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Pulmonary pathology of PRRS

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Abbreviations

ACD	Accidental cell death	MOMP	Mitochondrial membrane permeabilization
ADWG	Average daily weight gain	mRNA	Messenger RNA
APAF-1	Apoptotic protease-activating factor-1	NCCD	Nomenclature Committee on Cell Death
ASFV	African swine fever virus	NK	Natural killer cells
BAL	Bronchoalveolar lavage	OIE	World Organisation for Animal Health
BMDC	Bone marrow-derived dendritic cells	ORFs	Open reading frames
CASP	Caspase	P53	Tumor protein 53
CD95/Fas	Pre-ligand-associated transmembrane receptor	PAMs	Pulmonary alveolar macrophages
DCs	Dendritic cells	PBMCs	Porcine blood mononuclear cells
DGE	Digital gene expression tag profiling	PCD	Programmed cell death
DISC	Death-inducing signaling complex	pi	Post infection
DNA	Deoxyribonucleic acid	PIMs	Pulmonary intravascular macrophages
dpi	Days post infection	PRDC	Porcine Respiratory Disease Complex
EAV	Equine arteritis virus	PRRS	Porcine reproductive and respiratory syndrome
ELISA	Enzyme-linked immunoassay	PRRSV	Porcine reproductive and respiratory syndrome virus
FasL	Fas Ligand	RCD	Regulated cell death
HE/H&E	Hematoxylin-eosin stain	RNA	Ribonucleic acid
ICAM-1	Intercellular Adhesion Molecule 1	RT-PCR	Reverse transcription polymerase chain reaction
IFA	Indirect immunofluorescence assay	SHFH	Simian hemorrhagic fever virus
IFN-α	Interferon alfa	SWC3+	Specific myelomonocytic antigen
IFN-γ	Interferon gamma	TGF-β	Transforming growth factor beta
IL	Interleukin	TNFR	Tumor necrosis factor receptor
JNK	c-Jun N-terminal kinase pathway	TNF-α	Tumor necrosis factor alpha
LDV	Lactate-dehydrogenase elevating virus	TTF-1	Thyroid transcription factor-1
MA-104	Embryonic Rhesus Monkey Kidney Cells	TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
MARC-145	Subclone of African green monkey kidney-derived MA-104 cells	UPR	Unfolded protein response

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a disease globally affecting swine herds. This viral infection is triumphing on top of the lists accounting for disease-related costs annually in pig producing farms, affecting both breeding and fattening pig facilities. The disease has been around since its discovery in the 1980s, and new strains of higher virulence leading to more severe clinical signs and disease in production herds have been identified. This persists to be a major problem for large scale producing pig farms, where production parameters are affected as well as mortality seen depending on the isolate causing the infection. The pathogenesis of this viral disease is still not fully understood and is under continuous investigation by researchers. The immunomodulatory abilities of the virus after infection is an interesting topic, and the search for answers to how the virus interacts with the host is of major importance in the future prevention of disease. The involvement of cytokines and their importance during PRRSV have been devoted more research in the previous years. Pro- and anti-inflammatory cytokines are playing important roles in the regulation of immune responses following viral disease. Cytokines are key components in several other respiratory disease conditions, and dysregulation of expression and production of these cytokines have been suggested to be a part of the viral pathogenesis of PRRSV. Regulated cell death in form of both apoptosis and necrosis are frequently reported in the lung following infection with PRRSV and research in this area is trying to understand how and why this happens. Histopathological description of lung lesions following infection report similar characteristic findings but vary in some respects depending on the genotype and strain causing the disease. The aim of this review is to try and answer the following questions focusing on pulmonary pathology and shed light on the research carried out on these specific areas concerning PRRSV infection.

- What changes can be seen in the production of cytokines, specifically IL-10, TGF-β and TNF-α in response to PRRSV infection?
- Can PRRSV induce pro- and anti-apoptotic pathways in the infected cell?
- What pulmonary cellular damages are seen during PRRSV infection?

2. Literature review

2.1 PRRSV- Porcine reproductive and respiratory syndrome virus

PRRSV is a disease affecting swine populations worldwide that accounts for major disease-related economic losses in today's pork production industry (Neumann et al., 2005). In Europe, the first cases of this viral disease were detected in The Netherlands and Germany in 1991 affecting breeding sows and their piglets, as well as fattening pigs (Beyer et al., 2000; Wensvoort et al., 1991).

The causative agent is an enveloped single stranded non- segmented positive-sense RNA virus, that contain at least 10 open reading frames (ORFs) (Benfield et al., 1992; Johnson et al., 2011; Lunney et al., 2016; Meulenberg and Snijder, 1998; Snijder, 1998). It belongs to the order Nidovirales, family Arteriviridae and genus Betaarterivirus (Gorbalenya et al., 2018). Within the same family of viruses as PRRSV, we find lactate-dehydrogenase elevating virus (LDV), simian hemorrhagic fever virus (SHFV) and equine arteritis virus (EAV) among others (Payne, 2017).

There are two species responsible for PRRS, *Betaarterivirus suid* 1 (PRSSV-1) and *Betaarterivirus suid* 2 (PRRSV-2). These two species share approximately 55–70 % of the same nucleotide sequences (Chen et al., 2011; Forsberg et al., 2002; Gorbalenya et al., 2018). Previously PRSSV-1 was restricted to Europe and PRRSV-2 was found in North America and Asia, but recent studies show the coexistence of the two viral strains on all three continents (Amonsin et al., 2009; Balka et al., 2008; Bøtner et al., 1997; Dewey et al., 2000; Fang et al., 2007; Kim et al., 2009; Ropp et al., 2004; Thanawongnuwech, 2004; van Vugt et al., 2001).

Figure 1: Map showing the current prevalence of PRRSV based OIE reported cases, green; free, yellow; suspected, red; present, gray; no report available or no reported cases (map: https://wahis.oie.int/#/dashboards/country-or-disease-dashboard)

PRRSV, as the name suggests results in either disease of the reproductive or respiratory system in swine. The infection manifests in reproductive disease leading to abortion and birth of weak and stillborn piglets by pregnant gilts and sows (Cheon and Chae, 2000). The respiratory form of the disease can affect pigs of all age groups, but clinical signs and course of the disease are usually more severe in young piglets. Infection of the respiratory system commonly results in dyspnea, labored breathing and characteristic interstitial pneumonia (Cheon et al., 1997; Cheon and Chae, 1999; Halbur et al., 1996).

Replication of PRRSV happens primarily in pulmonary alveolar macrophages (PAMs), but also in other cells of the macrophage and monocyte lineage in the natural host Sus scrofa (Duan et al., 1997a; Gómez-Laguna et al., 2010; Su et al., 2021). *In vitro* the virus can infect primary cell cultures such as monocyte-derived macrophages, monocyte-derived dendritic cells as well as PAMs. Other than this infection of different cell lines are restricted to some monkey kidney derived cells such as MA-104 and its subclone MARC-145 cells (Charerntantanakul et al., 2006; Kim et al., 1993; Song et al., 2018; Wang et al., 2007; Wensvoort et al., 1991). In the bronchoalveolar tract, PRRSV induces an inflammatory

response that will affect alveolar macrophages at an early stage, this is due to them playing a vital role in the first line of defense against invading pathogens. Alveolar macrophages are able to carry out antigen-specific immune responses, because of this they will be the first cells to be affected by the intruding viral agent (Cheon et al., 1997; Cheon and Chae, 1999; Halbur et al., 1996). A study carried out by Chung and Chae (2003) on porcine lung tissue done by *in situ* hybridization showed that PRRSV nucleic acid was found to be exclusively detected in the cytoplasm of interstitial macrophages. The study was carried out on lung tissue originating from piglets where the PRRSV nucleic acid was detected from day 1 to 28 post-infection. Findings of another study report that PRRSV has even been found to persist as late as day 35 post-infection in alveolar macrophages. They concluded that the extent of the viraemia positively correlates with the virus titer found in the lungs and alveolar macrophages (Duan et al., 1997b). Beyer et al. (2000) concluded that their results showed that the lung macrophages are infected at an early stage of the disease, and that it appears the spread of the virus is happening at a lower rate in the lung tissue reaching a peak between days 14–28 post-infection.

The natural course of infection or oronasal inoculation results in a quickly developing viraemia in infected pigs. It is suggested that this is due to the replication of the virus in subepithelial macrophages found in nasal and pharyngeal mucosa (Beyer et al., 2000). It is previously established that the main replication of PRRSV during acute infection finds place in the lymphoid tissues and lung macrophages (Duan et al., 1997b). The formation of characteristic lung lesions is believed to be caused by a small number of macrophages infected during the viraemia. In the days following early infection more alveolar macrophages are likely infected by the continuous viraemia and locally, by means of other nearby infected macrophages releasing the virus. Multifocal spread of the infection in the lung supports this theory. The infection can also progress in the lungs by direct spread via the respiratory route (Beyer et al., 2000). Table 1 summarizes the 3 typical phases seen in a classical acute outbreak of PRRSV.

