



Experimental infection of pigs with European porcine
reproductive and respiratory syndrome virus:
correlations with pathology

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases of swine worldwide. Since its first emergence in 1987 the PRRS virus (PRRSV) has become particularly divergent with highly pathogenic strains appearing in both Europe and Asia. However, the underlying mechanisms of PRRSV pathogenesis are still unclear. This study sets out to determine the differences in pathogenesis between subtype 1 and 3 strains of European PRRSV (PRRSV-I), and compare the immune responses mounted against these strains in both the blood and the local tissues, ie the mediastinal lymph nodes and the lung. Piglets were infected with 4 strains of PRRSV-I: Lelystad virus, 215-06 a British field strain, a vaccine strain DV and SU1-bel from Belarus. Blood was collected at various time points for viraemia to investigate viraemia and immune responses. Post-mortem examinations were performed at 3, 7 and 35 days post-infection (dpi), and cells were collected from the alveolar spaces and the lymph nodes. The subtype 3 SU1-bel strain displayed greater clinical signs and lung gross pathology scores compared with the subtype 1 strains. This difference did not appear to be caused by higher virus replication, as viraemia and viral load in broncho-alveolar lavage fluid (BALF) were lower in the SU1-bel group. Infection with SU1-bel induced an enhanced adaptive immune response with greater interferon (IFN)- γ responses and an earlier PRRSV-specific antibody response, in both blood and BALF, which correlated with gross pathology. Of particular note was the large influx of cytotoxic T cells and production of IFN- γ . On the other hand those pigs in the SU1-bel group, which had much less pathology, had the highest number of regulatory T cells and levels of the immunomodulatory cytokine Interleukin-10. The results of this study indicate that the immune response has an important role in the pathogenesis of PRRSV infection. Although these enhanced immune responses clear virus from the serum and BALF more quickly than responses seen in other strains, if these responses go unchecked, as they seem to in this case, it can have disastrous consequences for the animal. It may be possible to look at which factors are leading to this viral clearance and enhanced cellular immune response and use this data to perhaps devise a new vaccine that can monopolise on these mechanisms without causing the complications observed here.

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Signed:

1	Introduction.....	3
1.1	Porcine Reproductive and Respiratory Syndrome	3
1.2	Porcine Reproductive and respiratory Syndrome Virus.....	5
1.3	Diversity of PRRSV	6
1.4	Virion Structure and Genome Organisation	7
1.5	Cellular Tropism and Virus Replication	9
1.6	PRRSV and the Immune System	10
1.6.1	<i>Innate Immunity.....</i>	<i>10</i>
1.6.2	<i>Humoral Immunity.....</i>	<i>13</i>
1.6.3	<i>Cell-Mediated Immunity.....</i>	<i>15</i>
1.6.4	<i>Immune Modulation.....</i>	<i>16</i>
1.7	Pathogenesis of PRRSV Infection	17
1.7.1	<i>Pathological Observations.....</i>	<i>18</i>
1.8	Aims and Objectives.....	19
2	Materials and Methods	22
2.1	Viruses.....	22
2.2	Animals and experimental design.....	22
2.3	Preparation of PBMC, BAL and LN cells	24
2.4	Microscopic lesion scoring.....	25
2.5	Flow cytometry.....	25
2.6	PRRSV Detection	30
2.6.1	<i>qPCR.....</i>	<i>30</i>
2.6.2	<i>IHC.....</i>	<i>31</i>
2.7	Serology	32
2.7.1	<i>PRRSV-specific Ab.....</i>	<i>32</i>
2.7.2	<i>ADV Ab.....</i>	<i>32</i>

2.8	Cytokine Analysis.....	33
2.8.1	<i>ELISpot</i>	33
2.8.2	<i>Multiplex Assay</i>	33
2.9	Statistical Analysis.....	34
3	Pathogenicity.....	37
3.1	Aims and Rationale	37
3.2	Results	37
3.2.1	<i>Clinical observations</i>	38
3.2.2	<i>Pathology</i>	40
3.2.3	<i>Viraemia and virus in tissues</i>	47
3.3	Discussion	50
4.	Peripheral Immune responses	56
4.1.	Introduction.....	56
4.2.	Results	57
4.2.1.	<i>PRRSV-Specific Ab</i>	57
4.2.2.	<i>Immune cell phenotyping</i>	58
4.2.3.	<i>IFN-γ responses</i>	60
4.3.	Discussion.....	62
5	Local immune responses	67
5.1	Introduction.....	67
5.2	Results	68
5.2.1	<i>Cellular Infiltration</i>	68
5.2.2	<i>Local humoral and cellular responses</i>	73
5.2.3	<i>Cytokine responses</i>	78
5.3	Discussion	82

6	Discussion	93
7	References	99

List of Figures

Figure 1.1.....	7
Figure 1.2.....	9
Figure 2.1.....	25
Figure 2.2.....	26
Figure 2.3.....	27
Figure 2.4.....	28
Figure 2.5.....	29
Figure 3.1.....	37
Figure 3.2.....	39
Figure 3.3.....	41
Figure 3.4.....	43
Figure 3.5.....	44
Figure 4.1.....	56
Figure 4.2.....	58
Figure 4.3.....	59
Figure 4.4.....	61
Figure 5.1.....	67
Figure 5.2.....	68
Figure 5.3.....	69
Figure 5.4.....	70
Figure 5.5.....	71
Figure 5.6.....	72
Figure 5.7.....	73
Figure 5.8.....	74
Figure 5.9.....	75

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List of Abbreviations

ADE	Antibody dependant enhancement
ADV	Ausjezkys disease virus
AM	Alveolar macrophages
BALF	Broncho alveolar lavage fluid
DCs	Dendritic cells
DP	Double positive
dpi	Days post-infection
dsRNA	Double stranded RNA
ELISA	Enzyme-linked immunosorbent assay
ELISPot	Enzyme-linked immunosorbent spot assay
FASL	FAS ligand
Foxp3	Forkhead box protein
GP	Glycoprotein
IFN	Interferon
IHC	Immunohistological staining
IL	iInterleukin
ILN	Inguinal lymph node
JS	Junction sequence
LAC	Leukocyte activation cocktail
LV	Lelystad virus
MHC	Major histocompatibility complex
MLN	mediastinal lymph node
NK	Natural Killer
ORF	Open reading frame
PAM	Porcine alveolar macrophages
PAMPs	Pathogen associated molecular patters
PBMC	Peripheral blood mononuclear cells
PRRS(V)	Porcine reproductive and respiratory syndrome (virus)
(q)PCR	(qualitative) Polymerase chain reaction
RLN	Retropharyngeal lymph node
ROS	Reactive oxygen species
S/P	Sample to positive
SLN	Sternal lymph node
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like resceptor
TLRs	Toll-like receptors
TNF	Tumour necrosis factor

Chapter I

Introduction

1 Introduction

1.1 Porcine Reproductive and Respiratory Syndrome

Porcine reproductive and respiratory syndrome (PRRS) was initially described in the USA during 1987 (Keffaber 1989). PRRS spread rapidly, with the first report of animals displaying similar signs appearing in Germany during 1990 (Lindhaus 1991). Through 1991-1992 the disease spread throughout Europe with outbreaks in the Netherlands, Spain, the UK, France, Belgium and Denmark (OIE 2010). PRRS is now found worldwide and is endemic in all major Pork producing countries (Zimmerman et al. 2012). Due to the endemic status of PRRS, it is no longer a requirement for producers to report outbreaks of the disease amongst their stock; therefore it is not known how many new outbreaks occur or how this compares to outbreaks in wild boar. A Spanish study looked at the prevalence of PRRS infection in wild boar surrounding high density domestic pig populations, and found that only seven of 294 boars sampled were positive for PRRSV (Rodríguez-Prieto & Kukiela n.d.), suggesting that transmission from farms to wild populations is not a major concern, and that PRRSV is perhaps a disease arising from modern farming methods.

Since its emergence, PRRS has become one of the most economically important diseases of swine causing an estimated \$664 million in production losses to the American pork industry each year, and closer to \$1 billion when taking into account veterinary and biosecurity costs (Holtkamp 2011)□. Due to the differences in pig farming practices between the different European countries, there is not an estimated cost of PRRS for Europe. A Dutch study has tried to quantify the cost to the industry in the Netherlands using nine swine farms, they found the cost to be €126 per breeding sow including both production and veterinary costs (Nieuwenhuis et al. 2012). If one were to apply these costs to the UKs estimated 470,000 breeding sows (DEFRA 2009) PRRS would be responsible for £47 million in losses. However, this value must be taken with a pinch of salt, as differences in farming methods between here and the Netherlands will influence costs.

The clinical signs of PRRS infection vary greatly between cases, due to the varying pathogenicity of the different strains, and responses of different animals. Respiratory signs are

seen mainly in young piglets and include tachypnea, dyspnea and coughing. Systemic signs are also predominantly seen in young piglets but may also be observed in sows and boars; pyrexia, anorexia, leading to a delay in reaching selling weights, and general lethargy are the most common observations (Nodelijk 2002). Reproductive failure in infected sows is the most dramatic result of PRRS infection and can manifest as abortions, stillbirths and mummified foetuses. Surviving piglets may be weak while some are born apparently normal. There are also reports of sows displaying infertility in particular a delayed return to oestrus. Affected boars may also have a loss of libido and a temporary reduction in semen quality (Nodelijk 2002). A study on infection demographics in Ontario found that the highest proportion of animals displaying clinical signs were in a farrow to finisher herd at 96.1% with nursery respiratory disease and nursery mortality being the most common signs (Young et al. 2010).

1.1.1 PRRSV Transmission and Control

As pigs are highly social creatures the predominant route of PRRSV transmission within a pig herd is direct physical contact between infected and naïve animals (Rossow 1998). Survival of PRRSV on fomites is not prolonged, although transmission via this route is still seen and should be considered when trying to control the virus, however, virus may survive for up to 11 days in water so drinking water is considered an important source of infection (Pirtle & Beran 1996). Mechanical transmission of PRRSV via non-biological insect vectors has been shown experimentally (S. A. D. K. D. R. R. D. M. C. P. Satoshi Otake 2002) but further studies have found that the virus may only survive for up to 12 hours in the digestive tracts of insects (S. A. D. R. D. M. K. D. R. C. T. M. F. C. P. Satoshi Otake 2003) perhaps limiting ability of insects to carry virus between farms. Simple steps can be taken to help reduce the risk of PRRSV transmission through a herd such as thorough disinfection of pens before bringing in new animals, quarantining and testing of animals coming onto the farm and perhaps appropriate insect control.

Due to the respiratory disease observed during PRRSV infection one would expect aerosol transmission both within and between farms to play a considerable role in the transmission of the virus. Experiments have shown that air-borne transmission of PRRSV can occur within

farm (Kristensen et al. 2004). Air sampling methods have shown that live PRRSV can be detected up to 4.7 km away from an infected farm (Scott Dee 2009) although transmission between farms would still depend on a number of environmental factors such as temperature, humidity and wind direction. Air filtration devices installed onto large-scale pig farms have shown to significantly reduce the risk of PRRS introduction to a naive herd as long as the farm has good biosecurity practises in place (Alonso et al. 2013).

Despite evidence that correct farm management, and biosecurity protocols can do a lot to reduce the burden of PRRSV infection on farms, controlling PRRS has proven difficult over the years with few countries claiming to be free of the disease. Both modified live and killed vaccines are commercially available but both come with their own limitations and risks. Killed vaccines are safe but have displayed poor efficacy due to a poor induction of humoral immunity (Kim et al. 2011). Modified live vaccines have proven to be much more efficacious than their inactivated counterparts, however, they pose more safety concerns as high mutation rates mean a reversion to virulence is much more likely (Charerntantanakul 2012). Current advice on vaccination strategies suggests that farmers in high-risk areas vaccinate breeding sows with a modified live vaccine, but not vaccination programme is enforced or subsidised by government. The MLV vaccines currently available rely upon adapting wild-type viruses to replicate in tissue culture, which renders them either unable to or inefficient at replicating in natural host cells, alveolar macrophages. The requirement for a safe and efficacious vaccine, which provides adequate cross protection between heterologous field strains, is still the holy grail of PRRSV research. Much work is being performed to develop more sophisticated methods for PRRSV vaccination including utilising the naturally conserved regions of the virus such as the N protein or GP5 in the hopes that one day we will have an effective and cross protective vaccine to help combat the PRRSV threat.

1.2 Porcine Reproductive and respiratory Syndrome Virus

PRRSV is an enveloped, single-stranded, positive sense RNA virus (Benfield et al. 1992) and was first isolated by (Wensvoort et al. 1991) in the Netherlands and was designated Lelystad virus (LV). In 1992 Benfield and others (1992) characterised an American isolate of

the then named swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). Both of these viruses are now used as the reference strains for their respective genotypes, described in section 1.3. PRRSV is an *Arterivirus* within the order *Nidovirales* family *Arteriviridae*, alongside equine arteritis virus, lactate dehydrogenase-elevating virus and simian haemorrhagic fever virus (Cavanagh 1997).

1.3 Diversity of PRRSV

PRRSV is divided into two very distinct genotypes, the European (PRRSV-I) and the North American (PRRSV-II), which have only 55-70% nucleotide identity; in fact the North American genotype is more closely related to lactate dehydrogenase-elevating virus than the European genotype (Forsberg et al. 2002). There are two hypotheses to explain this divergence between the two different genotypes. The first suggests that the viruses diverged shortly before emergence, followed by a very high substitution rate (Hanada et al. 2005)□. The second puts forth that the viruses diverged well before emergence and evolved independently on the two continents (Forsberg et al., 2002)□. Using a molecular clock model on an expanded set of open reading frame (ORF) 3 sequences estimated the most recent common ancestor to date back to 1979, more than 10 years before emergence, making it more likely that the two genotypes evolved separately. Presently the most popular theory on the evolution of the two genotypes is that an ancestor of lactate dehydrogenase-elevating virus in rodents made a species jump to wild boar in Europe. Then when wild boars from Europe were introduced to North America in 1912 the viruses evolved separately on the two continents (Shi et al. 2010)□. There is also great variation between virus strains within the two genotypes; these variants can have quite a difference in the outcome of clinical infection. The type I genotype of PRRSV is more diverse than type II. The type I viruses can be split into three subtypes, subtype 1 which is found globally with viruses of this type reported in five non-European countries, subtypes 2 and 3 have so far only been recorded within Eastern Europe (Stadejek et al. 2006). Stadejek also found that these viruses were not only different phylogenetically but also differed in pathogenesis and antigenicity discussed in section 1.7. The type II genotype is not as diverse as the European type, since its original identification in North America; it has spread to Asian and European countries. These viruses appear to be related to the Ingelvac PRRS

MLV vaccine and are found in eight countries, which permit the use of the vaccine (Egli et al. 2001)□. All strains isolated during the outbreak in the early 1990s were found to be related to VR2332 which was the first North American virus characterised, after this initial outbreak further isolated viruses were not related to VR2332 but instead large numbers of vaccine derivatives were found (Shi et al. 2010)□. In 2006 and a highly-pathogenic strain of PRRSV appeared in China, characterised by prolonged high fever, red discolouration on the body and high mortality rates in adult pigs, the disease spread to 20 provinces in China causing major economic losses (Tian et al. 2007).

1.4 Virion Structure and Genome Organisation

The PRRSV Virion is spherical in shape with a diameter of approximately 50-65nm (Wensvoort et al. 1991). The virus particle consists of an isometric core of 25-35nm surrounded by an envelope, which carries small projections (Snijder & Meulenberg 1998)□. The 15.1 kb genome is surrounded by a single nucleocapsid (N) protein with a mass of 12-15 kDa (Bautista et al. 1996). The genome contains nine open reading frames (ORFs) (figure 1.1), which encode the different structural and non-structural proteins of PRRSV.

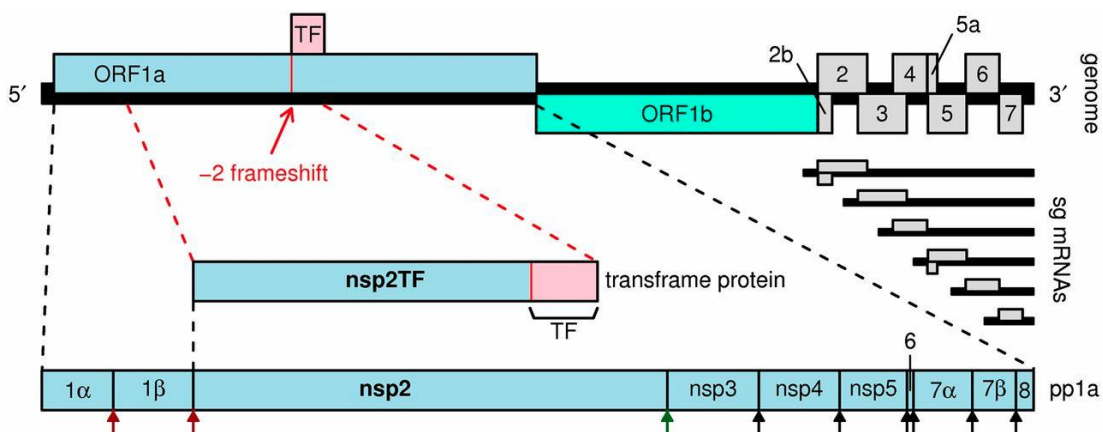


Figure 1.1 Genomic structure of porcine reproductive and respiratory syndrome virus

The N protein, which is encoded by ORF 7, contains five domains of antigenic importance in the North American ATCC VR2332 and four in the European LV strain (Dea et al. 2000)□. The non-glycosylated membrane (M) protein, encoded by ORF 6, is 18-19k Da in size and the most conserved of the structural proteins (Fields & Knipe 2007). It is thought to play an important role in virus assembly due to its close association with other viral envelope proteins, its membrane-associated function and similarity to the coronavirus M protein (Mardassi et al.

1996)□. Cells infected with an M protein free virus do not release viral particles suggesting that the M protein is indeed a required component of PRRSV assembly (Wissink et al. 2005). The ORF 2b or E protein is a newly defined structural protein of arteriviruses, first described in equine arteritis virus (Snijder & Meulenberg 1998). (Wu et al. 2001) were the first to report the presence of a 10 kDa protein in the virion of a North American PRRSV isolate and found that it was encoded by the same open reading frame as the small envelope protein identified in equine arteritis virus, ORF2b. E protein knockouts are unable to infect susceptible cells but do produce new virions in culture supernatant, indicating that that the structure is required for infectivity but not assembly (Lee & Yoo 2006). The major envelope glycoprotein (GP) is GP5 (ORF 5) and is the most diverse of the PRRSV structural proteins with only 50-55% nucleotide identity between the two genotypes and much variation within genotypes (Andreyev et al. 1997)□. The GP5 protein is one of the most immunogenic proteins with anti-GP5 MAbs reported to induce virus neutralisation (Pirzadeh & Dea 1997)□.

