages and TUNEL positive cells (expressed as the mean ± SD).



**Fig. 9.** Representative Haematoxylin-eosin (HE) of the thymus from (A) Control, (B) Infected with PR11 and dead at 10-14 dpi, and (C) infected with PR40 and dead at 10-14 dpi. Bar 100  $\mu$ m (left) and 50  $\mu$ m (right).

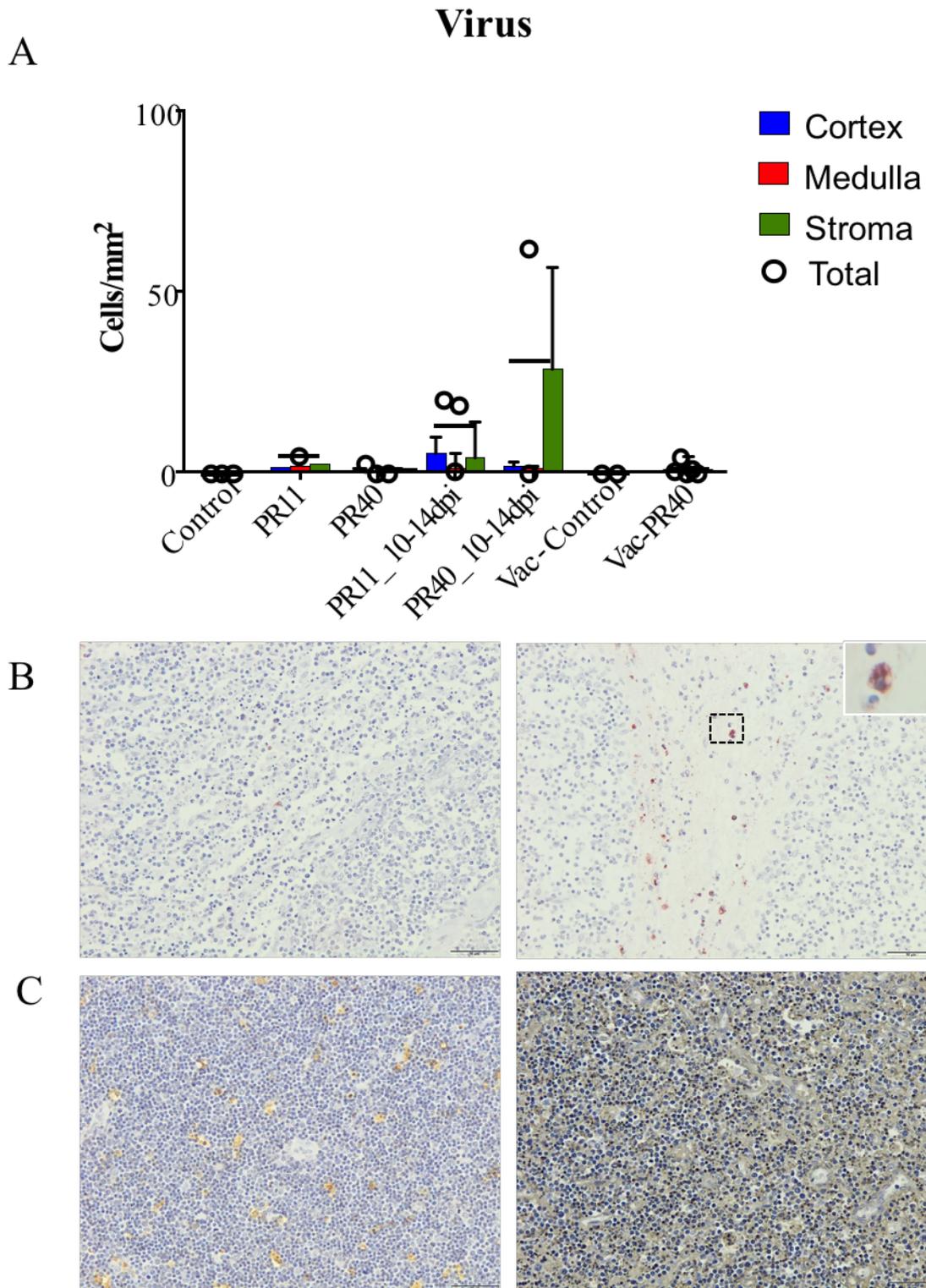
## **Virus**

PRRSV antigen was not detected in the thymus of control animals (groups i and iv). PRRSV antigen was observed in the cytoplasm of macrophages from the thymic cortex and the stroma, and in a lesser extent in macrophages from the medulla of the thymus of PR40 and PR11 infected animals at 35 dpi (Fig. 10A). Interestingly, PR40 and PR11 infected animals that died between 10-14 dpi, presented a marked increase in the number of PRRSV positive cells, mainly associated to a marked infiltrate of PRRSV positive cells within the inflammatory reaction observed in the stroma of these animals (Fig. 10B).

In case of PR40 vaccinated and inoculated animals, only 3 out of 5 animals presented PRRSV positive cells with a similar frequency and distribution than PR40 non-vaccinated but inoculated animals at 35 dpi.

## **TUNEL**

TUNEL labelling was mainly observed in apoptotic bodies within macrophages (“tingible body macrophages”) and occasionally in free apoptotic bodies. TUNEL staining was mostly observed in the cortex and, to a lesser extent, in the medulla of the thymus of all piglets. No differences were observed either between infected animals and controls or among infected groups at 35 dpi (Tab1). However, a marked increase of TUNEL labelling was observed in the cortex of PR11 and PR40 infected animals at 10-14 dpi which showed a diffuse labelling associated to an intense increase of cell death which occupied most of the cortex (Fig. 10C).