Clinical signs	Duration
Reduced food intake, fever, flu-like symptoms. Spread of the diseases happens quickly in the herd during this phase	1–3 weeks
Late-stage abortions, premature birth, birth of mummified-, stillborn or weak piglets, dysgalactia and increased mortality of newborn piglets. Cyanosis can sometimes be seen in sows on ears, ventral abdomen, and legs. Weaners and growers experience respiratory symptoms such as coughing, dyspnea, reduced ADWG and increased mortality.	Following 1–4 months
Normalization of reproduction and reduction in mortality. Infection can become persistent and can lead to immunosuppression that can result in secondary bacterial infections by ex. Pasturella multocida, actinobacillus pleuropneumonia ect. and lead to the development of PRDC.	Following 2–5 months

Table 1: (Sundell and Haukaas, 2020)

2.2 Cytokine involvement in viral pulmonary infection

Cytokines are important contributors to the regulation of immune responses in viral diseases. They are small proteins that mediate cell to cell communication essential for the coordination and activation of the different parts of the immune system (Rigbe, 2020). Macrophages and T-helper cells are the main cells producing these cytokines, but they can also be produced by other cell populations in the organism. This happens in reaction to stimuli generated by viral replication in the cells that induce cytokine gene expression, or the presence of virus or bacteria interacting with the cell membrane or cytoplasm of cells that result in gene activation and expression that triggers cytokine production (Van Reeth and Nauwynck, 2000). Cytokines produced and released during infection are grouped in various ways, for instance according to a kinetic or functional role as early or late cytokines, proinflammatory or anti-inflammatory as well as innate or adaptive (Mandal, 2019; Van Reeth and Nauwynck, 2000; Zhang and An, 2007).

Among the early cytokines we find interferon-alpha (IFN-α), interleukin-1 (IL-1), tumour necrosis factor-alpha (TNF-α), IL-6 and IL-8. Non-immune cells produce these cytokines at the site of infection and they generate a local inflammatory response (Van Reeth and Nauwynck, 2000). Late cytokines are a result of the recognition of antigens on the surface of antigen-presenting cells and are produced by T-cells. These cytokines are essential in the coordination and regulation of the adaptive immune response and consist of cytokines such

as IL-10, IL-2, IL-4, IL-5 and IFN-γ (Abbas and Lichtman, 2003, as cited in Rigbe, 2020; Van Reeth and Nauwynck, 2000).

It has been found that proinflammatory cytokines remain mostly at the site of production. In pulmonary infections production of cytokines takes place in the lung, the plasma concentration of cytokines have been found to be low or even undetectable, suggesting that cytokines produced during early infection in the lung only cross the blood-alveolar barrier in very small amounts to enter the circulation (Baarsch et al., 1995; Nelson et al., 1989). At the level of the lungs, the most potent effect exerted by the early cytokines are on the phagocytic cells (Van Reeth and Nauwynck, 2000).

TNF-α is a proinflammatory cytokine that recruits neutrophils and macrophages to the site of early infection and it plays an important role in acute inflammation. TNF-α and IL-1 stimulate the production of chemotactic cytokines that brings neutrophils to the site of infection by migration and they upregulate the expression of ICAM-1, E-selectin and P-selectin on vascular endothelium aiding in the extravasation of neutrophils (Strieter et al., 1993; Van Reeth and Nauwynck, 2000; Zhang and An, 2007).

IL-1 and TNF-α may have a stimulating effect on the function of neutrophils. Studies conducted with TNF-α added to porcine neutrophils showed degranulation, an increase in phagocytosis and respiratory burst activity (Coe et al., 1993; Van Reeth and Nauwynck, 2000). Non-specific antiviral effects are seen from TNF-α, IFN-α and IL-1. All of them are pyrogenic, and have been seen to cause fever when injected intramuscularly in rabbits (Dinarello et al., 1986; Van Reeth and Nauwynck, 2000). Catabolic effects on muscle cells and body reserves of fat are seen in chronic exposure to TNF-α. Clinically this is seen as wasting and cachexia. Calves exposed to prolonged treatment with TNF-α showed a decrease in body fat deposits (Ohmann et al., 1989).

The response of proinflammatory cytokines is controlled by the anti-inflammatory cytokines that act immunoregulatory. The immune response is carried out according to the signals generated by cytokines, specific cytokines inhibitors and their receptors (Zhang and An, 2007). IL-4, IL-10, IL-11, IL-13 and TGF-β are all important anti-inflammatory cytokines. IL-10 is a cytokine with a potent anti-inflammatory effect. It suppresses the expression of IL-1, IL-6 and TNF- α that are activated by macrophages and act as mediators of inflammation. IL-10 is also capable of down-regulating proinflammatory-, as well as up-

regulating anti-cytokine receptors within the organism. This gives IL-10 the opportunity to regulate proinflammatory cytokine response on many different levels (Zhang and An, 2007).

IL-10 as a cytokine of the adaptive immune system plays an important role in the limitation and termination of inflammatory responses. Furthermore, it inhibits the activation of T-cells, macrophages and monocytes and regulates the growth and differentiation of B-cells, natural killer cells (NK), cytotoxic- and helper T-cells among others (Moore et al., 2001). Inhibitory effects are seen on TNF production and this appears crucial for the anti-inflammatory activities carried out by IL-10, as these two work synergistically on inflammatory processes and pathways (Moore et al., 2001). A study conducted on mice showed that increased levels of IL-10 leads to impaired T-cell response (Brooks et al., 2006).

IL-10 acts to limit the damage of tissues caused by innate and adaptive immune cells during the process of inflammation. It is increasing evidence suggesting that viruses use this immunoregulatory mechanism carried out by IL-10 to suppress and create a tolerance to promote viral replication and survival in the host (Rojas et al., 2017; Suradhat et al., 2003). IL-10 administration iv. in pigs suffering from experimentally induced myocardial infarct showed a decrease in post-infarct inflammatory response, less extensive oedema and inflammatory cell infiltration of the injured tissues (Sendra et al., 2019).

TGF-β can act as a pro- or anti-inflammatory cytokine and is produced by platelets, neutrophils, macrophages and B- and T-cells. The cytokine has 3 isoforms, but TGF-β1 is the one predominantly involved with the immune system and possess similar immunoregulatory functions as IL-10 (Gómez-Laguna et al., 2012; Renukaradhya et al., 2010; Silva-Campa et al., 2009). Its activity as a promoter or inhibitor of inflammation vary and depend on the circumstances (Tizard, 2012 p. 521; Zhang and An, 2007). It is activated upon binding to integrins on the cell surface and act on macrophages, neutrophils, B- and T-cells as well as dendritic cells. The main activity of TGF-β is the regulation of macrophages, it can both simulate and inhibit, this is dependent on the activity of other cytokines. TGF-β has an effect on B- and T-cell activation and function by inhibiting proliferation and inducing apoptosis. It also exerts effect on macrophages in the regulation of integrin expression, phagocytosis and cytotoxicity, where the latter is reduced, and the others increased by TGF-β activation. It has an immunosuppressive effect, like IL-10 and aid in the regulation of cell division (Tizard, 2012 p. 218 and 521). Intramuscular injection of TGF-β in rats, by means

of adenovirus-mediated gene transfer in recipients, reduce the rate of acute lung rejection after transplantation (Suda et al., 2001).

2.2.1 TNF-α detection in the lungs of PRRSV infected pigs

Recently more studies are being carried out on the response of the immune system, especially the cytokine involvement during viral infection. TNF-α as mentioned previously has been studied and the detection, quantification, and role in PRRSV infection are of interest to better understand the defense mechanisms involved during early infection with PRRSV.

López-Fuertes et al. (2000) did a study on porcine alveolar macrophages (PAM) collected by means of alveolar lavage from 3-week-old healthy piglets. PAM incubated with PRRSV showed a decrease in the expression of TNF- α mRNA by use of RT-PCR. They also saw a slight and reproducible reduction in viral replication when recombinant porcine TNF-α was added to PAM cultures. Based on these findings they concluded that PRRSV-infected macrophages showed a reduced capacity to produce proinflammatory cytokines. A similar anti-viral response produced by the virus on PAM was also seen in another study conducted by RT-PCR (Zhang et al., 2012). Both concluded based on their results that TNF-α response was weak as a result of PRRSV infection (López-Fuertes et al., 2000; Zhang et al., 2012).

Van Reeth et al. (1999) conducted a study on early cytokine production in piglets being experimentally infected with the Lelystad strain intratracheally at 3–4 weeks of age. PRRSV infected piglets were euthanized between 3 and 10 days post-inoculation. Neither control nor infected piglets had detectable levels of TNF-α when BAL samples were analyzed that were collected between day 3 and 10 after inoculation. They concluded that this strain didn't provoke a massive immune response that led to damage of the airways or gross pathology of the lung. Due to the mild respiratory pathology seen they suggested this strain could be causing subclinical infection only, and that these infections "frequently pass without being noticed" (Van Reeth et al., 1999).