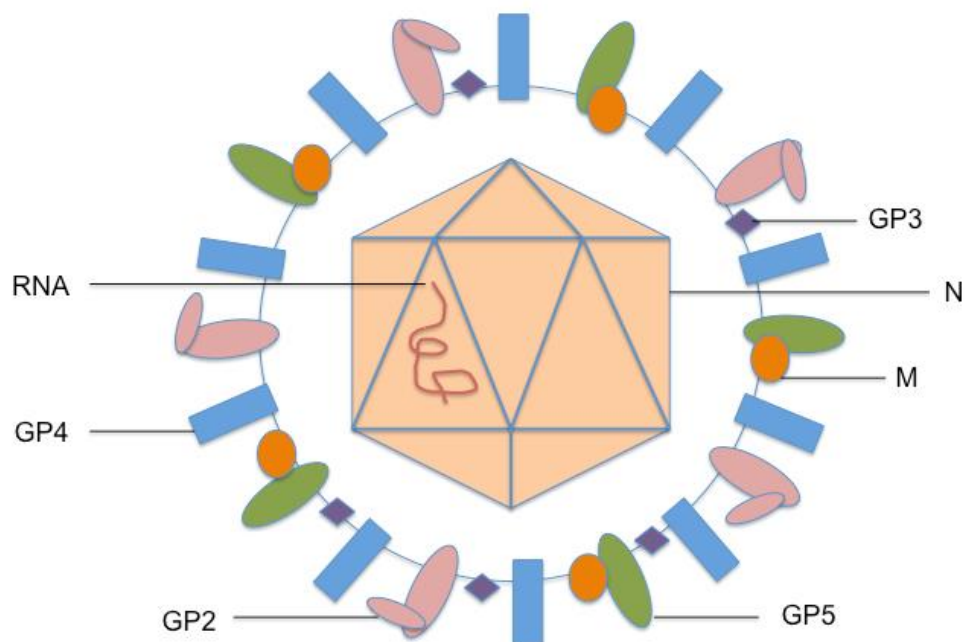


Figure 1.2 PRRSV structure. Schematic representation of the PRRSV virion (adapted from www.PorcillisPRRS.com).

PRRSV contains three minor structural glycoprotein's; GP2, GP4 and GP3. GP2, ORF 2a product, is around 29-30 kDa in size and is incorporated into the extracellular virion (Meulenberg 2000). GP4 (ORF 4) is a 19.6-20 kDa protein and contains 4 putative N-linked glycosylation sites, which are conserved between the two genotypes (Dea et al. 2000). GP4 has been shown to contain neutralising epitopes, which are highly variable between strains, however, anti-GP4 MAbs are less effective at neutralisation than those targeted to GP5 (Weiland et al. 1999)□. GP3, encoded by ORF3, has been difficult to characterise but is thought to be between 27-29 kDa (Gonin et al. 1998). Although the proteins role as part of the virion is also unclear it has been shown to induce neutralising Ab in conjunction with GP5 (Jiang et al. 2008)□. The first open reading frame of PRRSV is split into two sections, ORF 1a and ORF 1b makes up around 80% of the genome and encodes a replicase polyprotein, which is then cleaved to make a number of non-structural proteins (nsp) (Meulenberg 2000). Only the first two N-terminal cleavage products, nsp1a and nsp1b have been shown to be papain-like cysteine proteases (Boon et al. 1995). Nsp2, another cysteine protease and nsp 4 a serine protease are thought to cleave the ORF1 product into a further 12 nsps (Snijder & Meulenberg 1998). Little is known about the functions of the individual nsps.

1.5 Cellular Tropism and Virus Replication

PRRSV has a restricted cellular tropism *in vivo*, infecting cells of the macrophage lineage and with replication occurring primarily in porcine alveolar macrophages (PAMs) (Duan et al. 1997)□. *In vitro*, however, PRRSV has been shown to infect a number of different cell types including green monkey kidney cells, MA-104 and derivatives thereof (CL2621 and Marc-145) (Duan et al. 1997) monocyte-derived dendritic cells (mDCs) (X. Wang et al. 2007b) and mature DCs (Flores-Mendoza et al. 2008)□. (Delputte et al. 2002) found that a heparin like molecule on the surface of PAMs called heparan sulphate (CD163) is the first cell membrane target for PRRSV. The virus is thought to then bind to sialoadhesin (CD169) and is then internalised into the cell via the endocytotic route (Delputte et al. 2002). Once internalised the virus is transported towards an endosomal compartment where a drop in pH is required for efficient virus replication (Nauwynck et al. 1999). However, the sialoadhesin molecule is not present

on some permissible cells such as MARC-145 (Delputte & Nauwynck 2004). (Van Gorp et al. 2008) found that even though sialoadhesin is not essential for virus entry it did act as the attachment and internalisation receptor for PRRSV, but that the scavenger receptor CD163 may play a role in viral uncoating (required for genome integration) and that cells expressing both of these receptors are more susceptible to infection. The PRRSV replication cycle begins with expression of the replicase gene encoded by ORF1a and b both expressed from the genomic mRNA (Snijder & Meulenberg 1998). Translation of ORF1b requires a ribosomal frameshift just before translation of ORF1a ends (Boon et al. 1995). This pseudoknot structure allows the virus to translate the ORF1b portion of the genome, although this does not happen every time, producing more opportunity for variation. The next step involves the transcription of subgenomic mRNAs, which in arteriviruses consist of a leader and body part, which are non-contiguous in the genome sequence and transcribed from sequences in the 3' end and 5' terminal region (Snijder & Meulenberg 1998). The connection between the two sections is formed by a conserved junction site (JS) found at the 3' end of the common leader sequence and the 5' end of the mRNA body. The (+) JS at the 3' end can base pair with the (-) JS upstream of each transcription unit in the (-) template leading to the proposal of the leader-primed transcription model. Base-pairing of the (+) and (-) JS is followed by extension of the leader to give a subgenomic mRNA (Snijder & Meulenberg 1998). Analysis of mRNA JS sequences showed that the 3' side is more conserved than the 5' side suggesting that leader-to-body fusion mechanism may be imprecise (Godeny et al. 1998).

1.6 PRRSV and the Immune System

1.6.1 Innate Immunity

The innate immune system consists of a number of components, from the physical barrier provided by epithelial cells to the destruction of pathogens by specialised immune cells, such as macrophages, which together provide the first line of host defence. It provides a fast, non-specific response against invading pathogens, as well as acting as an activator of adaptive immunity. Although more primitive than the adaptive immune system the importance of the innate system during viral infection is becoming apparent. Although the innate immunity is described as non-specific, it does possess some capacity to identify invading pathogens.

Pathogen recognition receptors recognise different pathogen-associated molecular patterns (PAMPs) and activate pathways that induce immune responses.

Toll-like receptors (TLRs) are a form of pathogen recognition receptor important for anti-viral immunity, in particular TLRs 3, 7, 8 and 9. TLRs 7 and 8 are known to recognise single-stranded RNA (ssRNA), their endosomal location makes them important in the recognition of viruses which enter a cell via endocytosis (Seth et al. 2006), such as PRRSV. Activation of TLR7 and 8 induces the production of type I interferons (IFN) and proinflammatory cytokines through the MyD88 dependent activation of the IFN regulatory factor (IRF) 7 and NFκB (Kawai & Akira 2006). (Liu et al. 2009) found that expression of both TLR7 and 8 was increased in peripheral blood mononuclear cells (PBMC) from pigs infected with PRRSV, compared to mock-infected controls. TLR3 recognises double stranded RNA (dsRNA) (Seth et al 2006), which is produced as an intermediary during the PRRSV replication cycle (Snijder & Meulenberg 1998). TLR3 utilises a MyD88 independent pathway that signals through the adapter protein TRIF which in turn activates IRF3 and NFκB (Yamamoto 2003). (Sang et al. 2008) found increased expression of TLR3 in lung tissue from PRRSV infected pigs, but did not see the same up-regulation in PAMs infected *in vitro*. Expression of PRRSV RNA was also high in these samples, suggesting that the up-regulation of TLR3 did not have an inhibitory effect upon virus replication. In contrast a study by (Miguel et al. 2010) found that expression of TLR3 and 7 RNA was up-regulated in the tracheobronchial lymph nodes of PRRSV infected pigs but not in the lung.

Retinoic acid inducible gene I (RIG-I) and melanoma differentiation-association protein 5 have both been identified as intracellular receptors for dsRNA (Yoneyama et al. 2004). These receptors again initiate pathways leading to the activation of NFκB and IRF3 and subsequent production of type I IFN and proinflammatory cytokines (Fitzgerald et al. 2003) although in this case via interactions with mitochondrial anti-viral signalling protein (Kawai et al. 2005). (Luo et al. 2008) investigated the effect of PRRSV infection on the RIG-I pathway in MARC-145 cells and found that PRRSV infection alone did not significantly alter the RIG-I pathway but did inhibit later responses to dsRNA, this immunomodulation will be further discussed in section 1.6.4.

The type I IFN α and β cytokines are the hallmark of the innate anti-viral response (Pfeffer et al. 1998). Produced by an abundance of cell types, they trigger the expression of hundreds of different genes, which in turn induce a number of antiviral mechanisms, such as RNA degradation, and inhibition of translation and virus trafficking (Y. Sun et al. 2012). The susceptibility of PRRSV to IFN- α has been demonstrated both *in vitro* and *in vivo* (Buddaert et al. 1998). Although it is widely accepted that PRRSV inhibits the type I IFN response (Calzada-Nova et al. 2011; Patel et al. 2010; Van Reeth et al. 1999) discussed further in section 1.6.4, (Chung et al. 2004) has reported a significant induction of IFN- α producing cells in the lungs of PRRSV infected pigs.

Macrophages are mononuclear phagocytic cells of the myeloid lineage and are the major innate immune cells in the tissues. They are of particular importance in the lung as the large surface area of the pulmonary cavity is under constant assault from pathogens and toxins in the air. Alveolar macrophages originate from blood monocytes which migrate into the lungs, here they differentiate into parenchymal macrophages before entering the alveolar space (Landsman & S. Jung 2007). The roles of macrophages encompass a number of processes including maintenance of a homeostatic environment, tissue remodelling and host defence (Lambrecht 2006). Alveolar macrophages play a central role in innate immunity through phagocytosis of pathogens, which may occur with or without the help of complement, and subsequent production of a number of cytokines and chemokines which trigger inflammation and the adaptive response (Gordon & Read 2002). As the primary site of PRRSV replication, macrophages play an integral role during PRRSV infection. A flow cytometry study on broncho-alveolar cells by (Samsom et al. 2000) found that the numbers of PAMs in the lung of PRRSV infected pigs did not change, although due to lymphocytic infiltration, the percentage of PAMs decreased. (Gómez-Laguna et al. 2010) histological study showed that the total number of macrophages in PRRSV infected pigs increased above control animals, however, the number of PAMs decreased at 3 and 7 days post-infection (dpi). Their study also demonstrated that PRRSV infection induced the production of the proinflammatory cytokines interleukin (IL)-1, 6 and tumour necrosis factor (TNF)- α , anti-viral IFN- α and γ and the immunosuppressive cytokine IL-10, in interstitial but not alveolar macrophages. There has

also been evidence to show that PRRSV has a suppressing effect upon macrophages, discussed in section 1.6.4.

Natural killer (NK) cells are cytotoxic lymphocytes which differ from cytotoxic T cells by their ability to kill target cells without the need for specific antigen recognition (Andoniou et al. 2006). Despite this characteristic it is now believed that these cells also contribute to the adaptive immune response. A study by (J. C. Sun et al. 2009) found that in a mouse model of cytomegalovirus, NK cells not only had virus-specific receptors, but also underwent clonal expansion and had faster responses upon re-challenge, hallmarks of the adaptive response. PRRSV infection has been shown to increase the numbers of NK cells in both the blood (Dwivedi et al. 2012) and the lungs (Samsom et al. 2000) of infected pigs. However, functional studies have shown that the cytotoxicity of these cells is reduced as a result of PRRSV infection (Dwivedi et al. 2012; K. Jung et al. 2009). The field has thus far rather neglected research into the functions of NK cells in the lungs during PRRSV infection.

The respiratory epithelium is the first barrier encountered by pathogens entering the host through the airways. Their importance in innate immunity, through expression of the pattern recognition receptors described above and production of cytokines and chemokines (Parker & Prince 2011), has been largely overlooked in PRRSV immunology. This is surprising, as studies have identified PRRS antigen in the respiratory epithelium (Hu et al. 2012; Rossow et al. 1996). As these cells are able to recognise PRRSV RNA and dsDNA, and produce immune molecules in response, their role in local immune responses should be considered.

1.6.2 Humoral Immunity

Humoral immunity is the branch of the adaptive (antigen specific) immune system, mediated by B lymphocytes and their antibodies. B cells can be activated in either a T cell dependent or independent manner, and once activated, may differentiate in to antibody secreting plasma cells (Parker 1993). Antibodies possess both variable regions, which bind to pathogens, and constant (Fc) regions, Fc regions bind to a number of receptors on different immune cells triggering downstream responses. Antiviral mechanisms of humoral immunity include

production of neutralising antibodies, enhancement of phagocytosis, either directly or through complement activation, and antibody-dependant cellular cytotoxicity. Neutralising antibodies prevent virus entry into target cells by attaching to virus proteins that are involved in cellular tropism (Klasse & Sattentau 2002) hopefully restricting the replication of the virus at the later stages of infection. Antibodies bound to viral surface receptors enhance opsonisation by phagocytes through interaction with Fc receptors (R I Connor 1991) they also enhance complement mediated opsonisation by activating the complement cascade (Stoiber et al. 2001). Finally antibodies are able to induce cytotoxic responses of NK cells through antibody-dependant cellular cytotoxicity, the CD16 molecule on the NK cells surface can be activated through binding to the Fc region of an antibody leading to degranulation and perforin production (Caligiuri 2008).

Anti-PRRSV immunoglobulin (Ig) M antibodies appear in the serum of infected pigs by 5-7 dpi declining to undetectable levels after 2-3 weeks (Yoon et al. 1995). Levels of IgG reach a maximum at 21-49 dpi (Loemba et al. 1996). These early antibody responses are not neutralising and it has been suggested that they may enhance PRRSV infection of PAMs through antibody-dependent enhancement (ADE) of viral tropism (YOON et al. 1996) as has been shown for the related severe acute respiratory syndrome coronavirus (SARS-CoV) (Jaume et al. 2011). Although overall robust (Mulupuri et al. 2007) found that antibody titres declined overtime despite the continued presence of viral antigen in lymphoid tissues and that the humoral response to GP5 was delayed and weak compared to other viral proteins.

The development of neutralising antibodies is delayed during PRRSV infection, usually being detected by conventional tests after 28 dpi (Diaz 2005). Neutralizing antibodies are mainly targeted against the GP5 protein (Pirzadeh & Dea 1997) and have been shown to block the infection of PAMs by PRRSV although the receptor involved is yet to be identified (Delputte *et al.* 2004). Although natural infection with other swine diseases such as classical swine fever and swine influenza virus induce good humoral immune responses, the best protective vaccines in both of these cases do not induce an impressive Ab response but rather induce a potent T cell response, particularly when administered by the respiratory tract.

The cell-mediated response is antigen specific, T cells, which develop in the thymus, recognise processed antigen that is presented primarily by dendritic cells (DCs). DCs patrol the respiratory barrier and uptake viral proteins, migrate to the local lymph nodes where they present antigen to T cells, which are activated and proliferate. These cells then migrate to the site of infection where they mediate a number of anti-viral mechanisms, the notable of which is the production of IFN- γ .

The cytokine IFN- γ is a key indicator of the induction of a cell-mediated immune response. In pigs infected with PRRSV virus specific IFN- γ producing cells first appear at around 3 weeks post-infection with numbers fluctuating higher than uninfected animals, between 100-300 per million peripheral blood mononuclear cells (PBMCs) increasing to 400-500 per million PBMCs by the 48th week post-infection (Meier et al. 2003)□. These cells were mainly CD4+CD8+ T cells with a small number of CD8 cytotoxic lymphocytes. This cell-mediated response appears to be weaker than those observed in other porcine viral infections such as Aujeszky's disease virus, which at three weeks post-infection produces 200-300 IFN- γ producing cells per million PBMCs (Meier *et al.* 2003). Potent activation of T cells to induce a strong IFN- γ response. Pigs vaccinated with the C strain vaccine against classical swine fever and then challenged with live virus display a much more potent increase in cytokine secreting cells compared with virus infected alone controls (Suradhat et al. 2001).

Cytotoxic T cells are another potent producer of IFN- γ , re-stimulating PBMCs from PRRSV infected pigs has been found to induce the proliferation of cytotoxic T cells, although PRRSV specific cytotoxic activity was not observed until 56 dpi (Costers et al. 2009). Cytotoxic T cells are also able to kill virus-infected cells through the Fas and perforin pathways (Topham et al. 1997), lysis of virus-infected cells prevents viral replication, but destruction of macrophages in this manner is thought to contribute to immune suppression.

Due to these accounts of delayed cellular immunity during PRRSV infection, this has been an area of neglect in PRRSV research, although observations in the field that cell-mediated

immunity correlates with protection (Lowe et al. 2005) have re-ignited interest in this area and hopefully more detailed studies will be performed in the future.

1.6.4 Immune Modulation

When PRRS first appeared as a clinical disease, veterinarians and scientists had problems identifying the causative agent. Although a number of bacterial and viral agents were isolated from affected animals only PRRSV was able to recreate clinical disease under experimental conditions. The presence of a number of pathogens in affected animals brought about the question as to whether PRRSV had an immunosuppressive effect upon the host, leaving it susceptible to secondary infections.

As mentioned in section 1.3.1 PRRSV has been shown to suppress the production of a type I IFNs and TNF- α . It has also been shown that the virus up-regulates the expression of immunosuppressive cytokine interleukin (IL) 10 in porcine PBMCs (Suradhat et al. 2003)□. PRRSV has also been shown to have a regulatory effect on antigen-presenting cells (APCs). (Loving et al. 2007)□ found that PRRSV down-regulated the expression of major histocompatibility complex (MHC)-I, which present processed epitopes to lymphocytes, in DCs. Expression of both MHC-I and II as well as CD14, a co-stimulatory molecule for TLR4 which recognises gram-negative bacteria, in monocyte-derived DCs (X. Wang et al. 2007b). These alterations to APC gene expression have been shown to impair lymphocyte proliferation and which could explain the susceptibility to secondary infection seen in many cases of PRRS. How exactly PRRSV modulates the host immune response is still to be further elucidated.

Reports on PRRSV and regulatory T cells (Tregs) are conflicting. Studies have shown that North American PRRSV has the ability to stimulate inducible Tregs (Silva-Campa et al. 2012), but were unable to do so with European type strains (Silva-Campa et al. 2010). If PRRSV can induce Tregs, this may explain part of the IL-10 response that has been reported during infection. Tregs modulate the immune response through production of IL-10 and cytolytic agents (Vignali et al. 2008) and may play a role in the susceptibility to secondary infection noted in PRRSV infected pigs in the field.

1.7 Pathogenesis of PRRSV Infection

The pathogenesis of PRRSV infection is complex and as yet, not fully understood. Often the presence of secondary infection is attributed to the presentation of clinical disease, however, this does not explain the results observed during experimental infections using gnotobiotic pigs (Collins et al. 1992; Rossow et al. 1995). If steps are to be taken to further improve on current vaccines research must go back to the missing links in basic research and improve understanding of pathogenesis and immune responses to PRRSV.

Once viral particles have entered the lungs of piglets through inhalation, PRRSV enters alveolar macrophages by the endocytic pathway and replicates. During replication in the lung PRRSV induces apoptosis of both virus-infected and bystander cells (Labarque, Van Gucht, Nauwynck, Van Reeth & Pensaert 2003a; Sur et al. 1998). This loss of front line immune cells is thought to contribute to immune suppression, discussed in section 1.6.4. PRRSV has also been found to damage the ciliated epithelium of the pulmonary tract (Halbur et al. 1995), hence restricting the transportation of pathogens trapped within the mucosa, back towards the upper respiratory tract for expulsion.

Virus can be detected in the serum of infected pigs as early as 12 hours post-exposure (Rossow et al. 1995), however, the pattern and length of viraemia varies between strains, however, virus is normally detected for up to a month during acute infection. There have been reports of virus being detected in serum up to 210 post-exposure during persistent infection (Rossow 1998). PRRSV antigen is also detected in the tissues of infected pigs, although primarily found in organs of the lymph system such as the tonsils, spleen and lymph nodes, there are also reports of antigen detection in the kidneys, small intestine and adrenal glands (Halbur, Paul, Meng, et al. 1996b). Despite most tissues being confirmed to be free of virus eventually, PRRSV has been found to re-emerge within a few months after initial infection, whether this is the same virus which has lay dormant in a tissue, or if this is the introduction of a new strain to the herd, is not certain (Christopher-Hennings et al. 2002).