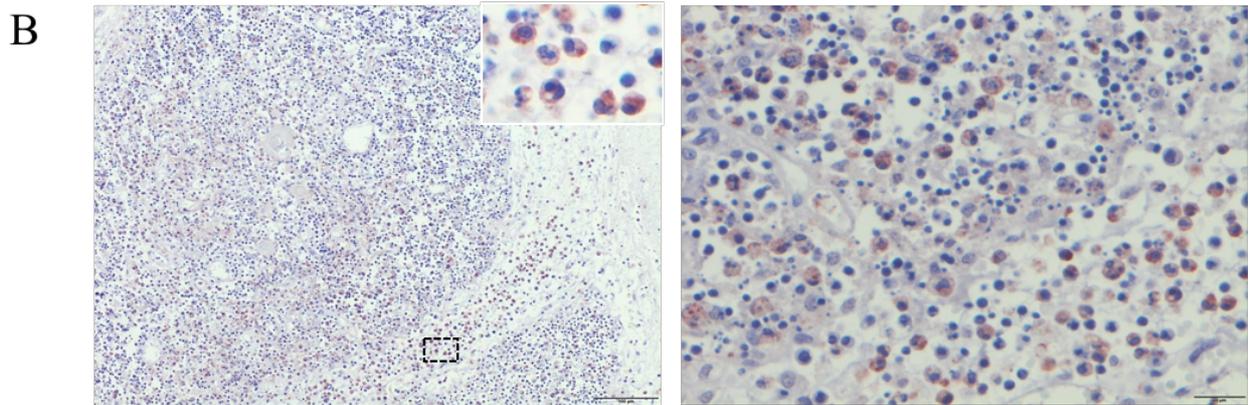
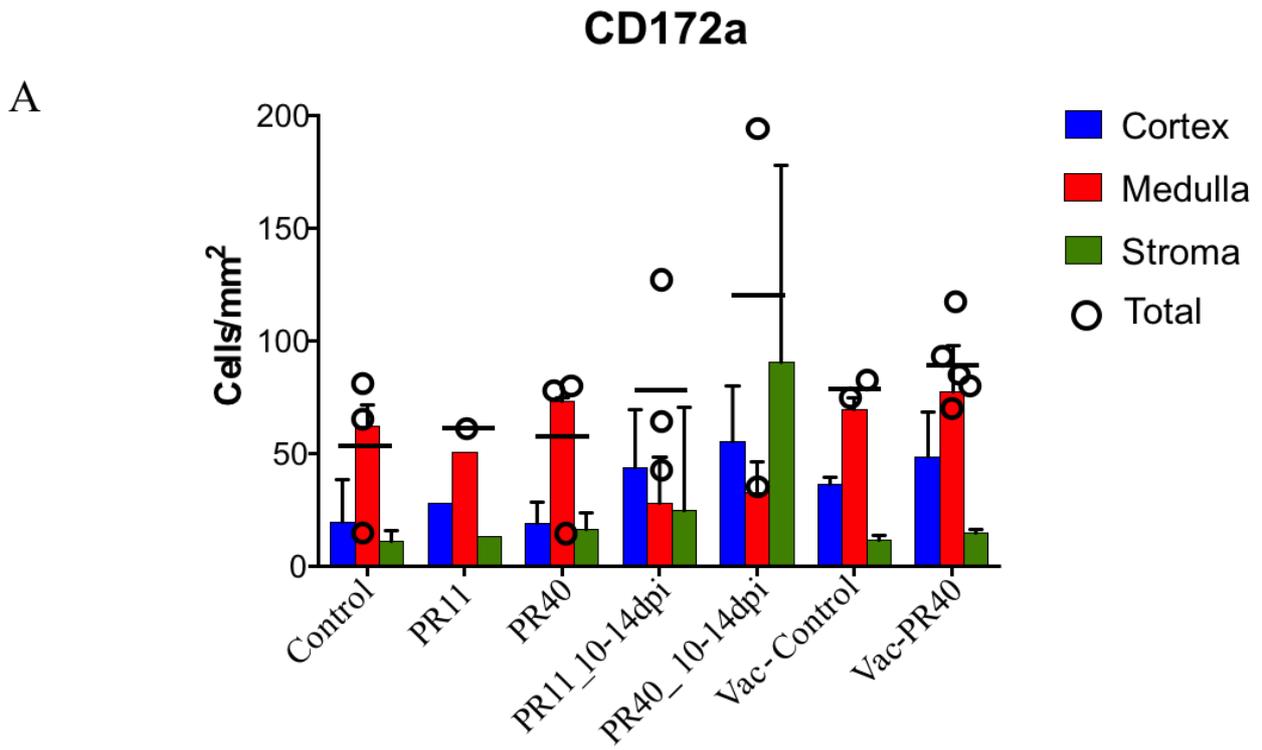


**Fig. 10.** A) Number of positive cells, performed by immunohistochemistry for PRRSV antigen. B) Representative immunohistochemistry of PRRSV antigen and.(C) TUNEL labelling. Bar 50  $\mu$ m.

## **CD172a**

Labelling against CD172a was mainly observed in the cell surface and cytoplasm of monocytes and macrophages as well as, in a lesser extent, in granulocytes (Fig. 11B). Tingible body macrophages did not stain for this marker. CD172a positive cells were more numerous in the thymic medulla than in the cortex and stroma of control animals. The thymus of the animals infected with PR11 and PR40 and killed at 35 dpi showed a similar distribution of CD172a positive cells than the control group (Fig. 11A). Interestingly, the expression of CD172a in PR11- and PR40-infected pigs that died at 10-14 dpi was dramatically different; specifically, these animals presented a major increase of positive cells in the stroma and minor in the cortex, together with a decrease of CD172a positive cells in the medulla (Fig. 11A). These changes were more pronounced in PR40 infected animals which presented a stunning increase in the number of CD172a positive cells in the stroma (Fig. 11A). In addition, a marked increase in the number of intravascular CD172a positive cells was observed within blood vessels of the cortex, medulla and stroma from both PR11 and PR40 infected animals dead at 10-14 dpi and from PR40 infected animals killed at 35 dpi (data not shown).