A study was done on 5 commercial pig lines intended to investigate the innate immune response to PRRSV infection using a H2 isolate. Among the breeds used were landrace, which interestingly showed a delayed response to PRRSV infection with significantly increased levels of TNF- α secretion. This study was carried out *in vitro* on porcine pulmonary macrophages obtained by bronchoalveolar lavage. These findings may suggest that there is also genetic differences that play a role in the responsiveness to PRRSV (Ait-

Ali et al., 2007). Yet another study also carried out on PAM *in vitro* using a Taiwan isolate (tw91) initially showed a decrease in the secretion of TNF-α up until 36 hours post infection, then the level of TNF- α started increasing, at 48-72 hours post infection there was an 26-33% increase respectively (Chiou et al., 2000).

Lungs of piglets infected with a Korean isolate (SNUVR970501) of PRRSV was investigated by means of *in situ* hybridization. Positive hybridization signals for TNF- α mRNA was detected as early as day 1 post infection up until day 10 post infection. Numbers used to score the estimated number of cytokines in the lung tissue peaked at day 5 and 7 post infection. Cells resembling macrophages were the ones expressing TNF-α, and expression of the cytokine was minimal in areas of the lung without lesions. Sections examined from the non-infected control group showed no hybridization signal for TNF-α. They write in their article that "the results strongly suggest that these cytokines are important mediators in the pathophysiology of PRRSV infection" (Choi et al., 2002).

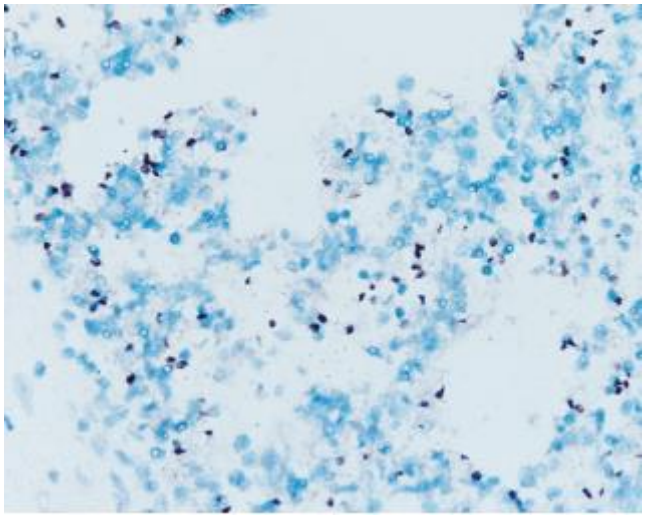

Figure 2: Lung from PRRSV infected pig 7dpi. Showing positive hybridization signals for TNF- α in the alveolar septa. Methylene green counterstain x 200 (Choi et al., 2002, p. 110).

Use of digital gene expression tag profiling (DGE) showed an upregulation of genes coding for TNF- α in pigs infected with a highly virulent strain of PRRSV. The study was conducted on 6-week-old piglets that had been taken from a high-health commercial Chinese farm. Piglets were experimentally infected with a highly pathogenic strain of PRRSV that caused major outbreaks of the so called "high fever" disease in China and Vietnam in 2006 and 2007. Piglets were euthanized 96 and 168 hours post infection. Their study found that expression of TNF-α seemed to be continuously upregulated when looking at mRNA and

protein levels analyzed with ELISA from serum samples. The author concluded that their data suggested that the severe pulmonary lesions caused by this highly virulent H-PRRSV infection was caused partially by the significant upregulation and production of TNF- α (Xiao et al., 2010).

Immunohistochemistry was used to detect proinflammatory cytokines during PRRSV infection in a study done by Amarilla et al. (2015). The researchers used 65 male piglets all at the age of 7 weeks at time of inoculation, originating from a specific pathogen-free farm in the Netherlands. Four strains of PRRSV were used in the experiment, Lelystad virus-Ter Huurne (LV), prototype PRRSV strain 215-06, SU1-bel and the attenuated vaccine strain (DV). The first two being low-pathogenic strains and SU1-bel being a highly pathogenic strain. The last animals were euthanized on day 35 post infection. They detected increased levels of TNF-α 7 days post infection with a higher expression in the piglets infected with LV and SU1-bel, compared to the DV group. There was no significant difference for the group infected with the 215-06 strain. Interestingly on day 35 the piglets infected with the low pathogenic strain LV had a higher expression of TNF-α in the lungs compared to SU1-bel and the ones infected with the 215-06 strain.

2.2.2 IL-10 detection in the lungs of PRRSV infected pigs

To better understand the role of the adaptive immune system in the clearance or progression of PRRSV infection cytokine involvement is investigated. Interleukin-10 presents as the main contributor; hence detection and quantification of this anti-inflammatory cytokine have been devoted more attention in the past decade.

Different immune cells have been used in studies for the replication of PRRSV during experiments, both porcine alveolar macrophages (PAM) and dendritic cells (DCs) are widely used.

A study was done on 4–5-week-old piglets experimentally infected intranasally with the Lelystad strain of PRRSV. BAL samples were used to detect IL-10 by means of ELISA. Infected piglets were euthanized between 1- and 52-days post infection with PRRSV. Their findings showed that IL-10 was detected already at day 5 post infection and reached a maximum of 139 pg/mL at 9 days post infection. Levels decreased and stayed low at 8–17 pg/mL until day 25 of the experiment (Labarque et al., 2003).

IL-10 mRNA has been found to be significantly upregulated in PRRSV infected porcine alveolar macrophages when compared to non-infected cells with a peak at 48 hours post infection. The study was done on PAM cells, obtained from a healthy PRRSV negative pig by bronchoalveolar lavage post mortem. IL-10 mRNA was detected using real-time quantitative RT-PCR every 12 hours post infection up until 48 hours (Zhang et al., 2012). Another study was also carried out by means of RT-PCR. 40 piglets aged 3 days were experimentally infected with a North American genotype (isolate SNUVR970501). The aim of the research was to prove the expression of IL-10 in the lungs post infection. Interleukin-10 mRNA was found to be persistently detected in the lungs from day 1 post infection up to day 28, with the group of negative control pigs failing to produce the same expression of IL-10 (Chung and Chae, 2003).

In the same study done by Chung and Chae (2003) *in situ* hybridization was also used to detect signals for IL-10. They noted an increase in IL-10 signals from day 3 to day 7 post infection, gradually decreasing from day 14 until the end of the study at day 28. The distribution within the lung of cells showing positive signals for IL-10 was patchy to multifocal and in connection with areas being affected by inflammation (Chung and Chae, 2003). Results from a different study reveal similar findings, that IL-10 was detected in higher numbers in areas with gross pulmonary lesions (Amarilla et al., 2015).

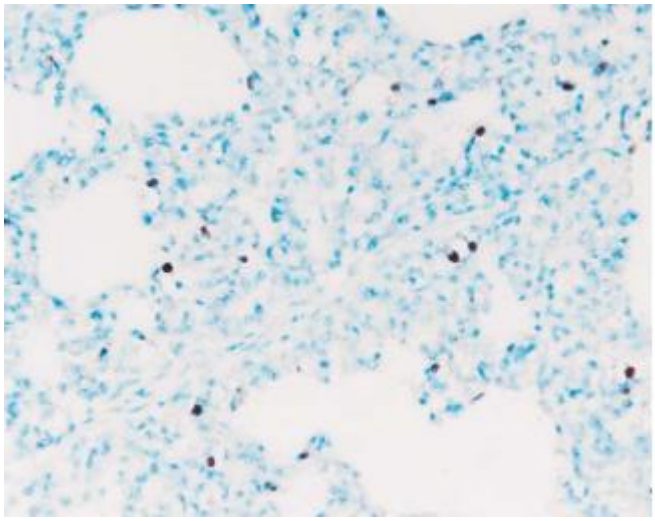

Figure: 3 Lung sample from piglet 7 dpi, showing positive hybridization signals for IL-10 in alveolar septa (black). Methyl green counterstain x 200 (Chung and Chae, 2003, p. 208).

On the other hand, another study on IL-10 expression *in vitro* do not show the same upregulation of interleukin-10 mRNA. This study was carried out on monocyte-derived

dendritic cells (DCs) originating from peripheral blood of a healthy sow infected with the SD-23983 PRRSV strain. DCs act as important antigen presenting cells as a part of the antiviral response that bridge between the innate and adaptive immune reactions to pathogens. ELISA was performed 24- and 48 hours post infection and IL-10 showed no obvious increase in PRRSV infected cells, compared to the uninfected control cells (Wang et al., 2007). Other results were obtained from a study carried out *in vitro* with DCs showing a significant increase in the upregulation of IL-10 when exposed to PRRSV, both when using immature and mature dendritic cells in the experimental setup and by means of ELISA or RT-PCR for cytokine measurement (Chang et al., 2008; Flores-Mendoza et al., 2008; Peng et al., 2009).

The reason for the contradictory results when looking at both TNF-α and IL-10 up- or downregulation in response to PRRSV infection is believed to depend upon multiple variables, for instance on the strain of PRRSV used. A study done by Gimeno et al. (2011) aimed to map the different cytokine profiles elicited by infection with different variants of PRRSV. They tested all together 39 isolates, all members of PRRSV 1, namely *Betaarterivirus suid* 1 (Gimeno et al., 2011; Gorbalenya et al., 2018).