Pyrexia and poor weight gain due to inappetance are the most common systemic signs of PRRSV infection. Pyrexia is caused by the effect of the virus as an exogenous pyrogen which, upon interaction with TLRs on macrophages, may induce the production of the main endogenous pyrogens such as IL-1, TNF- α and IL-6 (Dinarello 2004). As described in section 1.1 the respiratory signs of PRRSV infection include tachypnea, dyspnea, sneezing and coughing. During infection an inflammatory response in the lung causes infiltration of immune cells and tissue damage, which leads to varying degrees of interstitial pneumonia, described by (Halbur et al. 1995). This damage to the respiratory epithelia and particularly the thickening of the alveolar walls leads to the respiratory signs described during infection. Lesions can also be observed in the lymph nodes and lymphoid organs, typically PRRSV presents with follicular hypertrophy, hyperplasia, and necrosis (Halbur et al. 1995).

Pulmonary inflammation is probably one of the most significant observations during PRRSV infection. Inflammation is the complex immunological response to potentially harmful stimuli such as damaged cells, foreign objects or indeed invading pathogens. Upon entering the lung viruses may interact with TLRs, as mentioned in section 1.6.1 activation of TLRs initiates a pathway cascade, which leads to the production of a number of proinflammatory cytokines such as IL-1, IL-6 TNF and IL-8. The next step in inflammation is the recruitment of neutrophils, the hallmark of acute inflammation, to the site of infection or tissue damage by the chemokine IL-8. Once at the site of infection neutrophils contribute to inflammation by releasing a number of inflammatory mediators in a process called degranulation, as well as having antimicrobial properties degranulation can also cause tissue damage (Wright et al. 2010). Despite the role neutrophils have in exacerbating inflammation they are required for the initiation of tissue repair by macrophages later on (Butterfield et al. 2006). Alongside innate cells lymphocytes are also recruited to sites of inflammation from secondary lymphoid organs by the production of T cell chemoattractants (D'Ambrosio et al. 2001). Once at the site of infection effector T cells aid in the clearance of virus, but also contribute to inflammation either through direct cytolytic activity or by production of pro-inflammatory mediators (Monaco et al. 2004). Although not classically considered to be the main inflammatory cell type, the importance of these cells

in the pathogenesis of pulmonary inflammatory diseases such as chronic obstructive pulmonary disorder (COPD) is becoming apparent (Gadgil & Duncan 2008). A genome-wide study by (S. Xiao, Jia, et al. 2010a), using deep sequencing methods on lung tissue, found that infection with PRRSV-II upregulated the expression of pro-inflammatory cytokines and chemokines, adhesion molecules and complement activation all of which contribute to the inflammation observed during infection. Inflammation is resolved when apoptotic neutrophils are phagocytosed by macrophages; this process induces the release of anti-inflammatory cytokines such as TGF- β (Serhan & Savill 2005).

1.8 Aims and Objectives

As new subtype 3 viruses are emerging in Eastern Europe one of which has already been reported to be more pathogenic than the standard subtype 1 strains, characterisation of infections with more of these viruses is important to assess the threat to the European pork industry. The work will also investigate the immune responses to the separate viruses; comparisons of the response induced by the individual strains may shed light upon the pathogenesis of PRRSV infection and why different strains can induce such different clinical signs. As secondary infection during PRRSV infection appears to be a common observation we also investigate whether the ability of these strains to suppress the adaptive response to a secondary antigen differs. Reports of vaccine-like PRRSV viruses being isolated from commercial pigs in the UK have brought into question the safety of the current vaccines used in the field. The first aim of this study was to determine if the intranasal infection with the DV strain (used in the Porcillis PRRS vaccine) is able to infect pigs and cause disease *in vivo*.

Specific objectives;

1. Identify if another subtype 1 strain of PRRSV (SU1-bel) shows similarities to the pathogenic strain Lena (another subtype 3 strain). Investigate the role of immune modulated pathology by comparing local immune responses with less pathogenic strains (LV). PRRSV infected pigs will be tested for PRRSV-specific antibodies, IFN- γ responses, leukocyte populations and pro- and anti-inflammatory cytokine production.

-
2. Try and determine whether infection with any of these strains of PRRSV has a functional effect upon the response to a secondary antigen. Pigs will be inoculated with an Aujeszky's vaccine and antigen-specific humoral and cellular immune responses in the blood measured by IFN- γ .
 3. Determine whether a vaccine strain is able to infect animals and lead to clinical disease *in vivo* and compare these results with other subtype one strains and a newly emerged subtype 3 strain. Animals will be experimentally infected with four strains of PRRSV; clinical scores, viral loads and pathological changes will be investigated.

Chapter II

Materials and Methods

2 Materials and Methods

2.1 Viruses

Four genotype I PRRSV strains were used in this study: Lelystad virus-*Ter Huurne* (LV), the prototype PRRSV-I. Strain 215-06 was isolated at the Animal Health and Veterinary Laboratories Agency from the serum of a post-weaning piglet showing signs of wasting and poor condition on a farm in Suffolk, England in 2006. Strain SU1-bel was also isolated at AHVLA, from lung tissue homogenate provided by Dr Tomasz Stadejek, Warsaw University of Life Sciences, Poland, from a 30-day old piglet from a farm in Belarus in 2010. The final strain is the strain used in the commercially available vaccine Porcillis PRRS (MSD Animal Health). Live freeze-dried DV strain of PRRSV was reconstituted in media and cultured on Green monkey kidney cells (MARC145) for approximately 5 days in a humidified incubator at 37°C with 5% CO₂. Three strains were propagated in PAMs which were harvested from the lungs of piglets as described previously (Wensvoort et al. 1991) and cultured in RPMI-1640 medium (Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) (Autogen Bioclear, Calne, UK) and 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Oxford, UK) (cRPMI) for three days in a humidified incubator at 37°C with 5% CO₂. Virus titers were determined using an immunoperoxidase monolayer assay (Botner et al. 1994).□ Both the SU1-bel and 215-06 strains were used at the 4th passage, LV at the 8th passage and DV at the 3rd passage.

2.2 Animals and experimental design

A total of 96 Yorkshire cross Dutch Landrace 5-week old male piglets, negative for porcine circovirus type 2 (PCV-2), a common co-infection found in pigs infected with PRRSV which causes increased morbidity, were obtained from an isolated, specific-pathogen-free pig farm in the Netherlands. Animals were statistically blocked by weight and allocated as follows: the control group consisted of 16 pigs while each virus-infected group contained 20 animals. Groups were housed in 5 separate pens of a containment facility at the AHVLA, which allowed for the free flow of air from outside. No equipment was shared and staff changed in between rooms to prevent virus transmission between the groups. At seven weeks of age, after

acclimatising for 14 days, the piglets were inoculated intranasally with 10^5 TCID₅₀ of the respective virus (either LV, 215-06 or SU1-bel) in 1.5 ml of cRPMI with controls 'mock' infected with 1.5 ml of PAM cryolysate diluted in cRPMI.

Monitoring of clinical scores and rectal temperatures was performed daily from -3 days post-infection (dpi) until the end of the study, 35 dpi. Clinical signs associated with PRRSV infection were scored between 0-3, where 0 was normal and a score 1-3 represented increasing severity of each observation. Animals were also weighed on a weekly basis. Blood samples were taken at 0, 3, 5, 7, 9, 12, 14, 21, 28 and 35 dpi, these included coagulated blood for serum collection, which was aliquoted and stored at -70°C for later analysis, and heparinised blood, which was used immediately for immunophenotyping by flow cytometry and the quantification of T cell responses by interferon (IFN)- γ ELISpot assay.

At both 3 and 7 dpi, four pigs from the control group and 5 pigs from each infected group were euthanised. At 7 dpi half of the remaining animals in each group were vaccinated intramuscularly with an Aujeszky Disease vaccine (Suvaxyn Aujeszky I.N./I.M., Fort Dodge, Naarden, The Netherlands) with a booster vaccination at 21 dpi as per the manufacturer's instructions. All remaining animals were euthanized at 35 dpi. Humane end-points were set before the onset of the experiment and any animals that met these criteria were euthanised. Animals were euthanised using a lethal dose of pentobarbital followed by exsanguination. This experiment was approved by the AHVLA ethical review committee, and all procedures were carried out under the Animals (Scientific Procedures) Act, 1986, UK.

At post mortem, gross pathology scores of the lungs were performed in a blind fashion based on the method developed by Halbur et al. (1996). Briefly, lungs were divided into five sections and each section on both sides were given a score out of 10 for the amount of the lung surface to be affected by lesions, making the overall score for the lungs a percentage of total lung surfaces to be affected. Tissues were removed from the body in a precise order and placed onto sterile petri dishes to reduce the opportunity for contamination.

Heparinised blood from eight piglets (vaccinated and non-vaccinated controls) in each group was used to isolate PBMCs by layering density centrifugation over Ficoll (SIGMA-ALDRICH, Dorset, UK). Blood was diluted 1:2 with PBS (Invitrogen, Paisly, UK), the tubes were then centrifuged at room temperature for 20 minutes at 800 x *g*. After centrifugation the PBMCs were harvested from just above the porous barrier. The cells were then washed three times in PBS (Invitrogen) by centrifuging at 10°C for 15 minutes at 250 x *g*. Following the washing steps cells were resuspended in 1 mL of PBS (Invitrogen) and counted using a quantitative flow cytometer MACSQuant (Miltenyi Biotec, Bisley UK).

BALF cells were isolated from the left lung in a sterile environment, 200 ml of cold Dulbecco's phosphate buffered saline (DPBS) (Life Technologies, Paisley, UK). After massaging the lung 100 ml of fluid was recovered and centrifuged at 640x*g* for 10 minutes at 4°C. The broncho-alveolar lavage fluid (BALF) was removed from the cell pellets and stored at -70°C for subsequent analyses. The resulting fluid was deposited into 100ml falcon centrifuge tubes (Falcon Scientific, Seaton Delaval, UK). Following removal of the BALF cells were washed a further two times and cells were resuspended in 1 mL of PBS (Invitrogen) and counted using a quantitative flow cytometer MACSQuant (Miltenyi Biotec).

Mediastinal lymph node cells were collected in a sterile environment by breaking up the connective tissue over a cell strainer and collecting the resulting single cell suspension. Following removal of the BALF cells were washed a further two times and cells were resuspended in 1 mL of PBS (Invitrogen) and counted using a quantitative flow cytometer MACSQuant (Miltenyi Biotec).

2.4 Microscopic lesion scoring

The following samples were taken from all the pigs: right apical lung lobe, right middle lung lobe, right caudal lung lobe, Cranial mediastinal lymph node (LN), right medial retropharyngeal LN, sternal LN, right inguinal superficial LN, right tonsil, thymus and spleen. Samples were fixed in 10% buffered formalin for 24 h and processed for histopathological and immunohistochemical examination. Four-micron tissue sections were stained with haematoxylin and eosin (HE). Score systems were used to evaluate the histopathological changes in tissue sections. The severity of histopathological lesions in the lung was scored as previously described by Halbur et al., 1996: 0, no microscopic lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia. The severity of the lesions within lymphoid organs was scored as follows: 0, within normal limits; 1, light hyperplasia of lymphoid follicles, with isolated cellular necrosis; 2, moderate follicular hyperplasia with multifocal cellular necrosis and 3, severe follicular hyperplasia with abundant cellular necrosis.

2.5 Flow cytometry

50µl of whole blood and 2×10^5 isolated BALF cells were triple stained with mAbs directed to porcine SWC1, SWC3 and SWC8 for identification of leukocyte populations or CD3, CD4 and CD8 for identification of T-cell subpopulations. The staining with primary antibodies was followed by a combination of PerCP, FITC and PE labelled secondary antibodies and dilutions were performed in PBS. Primary antibodies used were: mouse anti-porcine-CD3: IgG₁ (1:2); mouse anti-porcine-CD4: IgG_{2b} (1:2); mouse anti-porcine-CD8: IgG_{2a} (1:2), mouse anti-porcine-SWC1a: IgG₁ (1:5) (all AbD Serotec, Oxford UK), mouse anti-porcine-SWC3: IgG_{2b} (1:10) (VMRD, Pullman, WA, USA), mouse anti-porcine-SWC8: (neat) IgM (AbD Serotec). Secondary antibodies used were: rat anti-mouse IgG₁ PerCP (BD), goat anti-mouse IgG_{2b} FITC (1:10) (Southern Biotech, Birmingham, Alabama, USA) and goat anti-mouse IgG_{2a} PE (neat) (Southern Biotech) and goat anti-mouse IgM PE (Southern Biotech). Neutrophils were identified as SWC1⁺SWC3⁺SWC8⁺, monocytes as SWC1⁺SWC3⁺SWC8⁻, and B-cells as SWC1⁻SWC3⁻SWC8^{high} (Summerfield et al., 2001) figure 2.1.

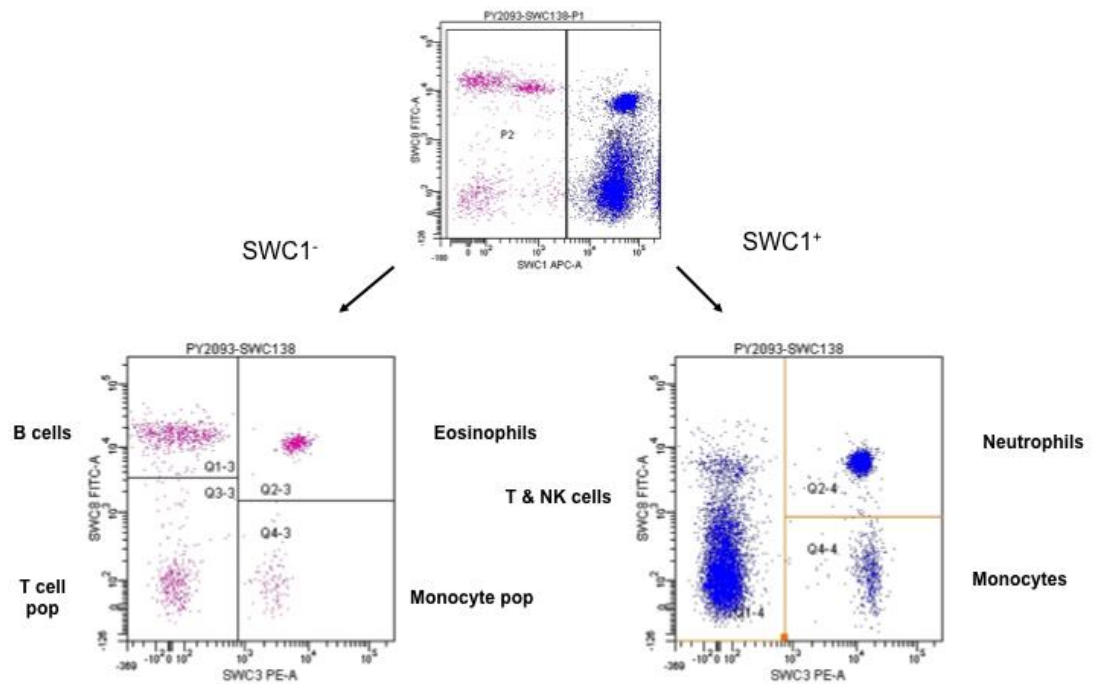


Figure 2.1 Gating strategy for SWC1, 3 and 8 staining. Neutrophils were identified as SWC1⁺SWC3⁺SWC8⁺, monocytes as SWC1⁺SWC3⁺SWC8⁻, and B-cells as SWC1⁻SWC3⁺SWC8^{high}

The cytotoxic T-cell sub-population (Tc) was identified as CD3⁺CD4⁻CD8^{high}, $\gamma\delta$ -T-cells as CD3⁺CD4⁻CD8⁻, naïve T-helper cells as CD3⁺CD4⁺CD8⁻ (T-helper cells), memory T-helper cells CD3⁺CD4⁺CD8⁺ and NK cells as CD3⁺CD4⁻CD8⁺ cells (Nielsen et al., 2003; Gerner et al., 2009).

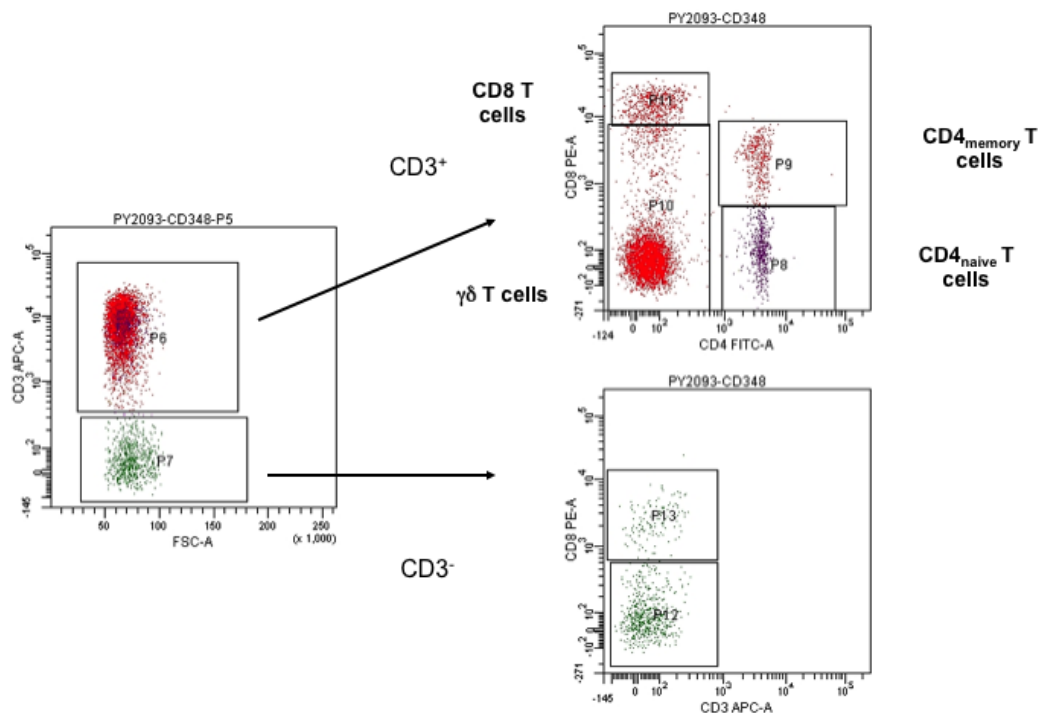


Figure 2.2 Gating strategy for CD3, 4 and 8 staining. Lymphocytes are discriminated from other populations by their expression of CD3, cells high in expression with CD8 are cytotoxic T cells, and cells positive for both CD4 and CD8 are memory/effector cells.