Both vaccinated groups (VAC-C and VAC-PR40) showed a similar dispersion of CD172a positive cells than control animals (CON), with a mild increase in the cortex and medulla of VAC-PR40 animals (Fig. 11A).

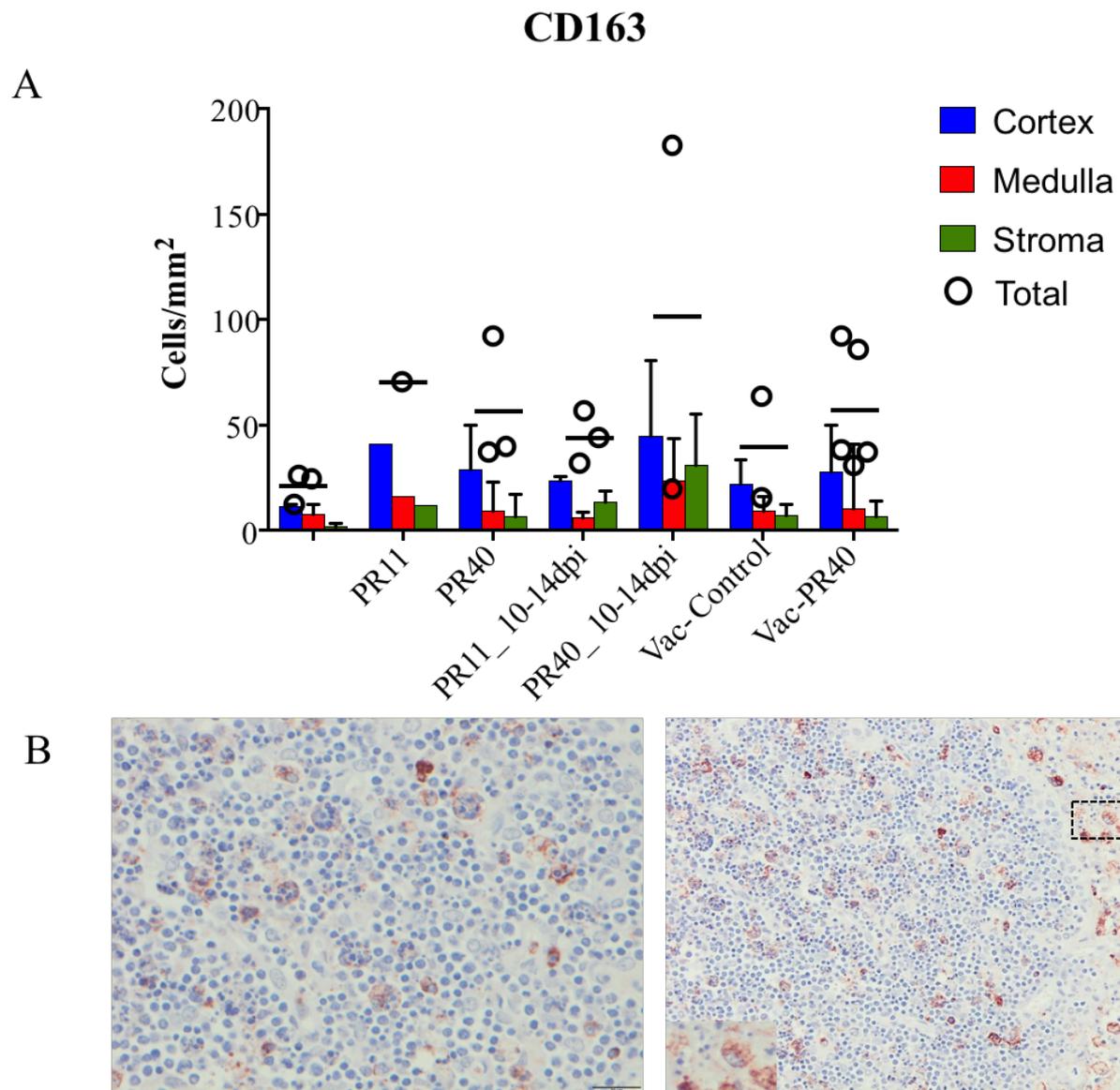


**Fig. 11.** A) Number of positive cells, performed by immunohistochemistry, for CD172a.  
 B) Representative immunohistochemistry of CD172a. Bar 100  $\mu$ m and 20  $\mu$ m.

## **CD163**

CD163 positive immunolabelling was visualized in the cytoplasm and cell surface of positive macrophages. Tingible body macrophages from the cortex were also stained against CD163 antibody (Fig. 12B). The major number of cells expressing CD163 was found in the thymic cortex for all groups. In the control group, the expression of CD163 was also detected in the medulla and, secondly, in the stroma. A general increase in the number of CD163 positive cells was observed in the cortex and in the stroma of infected animals; particularly, in PR40-infected pigs that died at 10-14 dpi, which showed an overall enhancement in the three compartments (cortex, medulla and stroma) together with a moderate increase in the frequency of intravascular CD163 positive cells in the cortex and the medulla (Fig. 12A).

No changes were observed in the distribution of CD163 positive cells in the thymus of VAC-C and VAC-PR40 animals (Fig. 12A).