Their study showed significant differences in the cytokine response after testing each isolate in four different cell systems, PAM, peripheral blood mononuclear cells (PBMC), bone marrow-derived dendritic cells (BMDC) and $SwC3^+$ blood mononuclear cells. They concluded that their study "primarily revealed that every cell type has a different sensitivity in terms of the cytokine response against PRRSV", with BMDC and $SwC3^+$ blood mononuclear cells showing the most efficient cytokine production. Their most surprising finding however was that "all four possible cytokine inducing phenotypes for IL-10 and TNF-alpha were detected (from negative-negative to positive-positive". Giving us an idea of the major differences in the cytokine profiles that can be expected within the same viral genotype in response to infection (Gimeno et al., 2011).

2.2.3 TGF-β detection in the lungs of PRRSV infected pigs

Both TGF-β and IL-10 can downregulate the immune response and act as immunomodulatory cytokines (Letterio and Roberts, 1998). There are limited studies carried out on the involvement of TGF-β in PRRSV infections, but there is reason to believe it might play a role in the ability of the virus to avoid getting eliminated by the host immune system

and aid in the prolonged viraemia that is typically seen in infected pigs (Darwich et al., 2010).

Detection of TGF-β in porcine lungs were done by Gómez-Laguna et al. (2012). 28 five-week-old piglets were experimentally infected with field isolate 2982 intramuscularly. Euthanasia was performed at different time points between 3- and 24-days post inoculation. Immunohistochemistry was performed to detect PRRSV and TGF-β, where viral antigen was found to be mostly present in PAMs and septal macrophages. PRRSV was detectable in lung tissue from day 3 post inoculation, reaching a peak at day 7. TGF-β expression reached a maximum on day 3 and decreased thereafter. Inside the cells TGF-β was found to be in the cytoplasm of PAMs, septal macrophages, lymphocytes and neutrophil granulocytes. It is also interesting that the same study investigated serum samples from the same individuals, and there were no detectable levels of TGF-β found. This despite that the cytokine was detected in the lung samples of the infected pigs, but not in mock infected pigs. The research group concluded that their results showed a significant correlation between the presence of viral antigen and expression of TGF-β in the lungs, and it is suggested that PRRSV replication in the lung has a direct effect on the production of TGF-β.

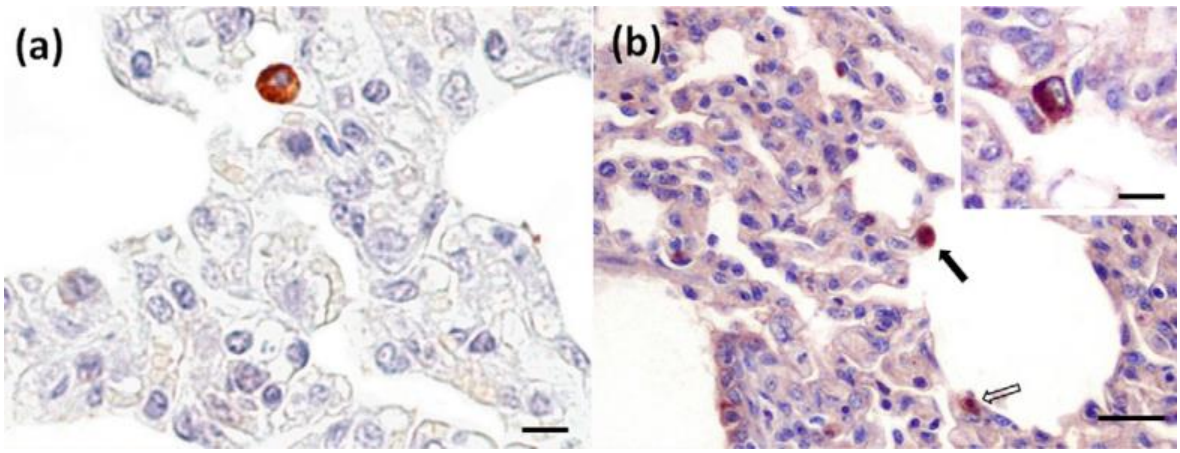

Figure 4: Positive immunohistochemical labelling of PRRSV (a) bars, 10 µm and TGF-β (b) bars, 20 µm in lungs of pigs (Gómez-Laguna et al., 2012, p. 190).

Results from a study done *in vitro* suggest that PRRSV raised the number of T regulatory cells that produced TGF-β (Silva-Campa et al., 2009). This can give clues as to the role of TGF-β and that it might be expressed in higher numbers during PRRS infection for the virus to escape the detection and elimination by immune cells during early phase of the disease.

A research group investigated what would happen if TGF-β was silenced during PRRSV infection. Even though the study was conducted *in vitro* on PRRSV infected PBMCs, their results suggest increased viability of infected cells and a significant reduction in viral replication when TGF-β expression was inhibited. They also stated that a knockdown of the TGF-β expression lead to a better responsiveness by the immune system, noted by an increase in expression of proinflammatory cytokines (Wang et al., 2019).

2.3 Regulated cell death

Another method of host defense, alongside the production of cytokines in response to viral infection, include regulated cell death (RCD). To fight against the viral agent as a part of the innate response, RCD are important processes carried out to limit viral replication as well as destroy already infected cells (Orzalli and Kagan, 2017).

Cell death can be classified into two distinct groups based on functional aspects. According to the Nomenclature Committee on Cell Death (NCCD) these are accidental cell death (ACD) and regulated cell death (RCD). Further I will focus only on the RCD, more specifically on intrinsic- and extrinsic apoptosis. RCD is controlled by means of strict signaling cascades that uses transduction molecules for activation. To clarify, RCD is termed programmed cell death (PCD) when taking place during physiological conditions (Galluzzi et al., 2018).

Intrinsic apoptosis is initiated by damage to the DNA and other cellular stressors and carried out by activation of caspases. This process starts with mitochondrial membrane permeabilization (MOMP) that enables cytochrome c to move from the inner mitochondrial membrane through the outer membrane into the cell cytoplasm. Once in the cytoplasm cytochrome c attaches to the apoptotic protease-activating factor-1 (APAF-1) and the two together form the apoptosome that first activate caspase-9 (CASP9) that again goes on to activate caspase-3 (CASP3). This initiates the cellular process and morphological changes characteristic for apoptosis. These include shrinkage of the cell followed by nuclear pyknosis and karyorrhexis, chromatin condensation and nuclear fragmentation. The final result is that the cell disintegrates into small vesicles called apoptotic bodies that in turn are phagocytosed by other cells, mostly by macrophages (Chipuk et al., 2006; Von Ahsen et al., 2000).

Extrinsic apoptosis is carried out with the help of membrane receptors. The pathway is stimulated by cytokines, for instance TNF-α. Two common death receptors are CD95 and

TNFR. These death cell receptors contain a death domain which is a sequence of amino acids found in the cytoplasm. Activation of death receptors happens via ligands, that that are mostly located on cytotoxic T-cells. After ligand binding to the death receptor, proteins will be assembled to create DISC. In turn this complex will activate CASP8 and CASP10 and initiate apoptosis (Tizard, 2012 p. 191).

Viral pathogens have proved able to alter the regulation of apoptosis. Dysregulation, both up and down regulation of apoptosis might be favorable to the virus. Viruses have developed methods to hinder apoptosis during early stages of infection, this helps the virus complete its replication cycle and ensures progeny production. When viral replication is successfully carried out inside the cell, several viruses are known to trigger apoptosis and subsequently the death of the infected cell. This process is beneficial for the virus because it aids in the release and assembly of the newly created viral progenies, and it can also help with the spread without inducing inflammation (Best and Bloom, 2004; Carthy et al., 2003; Fadok et al., 2001; Teodoro and Branton, 1997; Thomson, 2001).

Galluzzi et al. (2008) suggest that viruses can be classified into four different categories based on the way they interfere with the regulation of apoptosis. The first group of viruses possess proapoptotic proteins that alter mitochondrial membrane permeabilization when attaching to the mitochondrial membrane. The second category indirectly activate mitochondrial membrane permeabilization or uses other antiapoptotic modulators. In the third group we find viruses that have structures or sequences showing similarities with the Bcl-2 family proteins found in the outer mitochondrial membrane and lastly the fourth category is composed of viruses that prevent apoptosis in other ways, for example the ASFV that inhibit apoptosis by means of blocking the effect of CASP3 (Dixon et al., 2017). Hence the first two categories are methods used by viruses to activate-, and the last two to inhibit apoptosis.

Other pathways suggested to be involved in the regulation of apoptosis during PRRSV infection include oxidative stress, P53, JNK, autophagy and UPR (Fan, 2019).

2.3.1 Methods detecting apoptosis

Studies related to PRRSV and induction of apoptosis is an interesting area of research that can help us to better understand the pathogenesis of the viral infection. There are several methods used to detect apoptosis in experimental setups, for instance TUNEL assay, electron

microscopy, fluorescence staining, detection of DNA fragmentation by means of gel electrophoresis, western blotting, flow cytometry, caspase detection and by measuring the mitochondrial membrane potential amongst others (Martinez et al., 2010).