Regulatory T cell (Treg) populations in the blood and BALF were also examined by flow cytometry, 50 μ l of whole blood and 2×10^5 BALF cells were stained with anti-pig CD4-FITC, anti-pig CD25-PE (both AbD Serotec, Oxford, UK) and LIVE/DEAD® Fixable Near-IR Dead Cell Stain (Life Technologies) after which the red blood cells were lysed by adding 1ml of Pharmlyse (BD Bioscience, Oxford, UK). Single colour controls that did not require subsequent processing were lysed and fixed using FACS lysing solution (BD Bioscience). Forkhead Box p3 (Foxp3) staining was carried out using a commercially available kit (eBioscience, Hatfield, UK). Briefly, cells were fixed using Foxp3 Fixation/Permeabilization solution, after which they were washed in Permeabilization buffer and stained with cross-reactive anti-human CD3-Pacific Blue (AbD Serotec) and anti-mouse Foxp3 (clone FJK-16s, rat IgG2a; Käser et al. (2008)). To control for FoxP3 staining, an isotype control mAb was used according to the manufacturer's instructions. After a final wash in Permeabilization buffer, the cells were

resuspended in DPBS prior to acquisition on a MACSQuant flow cytometer. Tregs were identified as CD3+CD4+CD25^{hi}Foxp3⁺ (see figure 2.3)

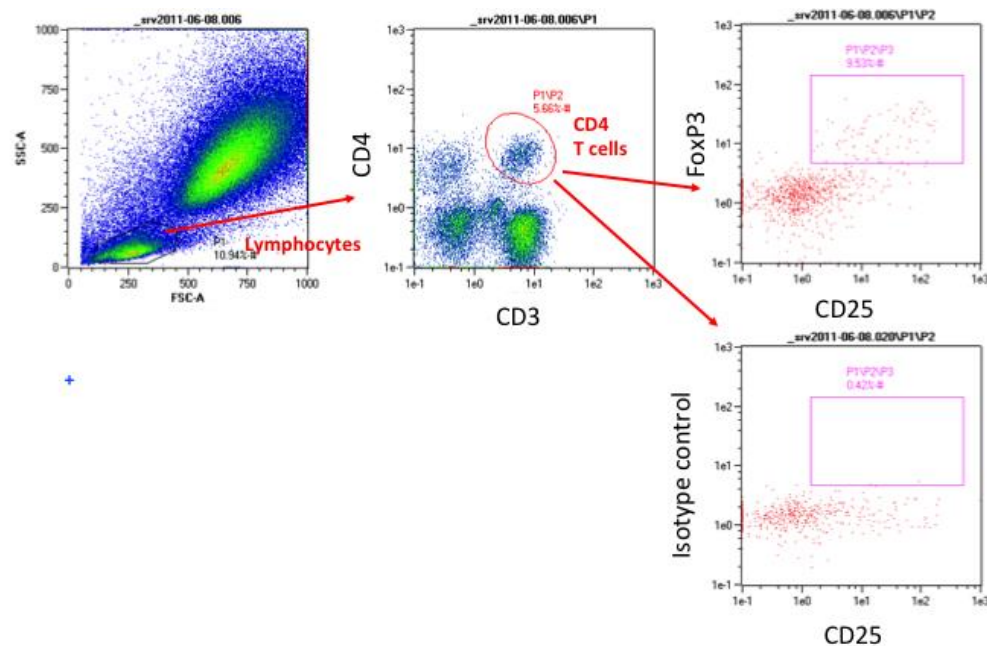


Figure 2.3 Gating strategy for Regulatory T cell stain. Lymphocytes are gated on to distinguish them from myeloid cells, then cells positive for both CD3 and CD4 are selected. From here Foxp3 is plotted against CD25. Double positive cells are regulatory T cells. The Foxp3 isotype used in this assay is also shown.

For intracellular IFN- γ staining, BALF cells were stimulated with mock supernatant in the presence of BD GolgiStop (BD, Oxford, UK) at 4 μ l per ml of cell culture and BD GolgiPlug (BD) at 1 μ l per ml of cell culture, or leukocyte activation cocktail (LAC) (BD) at 2 μ l per ml of cell culture in the presence of BD GolgiStop at 4 μ l per ml of cell culture. Cultures were left for eight hours in a humidified incubator at 37°C with 5% CO₂. Cells were then washed twice in PBS by centrifuging at 640 \times g for 2 minutes cells were resuspended in PBS and surfaced stained, 2 \times 10⁵ BALF cells were stained with anti-pig CD4-PerCPCy5.5 (1:10) (BD) anti-pig CD8- (1:2) (BD) and LIVE/DEAD® Fixable Near-IR Dead Cell Stain (1:100) (Life Technologies). Single colour controls that did not require subsequent processing were lysed and fixed using FACS lysing solution (BD Bioscience). Permeabilization solution, after which they were washed in Permeabilization buffer (BD) and stained with cross-reactive anti-human CD3-Pacific Blue (1:5) (AbD Serotec) and anti-pig Foxp3 IFN- γ -APC (1:10). After a final wash

in Permeabilization buffer, the cells were resuspended in DPBS prior to acquisition on a MACSQuant flow cytometer. IFN- γ cell expression was measured as the percentage of CD3+CD4+ and CD3+CD8+ cells (see figure 2.4).

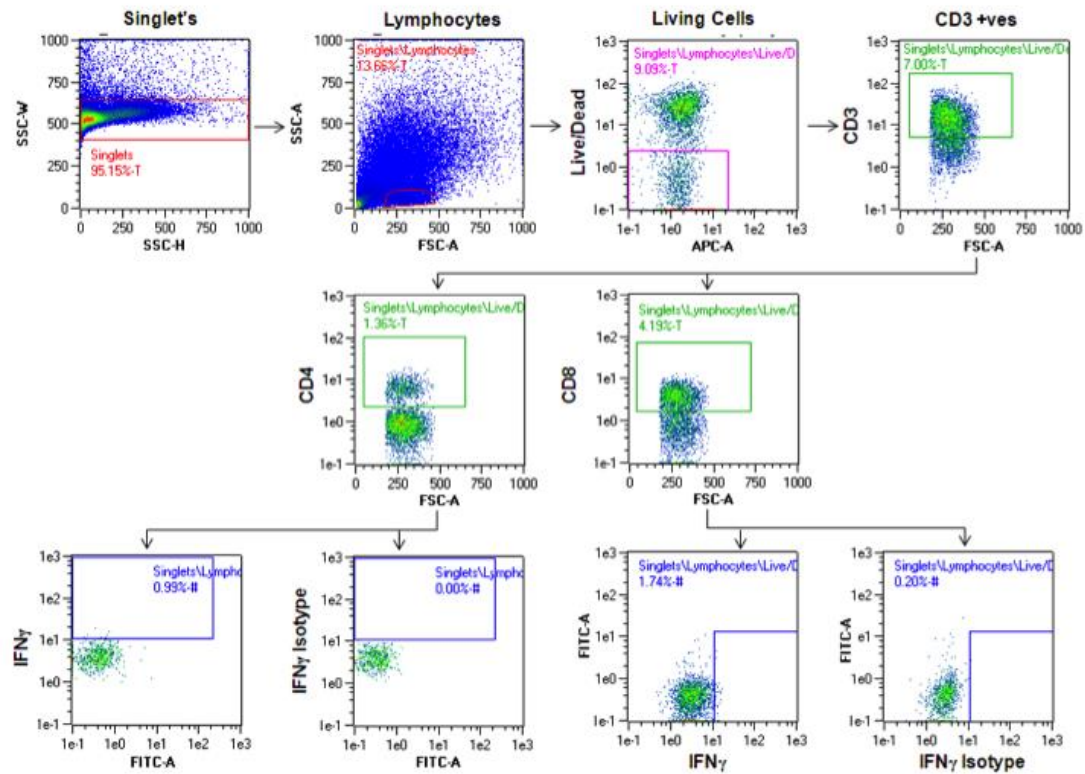


Figure 2.4 Gating strategy for interferon-gamma staining. Firstly a gate is placed on the singlets to remove any cells which may have stuck together causing improper fluorescence. Lymphocytes are then selected based on forward side scatter and dead cells discarded by Live/Dead. The CD3 positive population is then divided into CD4 and CD8 positive cells. IFN- γ can then be analysed and expressed as either the percentage of CD4 or CD8 T cells. Isotype controls for the enzyme are also shown.

Alongside isotype controls it is important to have unstained cell controls, to check the autofluorescence of the cell types being used. Also fluorescence minus one controls can be very useful for drawing gate boundaries, as they use all but one channel and more accurately predict where populations will begin.

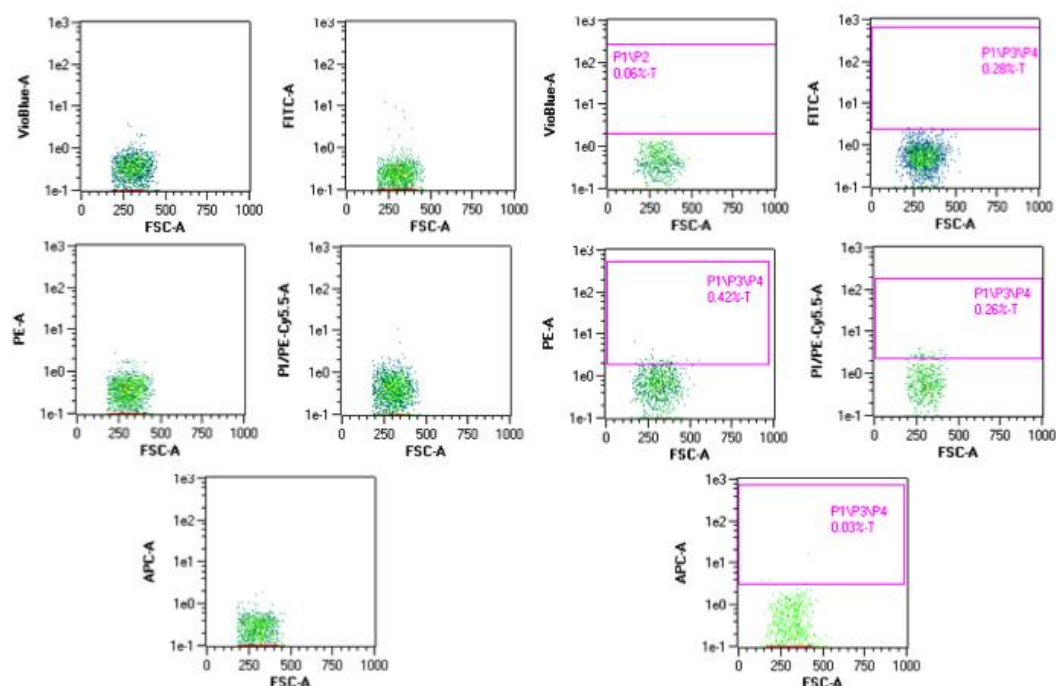


Figure 2.5 Flow cytometry controls. Unstained cells are used to ensure the cells you are running do not have autofluorescence that may produce false positives. Fluorescence minus one controls are useful for helping to set gate positions as they incorporate the combination of each fluorochrome.

2.6 PRRSV Detection

2.6.1 qPCR

RNA was isolated from serum and BALF using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, UK) according to the manufacturer's instructions. The quantitative real-time reverse transcription PCR (qRT-PCR) was performed using the QuantiTect® Probe RT-PCR kit (Qiagen) as per the manufacturer's instructions. Briefly the reaction mastermix (for one reaction), was prepared using 0.5x QuantiTect® Probe RT-PCR master mix, 0.25 µl of RT enzyme, 6.75 µl of nuclease-free water, 0.4 µM of each primer (Fw: 5'- GAT GAC RTC CGG CAY C -3' and Rev: 5'- CAG TTC CTG CGC CTT GAT -3') and 0.2 µM of probe (5'- FAM-TGC AAT CGA TCC AGA CGG CTT-Tamra-3'). Samples and standard RNA dilutions were added in 2 µl volumes per reaction, alongside negative and no RT controls. Standard RNA was synthesised by amplifying complementary DNA (cDNA) of the PRRSV ORF 7 region of both

LV and SU1-bel by conventional PCR using an ORF7 forward primer – 5'- GAA ATT AAT ACG ACT CAC TAT AGG GGC CGG TAA AAA CCA GAG -3' with a T7 promoter region (Cazenave and Uhlenbeck 1994) and an ORF7 reverse primer – 5'- CGC ACT GTA TGA GCA ACC- 3' (both from Sigma, Poole, UK). cDNA was amplified under the following cycling conditions; 94°C 2 minutes, 46°C 1 minute and 72°C 2 minutes for 3 cycles followed by 94°C 30 seconds, 53.4°C 1 minute, 72°C 1 minute, 72°C 5 minutes for 35 cycles. The amplification product was visualized in a 2% agarose–Tris-acetate-EDTA (TAE) gel by GelRed™ (Biotium, Hayward, CA, USA) staining and UV transillumination. DNA was purified from the gel with a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions and quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Loughborough, UK). ORF7 DNA was *in vitro* transcribed into RNA using the Ambion® MEGAshortscript™ T7 Kit and then purified using the Ambion® MEGAclean™ kit (both Life Technologies) according to the manufacturer's instructions. The product was quantified and assessed for integrity using the 2100 Bioanalyzer (Agilent, Wokingham, UK). The RNA was serially diluted 10-fold in nuclease-free water (Promega, Southampton, UK) and stored at -70°C. The thermal profile consisted of 30 minutes at 50°C for one cycle, one cycle at 95°C for 15 minutes, 94°C for 20 seconds and 17 cycles of 72°C for 45 seconds with the annealing temperature decreasing by increments of 1°C every cycle and finally 38 cycles of 93°C for 20 seconds and 55°C for 45 seconds. The reaction was performed on the Stratagene Mx-3000P platform and data was analysed with the MxPro software (Stratagene, Leicester, UK), briefly the software uses the ct values obtained from the RNA standard curve of known copy numbers to devise a standard curve and unknown sample values are interpolated.

2.6.2 IHC

4-micron sections were cut and stained for immunohistochemistry. Primary Antibody against PRRSV (SDOW17, Rural Technologies), was applied 1/70 for 2 hours at room temperature. Epitope demasking consisted of enzymatic digestion with trypsin (0.5%)/chemotrypsin (0.5%) at 37°C for 10 min. Chemmate Dako Envision Detection kit (Dako, Burlingame, CA, USA) was applied for 30 minutes at RT with the addition of 5% Normal Swine Serum. TBST (0.005 M Tris-buffered saline, pH 7.6 with 0.05% Tween20) was used as wash and diluent buffer, slides

were washed three times in between each step. Immunostained sections were analysed under light microscopy and Image analysis software (Lucia®, Prague, Czech republic) to determine the number of positive cells per mm².

2.7 Serology

2.7.1 PRRSV-specific Ab

PRRSV-specific antibodies in serum samples were tested with an antibody ELISA (HerdCheck PRRS X3, IDEXX laboratories), according to the manufacturer's instructions. A sample-to-positive (S/P) ratio equal to or greater than 0.4 was considered positive.

To detect virus neutralizing antibodies against PRRSV, serum samples from day 33 p.i. were heat-treated for 30 min at 56 °C and serial 2-fold dilutions (50 µl volumes) of the test serum were made in cRPMI. An equal volume of the homologous PRRSV strains containing 10² TCID₅₀ in cRPMI were added to each serum dilution and serum-virus mixtures were incubated at 37°C for 60 min. Finally, 2.5x10⁵ PAM cells were added to each well and plates were incubated for 3 days at 37°C before IPMA staining of virus positive cells as described above.

2.7.2 ADV Ab

ADV-specific IgG1 antibody responses were detected using an indirect ELISA (Kimman et al., 1992). Briefly, micro-ELISA plates were coated with mAb directed against ADV by incubation overnight at 37°C. After each incubation step, plates were washed 10 times with 0.05% (w/v) Tween 80 in water. Plates were then incubated with antigen (NIA3) at 37 °C for 1 h, followed by incubation with test samples at 37 °C for 1 h. Serial two-fold dilutions of test samples were made in the plate starting at 1:20. In each plate a standard positive and negative serum were included. Next, plates were incubated at 37 °C for 1 h with peroxidise labelled mAb directed against porcine IgG1. Each ELISA procedure was completed by the addition of the substrate solution, which consisted of 3,3' 5,5'-tetramethylbenzidine solution (DIARECT). Colour development was stopped after 15 min by adding 100 µl of 0.5 M H₂SO₄. Absorbance was measured with at 450 nm. The titer of a sample was expressed as log₁₀ of the reciprocal of the highest dilution yielding an S/P ratio > 0.4.

2.8 Cytokine Analysis

2.8.1 ELISpot

The number of antigen-specific IFN- γ -secreting cells per 2.5×10^5 PBMC was determined using a ELISPOT assay. Briefly, MultiScreen_{HTS}-IP Filter plates (Millipore) were coated overnight at 4°C with 10 μ g/ml of anti-pig IFN- γ mAb (BD) and blocked with DMEM GlutaMAX™ medium supplemented with 4.5 g/L glucose, 25 mM HEPES, 10% FBS, 100 IU/ml penicillin, 100 μ g/ml streptomycin and 5 μ M β -mercaptoethanol (all from Gibco, Invitrogen) for 2 h at 37 °C. Nine wells per pig were seeded with 2.5×10^5 PBMC/well. PBMC were stimulated in triplicate wells by addition of homologous PRRSV at a multiplicity of infection (MOI) of 0.01, 20 μ g/ml Concanavalin A (ConA; Sigma) as a positive control, and culture medium as negative control. Plates were incubated at 37 °C, in a 5% CO₂ humidified atmosphere. After 20-24 hours, plates were washed with PBS containing 0.01% Tween20 and 0.1% FBS (wash buffer), and incubated with 100 μ l of anti-pig IFN- γ biotin-labelled mAb (BD) at a concentration of 0.17 μ g/ml for 1 h at 37 °C. Plates were washed again with wash buffer and then incubated for 1 hour at room temperature with 1/60 of streptavidin-alkaline phosphatase enzyme conjugate (R&D Systems) in PBS containing 0.01% Tween20 and 1% FBS. After washing, plates were incubated for 15 min with BCIP/NBT substrate solution (R&D systems), the reaction was stopped with distilled water and the plates were air-dried. The number of specific IFN- γ secreting cells, as determined using an ImmunoSpot® S4 Analyzer (Cellular Technology Ltd.), were calculated as the average number of spots in the triplicate PBMC cultures stimulated with virus, minus the number of spots in triplicate PBMC cultures exposed to culture medium only. The data were expressed as the background corrected number of IFN- γ -secreting cells per 2.5×10^5 PBMC.

2.8.2 Multiplex Assay

For the cytokine assay a multiplex porcine cytokine array was used, SearchLight (Aushon Biosystems, Billerica, USA). Prior to analysing samples 2.5×10^5 BALF and mediastinal lymph node cells were stimulated with mock supernatant and virus at an MOI of one. Cells were cultured overnight before supernatants were collected for testing. Test standards were made up as per the manufacturers instructions. 50µl of standard and sample was added to the appropriate wells and incubated for three hours at room temperature on a shaker at 200rpm. The plate was then washed four times with wash buffer (provided in the kit). 50µl of prepared biotinylated antibody reagent was then added to each well and the plate left to shake again at room temperature for 30 minutes. Plates were washed again four times in wash buffer before adding 50µl streptavidin-HRP peroxidase to each well. The plate had a final incubation room temperature with shaking for a further 30 minutes. The plates were washed four times in wash buffer before being read by the Aushon CCD camera plate reader. Aushon software was used to determine the standard curve for each individual cytokine and calculate sample values. A number of controls were included in the assay, these included wells containing no cells and just media, wells with no cells but mock supernatants and wells with cells and just media. All samples and standards were performed in duplicate.

2.9 Statistical Analysis

Graphpad Prism 5.01 (Graphpad software, LaJolla, CA, USA) was used for graphing and statistical analysis. Two-way analysis of variance (ANOVA) was used for the analysis of fixed effects upon different traits with the Bonferroni for multiple comparisons as the post-test for clinical data, viraemia, serum antibodies and peripheral IFN- γ production. For all other mean comparisons the conservative non-parametric kruskal-wallis test was used to determine significance within the data and the non-parametric mann whitney-U test was used to compare individual groups. Non-parametric tests were used due to small numbers of biological replicates making it not possible to assume data fits a normal distribution. For correlation data the non-parametric Spearman rank test was used.

Chapter III

Pathogenicity of PRRSV-I Infection

3 Pathogenicity

3.1 Aims and Rationale

The responses of weaned piglets to PRRSV infection may range from subclinical disease to inappetance, lethargy, respiratory problems, and in some cases even death. Although the pathogenesis of PRRSV infection is not fully understood a number of factors are known to contribute to the outcome of infection, including age, the virus strain involved and host genetics. PRRSV is evolving rapidly with reports of highly pathogenic variants appearing in both Asia (Tian et al. 2007) and Europe (Karniychuk, Geldhof, Vanhee, Doorselaere, Saveleva & Nauwynck 2010a). Although a number of pathogenesis studies comparing different type II strains have been performed, studies with European strains are less common. The higher diversity observed within genotype I, and the discovery of more pathogenic strains highlight the need for comparative type I pathogenesis studies.