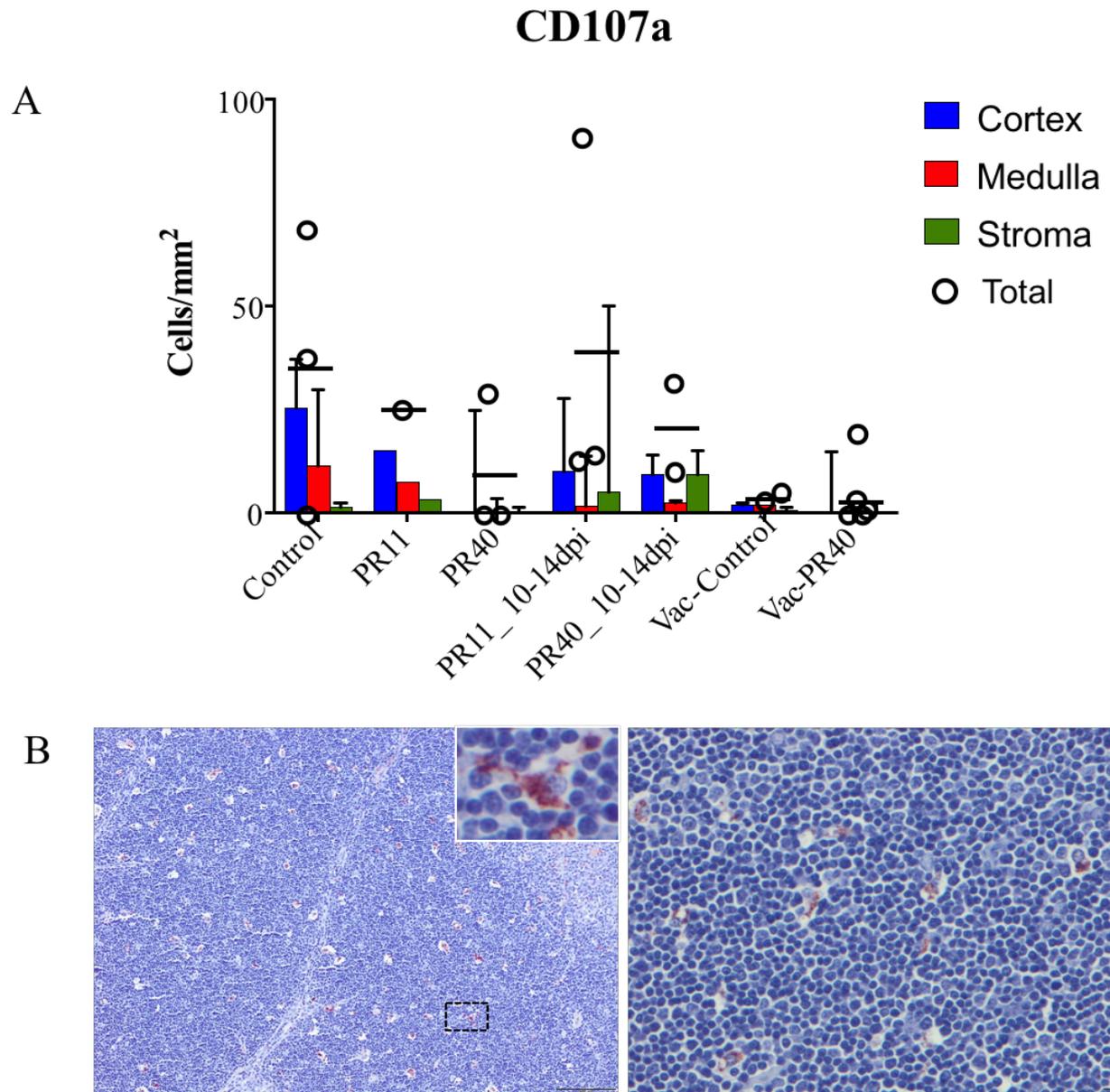


**Fig. 12.** A) Number of positive cells, performed by immunohistochemistry, for CD163. B) Representative immunohistochemistry of CD163. Bar 50  $\mu$ m and 20  $\mu$ m.

## **CD107a**

The staining for CD107a was mainly observed in the cytoplasm of macrophages, being also observed in tingible body macrophages from the cortex (Fig. 13B). The number of CD107a positive cells in all experimental groups, except for the control group (CON), was lower than the one detected for CD172a and CD163 positive cells (Fig. 13A). In control animals, the expression of CD107a was mainly found in the thymic cortex, with lower expression in the medulla and only few positive cells in the stroma. A general decrease in the number of CD107a positive cells was observed in all infected animals with only a moderate increase being observed in the stroma of PR11- and PR40-infected animals at 10-14 dpi (Fig. 113A).

Interestingly, vaccinated groups (VAC-C and VAC-PR40) presented a similar trend among them showing the lowest number of CD107a positive cells (Fig. 13A).