A study done *in vitro* on PRRSV infected MARC-145 cells showed DNA fragmentation 48 and 60 hours post infection when compared to mock infected cells using ELISA. DNA fragmentation is a marker used to detect late stages of apoptosis. The same study also detected early stages of apoptosis by detecting the translocation of PS on the leaflet of the plasma membrane by using annexin V staining, a type of fluorescence followed by flow cytometry. Their results concluded that early apoptosis could be seen in virus infected cells at 48- and 60 hours post infection (Lee and Kleiboeker, 2007).

2.4 Pro- and anti-apoptotic pathway activation by PRRSV

2.4.1 Induction of apoptosis

PRRSV has proven capable, mostly during *in vitro* experiments, to induce pro-apoptotic pathway activation. I will try to focus mainly on the experiments carried out *in vivo* on lung tissue samples.

A study done by Sur et al. (1998) found that during PRRSV infection there was evidence of apoptosis. They detected this by means of TUNEL and DNA electrophoresis and they also detected the presence of viral antigen by means of immunohistochemical methods. The results indicated that PRRSV negative cells in the lung were the ones showing the highest incidence of apoptosis, a so called bystander effect, a phenomenon where apoptosis is seen in neighboring uninfected cells (Sur et al., 1998).

Sirinarumitr et al. (1998) conducted a study on piglets experimentally infected with ATCC VR2385 strain of PRRSV that were euthanized at 1,3,5 and 10 dpi. They used TUNEL assay in situ and ultrastructural morphology to investigate apoptosis in the infected lungs. Dual labelling was performed to detect whether apoptotic cells were indeed infected or bystander cells. They also concluded like the previously mentioned research group that the apoptotic cells were almost exclusively uninfected cells.

An attempt was made to detect apoptosis together with TNF-α in lung tissue samples using a combination of TUNEL assay and immunohistochemistry labelling. The isolate used was SNUVR970501, introduced intranasally to 3-day old piglets. Euthanasia found place on day

1, 3,5 and 7 post inoculation. Their results showed that apoptosis could be detected as early as 1 dpi, with the highest number of apoptotic cells detected at day 5, decreasing by day 7. Double labelling concluded that cells were either positive for PRRSV or apoptosis, and that positivity for both did only occur in very few cells. Similarly, overall, cells showed positive signals for either TNF-α or PRRSV and those cells found to be positive for TNF-α were mostly confirmed to be macrophages (Choi and Chae, 2002).

Whether PRRSV could induce apoptosis in the porcine lung was also studied in 4–5 week old piglets infected with the Lelystad strain. Inoculation of the virus was done intranasally and piglets were euthanized on selected days starting at day 1, where the last piglets were put down at day 52. Tissue samples were harvested from the lungs and BAL samples were also obtained from all piglets. Both the lung and the BAL cells were evaluated for the presence of viral antigen and signs of apoptosis. Additionally, detection of IL-10 and TNF-α was carried out on the BAL samples. Similarly, apoptosis was found to be present in both PRRSV infected and non-infected cells, the latter accounting for more than 99% of detected apoptotic cells in the lung. Amongst apoptotic cells macrophages and monocytes were the mostly represented. TUNEL assay was used on both BAL and lung samples. Infected apoptotic cells in the lungs between days 5 and 25 post infection were found to be between 9-39 %, and in BAL samples the numbers ranged from 13-30% from day 3 to 25. A peak of IL-10 was noticed at day 9 followed by the highest measurement of apoptotic cells at day 14 post infection (Labarque et al., 2003).

Lee and Kleiboeker (2007) demonstrated by help of a colorimetric method the detection of activated CASP8 and CASP9 in PRRSV infected MARC-145 cells were both caspases were seen to have an increase in activity from 48 hours post infection. Their number was two-fold that of mock infected cells at 60 hours pi. Since CASP9 and CASP8 activate the intrinsic and extrinsic pathway respectively, by in turn activating CASP3 which act as the executioner of the apoptotic process, they also measured the level of activated CASP3. They found CASP3 to be at elevated 48- and 60-hours pi, at the latter measurement as much as 4-fold higher than in control cells. They concluded that PRRSV infection stimulate the activation of CASP3, 8 and 9 that are key elements in both the extrinsic and the intrinsic pathway of apoptosis. At 60 hours pi they also found with the help of IFA, that there was a rise in expression of TNFR-1 and FasL in infected cells. Further linking the involvement of the extrinsic pathway in the PRRSV associated apoptosis. In their study most cells undergoing apoptosis at 48- and 60 h post-infection were virus positive cells.

Research carried out by Sánchez-Carvajal et al. (2021) noted apoptosis *in situ* by means of TUNEL assay on lung samples obtained from piglets infected with two different subtypes of PRRSV intranasally. Both a virulent and a low-virulent strain were used, namely the Lena strain and field isolate 3249 respectively. The study lasted 13 days, and piglets were euthanized at different timepoints post inoculation. Their result detected DNA fragmentation with the peak of positive labelling at day 8 post infection for both strains, but piglets infected with the virulent strain showed a higher number of positive signals compared to the low-virulent strain already at day 3 pi. The positive signals were detected in PAMs, PIMs and interstitial macrophages. The same study investigated caspase expression by means of immunohistochemistry assays, and CASP3 was found to be significantly higher in both the infected groups compared to the mock infected piglets. Lena infected piglets showed a peak at day 8 whereas the 3249-infected piglets had a peak of CASP3 at day 13 pi. CASP8 was found to be at its maximum at day 8 in Lena infected piglets and interestingly in the 3249-infected group the levels stayed below that of the mock infected group until it suddenly increased at day 13 pi. CASP9 was showing a slight rise from day 8 until the termination of the study at day 13 in both groups, with Lena infected piglet's having a slightly higher incidence of positive cells. These results indicate the involvement of both the extrinsic and the intrinsic pathway in the PRRSV induced apoptosis.

2.4.2 Inhibition of apoptosis

A study conducted on PAMs and MARC-145 cells showed interesting results looking at possible anti-apoptotic effect of PRRSV during early stages of viral infection. Cells were infected with the prototype European Lelystad strain, and the researchers tried to induce apoptosis at different time points post infection by help of staurosporine, an agent use to induce apoptosis across a wide variety of cell types. Before initiating the apoptosis, the completion of the viral replication cycle was determined by analyzing the intra- and extracellular virus titers, it was found to be increased in both cell types from 12 hours pi indicating the release of new virus from the cells at this time point. For macrophages the extracellular virus titers where higher than for the MARC-145 infected cells and it was concluded that release of the virus was more efficient in PAMs. Keeping in mind that new virus was detected extracellularly after 12 hours pi, infected cells were treated with the apoptosis inducing agent at 2, 3, 4, 5, 6, 8 and 12 hours pi in PAMs. Results showed that compared to mock cells, there was no significant reduction in apoptosis at 2 and 3 hours post

infection, but at 4, 5, 6, 8 hours pi a significant reduction of apoptosis was seen. In infected cells, percentages of cells showing signs of apoptosis reduced significantly, ranging from 13,3–24.8% at different time points post infection compared to 68.2–74.0% at the same time points in uninfected cells treated with the apoptotic agent. 12h pi the infected cells no longer showed protection against the apoptotic agent. Similar results were obtained from the MARC-145 infected cells (Costers et al., 2008).

2.5 Cellular lung damage

2.5.1 Gross and microscopic lung pathology

Lesions seen in lungs of PRRSV infected pigs vary and depend on the virulence of the strain causing disease (Wensvoort et al., 1991). Some virulent strains can cause severe and extensive destruction of the lung tissue whereas other low-virulent strains can go unnoticed as subclinical infections. During field conditions many virulent PRRSV infections are complicated by secondary bacterial infections and other viral diseases. This further diversifies the lesions seen as compared to what can be observed in studies carried out under experimental conditions (Amadori and Razzuoli, 2014).

Gross pathology of lungs infected with PRRSV has been described by several researchers. Morgan et al. (2016) found that interstitial pneumonia results in lungs that fail to collapse with mottled tan areas having a rubbery consistency. It is reported elsewhere that the color of the lungs can vary from tan to dark red-purple. Edema can be mild to severe where separation of the lobules can be observed. If caused by a highly virulent strain, there might also be hemorrhages visible. Interstitial pneumonia following infection vary in severity and distribution are mostly seen in cranioventral lung lobes, but lesions can also be diffuse affecting the entire lung (Zimmerman et al., 2019). Morgan et al. (2016) also concluded that the apical lung lobe was the most affected area based on macroscopic pathological findings.