Modified-live vaccines for PRRS are licensed for use in many countries worldwide including the UK where the modified-live Porcillis PRRS is the most commonly used vaccine in our pig farms. However there is a safety concern surrounding the use of these vaccines, which may return to virulence through mutations and/or recombination with field strains (Murtagh et al, 2010) that may already be present within the herd. For this reason it is important to determine the impact that the use of these vaccines may have upon animal health.

This chapter compares the pathogenesis of four European PRRSV strains; the prototype LV, the Porcillis PRRS strain DV and a recent clinical UK field isolate (215-06), all subtype 1, and a divergent Eastern European subtype 3 strain (SU1-bel). We also aim to determine whether the vaccine strain DV is able to infect pigs and cause clinical disease in an experimental setting. Finally the viral replication of the different strains will be determined within the serum and various tissues and its link with pathology investigated. Specifically we hypothesise that subtype 3 strains are generally more pathogenic than their subtype 1 counterparts.

3.2 Results

3.2.1 *Clinical observations*

All animals had rectal temperatures and clinical scores recorded daily up until euthanasia. SU1-bel infected animals had mean temperatures (Figure 3.1 A) significantly higher than controls at 3 and between 6 and 10 dpi. Mean temperatures of control, DV, 215-06 and LV groups remained below 40°C, and those of the LV, DV and 215-06 groups were not significantly different from controls.

Clinical observations showed a clear difference between the SU1-bel group and the others, having higher mean respiratory (Figure 3.1 B) and systemic (Figure 3.1 C) clinical scores between 5 and 17 dpi, peaking at 8 dpi. Clinical scores of the LV, DV and 215-06 infected animals were comparable to those of controls. Two animals in the SU1-bel group displayed a prolonged fever along with high clinical scores and were euthanised for welfare reasons at 12 and 13 dpi. None of the other groups had mean systemic scores that increased above 3 for the duration of the study.

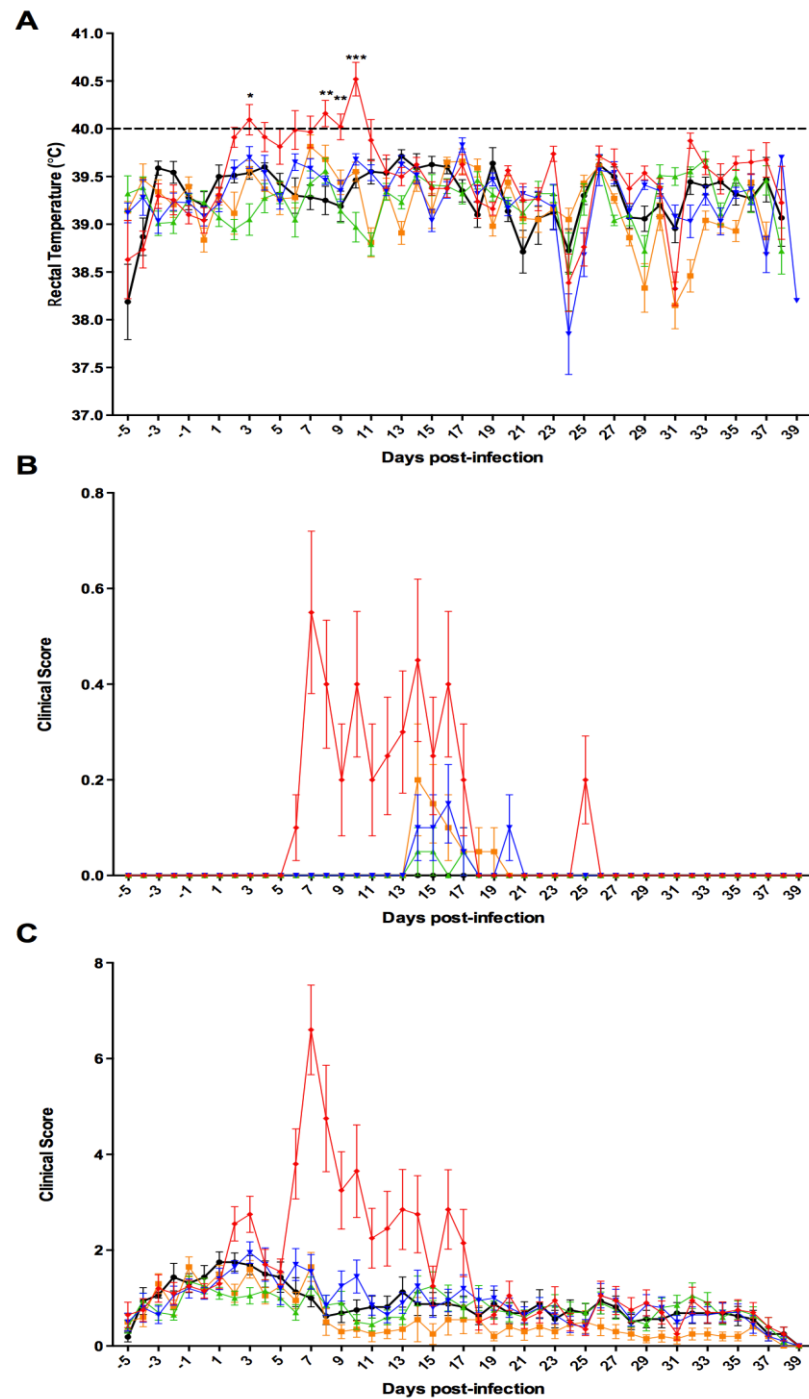


Figure 3.1 SU1-Bel infected animals display higher rectal temperatures and greater clinical scores compared with those infected with LV, DV and 215-06. Animals infected with four strains of PRRSV, LV (■), DV (▲) 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●) had rectal temperatures and clinical scores monitored daily. Temperature above 40° were considered febrile (dashed line). Data shows the mean temperature (A) and respiratory (B) and systemic (C) clinical scores with error bars representing \pm SEM for n=20 (infected groups) and n=16 (controls) between -5 and 3 dpi, n=15 (infected groups) and n=12 (controls) between 4 and 7 dpi (12 controls) and n=10 (infected groups) and n=8 (controls) between 8 and 35 dpi. Significance is indicated by: ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05

Macroscopic, or gross, lung lesions were scored from the amount of grossly visible pneumonia and individuals were given a score based upon the estimated percentage of the lung surface to be affected, with a maximum of 100 points. At 7 dpi all LV, 215-06 and SU1-bel infected animals displayed gross pathology (Figure 3.2 B). The SU1-bel group had the highest gross pathology score, with individual scores ranging between 13 and 45, this was significantly higher than that observed in the LV but not 215-06 groups. The lowest mean score was observed in the LV group with values ranging from 3 to 20. 215-06 pigs had pneumonia scores between 9 and 19%. By 35 dpi gross pathology had cleared from the lungs. At 3 dpi the SU1-bel group had the highest mean score, although three out of five animals from 215-06 displayed gross pathology, ranging from 2 to 11%, compared to two SU1-bel infected animals, scoring 7 and 32, (Figure 3.2 A). Infection of piglets with the DV strain did not produce any gross pathological changes in the lung. Two animals from the control group showed minimal background pathology (1 score point) at 7 dpi, and remained free from grossly visible pneumonia at 3 and 35 dpi. The SU1-bel infected pigs that were euthanised at 12 and 13 dpi had very high gross pathology scores of 66 and 39 respectively.

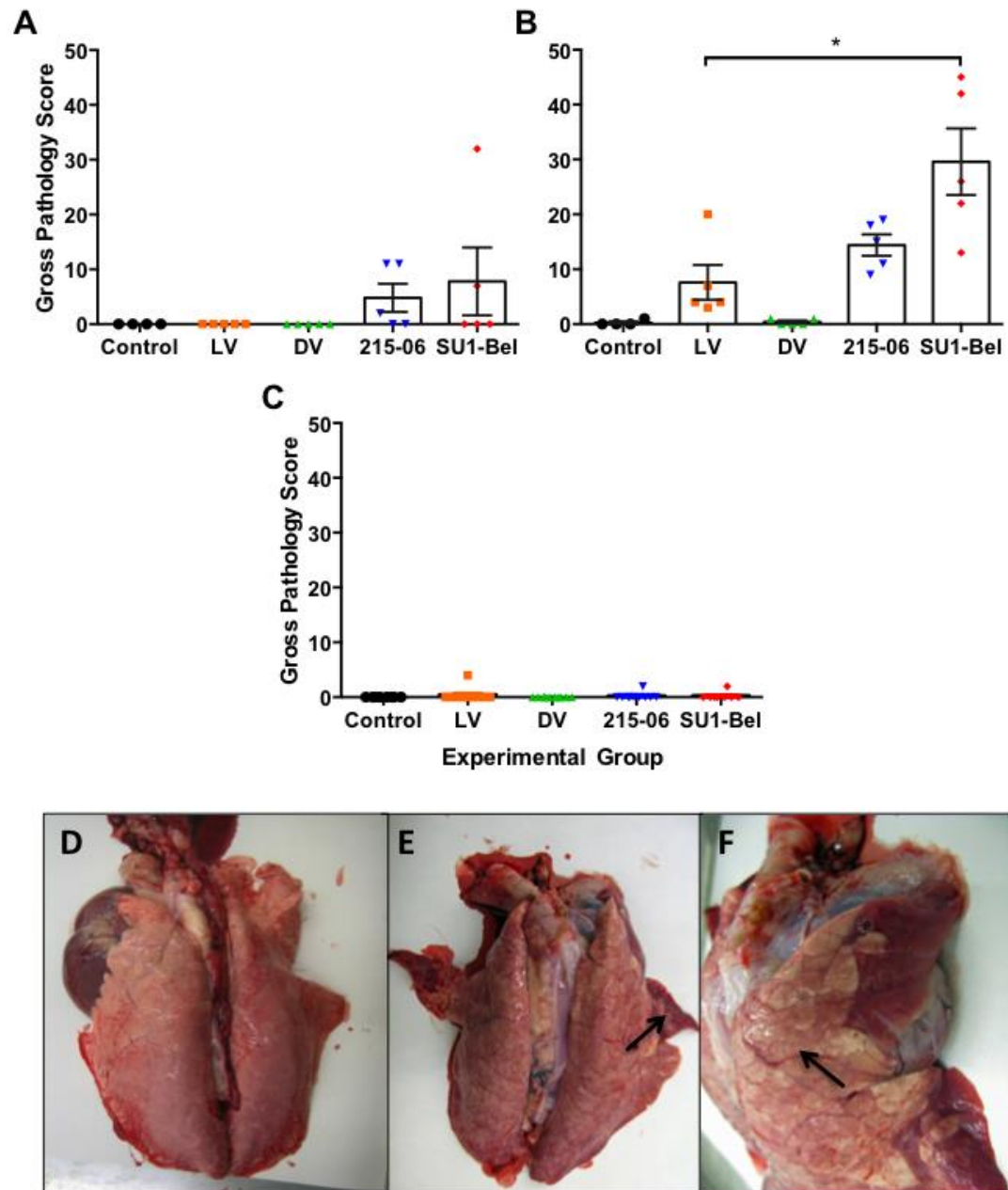


Figure 3.2 PRRSV infection induced gross pathological changes, which were greatest in the SU1-bel infected group. Lungs were removed and scored from animals infected with four strains of PRRSV; LV (■), DV (▲) 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●). Gross pathology was scored based on the percentage of the lung surface to be affected by grossly visible pneumonia at 3 (A), 7 (B) and 35 (C) dpi. Lungs with a gross score of zero (D) show no visible pneumonia (D), while higher scoring lungs displayed severe consolidated red-tan discoloration (E) and mild multi-focal mottling (F). Data points show the individual values for each animal and bars display the mean gross pathology score with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls) at 3 and 7 dpi and n=10 (LV, DV and 215-06 groups) and n=8 (SU1-bel and control groups) at 35 dpi. Significance is indicated by: * p <0.05.

Sections from the apical, medial and caudal lobes of the right lung were sectioned, stained and given a microscopic lesion score between 0 and 4. The median values of all three lobes were taken from each pig to provide a representative score for the lung as a whole. Both the control group and the DV infected group did not show any histological changes in the lung at any time point, with all animals having a lesion score of 0 (Figure 3.3 D). At 3 dpi (Figure 3.3 A) microscopic changes were observed all individuals within both the 215-06 and SU1-bel groups being significantly higher than that seen in the LV group, four animals in each group had mild interstitial pneumonia (Figure 3.3 E), score of 1, and one animal in each group had moderate interstitial pneumonia (Figure 3.3 F) scoring 2. Only one animal in the LV group displayed mild interstitial pneumonia with the remaining individuals having normal lung histopathology, this group had significantly lower lesion than both the 215-06 and SU1-bel groups.

At 7 dpi the SU1-bel group displayed significantly greater (Figure 3.3 B level of pneumonia with three pigs displaying moderate diffuse interstitial pneumonia, with a lesion score of 3 (Figure 3.3 G). One pig within the SU1-bel group had severe interstitial pneumonia, score of 4 (Figure 3.3 H), at 7 dpi. Pneumonia scores of the SU1-bel group were significantly higher than those observed in the LV, but not the 215-06 group. All LV animals had pneumonia with four pigs scoring 1 and one animal with a score of 2, the 215-06 group had three animals with a score of 2 and only two with a score of 1.

Lesion scoring at 35 dpi shows no significant differences between any of the groups (Figure 3.3 C) but with all animals testing positive for virus having some degree of microscopic pathology.

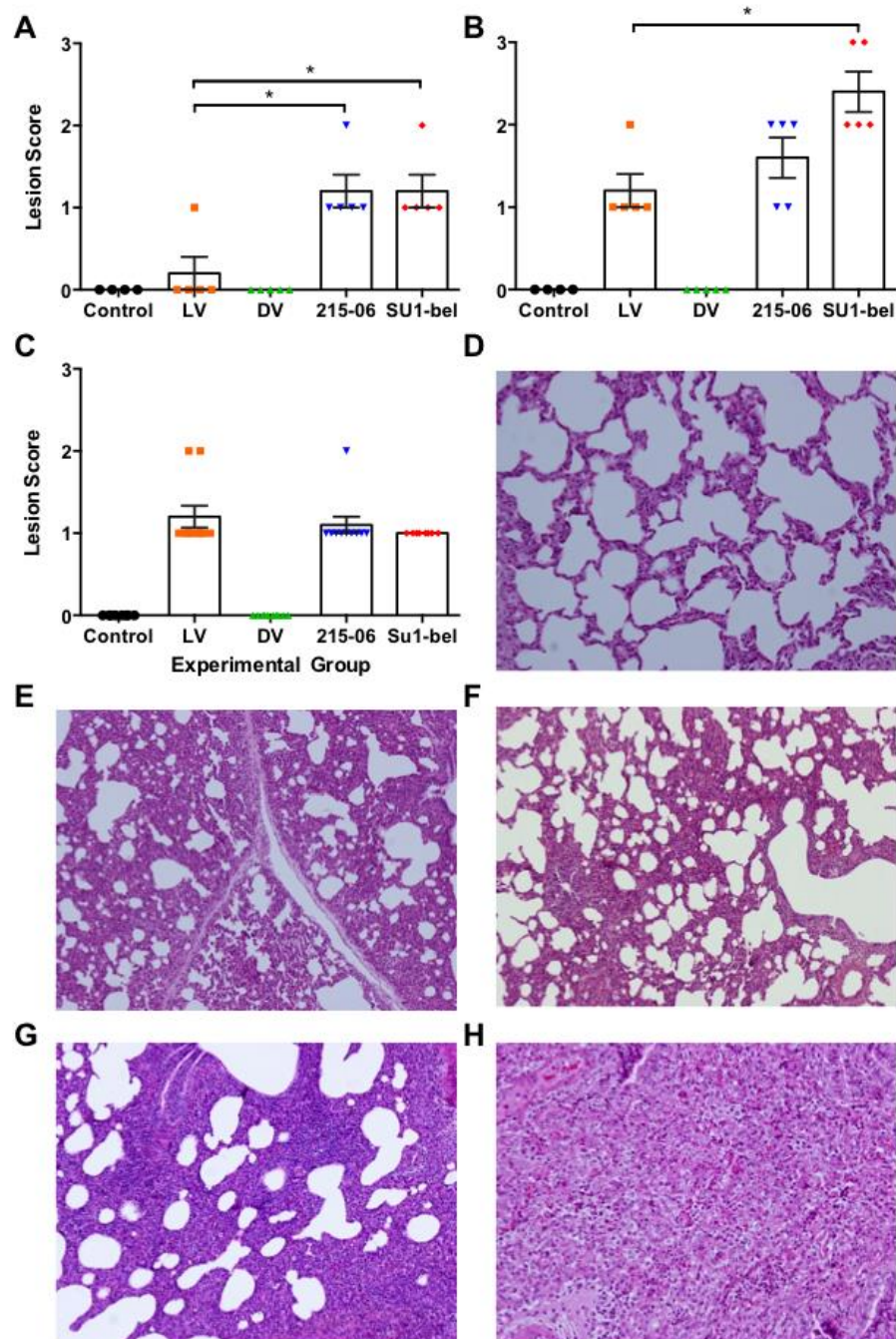


Figure 3.3 Pigs infected with both 215-06 and SU1-bel PRRSV strains display higher microscopic lung lesion scores compared to LV infected animals. Sections from the apical, medial and caudal lobes of the lung were examined for the presence of microscopic lesions; the median values from the 3 areas were taken for each at animal at 3 (A), 7 (B) and 35 (C) dpi. 0 = no microscopic lesions (D); 1 = mild interstitial pneumonia (E); 2 = moderate multifocal interstitial pneumonia (F); 3 = moderate diffuse interstitial pneumonia (G); 4 = severe interstitial pneumonia (H). Data points show the individual values for each animal and bars display the mean lesion score with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls) at 3 and 7 dpi and n=10 (LV, DV and 215-06 groups) and n=8 (SU1-bel and control groups) at 35 dpi. Significance is indicated by: * p<0.05. HE stain, ×10 objective.

In the tonsils, spleen, mediastinal, medial retropharyngeal, sternal and inguinal lymph nodes, hyperplasia of germinal centres of the lymphoid follicles with an increase in the number of mitotic figures and lymphoblasts were observed (Table 1). Among the lymph nodes, the sternal, mediastinal and retropharyngeal were the most severely affected, while the inguinal lymph node showed very mild lesions. In the thymus, a mild to moderate lymphoid depletion was observed with the presence of a mild 'starry-sky' appearance. The spleen from the virus-inoculated groups showed only a mild hyperplasia of the lymphoid follicles. The severity of lesions was higher in the BE group followed by the UK and the LV groups. Some animals from the control group showed minimal background lesions. Moreover, the greatest severity was found at 7 dpi followed by 35 dpi and then 3 dpi. Pyknosis and cell debris were also observed in the septal interstitium of the lung and lymphoid follicles, associated with lymphoid depletion.