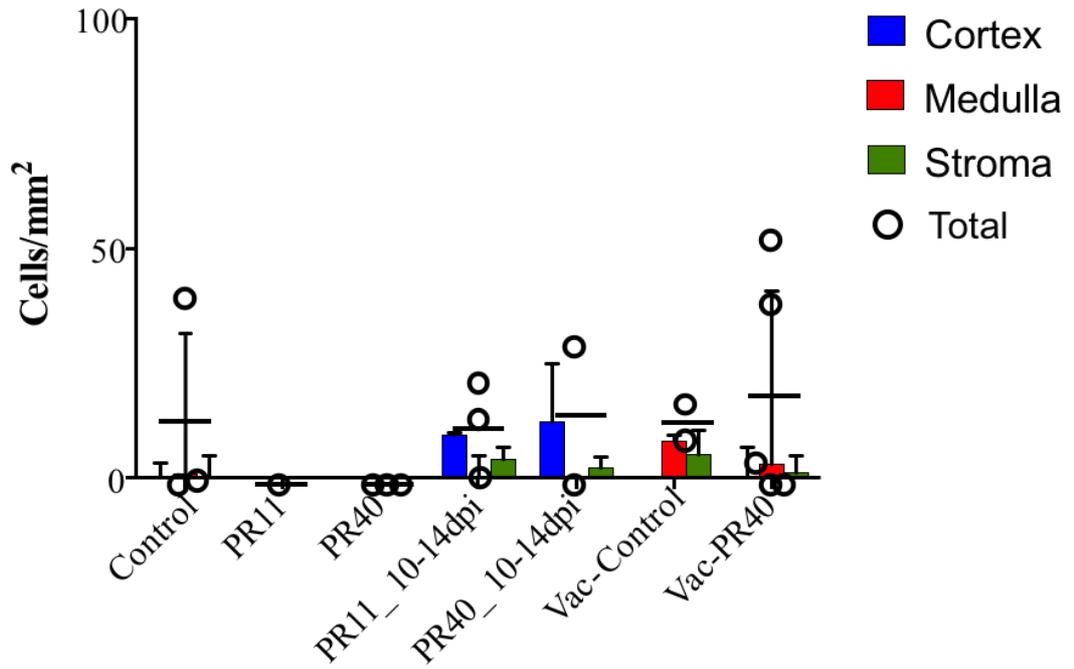
**Fig. 13.** A) Number of positive cells, performed by immunohistochemistry, for CD107a.  
 B) Representative immunohistochemistry of CD107a. Bar 100  $\mu$ m and 20  $\mu$ m.

## **BA4D5**

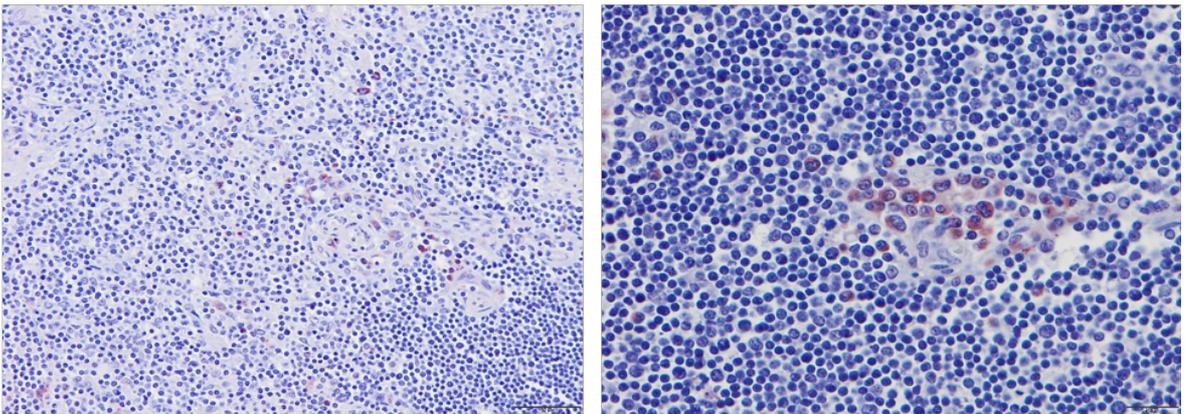
The staining for BA4D5 was very low in all experimental groups being observed in the cytoplasm of macrophages of cortex, medulla and stroma of the thymus (Fig. 14A). Perivascular positive cells were found in the thymic medulla of some animals, whereas intravascular positive cells were scattered (Fig. 11B). No changes were observed in PR11- and PR40-infected animals at 35 dpi when compared with control animals. The animals infected with PR11 and PR40 that died at 10-14 dpi showed an increase of BA4D5 positive cells in the cortex and in a lesser extent in the stroma (Fig. 14A). Vaccinated groups displayed an increase in the number of positive cells to this marker in the medulla and in the stroma, being more pronounced in the VAC-C group (Fig. 14A).

# BA4D5

A



B



**Fig. 14.** A) Number of positive cells, performed by immunohistochemistry, for BA4D5. B) Representative immunohistochemistry of BA4D5. Bar 50 µm and 20 µm.

# *Discussion*

This doctoral thesis mainly aimed at analyzing the response of the functionally macrophages to the infection due to different strains of Porcine Reproductive and Respiratory Syndrome Virus, both *in vivo* and *in vitro*.

### **In vitro studies**

Two *in vitro* study were performed by using two different models: monocytes derived macrophages (MDMs) and polarized porcine alveolar macrophages (polarized-PAMs). For the *in vivo* study, samples of thymus, obtained during an *in vivo* experimental infection, were analysed to characterize the macrophage populations.

Macrophages play a central role in the innate immune response as they carry out many defensive functions effective against the pathogens: antigen presentation (APC); killing and phagocytosis of the extracellular pathogens, phagocytosis of apoptotic cells and cell debris, synthesis and secretion of a wide pattern of cytokines. (Pearse et al., 2006a; Geissman et al., 2010). They are also involved in the control of inflammatory response and in promoting tissue repair.

PAMs represent the main target of the PRRSV *in vivo*, but viral replication can occur also in other macrophage subpopulations from lungs, as well as from lymphoid organs of infected animals (Duan et al.,1997; Gómez-Laguna et al.,2010; Barranco et al.,2012).

PRRSV is also able to infect and replicate in monocyte-derived cells as MØ and DC generated *in vitro* (Wang et al.,2007; Van Breedam et al.,2010). Moreover, Garcia-Nicolas et al. (2014) demonstrated that cytokines-primed MØ cultures are an efficient model to study the PRRSV infection and to evaluate the virulence of different strains. Classically activated M1, alternatively activated M2 and regulatory MØ (M2b) are the three distinct functional conditions proposed for the macrophages. M1 “classical” macrophages are involved in the inflammatory response by the production of pro-inflammatory cytokines and by inducing the activation of other innate cells including immature dendritic cells (iDC); conversely, M2 and M2b have more anti-inflammatory effect and promoting the tissue repair.