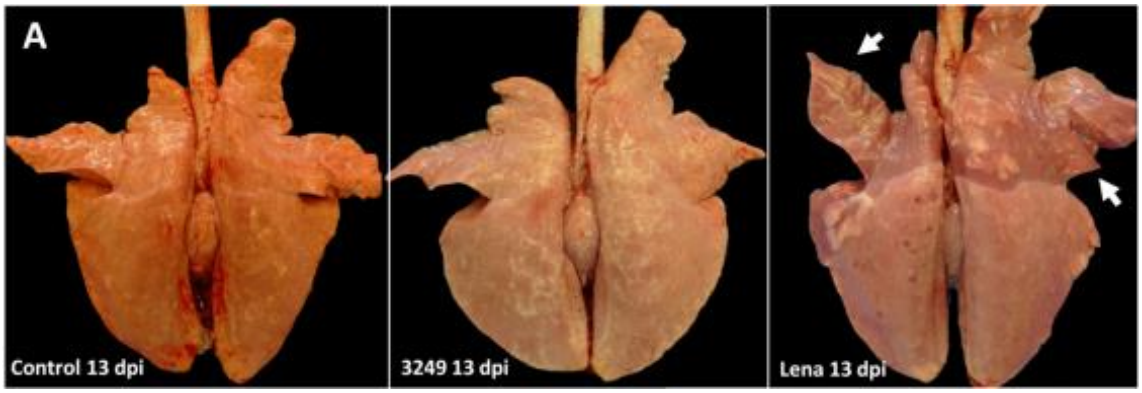

Figure 5: Gross lung pathology showing characteristic interstitial pneumonia 13 dpi in mock-, low- and highly virulent infected pigs with PRRSV. White arrows show consolidated areas in the cranioventral and middle lung lobes (Sánchez-Carvajal et al., 2021, p. 5).

2.5.2 Pneumocytes

Pneumocytes exist in the lung as type 1 and type 2. Type 1 pneumocytes are sensitive to injury, for instance by cytokines produced during acute inflammation in the lung. When injured, these type 1 pneumocytes swell and vacuolization can be seen. Once injured they detach from the basement membrane and during this process alveolar edema develops due to an increase in alveolar permeability. Because of this proteins, plasma and fibrin accumulate in the alveolar space. Under physiological circumstances this fluid is cleared quite quickly by lymphatic and alveolar absorption as well as phagocytosis of sloughed cells by alveolar macrophages. Type 2 pneumocytes as precursor cells that can replace the lost type 1 pneumocytes by undergoing mitosis and differentiation into new mature type 1 pneumocytes (Zachary, 2017 p. 478-480). During the course of type 2 pneumocytes replacing the injured type 1 pneumocytes proliferation leads to significant thickening of the alveolar walls (Beyer et al., 2000; Zachary, 2017 p. 521).

Infection with PRRSV induces hypertrophy and hyperplasia in type 2 pneumocytes (Amarilla et al., 2015; Beyer et al., 2000; Chung and Chae, 2003). A study done on type 2 pneumocyte proliferation after infection with a subtype 1 virulent field isolate showed increased numbers of type 2 pneumocytes from day 10 pi. These cells were still present in higher numbers at the end of the study at 21 pi (Balka et al., 2013). Thickening of the alveolar wall is also documented after infection with PRRSV, partially due to the proliferation of type 2 pneumocytes (Nazki et al., 2020). When piglets of different age groups suffering from

PRRSV was examined, type 2 pneumocyte proliferation was seen in weaners as the lowest age group (Darbès et al., 1996).

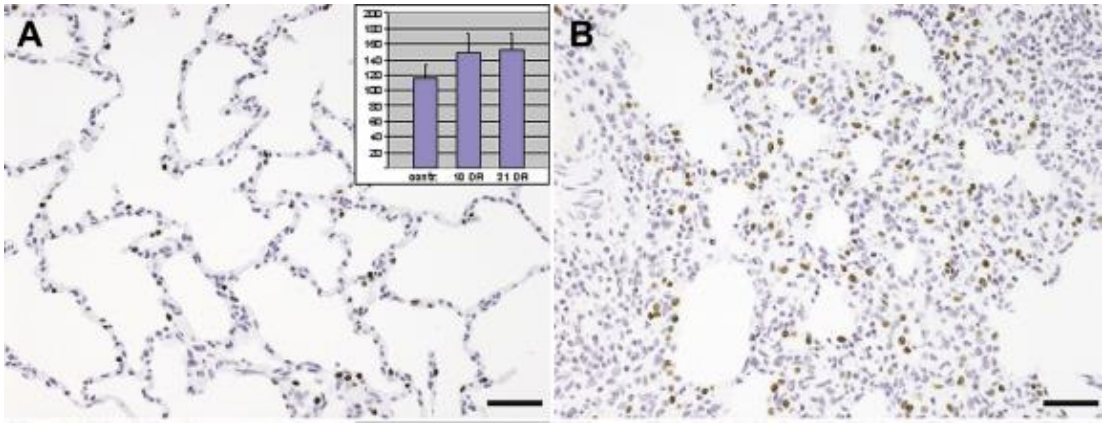

Figure 6: Identification of type 2 pneumocytes in control and PRRSV infected lungs of pigs. "(A) TTF-1 labelling of control lung. (B) TTF-1 labelling of infected lung". Bars, 50 µm (Balka et al., 2013, p. 327).

2.5.3 Mononuclear infiltration of the septa

It is a common finding in articles where researchers have conducted histopathological investigations on lung tissue or analysis of BAL samples in relation to PRRSV infection that mononuclear cell infiltration of the alveolar septa is mentioned. To clarify when mononuclear cells are mentioned, I refer to lymphocytes, plasma cells and macrophages. One of the circulating hypotheses regarding the significant septal cellular infiltration seen after the first week of infection with PRRSV has to do with the replacement of macrophages by cells from the monocyte/macrophage lineage. It is believed that following destruction of the alveolar macrophages by means of apoptosis (or necrosis) after PRRSV replication, these cells seen infiltrating the septa is an attempt to replace the declining cell population of PAMs as a way of restoring balance and homeostasis by the host body (Labarque et al., 2000).

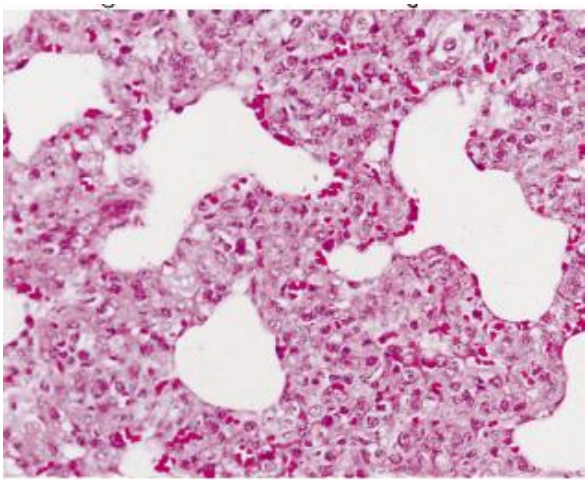

Figure 7: Lung from PRRSV infected pig 7 dpi, thickening of alveolar septa by infiltration of mononuclear cells. HE. x400 (Choi et al., 2002, p. 109).

Other opinions are that these cells are attracted to the lungs by the mediators of inflammation, for example by cytokines produced by macrophages infected with the virus, and that this leads to a strong influx of mononuclear cells in the lung tissue (Van Reeth et al., 1999). This hypothesis is supported by the findings of Gómez-Laguna et al. (2010), that experienced an increase in the expression of local cytokines in the lung, among these TNF-α. They concluded that "there is a direct correlation between this expression and the infiltration of the pulmonary interstitium by macrophages" (Gómez-Laguna et al., 2010).

Histopathological lesions in weaners infected with PRRSV describes diffuse infiltration of the alveolar walls by mononuclear cells and infiltration of the interlobular septa with lymphocytes and plasma cells (Darbès et al., 1996).

During experiments conducted *in vivo* on lung tissue and BAL samples, results showed that from day 9 to 52 pi there was a two- to fivefold increase in the cellular content of the BAL samples, reaching a peak at day 25 pi. The cell fractions obtained from the BAL samples after analysis reviled that 55–92% of the cells were of monocyte/macrophage lineage, 1-15% neutrophile granulocytes and 6–31% were non-phagocytes, presumably lymphocytes (Labarque et al., 2000).

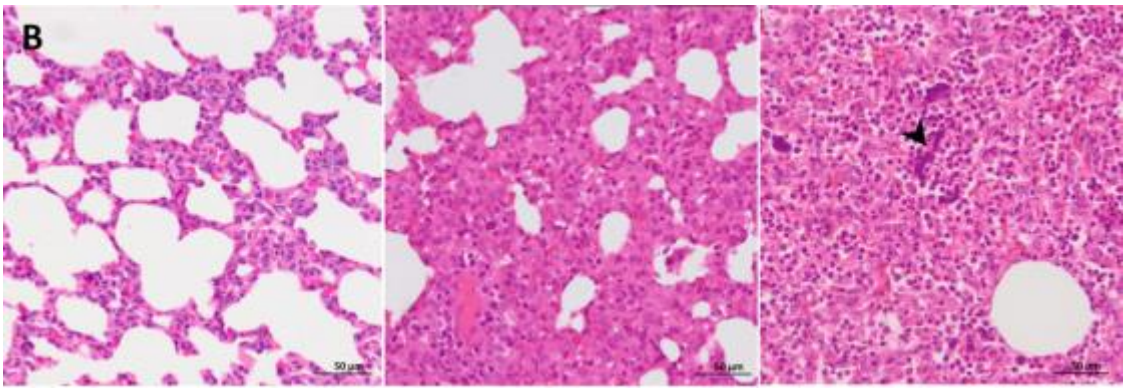

Figure 8: B: "from control (left), 3249 (middle) and Lena (right) infected pigs euthanized at 13 dpi (H&E; bars 50 μm)" (Sánchez-Carvajal et al., 2021, p. 5). Histopathology shows characteristic interstitial pneumonia with thickened alveolar septa, in Lena infected lungs foci of suppurative bronchopneumonia can also be seen (arrowhead).