Table 2. Microscopic lesions in the lymphoid organs of pigs from each group at 3, 7 and 35 dpi, in relation to the total number of pigs per group, classified according to the scores obtained

Tissue	Score	Number of affected/total pigs analysed per group											
		3 dpi				7 dpi				35 dpi			
		C-	UK	LV	BE	C-	UK	LV	BE	C-	UK	LV	BE
Mediastinal LN	0	4/4	3/5	4/5	0/5	3/4	0/5	0/5	0/5	7/8	1/10	0/10	0/8
	1	0/4	2/5	1/5	4/5	1/4	5/5	5/5	2/5	1/8	7/10	10/10	7/8
	2	0/4	0/5	0/5	1/5	0/4	0/5	0/5	3/5	0/8	1/10	0/10	1/8
	3	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
Retropharyngeal LN	0	3/4	2/5	3/5	0/5	4/4	1/5	1/5	0/5	7/8	1/10	3/10	0/8
	1	1/4	3/5	2/5	4/5	0/4	3/5	5/5	1/5	1/8	8/10	5/10	7/8
	2	0/4	0/5	0/5	1/5	0/4	1/5	0/5	4/5	0/8	1/10	2/10	1/8
	3	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
Sternal LN	0	3/4	3/5	4/5	0/5	4/4	0/5	2/5	0/5	7/8	2/10	4/10	0/8
	1	1/4	2/5	1/5	4/5	0/4	5/5	3/5	1/5	1/8	8/10	4/10	5/8
	2	0/4	0/5	0/5	1/5	0/4	0/5	0/5	4/5	0/8	0/10	2/10	3/8
	3	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
Inguinal superficial LN	0	3/4	5/5	5/5	0/5	3/4	4/5	3/5	0/5	7/8	6/10	6/10	2/8
	1	1/4	0/5	0/5	3/5	1/4	1/5	2/5	5/5	1/8	4/10	4/10	5/8
	2	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	1/8
	3	0/4	0/5	0/5	1/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
Tonsil	0	4/4	2/5	4/5	2/5	4/4	0/5	1/5	0/5	8/8	9/10	9/10	6/8
	1	0/4	3/5	1/5	2/5	0/4	5/5	4/5	4/5	0/8	1/10	1/10	2/8
	2	0/4	0/5	0/5	1/5	0/4	0/5	0/5	1/5	0/8	0/10	0/10	0/8
	3	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
Thymus	0	4/4	5/5	4/5	0/5	4/4	0/5	0/5	0/5	7/8	0/10	0/10	0/8
	1	0/4	0/5	1/5	5/5	0/4	4/5	5/5	1/5	1/8	10/10	10/10	6/8
	2	0/4	0/5	0/5	0/5	0/4	1/5	0/5	4/5	0/8	0/10	0/10	2/8
	3	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
Spleen	0	4/4	5/5	5/5	4/5	3/4	2/5	3/5	3/5	7/8	7/10	10/10	4/8
	1	0/4	0/5	0/5	1/5	1/4	3/5	2/5	2/5	1/8	3/10	0/10	4/8
	2	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8
	3	0/4	0/5	0/5	0/5	0/4	0/5	0/5	0/5	0/8	0/10	0/10	0/8

0, within normal limits; 1; light hyperplasia of lymphoid follicles, with isolated cellular necrosis; 2, moderate follicular hyperplasia with multifocal cellular necrosis; and 3, severe follicular hyperplasia with abundant cellular necrosis.

Viraemia was determined by qRT-PCR on serum samples. All animals were negative for viral RNA on 0 dpi and control animals remained negative throughout the study. Viral RNA was detected in all LV, 215-06 and SU1-bel-infected groups on 3 dpi, peaked at 7 dpi and had

cleared from the serum by 35 dpi (figure 3.4A). The LV group had significantly higher RNA copy numbers compared with both the SU1-bel and 215-06 groups at 3 and 7 dpi. At 35 dpi viral RNA was significantly lower in the SU1-bel group compared with the LV animals. Although the fall in SU1-bel RNA was not mathematically significant, the 2-log difference at 21 dpi may have been biologically significant and by 35 dpi six out of eight of the SU1-bel animals had cleared virus. No viral RNA was detected in the serum of DV infected animals at any time point. Viral RNA was detected in BALF at 3, 7 and 35 dpi (Figure 3.4B, C and D, respectively) of LV, 215-06 and SU1-bel groups, and was highest at 7dpi. No virus could be detected in the BALF of the control pigs at any time point, and the group infected with the vaccine strain did not have any viral RNA in either the serum or the BAL at any time point.

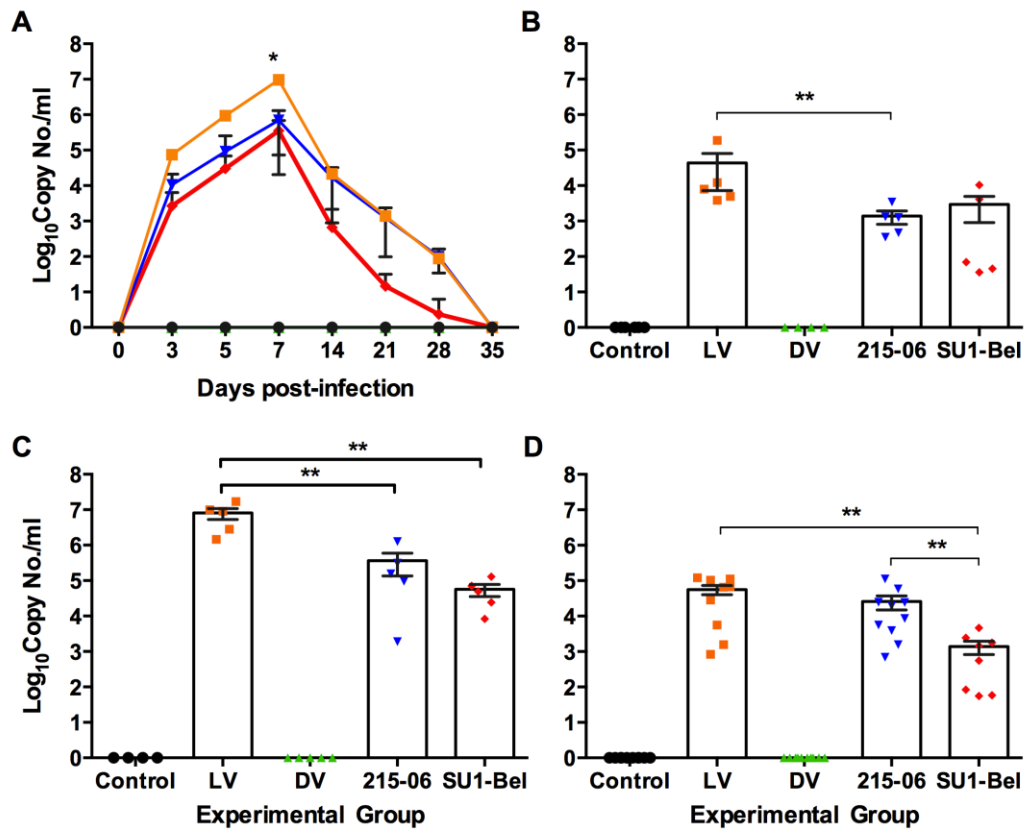


Figure 3.4 Viraemia and viral loads in BALF were lower in pigs infected with PRRSV SU1-bel strain compared with subtype 1 groups. Viral loads were determined by qRT-PCR in sera from animals infected with PRRSV LV (■), DV (▲) 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●) for viraemia (A). Viral load in the BALF was determined at 3 (B), 7 (C) and 35 (D) dpi. Data shows the mean viral RNA copy number/ml of serum with error bars representing \pm SD for n=20 (infected groups) and n=16 (controls) between -5 and 3 dpi, n=15 (infected groups) and n=12 (controls) between 4 and 7 dpi and n=10 (infected groups) and n=8 (controls) between 8 and 35 dpi in the serum. BALF data shows the \pm SEM and n=5 (infected groups) and n=4 (controls) at 3 and 7 dpi and n=10 (LV, DV and 215-06 groups) and n=8 (SU1-bel and control groups) at 35 dpi. Significance is indicated by: **p<0.01 and *p<0.05.

Viral antigen was detected by immunohistochemistry in the lung, mediastinal, medial retropharyngeal, sternal and inguinal superficial lymph nodes, tonsils, spleen and thymus from the virus-inoculated groups. However, the number of immunostained cells was small and irregularly distributed, occasionally appearing as groups of positively stained cells. In the lungs, viral antigen was mainly detected in alveolar, septal and intravascular macrophages (Fig. 3.5 A) with a few positive cells within the lamina propria of bronchioles. In the lymphoid organs, positive immunoreaction was detected in macrophages, but also in a few dendritic like

cells. No differences were observed in the other tissues analysed, this data again perhaps supports the idea that it is not an enhanced viral replication that is responsible for the marked increase in clinical disease and pathology resulting from infection with the SU1-bel strain.

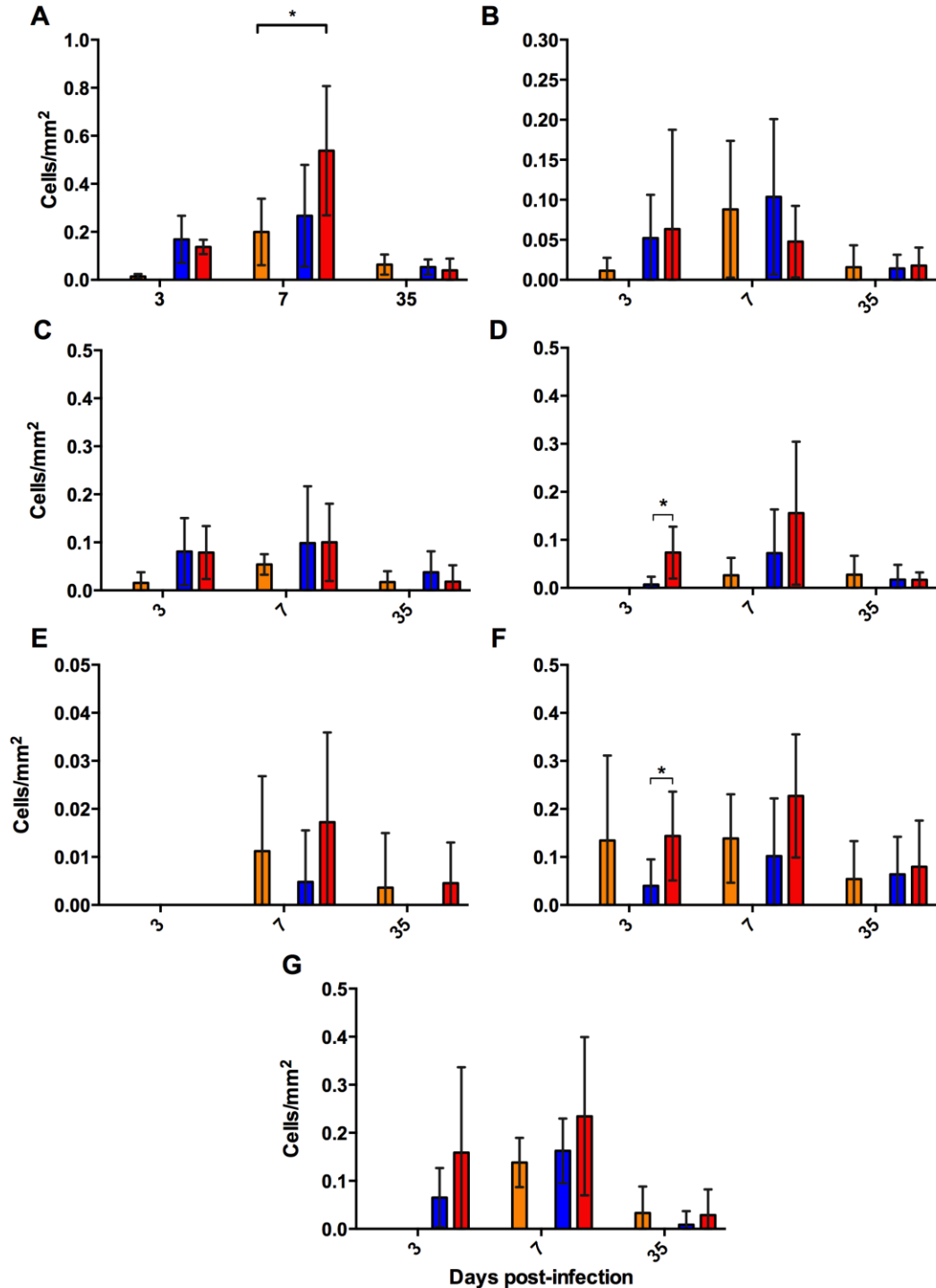


Figure 3.5 Number of virus infected cells in the lung and lymphoid tissues were low in all PRRSV-infected groups and not present at all in DV infected animals. Sections from the lung (A), MLN (B), RLN (C), SLN (D), ILN (E) thymus (F) and tonsil (G) were stained by immunohistochemistry for PRRSV antigen and virus quantified by counting the number of positive cells per mm². Data shows the mean number of cells/mm² with error bars representing \pm SD for n=5 (infected groups) and n=4 (controls ■) at 3 and 7 dpi and n=10 (LV (■), DV (■) and 215-06 (■) groups) and n=8 (SU1-bel (■) and control groups) at 35 dpi. Significance is indicated by: *p<0.05.

This chapter compares the pathogenesis of a PRRSV-I subtype 3 strain, isolated from pigs displaying respiratory disease in Belarus, with strains of the pan-European subtype 1. Although other pathogenesis studies on PRRSV-I have been conducted, with this being a highly diverse and rapidly evolving virus (Stadejek et al. 2006), and new subtypes being described, it is important to reassess the impact of infection with such strains under controlled conditions.

As expected, the temperature data from the subtype 1 strain infected animals in both experiments remained similar to controls and below 40°C. The SU1-bel strain caused a fever in infected animals, lasting around 10 days. These pigs also displayed much greater clinical scores compared with other groups between 6 to 17 dpi. The LV, DV and 215-06 groups temperatures and clinical scores that were comparable to the control group. This is perhaps unsurprising as sub-clinical disease is a common result of experimental infection with PRRSV-I, so the severity of disease seen during infection with SU1-bel poses a concern to producers. In 2010, Karniyuchuk et al. found that experimental infection with the Lena strain resulted in high temperatures lasting for a period similar to that seen in this study. This period of fever observed during infection with these subtype 3 strains is similar to that observed during experimental infection with Asian highly-pathogenic PRRSV strains (Zhou et al. 2008).

In line with these clinical observations the SU1-bel group also displayed higher lung gross pathology compared to the 215-06 and the LV group; although at 3 dpi the 215-06 group had three of five pigs with gross pathology whereas the SU1-bel group had only two. The LV infected pigs did not display any gross pathology at 3 dpi and had an average score lower than both the SU1-bel and 215-06 groups at 7 dpi. These higher gross pathology scores in the SU1-bel group are more comparable to those observed after infection with North American strains as seen in the study by Martínez-Lobo et al. (2011) when compared with European strains. As inflammation, reflected in gross lesion scores, is a key mediator of fever, this observation may explain the fever also observed in the SU1-bel group. The lungs of SU1-bel infected pigs also displayed extensive microscopic lesions, which were again greater in this

group compared with the LV and 215-06 groups. These findings are perhaps unusual given that these animals did not present with any significant respiratory disease. Although this is the first time that the 215-06 and SU1-bel strains have been used in an experimental infection, pathology data from the lungs of LV infected animals is comparable to previous studies (Halbur et al. 1995; Weesendorp et al. 2012). The DV inoculated animals did not display any gross nor microscopic pathology in the lung. This result matches that seen in a study completed by (Opriessnig et al. 2002) who looked at the modified live vaccine Ingelvac and found that it did not produce any pathological changes in the lung of infected pigs.

Microscopic lesions were also scored in a number of lymphoid tissues. In general a trend towards the SU1-bel group having more severe pathology was observed in all tissues, and this was most pronounced in the MLN and RLN, the lymph nodes associated with the respiratory tract. Interestingly only the SU1-bel group had lesions in the ILN, despite their location far from the site of infection. Lesions were also observed in the thymus and tonsil of PRRSV infected pigs. Cellular damage to the thymus in particular can pose problems in mounting an adequate immune response to infection. The spleen did not show any significant pathology, which is unusual as PRRSV infection often results in follicular hyperplasia and necrosis of the spleen (Halbur et al, 1995). The spleen is also thought of by many as being one of the best places to detect, however in this study only two animals were found to have very low numbers of PRRSV positive cells in the spleen (Cheon & Chae 2000).

Virus quantification in the tissues was performed using immunohistochemistry, in general the counts of PRRSV positive cells per mm² were very low, as can be seen by comparing the results obtained from the LV group to past studies (Halbur, Paul, Frey, et al. 1996a). In general however, the SU1-bel strain displays a trend to have higher mean numbers of positive cells. Interestingly the ILN, which showed lesions at 3 dpi in the SU1-bel group is free from viral antigen at the same, time point, why this may be and what is causing the tissue damage are yet to be determined. This is particularly apparent in the lung at 7 dpi. This is in stark contrast to the results obtained from the PCR performed on BALF, where the SU1-bel group had the lowest amount of viral RNA. When the counts from individual animals are plotted against their gross pathology score however, there is no significant correlation observed between virus and

pathology, in fact in the SU1-bel group there is no correlation observed, significant or otherwise. Martinez-Lobo et al (2011) also observed that pathogenesis of PRRS infection was not dependant on viral loads. Taken with the lower viral load in the BALF of SU1-bel infected pigs, this data suggests that it is not viral replication that is influencing pathogenesis but some other factor. The viraemia data shows that virus is actually cleared more quickly from the serum of SU1-bel infected animals compared with the LV and 215-06 groups, suggesting that these animals may actually be mounting a more efficient immune response against the virus despite showing the most severe clinical signs and pathology.

Despite concerns over commercial modified live vaccines reverting to virulence and inducing disease the Porcillis PRRS strain did not result in the induction of clinical disease or any significant pathology. Also virus could not be detected in any medium by either qPCR or IHC. We have shown that intranasal inoculation of the DV strain does not induce a productive infection in piglets. This may be because the virus has been adapted to grow on MARC-145 cells and so is no longer permissible to infection of PAMs. In fact the author attempted to culture this virus on PAMs *in vitro* with no success. It is possible that in a farm setting, where the virus may mix with field viruses that a return to virulence may still be possible. There are a number of reports that field isolate sequencing has discovered a number of clinical strains that are closely related to the vaccine strain. Future studies may also want to look at whether virulent infection is possible after intramuscular inoculation, as would be performed in the field, in a farm environment where animals are exposed to a number of PRRSV strains and so the chance for virus mutation is much higher than in the controlled experimental environment. It would also be interesting to see how these so called “vaccine-like” strains behave in an experimental setting and if they compare with current field strains.

The results from the first portion of this project reveal that this subtype 3 virus is in fact more pathogenic than its subtype 1 relatives. That animals infected with this virus show increased clinical disease as well as more severe tissue pathology. However, we have also shown that this difference may not be due to greater virus replication, but rather other factors. As it is well known that individual responses to infection with PRRSV vary greatly, it is likely to be a host factor contributing to these differences. As the immune system has long been established as

a driving force in the pathogenesis of many different diseases this seems like a logical area to investigate further.

Peripheral immune responses

4. Peripheral Immune responses

4.1. Introduction

Infection of piglets with the PRRSV subtype 3 strain SU1-bel resulted in clinical disease, whereas subtype 1 infected animals developed subclinical infections. Su1-bel infected animals also displayed greater gross and microscopic pathology compared with the LV and 215-06 groups. Virus detection in both the serum and BALF showed that the SU1-bel group did not have the highest viral load as one may expect. Although viral Ag detected by IHC within the lesions of lung tissue was greatest in the lungs of SU1-bel pigs. However, amounts of viral Ag detected by either qPCR or IHC did not correlate with the gross pathology observed in the SU1-bel group.

It is known that immunopathology is the cause of cellular damage for a number of viral infections, such as Ebola virus (Zampeiri, et al 2007). As the data from virus detection indicated that it may not be greater virus replication causing the difference seen in disease outcome, the immune response to the different viruses are to be compared. It is also suggested that PRRSV suppresses the immune response to secondary infection, another factor that could increase the pathology observed after infection with PRRSV.

This chapter tests the hypothesis that infection with the different strains of PRRSV induces altered immune responses in the blood. As the pigs that were inoculated with the DV strain of PRRSV did not become infected they have been removed from this chapter. We aim to compare the humoral and cell mediated response to PRRSV infection between the groups, through detection of PRRSV-specific antibody and IFN- γ secretion. The ability of the different strains to suppress the immune response to secondary infection will also be tested by vaccinating animals with an Aujeszky's disease virus (ADV) vaccine. The ADV-specific antibody and IFN- γ responses post vaccination will be measured.