By using MDMs as a model, the *in vitro* characteristics of PRRSV isolates belonging to different

subtypes within PRRSV-1, and with different *in vivo* pathogenicity, were evaluated, through assessment of infection capacity, replication and cytokine production. It has already been demonstrated that IFN $\gamma$  and IFN $\beta$  are able to determine an antiviral status, depending on the virulence of the strain employed, and that IL-4-M $\emptyset$  does not interfere with the viral replication (Garcia-Nicolas et al.,2014). Moreover, another study showed that the presence of IFN- $\alpha$  before or during the infection, determines a significant difference in the promotion of viral clearance and adaptive immune responses (Brockmeier et al.,2009, 2012). In the present study, the effect of interferon treatment was marked with Lelystad strain and the Italian low pathogenic strain PR11, while the more pathogenic viruses ILI6, Lena, PR40 and BOR59 were relatively resistant. The greatest capacity of IFN $\beta$  to determine an antiviral status was confirmed.

Moreover, this study tried also to verify if the *in vivo* evidence founded with the comparison of the strains used, particularly among Lena, ILI6 and BOR59 (Stadejek et al.,2017), and between the two Italian strains PR11 and PR40 (Canelli et al.,2017) could be also evident in the *in vitro* model.

Stadejeck et al. (2017) described BOR59 as a strain with a higher virulence compared with ILI6, described indeed as a less pathogenic strain. The *in vitro* behaviour of these two strains did not statistically differ from each other. BOR59 was similar in the infectivity ability on MDMs to Lena, underling the hypothesis already proposed, of a similar pathogenicity of these two strains. Moreover, BOR59 consistently released less infectious particles. It would be interesting to investigate whether the growth is simply delayed, or if BOR59 compensates this shortcoming with other mechanisms.

Currently, no study has compared the *in vivo* virulence of Lena and the PR40, but the *in vitro* comparison showed that the PR40 infectivity was inferior to Lena, and also to BOR59. Interesting, PR40 growth ability was similar to Lena and this evidence was particularly accentuated with IFN $\beta$  M $\emptyset$ .

PRRSV infection and persistence are related to a dysregulation of the immune response to the virus; one mechanism of that is the subversion of cytokine production by M $\emptyset$  and other immune cell effectors (Beura et al.,2010; Chand et al.,2012). Cytokines play an important role in the efficient onset

of the immune response during viral infection and in the present work the production of four main cytokines such as IFN $\alpha$ , IL-1 $\beta$ , IL-10 and TNF, produced by macrophage, was tested.

Data regarding the modulation of the cytokines production by PRRSV infection are controversial.

None of the PRRSV-1 isolates tested induced production of IFN $\alpha$  or IL-10.

Many *in vivo* studies reported the production of IFN $\alpha$  during PRRSV infection (Gómez-Laguna et al.,2010; Barranco et al.,2012; Garcia-Nicolàs et al.,2014), and that different strains showed different capacity to affect IFN- $\alpha$  pathway (Lee et al.,2004; Lunney et al.,2016). The inhibition of IFN- $\alpha$  may be mediated by post-transcriptional mechanisms (Lee et al.,2004), and it also maybe correlated with a significant inhibition of NK cell activity (Lee et al.,2000; Lunney et al.,2016).

Another mechanism for PRRSV persistence, may be the interference with the immune response cells through the induction of immunosuppressive cytokines like IL-10, by the NF- $\kappa$ B inhibition (Johnson et al.,1999).

Similarly, previous studies, using different viral strains, have reported that PRRSV infection may induce or block IL-10 production in dendritic cells, PAMs or CD172a<sup>+</sup> (Charemtantanakul et al.,2006; Gimeno et al.,2011; Wang et al.,2007, Amarilla et al.,2015).

Interestingly, in the present study, a statistically significant production of TNF and IL-1 $\beta$  was observed following *in vitro* infection with the Italian strain PR40, when compared to the other viruses. The IL-1 $\beta$  function during PRRS infection is not totally understood, but some studies reported IL-1 $\beta$  expression both *in vivo* e *in vitro* (Thanawongnuwech et al.,2004; Qiao et al.,2011; Lunney et al.,2010; Borghetti et al.,2011), and a low IL-1 $\beta$ -gene expression also in the early phase of the infection (Darwich et al.,2010).

In the same way, other studies have reported both an increase and a suppression of TNF during PRRSV infection. In some cases, it has been detected a TNF *in vivo* and *in vitro* increase during the infection (Borghetti et al.,2011; Darwich et al.,2010), while, it has been also shown that PRRSV is able to suppress the *in vivo* and *in vitro* production of this cytokine (Lopez-Fuertes et al.,2000; Van Reeth et al.,1999; Choi and Chae 2002), and it has been reported that the non-structural protein could

be play an important role in the TNF suppression (Chen et al., 2010; He et al.,2015).

A further *in vivo* study showed that HP-PRRSV-2 infection resulted in the expression of TNF, IL-1 $\beta$ , IL-6, and IL-10, with an inflammatory response within the first week of infection (Fan B et al.,2016).

The high production of TNF described here for the PR40 strain may be related to the genetics of this virus. It has been shown that the non-structural protein 2 (nsp2) interferes with the TNF pathway (Wang, et al.,2014). The genome analysis of PR40 shows two amino acidic deletions (one large of 121 aa) in the sequence coding for the non-structural protein 2 (nsp2) (Canelli et al.,2017). Further analyses would be necessary in order to evaluate the specific association between amino acidic deletions in PR40, the effect on the TNF pathway and its correlation with the pathological lung lesions observed after infection (Canelli 2017).