2.5.4 Necrotic debris, perivascular and interalveolar accumulation of inflammatory cells

Following injury to the type 1 and 2 pneumocytes during the course of acute interstitial pneumonia that develops from the PRRSV infection, a disruption in the blood-air barrier as mentioned previously leads to the filling of the alveolar lumen with protein rich fluid. In addition to this, alveolar walls become thick due to the edema resulting from the inflammation and neutrophil granulocytes infiltrating the alveolar interstitium (Zachary, 2017 p. 521).

Consequently, because of the release of proinflammatory cytokines by PRRSV infected macrophages more inflammatory cells are recruited to the lungs during acute inflammation. During this process cell lysis is seen because of the enzymes and mediators released. Neutrophil granulocytes, edema fluid and necrotic cell debris collect in the alveoli after cells are killed off by enzymes released from the inflammatory cells (Zachary, 2017 p. 210). Characteristic filling of the alveoli with proteinaceous and karyorrhectic debris is a common finding in PRRSV infected lungs of pigs (Rossow, 1998). Cells found in the alveolar space is usually a combination of macrophages, neutrophile granulocytes, lymphocytes and necrotic cells that display signs of nuclear changes such as pyknosis or free chromatin (Gomez et al., 2018).

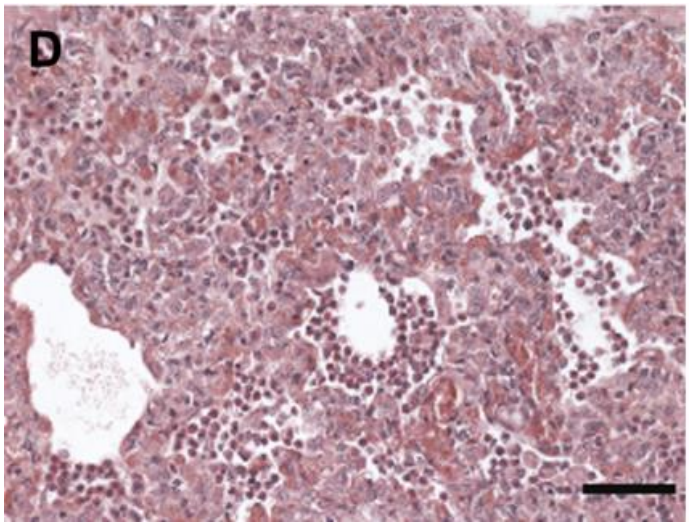

Figure 9: Inflammatory cells accumulating in the alveolar space in PRRSV infected porcine lungs. HE. Bars, 50 µm (Balka et al., 2013, p. 326).

The finding of perivascular cuffing is another lesion that is frequently seen in the lung of infected pigs. Lymphocytes and plasma cells are mostly responsible for this histopathological finding during PRRSV infection (Zimmerman et al., 2019 p. 695). During the inflammatory processes lymphocytes arrive within the first 24–48h from the onset of acute inflammation in response to stimuli such as cytokines, and these cells will often gather around blood vessels (Zachary, 2017 p. 113). Periarteriolar accumulation of plasma cells and lymphocytes were seen in weaners infected with PRRSV (Darbès et al., 1996).

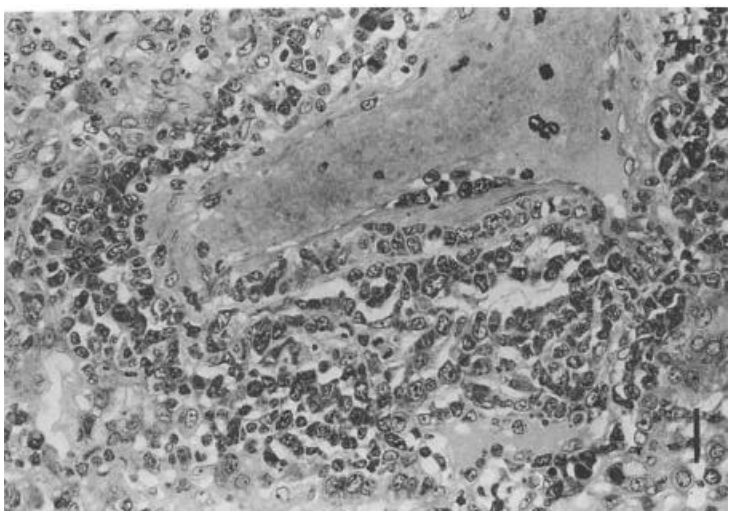

Figure 10: PRRSV infected lung tissue obtained from a weaner, stained with giemsa showing perivascular lymphoplasmacytic infiltration. BAR 25 µm (Darbès et al., 1996, p. 358).

Histopathological findings are many times evaluated using the above-mentioned characteristics to determine the severity of the lesion in question. Lungs are typically given a score, and finally a total overall score is calculated. Jung et al. (2007) have suggested a scoring system: " (i) thickening of alveolar septa by infiltration of inflammatory leukocytes, principally mononuclear cells, and type 2 pneumocyte hypertrophy and hyperplasia (ranging from 0 to 6, where 0 is normal appearance, 1 is mild focal or multifocal, 2 is mild diffuse, 3 is moderate focal or multifocal, 4 is moderately severe, 5 is severe focal or multifocal, and 6 is severe diffuse interstitial pneumonia); (ii) accumulation of necrotic cells and inflammatory leukocytes in alveolar and bronchiolar lumina due to airway epithelial necrosis (ranging from 0 to 3, where 0 is normal appearance and 3 is severe); and (iii) peribronchiolar or perivascular lymphohistiocytic inflammation (ranging from 0 to 3, where 0 is normal appearance and 3 is severe)" (Jung et al., 2007). This system and modified versions of this is widely used to determine the severity of pulmonary lesions and damage following infection with PRRSV.

When investigating a virulent strain, VR-2385 Sirinarumitr et al. (1998) described moderate multifocal interstitial pneumonia, occurring 3–5 days post infection that turned into severe diffuse interstitial pneumonia 10 days post infection. Lesions accompanying the interstitial pneumonia was infiltration of the septa with a mixture of mononuclear cells, hypertrophy, and hyperplasia of type 2 pneumocytes and in the alveolar space accumulation of macrophages that were both necrotic and of normal in appearance.

The Lelystad strain, which is a low-virulent strain produced thickening of the alveolar septa, hypertrophy, and hyperplasia of type 2 pneumocytes and necrotic and apoptotic debris together with macrophages were seen to fill the alveoli. Some animals also showed perivascular lymphohistiocytic cuffing (Amarilla et al., 2015). Lesions that can be observed following PRRSV infection overlap between the different strains, but the severity is different between the virulent and low-virulent strains and a system made to grade it is necessary to categorize the pulmonary histological findings.

3. Discussion

PRRSV is a significant disease in the swine producing industry that can lead to subclinical disease accounting for poor production parameters in all pig producing facilities. It can lead to mortality or make the animal susceptible to secondary infections that can contribute to development of multifactorial diseases such as PRDC. Since the disease was discovered, a lot of research has been carried out to fully understand the pathogenesis and lesions detected during infection. There are still parts of the viral pathogenesis that are not fully understood, one of which is the ability of the virus to interfere with the production of different cytokines and if it can manipulate this production for its advantage.

Studies previously mentioned are somewhat contradictory when it comes to changes in the production of specific cytokines. We could see that TNF-α has shown to be both upregulated and downregulated during infection with PRRSV. It was hypothesized that viral infection reduced the ability of the infected cells to upregulate proinflammatory cytokine expression during the early stages of disease to reduce the inflammatory response and essentially detection and elimination by the host immune cells. In line with this prediction there were reports of weak TNF-α response, and also almost undetectable levels of the cytokine that seem to support this hypothesis (López-Fuertes et al., 2000; Van Reeth et al., 1999; Zhang et al., 2012). At the same time, other authors presented evidence of increased expression of TNF-α (Ait-Ali et al., 2007; Choi et al., 2002; Xiao et al., 2010), but regarding these studies, the infection was carried out with a highly virulent strain of PRRSV as compared to the experiments reporting low or absent TNF-α expression that used strains of lower virulence. Although this might seem logical, Amarilla et al. (2015) found a low-virulent strain to cause a higher expression of TNF-α than a virulent one, leaving us unable to conclude if this is indeed a trend. Unfortunately, these articles are not able to give us clear answers to the cause of the low expressions reported by some research groups. Since this could on one hand be due to that these low virulence strains simply cannot elicit a strong enough response by the immune system resulting in higher levels of TNF-α production by the host or it could on the other hand indeed be the active work of the virus to regulate the expression and keep the proinflammatory cytokine production to a minimum.