4.2. Results

4.2.1. PRRSV-Specific Ab

Levels of PRRSV-specific Ab levels were measured in the serum of virus-infected animals (Figure 4) and control animals were tested on both 0 and 35 dpi. All animals were seronegative (S/P ratio <0.4) for PRRSV-specific Ab prior to infection and controls were still negative at the end of the study. Levels of Ab increased above the cut-off point in the SU1-bel group by 7 dpi, earlier than both the LV and 215-06 groups at 9 dpi (Figure 4A). At 28 dpi the SU1-bel group had significantly lower levels of Ab compared with the LV and 215-06 groups. PRRSV-specific Ab were detected only in the BALF of SU1-bel infected pigs at 7 dpi (Figure 4B), but by 35 dpi all groups had comparable Ab levels (Figure 4C). Surprisingly, one animal in the 215-06 group did not seroconvert by the end of the study, although viral RNA was detected in both the serum from 3 dpi and BALF at 35 dpi.

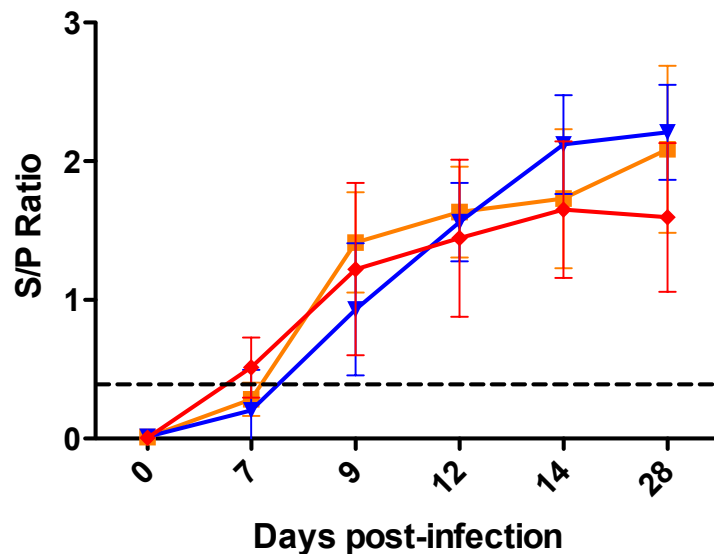


Figure 4.1 PRRSV-specific antibody responses were detectable earlier in animals infected with SU1-bel compared with subtype 1 strains. PRRSV-specific Ab responses of animals infected with LV (■), 215-06 (▼) and SU1-bel (◆) were measured by ELISA in the serum. An S/P ratio above 0.4 (dashed line) was considered to be a positive result. Data shows the mean S/P ratio with error bars representing \pm SD for n=10.

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4.2.2. Immune cell phenotyping

Volumetric flow cytometry was used to determine the changes in blood leukocyte subsets during the course of infection (figure 6). In all cell types, fluctuations in the absolute number of cells were observed over the course of the experiment. Monocytes (SWC1⁺SWC3⁺SWC8⁻) fell below controls in the LV and SU1-bel groups at 3 and 7 dpi. Numbers of B cells (SWC1⁻SWC3⁻SWC8^{hi}) in the blood of SU1-bel infected animals fell below controls at 3 dpi and remained lower until the end of the study. A peak in naïve CD4 T cells (CD3⁺CD4⁺CD8⁻) was seen in SU1-bel and 215-06 groups at 7dpi and not in control or LV groups. A peak in memory CD4 T cells (CD3⁺CD4⁺CD8⁺) was seen in virus-infected groups at 7 dpi. A second peak was observed on 21 dpi in the SU1-bel group, which was significantly higher than controls. CD8 T cells (CD3⁺CD4⁻CD8^{hi}) in virus-infected groups increased above control levels at 7 dpi until 21 dpi. NK cells (CD3⁻CD4⁻CD8⁺) also increased above controls in virus-infected groups at 14 and 21 dpi. No differences were seen in populations of neutrophils, $\gamma\delta$ -T cells (CD3⁺CD4⁻CD8⁻/lo) or Tregs (CD3⁺CD4⁺CD25^{hi}Foxp3⁺).

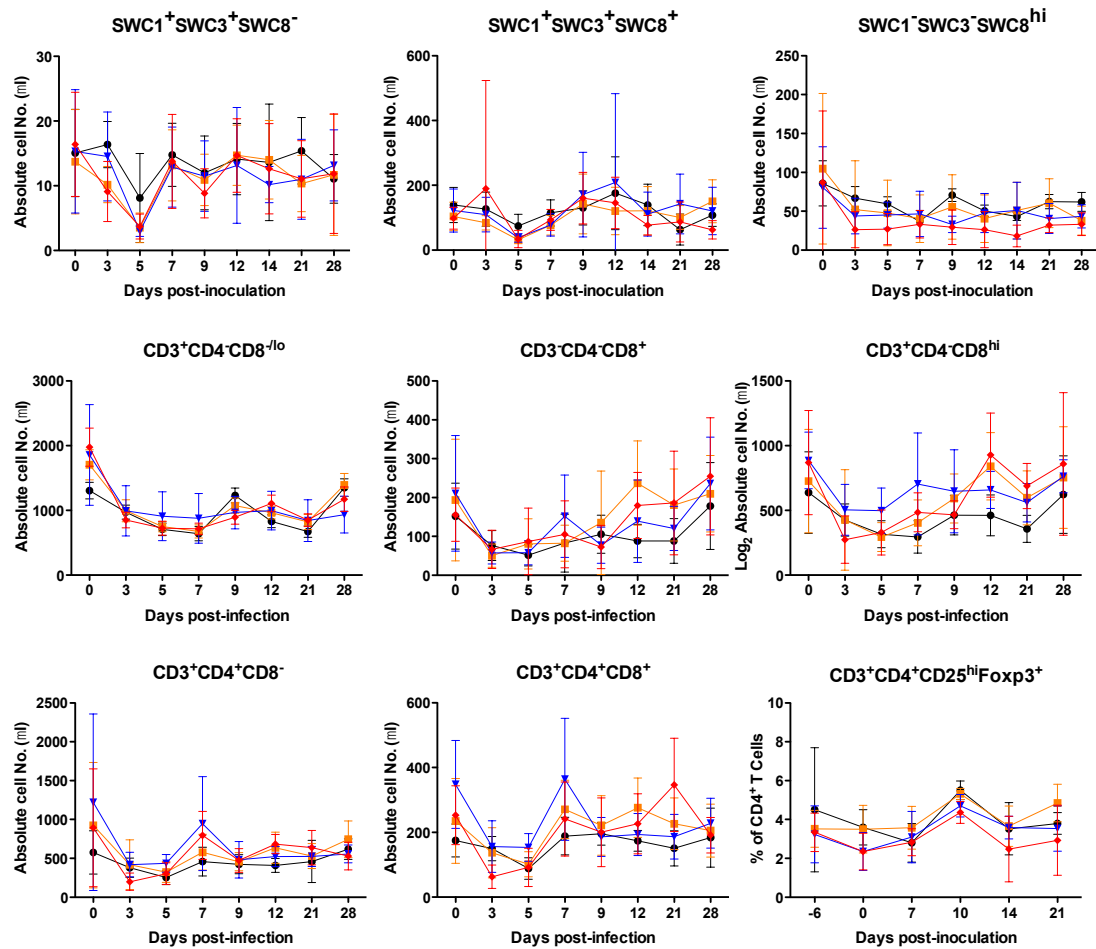


Figure 4.2 Population kinetics of leukocyte subsets in the blood displayed only subtle changes between PRRSV infected animals and controls. Whole bloods from animals infected with LV (■), DV (▲) 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●) were used to monitor changes in immune cell populations using flow cytometry. Myeloid and B cell populations were determined using antibodies against SWC1, SWC3 and SWC8 (A, B and C) and antibodies against CD3, CD4 and CD8 were used to phenotype other lymphoid cells (D, E, F, G and H). Treg cells were distinguished by a phenotype positive for CD3, CD4 and Foxp3 with high expression of CD25 (I). Data shows the mean absolute number of cells per μ l blood with error bars representing \pm SD for n=10 (infected groups) and n=8 (controls).

4.2.3. IFN- γ responses

IFN- γ responses of the pigs were determined by weekly stimulation of PBMC with PRRSV *ex vivo*. The SU1-bel group displayed a significantly greater response to homologous virus compared to LV at 21 dpi (Figure 4.3B) and higher levels than both 215-06 and the LV groups at 14, 21 and 26 dpi (Figure 4.3A). Neither the LV nor 215-06 groups showed a significant increase in IFN- γ secreting cells, their response was weak and only occasionally above the control group.

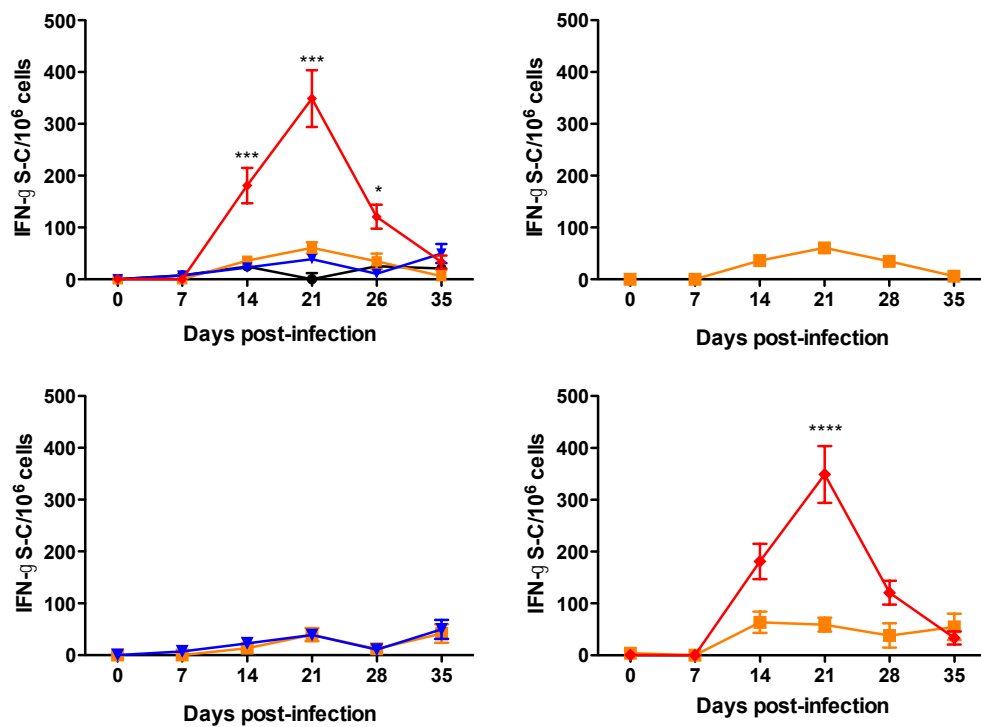


Figure 4.3 PRRSV-specific IFN- γ responses in the blood of SU1-bel infected animals were greater than those observed in both LV and 215-06 groups. Animals infected with LV (orange square), DV (green triangle), 215-06 (blue inverted triangle) and SU1-bel (red diamond), and mock-infected controls (black circle) were bled weekly and PBMC were used in an ELISpot assay to detect PRRSV-specific IFN- γ responses (A). PBMC from SU1-bel infected animals were also stimulated with the heterologous LV (red diamond dashed line) to look at cross-reactive responses (B). Data shows the mean number of IFN- γ secreting cells after correcting for responses to mock stimulation, with error bars representing \pm SD for n=10 (infected groups) and n=8 (controls). Significance is indicated by: ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05.

At 7 dpi five pigs from each virus-infected group (and four from the control group) were immunised with an attenuated ADV vaccine, with a booster at 21 dpi. To investigate the response to vaccination, levels of ADV-specific Ab and IFN- γ responses (Figure 4.4A and B, respectively) were measured in the blood at weekly intervals. The results showed that infection with any of the PRRSV strains used in this study did not result in a significant change in either Ab or IFN- γ responses to vaccination, compared with uninfected controls. Vice versa the vaccination against ADV did not affect the progression of PRRSV infection (data not shown).

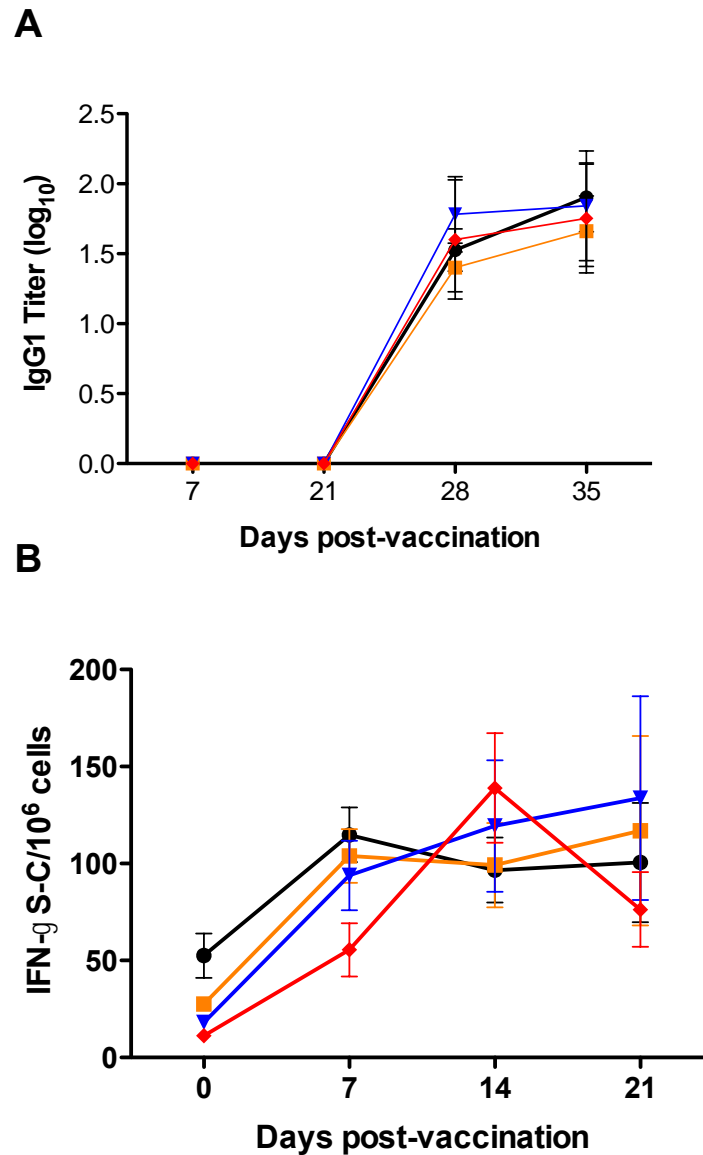


Figure 4.4 Infection with PRRSV did not have an effect on the immune response to ADV vaccination. Animals infected with PRRSV LV (■), DV 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●) were vaccinated with an inactivated ADV vaccine 7 dpi. Immune responses to vaccination were measured using an ELISA for ADV-specific Ab response (A) and an ELISpot assay for ADV-specific IFN- γ . Data shows the mean Ab titre (A) and mean number of IFN- γ producing cells (B), with error bars representing \pm SD for n=5 (infected groups) and n=4 (controls).

4.3. Discussion

Ab levels in the SU1-bel group increased above the cut-off by 7 dpi earlier than the other two groups, and although the final Ab concentration was lower in this group, this did not affect viral clearance. This earlier response was not seen in a study by Weesendorp et al (2012) who performed a similar experiment with the subtype 3 strain Lena, these, suggesting that this enhanced response may not be common to all subtype 3 strains. Ab responses in the serum of pigs infected with the 215-06 and LV viruses were comparable to previously reported humoral response to PRRSV (15). No neutralising Ab could be detected at any time point up to 35 dpi in any of the experimental groups. Considering some studies have reported that neutralising Ab cannot be detected until 5 or more weeks post-infection (16) this may not be surprising.

As well as an earlier humoral response, significantly greater numbers of PRRSV-specific IFN- γ producing cells were detected in the blood of SU1-bel infected pigs compared with other groups. These cells peaked at 21 dpi, which corresponds with a significant peak in memory CD4 T cells in this group, and returned to the level of the other groups by 35 dpi. IFN- γ has antiviral properties, and has been shown to inhibit replication of PRRSV *in vitro* (Bautista et al. 1996 and Rowland et al. 2001). This may be an important contributing factor to the apparent faster clearance of SU1-bel from the serum. IFN- γ is also involved in the inflammatory response, which may explain the greater gross pathology in the lung, if they are occurring in the lungs without the necessary anti-inflammatory mediators to modulate inflammation. PBMC from the SU1-bel group were stimulated with the heterologous LV strain, the IFN- γ response was significantly weaker at 21 dpi compared to stimulation with homologous virus, which may reflect that only a proportion of T cell epitopes are conserved between these viruses. Taken together the results suggest that enhanced immune responses to the SU1-bel strain may lead to the more severe clinical disease and gross pathology observed in this study, but also support an enhanced clearance of virus.

PRRSV infection has been reported to enhance susceptibility to secondary bacterial infections (Collins. & Rossow, 1993 and Zeman et al. 1993). There is some evidence of PRRSV modulating the host immune system, although the underlying mechanisms are not fully understood. A study by Li et al. (2003) reported that PRRSV infection impaired the response

of pigs to vaccination against classical swine fever. In an attempt to determine whether any of our PRRS strains induced such immunomodulatory effects, animals were vaccinated with a commercial ADV vaccine, and the subsequent humoral and cell-mediated responses were analysed. Although Weesendorp et al (2012) found that the Ab response to ADV vaccination was slightly delayed in the Lena strain group, none of the strains used in this experiment appeared to influence either the Ab or the IFN- γ response to vaccination. Furthermore, Treg cells have been suggested to play an important role during infection with PRRSV-II (Silva-Campa et al. 2012). However, we could not detect any evidence for the expansion of the Treg population in blood or infiltration into the lungs (data not shown) following infection with these three PRRSV-I strains.

This data demonstrates that the immune response to the SU1-bel strain is enhanced compared to that observed in the 215-06 and LV groups. As it is well known that immune responses contribute a great deal to disease pathogenesis this could be the cause of the increased pathology and greater clinical presentation after infection with this strain. For this reason the local immune responses in the lung of infected pigs will be investigated for a link with PRRSV pathogenesis.

Local immune responses and pathology

5 Local immune responses

5.1 Introduction

This chapter aims to further dissect the immune response to these viruses by focussing on the local response in the lung and mediastinal lymph nodes, and relate this to gross pathology. The chapter focuses on the tissues from 7 dpi as this was the day when the greatest levels of pathology were observed and when virus-specific immune responses can be detected. Cellular infiltration into the BAL is measured by flow cytometry, and local cytokine and Ab responses are measured to determine if local immune responses are more pronounced than those observed in the periphery.

Infection of pigs with the SU1-bel strain of PRRSV resulted in marked clinical signs and gross pathology, particularly compared with the subtype 1 strains. However, viral loads were comparable to, or greater than those seen in the LV and 215-06 groups. Analysis of the peripheral immune response revealed a surprisingly early cell-mediated response to SU1-bel infection (measured by levels of IFN- γ), compared to the subtype 1 strains and previously reported findings. The humoral response was also greater in this group with antibodies being detected earlier than in the LV and 215-06 infected pigs. These animals did also appear to clear virus more quickly from the serum.

Following these findings it was decided to investigate the relationship between the local immune responses in the lung and mediastinal lymph node and gross pathology. The immune response can play a significant role in disease pathology. As the immune system tries to eradicate an invading pathogen, the bodies own cells inevitably become caught in the cross fire. This is particularly true in the case of viral infection, as the very nature of the viruses necessity to reproduce within the animals cells means the immune system must destroy the bodies own cells to remove the virus.