The induction of cytokine production may also explain a particular/uncommon *in vivo* behaviour of HP-PRRSV infection. PR40 induces high mortality and fever, severe inflammation and thymus atrophy, leads to T-cell depletion, and a low-level neutralizing antibody response (Canelli et al.,2017); ongoing *in vivo* studies would suggest the involvement of TNF during the early stage of infection (unpublished data).

In conclusion, the results confirm that MDMs represent an adequate and useful model for *in vitro* analyses of PRRSV isolates with different pathogenicity (García-Nicolás et al.,2014), in particular data obtained indicate that strain with a major *in vivo* pathogenicity showed a certain *in vitro* resistance to the IFN treatment. Additionally, obtained results underline that different PRRSV isolates induce differential patterns of cytokine release (Gimeno et al.,2011), and suggest that these different patterns may be associated both with genetics and with uncommon *in vivo* pathogenic mechanisms of HP-PRRSV stains.

Beyond investigating the *in vitro* infection of PRRSV, another *in vitro* model: polarized porcine alveolar macrophages, was used.

Polarized PAMs are lung tissue-resident and antigen-presenting cells that play an important role in the host defense.

The respiratory distress, in the acute phase of the PRRS infection, is characterized by virus replication in the lung that determines interstitial pneumonia, release of inflammatory cytokines (Chand et al., 2012), and a quantitative and functional decrease of PAMs and pulmonary intravascular MØ (Chand et al., 2012; Halbur et al., 1995a; Molitor et al., 1996).

PAMs polarization was performed as for the MDMs. M1 and M2 phenotypes were obtained by using IFN $\gamma$  and IL-4 respectively, while IFN $\beta$  was used to test its antiviral effect.

This in vitro model was used to compare the infection with two PRRSV-1.1 Italian strains, particularly the PR11 strain, considered as a “conventional virus” and the HP-PRRSV PR40.

Interestingly, the levels of N protein detected by cytometry analysis did not show a difference between the two strains tested with all the treatments.

Despite this result, it was observed that PRRSV is sensitive to the action of type I interferon (Sang et al., 2010; Garcia-Nicolas et al., 2014) and also to IFN $\gamma$  (Rowland et al., 2001; Wang et al., 2014). In the present study an increase of the infected cells treated with this cytokine was not observed, but the percentage of N protein was similar to Mock and IL-4 treated PAMs. On the other hand, the growth of both viruses was totally blocked by IFN $\beta$ , confirming the higher antiviral action of this cytokine.

The production of IFN $\alpha$ , IL-10 and TNF was also tested. Both strains, PR11 and PR40, did not induce the production of these cytokines. These results confirmed the controversial differences in the cytokines modulation induced by PRRSV infection.

A recent study showed that polarized macrophages (M1 and M2) present different susceptibility when infected with different PRRSV-2 strains (Wang et al., 2017). This evidence is not in accordance with results obtained in the present study, nevertheless, unpublished data demonstrated a re-polarization from the M0-M1 to M2 phenotype, after the HP-PRRSV infection (Wang et al., 2017).

This evidence could represent an explanation for obtained results, in which no differences were found in the infected M1 and M2 polarized-PAMs.

## **In vivo study**

This study evaluated the effect of an *in vivo* experimental infection by two Italian PRRSV-1 subtype 1 strains (PR40/2014 and PR11/2014) (Canelli et al.,2017) on the macrophages population of the thymus considering that this primary lymphoid organ is the target of PRRSV infection and replication and that it triggers off the host immune response. In addition, the effect in PRRSV infected animals was compared to the effect in uninfected control animal and in infected animals previously vaccinated with heterologous PRRSV vaccine.

The thymic lesions were analysed by using histopathology and immunohistochemistry techniques, and the macrophages population were characterized by targeting specific markers.

Despite the main cell target for the viral replication are PAMs, PRRSV replication has also been reported in the macrophages of the lymphoid organs of infected animals like the thymus (Duan et al., 1997; Gómez-Laguna et al.,2010; Barranco et al.,2012).

This organ plays an important role in development and the functional efficiency of the immune system through the differentiation and maturation of T cells (Pearse et al.,2006b), and PRRS infection may have a negative impact in thymic cellularity (Guo et al.,2013).

Thymus atrophy, with a wide decrease in the numbers of the cortex thymocytes, T cell depletion and impairment of the development of naïve T cells (Butler et al.,2014) are the consequence to PRRSV infection. The intensity of these thymus lesions is dependant to the virulence of the strain (Amarilla et al.,2016), with HP-PRRSV strains determining more severe damages.

In order to described the macrophages population of the thymus, the CD172a, CD163, CD107a and BA4D5 immunolabelling was analyzed.

These markers have been previously used in many studies to characterize porcine tissue macrophages (Bullido et al.,1997; Domenech et al.,2003; Pérez et al.,2008).

A restricted marker to monocyte and macrophages is CD163, a member of the family of proteins with scavenger receptor cysteine-rich domains (Law et al.,1993). CD163, identified as a major receptor for PRRSV internalization (reviewed in Van Breedam et al.,2010), could also contribute, during the

PRRSV infection, to the expression of the anti-inflammatory cytokine IL-10 (Philippidis et al., 2004). CD172a is one of the markers most commonly used and identifies myeloid cells from precursor stages until cellular differentiation (Summerfield et al., 1997).

Moreover, CD107a, or lysosomal-associated membrane protein 1 (LAMP-1), despite not restricted to macrophages, has been demonstrated to be useful for identifying macrophages populations in tissue sections (Bullido et al., 1997; Domenech et al., 2003).