When investigating anti-inflammatory cytokine expression we found that overall, the IL-10 and TGF-β expression be upregulated in PRRSV infected animals. Reports of increased levels of IL-10 (Amarilla et al., 2015; Chung and Chae, 2003; Zhang et al., 2012) and TGF-β (Gómez-Laguna et al., 2012) is also thought to be a way for the virus to escape detection and destruction by the immune system. It would be interesting in future challenges to block or silence the expression of both of these cytokines during PRRSV infection to see if one would witness a better and more efficient clearing of the viral disease by the host since it seems the virus upregulates these anti-inflammatory cytokines in order to stay hidden or to slow clearance and aid in the persistence of the virus in the host.

Apoptosis is always seen to some extent even in control animals when research is carried out due to this being a natural process that takes place also during physiological conditions. Apart from this, apoptosis is seen to be of higher prevalence in animals infected with PRRSV. Apoptosis can potentially be initiated directly by the viral replication in the susceptible cells or indirectly utilizing cytokines produced by infected cells that exert an apoptogenic effect in *bystander* cells. One of the cytokines that can trigger apoptosis is the TNF-α, and we have seen evidence that this cytokine can be found in higher levels, especially during infection with highly virulent strains of PRRSV, which might partially be the explanation for the severe lesions seen in the lungs of these animals. TNF-α produced by infected macrophages that act locally in the lung, inducing apoptosis in bystander cells was suggested in previous research to be the reason for cell death among uninfected cells seen in PRRSV infections (Choi ad Chae, 2002).

TUNEL assay and the detection of DNA fragmentation is a frequently applied method to detect apoptosis on tissue samples, and positive signals were described in the above-mentioned studies. The drawback of using this test exclusively is that necrosis will also result in positive signals and it is not able to detect the early stages of the apoptotic process (Lee and Kleiboeker, 2007). Therefore, the need for more specific methods to detect more than just the end result of regulated cell death is needed when investigating PRRSV induced apoptosis.

Caspases are good indicators for apoptosis since they are not activated in necrosis (Cryns and Yuan, 1998). Although false predictions can be made when experiments are carried out *in vitro*, because in the host, apoptotic cells will be phagocytosed, but in cell cultures

apoptosis will be followed by secondary necrosis (Wu et al., 2001). Higher incidence of necrosis might be recorded that could differ if the experiment was conducted *in vivo*.

PRRSV can interfere with the activation of pro-apoptotic pathways. Focusing on the intrinsic and extrinsic pathways of apoptosis significant upregulation of both CASP8, CASP9 and the executioner CASP3 could be seen in infected cells along with positive TUNEL assays. Other viruses known to induce apoptosis via extrinsic and intrinsic pathways are human immunodeficiency virus and vesicular stomatitis virus respectively (Bartz and Emerman, 1999; Gadaleta et al., 2005). It is still to be determined if CASP9 activation is a result of CASP8 activation or if these two pathways are activated independently from each other. Lee and Kleiboeker (2007) also witnessed an increase in the expression of death receptors of the cell surface, and the ligation of these could also have been the cause of the increased CASP8 measured, resulting in apoptosis by the extrinsic pathway. Whether the intrinsic pathway is activated independently still needs further research, since crosstalk between the pathways can interfere with the results obtained.

There is a lack of studies investigating the anti-apoptotic properties of PRRSV, in fact, none that could be found by the author that was carried out *in vivo*. Costers et al. (2008) did produce results suggesting that PRRSV indeed can protect infected cells against apoptosis within a specific timeframe during viral replication under laboratory conditions, with the addition of apoptotic agents to cell cultures. It would be interesting to see if these findings could also apply for *in vivo* experiments, and if apoptosis is blocked by the virus during its replication in the infected cells and if this is a strategy used by the virus to successfully carry out viral progeny production. More research has been devoted to trying and explaining the reasons for the high incidence of apoptosis seen, and by what means this is initiated. The ability of the virus to actively block apoptosis in the first 12 h pi has been less studied.

A reasonable question is whether apoptosis is induced by the host as a protective mechanism or by the virus as a part of its pathogenesis. It seems that PRRSV can activate extrinsic, possibly also the intrinsic pathway of apoptosis and that this might be a way to efficiently release progeny after replication is carried out in susceptible cells. So, this can potentially be a strategy from the virus point of view. What we also have touched on is that most cells found to be apoptotic are uninfected, which is interesting since that doesn't directly favor the virus in any way having increased loss of healthy surrounding cells. The virus wasn't directly targeting these cells, but they still undergo apoptosis. It is suggested that this is due

to the proinflammatory cytokines produced by the infected cells themselves in response to viral replication, acting on the neighboring cells and hence triggering their apoptosis. If this is a consequence of viral interference of cytokine production or a host defense mechanism is still unknown. A virus that is known to cause bystander apoptosis is the previously mentioned HIV (Garg and Joshi, 2017).

Pulmonary pathology seen in PRRSV is characteristic of interstitial pneumonia, but the severity of lesion very much varies. Gross pathology and histopathological lesions are seen to a lesser or greater extent, depending on the strain and genotype of the virus that caused the infection. In experimental conditions, this is highly regulated since experimentally infected animals usually don't harbor other viral or bacterial diseases. In field conditions, PRRSV is many times complicated by other pathogenic agents giving lesions a different appearance. The virulence of the strain plays a significant role in the development of pulmonary damage, and as discussed different stains also seemingly elicit different cytokine profiles, giving them different immunomodulatory capabilities, that will further decide the lung damage resulting from the infection. Apoptosis of infected macrophages in the lung and the denudation of the basement membrane resulting from apoptosis of bystander will contribute to the increased vulnerability of the lung tissue to secondary infection in field conditions as well as the depletion of the alveolar macrophages. The release of cytokines, induction of apoptosis and lung damage is very much related, and further research should be conducted to understand the complexity and the interaction among them (Figure 11).

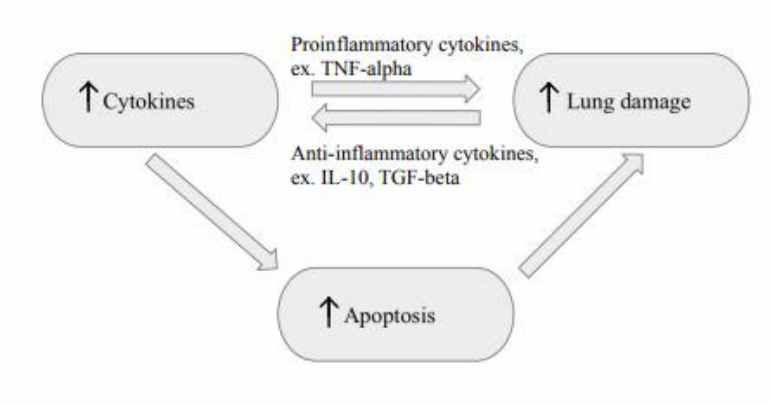

Figure 11: Simplified figure suggesting the relationship between different components of PRRSV infection (own illustration).

4. Conclusion

PRRSV and the viral interaction with the host are continuously under investigation. Since the specific mechanisms of pathogenesis are not yet fully understood it is of interest to map these events to prevent the disease from being one of the costliest agents affecting pig production. One of the main issues is the lack of uniformity when it comes to the investigation of cytokine involvement, occurrence and frequency of apoptosis as well as a description of pulmonary lesions. Most experiments differ in the experimental setup and the strain used for infection, which lead to results that are generally hard to compare even though they are conducted to investigate the same parameters. As we have seen pathogenesis and lesions very much depend on the isolate that is causing the infection.

Future studies should focus on the thorough investigation of PRRSV and its possible anti-apoptotic properties, since the amount of research on this area is scarce. It is evident that PRRSV can induce cell death, but more studies on RCD in the lungs focusing on apoptosis not only employing TUNEL assay would help towards understanding the pathways involved and potentially activated by the virus.

Finally, I will answer the questions proposed in the introduction of this review.

- What changes can be seen in the production of cytokines, specifically IL-10, TGF-β and TNF-α in response to PRRSV infection?

IL-10 and TGF-β have in the experiments mentioned been seen to be either upregulated or has remained undetectable.

- Can PRRSV induce pro- and anti-apoptotic pathways in the infected cell?

PRRSV can induce apoptosis in the infected cells via the extrinsic and possibly the intrinsic pathway. Whether it has the ability to inhibit apoptosis during replication in the cell is yet to be determined since there is currently little published material on this matter.

- What pulmonary cellular damages are seen during PRRSV infection?

Hypertrophy and hyperplasia of type 2 pneumocytes, thickening/infiltration of monocytes of the alveolar septa, necrotic debris and inflammatory cells filling the intraalveolar space, and perivascular cuffing is seen that accounts for the most severe pulmonary changes in response to PRRSV induced interstitial pneumonia.

5. Acknowledgements

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6. References

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