Another interesting marker is BA4D5, that is thought to be specific for porcine CD68, which is mainly expressed by cells from the monocyte lineage, by circulating macrophages and by tissue macrophages (Taylor et al., 2005). Among other functions CD68 plays a role in the cytotoxic activity, with a predominant intracellular location in phagolysosomes (Kurushima et al., 2000); phagocytic activity, associated to the scavenger receptor family and promoting cellular debris clearance (Taylor et al., 2005) and mediating the recruitment and activation of macrophages through binding to specific lectins and selectins (Song et al., 2011).

Thymic atrophy was observed in both PR11- and PR40-infected animals with more intense changes in animals dead at 10-14 dpi. Microscopically no differences were observed among both infected groups, which presented disappearance of the cortico-medullary boundary, extensive cell death phenomena in the cortex and a stunning oedema and interstitial infiltration of the stroma at 10-14 dpi. Nevertheless, due to this damage to the structure, it was not possible to make the histomorphometric analysis by the count of the tangible body macrophages and the TUNEL positive cells. TUNEL labelling has been already used for measuring PRRSV-induced cell death (He et al., 2012; Amarilla et al., 2016). For the other subjects, the labelling was mainly found in the tangible body macrophages of the cortex, but a remarkable increase of positive cells was observed in the animal infected PR11 and PR40 at 10-14 dpi. The severity of cell death phenomena in these animals, together with the number and location of PRRSV-positive cells support the role of both direct and indirect induction of cell death by PRRSV (revised in Rodríguez-Gómez et al., 2013).

The remarkable inflammatory reaction observed in the stroma of the thymus at 10-14dpi was

associated with a high number of PRRSV positive cells, both in the cortex and in the stroma of the thymus. These findings could be explained with an occurred active replication and dissemination of the viruses, suggested also by peaks of viremia at 10 dpi in the PR11 group, and 7 dpi in the PR40 group, observed in the comparative experimental infection performed by Canelli et al. (2017).

The main observed result was the increase of the staining for all the macrophages marker tested in the animals infected with both PR11 and PR40, particularly in the animals dead at 10-14 dpi. The infected animals present an increase of CD163, particularly in the ones PR40-infected and dead at 10-14 dpi, with a general increase of the labelling in all the compartments, blood vessels included.

The immunolabeling with CD172a increased during the infection and an interesting difference of the compartments distribution in the animal infected with both viruses, and dead at 10-14 dpi was detected. In-fact these animals showed a higher CD172a positive cells in the stroma instead of the cortex, as was detected in the other group and additionally, the presence of positive cells in the blood vessels was observed.

The increase in the number of CD172a and CD163 positive cells observed in both infected groups at 10-14 dpi was associated with the marked inflammatory infiltrate of the stroma of the thymus as well as with the extensive cell death of cortical thymocytes. Thus, monocytes/macrophages may be migrating from the bloodstream and other tissues to the thymus through chemotaxis from inflammatory foci as well as from a high demand of phagocytosis of cell death debris in the thymic cortex. The identification of intravascular CD172a and CD163 positive cells observed in the present study supports this hypothesis. Furthermore, the increase in the number of CD163 positive cells may also get along with the induction of this surface molecule in resident tissue macrophages from the thymus.

CD107a immunolabeling was mainly found in the control group, while in the infected animals a general decrease of positive cells was observed, with the only exception of the infected animals (of both groups) dead before the end of the experiment.

In comparison with the others markers, the cytoplasmatic marker BA4D5, was less expressed; but, also in this case the animal infected with PR11 and PR40 (10-14dpi) showed an increase of positive cells in the cortex and in a lesser extended in the stroma.

BA4D5 positive cells highlight different mechanism of regulation of the cytotoxic activity not only in infected pigs but also in vaccinated animals, which points out potential mechanisms involved in the modulation of the host immune response.

The thymic macrophages population of pigs vaccinated with MLV PRRSV-1 uninfected and infected was also evaluated.

The uninfected group did not show any remarkable differences in comparison with vaccinated infected pigs. The labelling with CD172a showed a mild increase of positive cells in the vaccinated infected group; with the markers CD163 and CD107a, the same behaviour of the control group was observed. While, BA4D5 was the only labelling that showed an increase of positive cells in both medulla and stroma, particularly in the vaccinated-control group.

The main evidence observed in the present study was the presence of severe lesions in the thymus of the animals infected with PRRSV strains PR11 and PR40 and dead at 10-14 dpi, and the higher number of macrophages in all the compartments of the thymus gland.

The different markers used in this study allow us identifying the recruitment of macrophages associated to the strong and early inflammatory response in the stroma of the thymus, the increase in the expression of the major receptor of PRRSV and the regulation of the host cytotoxic activity by macrophages.

In the animals survived until the end of the experiment, it was observed a little variation of the macrophages population in the thymus infected with HP-PRRSV strain, on the contrary a markedly reduction of T cells population has been already demonstrated (Canelli et al.,2017). Remarkably, no significant differences were observed between the low virulent (PR11) and highly pathogenic (PR40) PRRSV strains used in this study.

It has been demonstrated how PRRSV infection determines a dysregulation of the immune state in the host by altering the expression of molecules involved in the antigen presentation (Charerntantanakul et al.,2006), and that the APCs infection determined a negative effect on the development of naive T cells (Butler et al.,2014).

The results showed that this alteration of the macrophages population during the early phases of the infection could strongly influence the decrease of the T population.

Based on the studies presented, it can be confirmed the importance of the interaction between the virus and the macrophages population to better understand the complex pathogenicity of PRRSV infection. Moreover, the macrophages polarization, particularly the antiviral effect determined by Interferon, could be a base for the development of strategies able to contrast PRRSV infection.

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*“Se non credi in te stesso, chi ci crederà?”*