5.2 Results

5.2.1 Cellular Infiltration

The total number of cells per μl in the BALF was determined using volumetric flow cytometry. Although no statistically significant differences were observed between the experimental groups the controls had the lowest mean total number of cells, followed by the LV group and then the 215-06 group. The SU1-bel infected group displayed the highest mean number of cells in the BALF (Fig 5.1A). The SU1-bel group also had the highest percentage of lymphocytic infiltrate this was significantly higher compared to both controls and the 215-06 group. Interestingly the percentage of lymphocytes in the BALF of 215-06 infected animals was significantly lower than controls ($p = 0.0317$). The LV group showed a small increase above controls but was only significantly different from the 215-06 group.

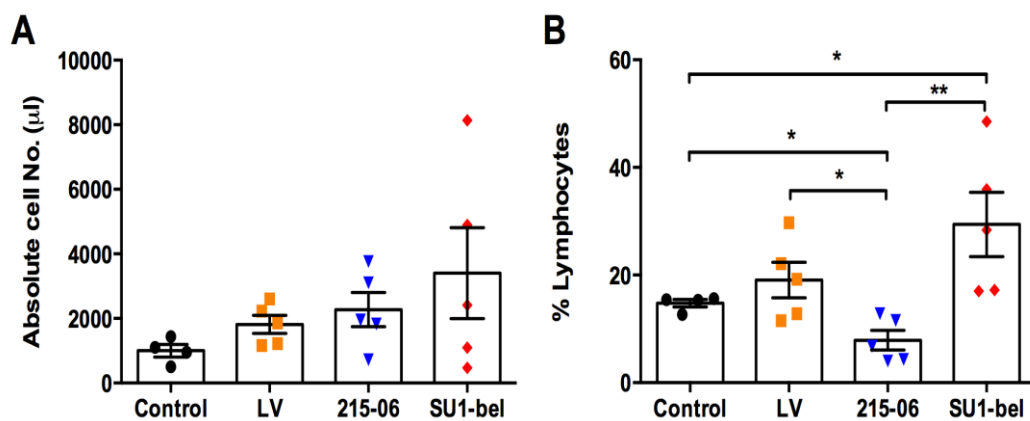


Figure 5.1 Total number of cells in the BALF of PRRSV infected pigs increased above controls, this increase was greatest in the SU1-bel infected group. Cellular infiltrate in the BALF of animals infected with three strains of PRRSV; LV (■), 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●) was determined using volumetric flow cytometry. Total cell density is expressed as absolute cell number per μl (A) and lymphocytic infiltrate is quantified as a percentage of the total cell density (B). Data points show the individual values for each animal and bars display the mean cell counts with error bars representing $\pm\text{SEM}$ for $n=5$ (infected groups) and $n=4$ (controls). Significance is indicated by: * $p < 0.05$, ** $p < 0.01$

Volumetric flow cytometry was used to determine absolute innate cell counts in the BAL. The SU1-bel group had significantly higher numbers of both immature (figure 5.2C) and mature (figure 5.2D) neutrophils compared with controls. As neutrophils have a major inflammatory role in the innate immune response this increase might make sense, however, the LV group

which had less pathology in the lung compared with the 215-06 groups also had significantly higher numbers of neutrophils whereas the British strain remained comparable with controls. Perhaps this trend may be due to the effect of infection upon the alveolar macrophage population (figure 5.2A), macrophages remove spent neutrophils from sites of inflammation to prevent inflammatory processes from causing too much damage. The SU1-bel group with the highest proportion of neutrophils had the lowest number of macrophages compared with the two virus infected groups, with the 215-06 animals displaying significantly higher macrophage counts compared with SU1-bel. Significant differences were seen in the monocyte population (figure 5.2B) with SU1-bel and the LV groups being significantly higher than controls.

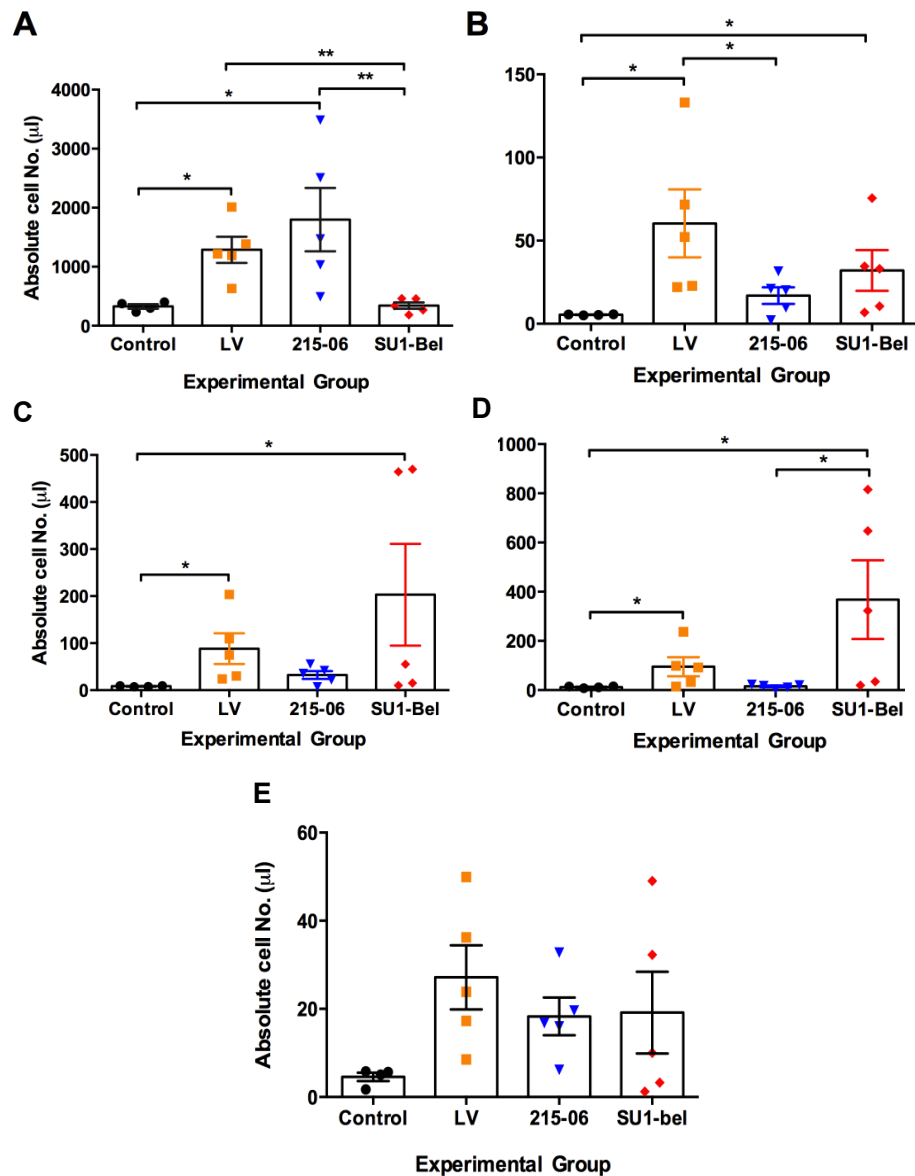


Figure 5.2 Absolute adaptive cell numbers in the BAL of pigs experimentally infected with PRRSV. Animals were intra-nasally infected with three strains of PRRSV; LV (■), 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●), absolute cell numbers were determined by flow cytometry for macrophages (A), monocytes (B), immature (C) and mature (D) neutrophils and NK cells (E). Data points show the individual values for each animal and bars display the mean cell number with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls). Significance is indicated by: * p < 0.05, ** p < 0.005.

Despite showing the same trend for the SU1-bel to have greater number of B and T cells in the BAL compared with the other infected groups only gamma-delta T cells show a significant increase (figure 5.3B). The LV group however, shows a significant increase in the three main T cell populations, C4+CD8+ (figure 5.3C), CD8hi (figure 5.3D) and CD4+ cells (figure 5.3D).

Although the SU1-bel group does not show significant changes over other groups, these cell counts do correlate with pathology with both CD8hi and CD4+CD8+ being higher in individuals displaying the greatest gross pathology.

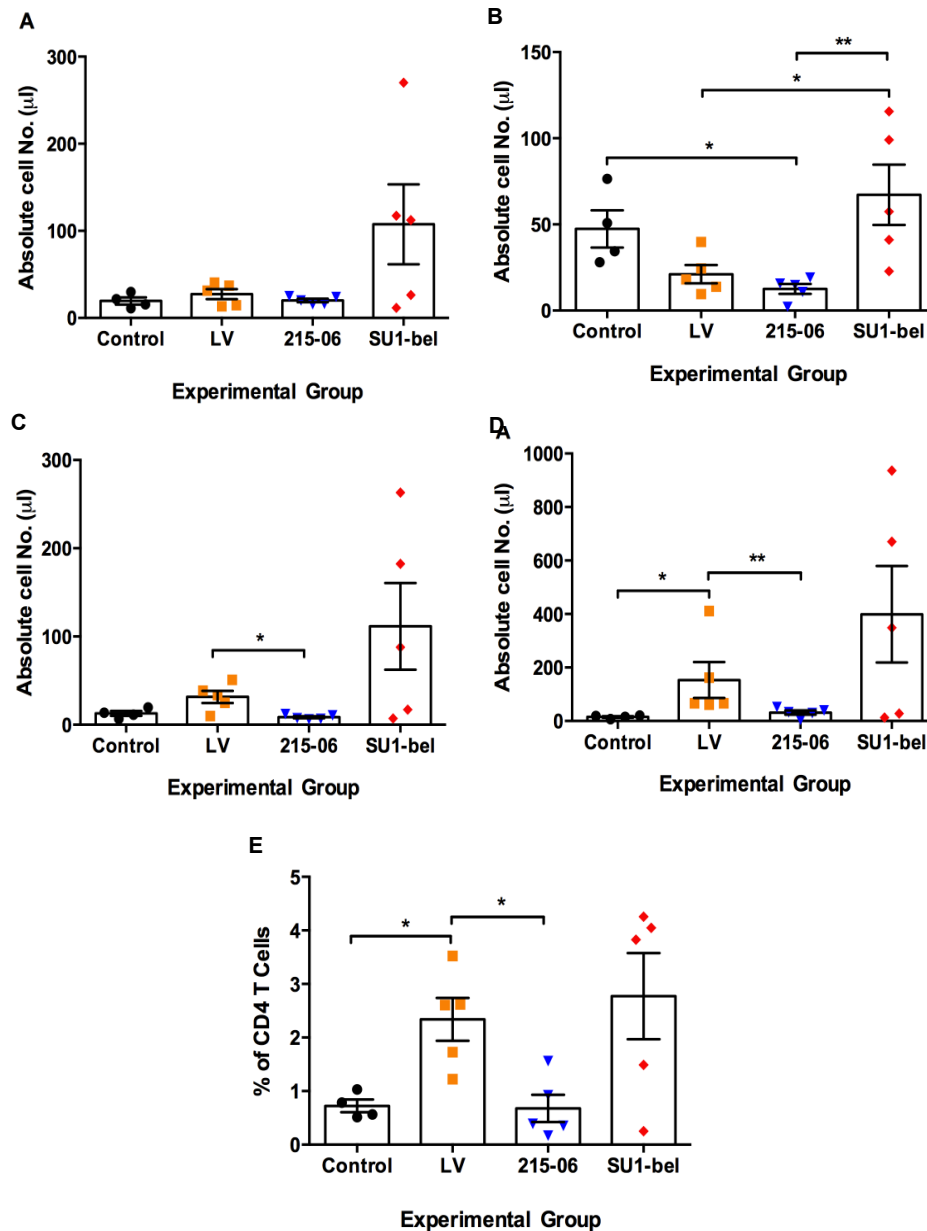


Figure 5.3 Absolute innate cell numbers in the BAL of pigs experimentally infected with PRRSV. Animals were intra-nasally infected with three strains of PRRSV; LV (■), 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●), absolute cell numbers were determined by flow cytometry for B cells (A), gamma delta (B), CD4+CD8+ (C) CD8+ (D) and CD4+ (E) T cells. Data points show the individual values for each animal and bars display the mean cell number with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls). Significance is indicated by: * p < 0.05, ** p < 0.005

Regulatory T cells (Tregs) were quantified as the percentage number of total CD4+ T cells in the BAL of virus-infected and control pigs (Fig. 5.4). Data reveals that the LV group had a significant increase in the percentage of Tregs in the BALF compared with both the 215-06 group and controls. Despite having the highest mean percentage of Tregs the SU1-bel group was not significantly higher statistically than the other groups. However, whereas these groups did not display any correlation between Treg population and pathology, the SU1-bel group showed a inverse correlati~~on~~n between Tregs and gross pathology.

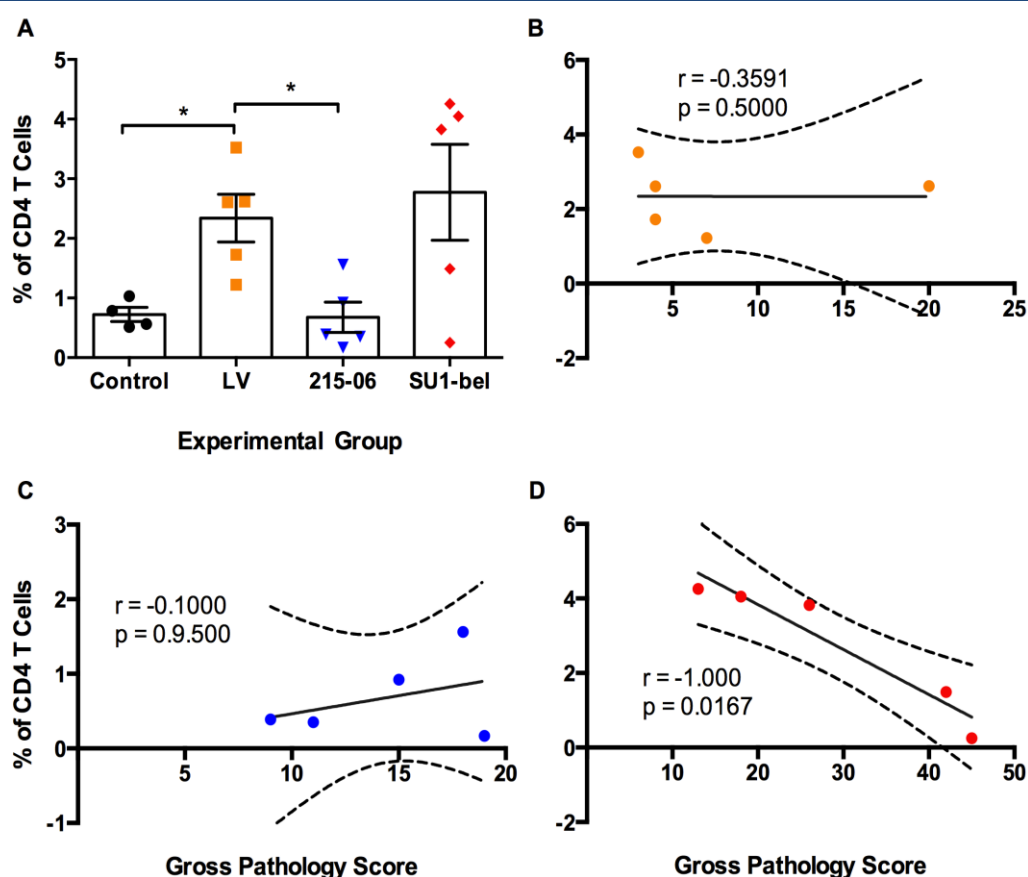


Figure 5.4 Regulatory T cell numbers in the BAL of the SU1-bel infected animals negatively correlated with pathology. Regulatory T cells in the BAL of animals infected with three strains of PRRSV; LV (■), 215-06 (▼) and SU1-bel (◆), and mock-infected controls (●), absolute cell numbers were determined by flow cytometry (A). Data points show the individual values for each animal and bars display the mean cell number with error bars representing \pm SEM for $n=5$ (infected groups) and $n=4$ (controls). Significance is indicated by: * $p < 0.05$. Regulatory T cell populations were correlated with gross pathology scores in the LV (B), 215-06 (C) and SU1-bel (D) infected groups. Data points show absolute cell numbers plotted against gross pathology score of individuals, the solid line shows linear regression and the dashed lines the 95% confidence interval. The Spearman r value and the p value for each data set is displayed on the graph.

5.2.2 Local humoral and cellular responses

The BALF of pigs infected with LV and 215-06 was tested for PRRSV-specific Ab (Fig. 5.5). However at 7 dpi only the SU1-bel group contained Ab positive (an S/P ratio of over 0.4) animals (four of five). PRRSV infection has been reported to be enhanced by none-neutralising local antibody responses, so we looked to see if there was any correlation between BAL Ab and viral replication (figure 5.5B). No correlation was seen, however, there was positive correlation between local Ab and gross pathology. When individual S/P ratios of these animals were plotted against viral copy number and gross pathology the results showed that there was no

correlation between Ab and viral copies but a significant positive correlation between Ab and gross pathology.

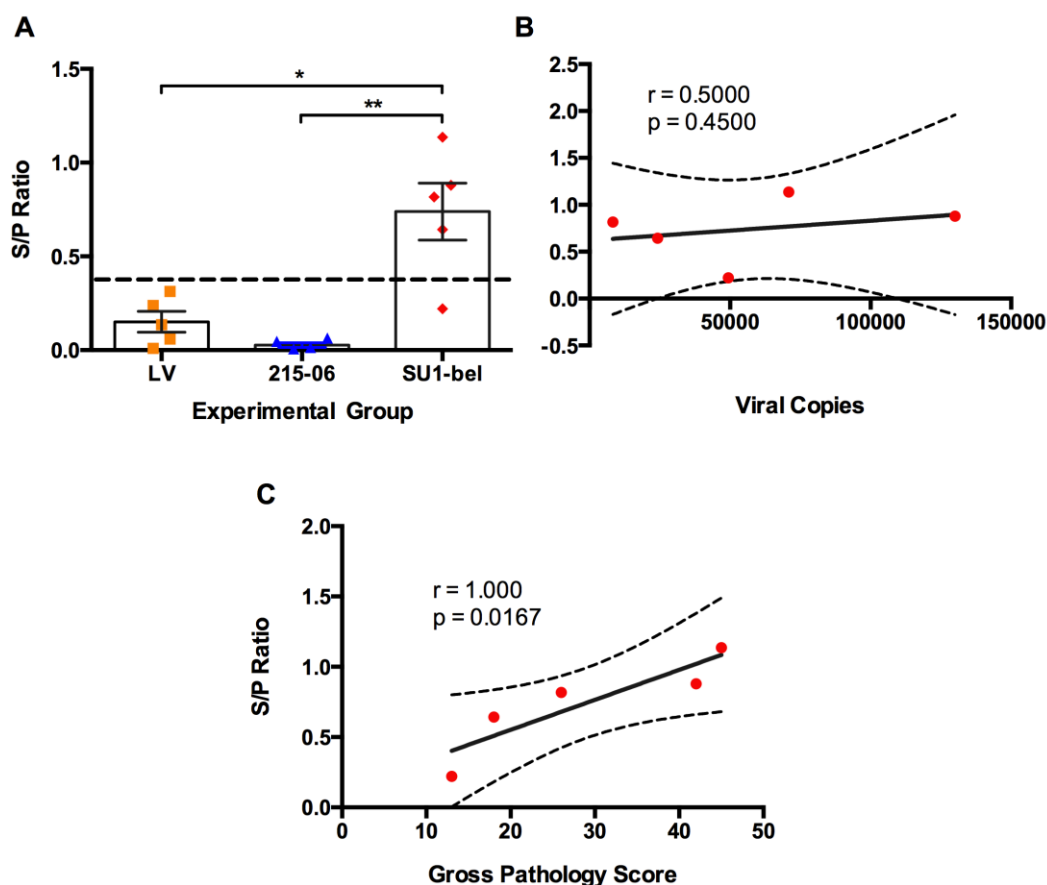


Figure 5.5 PRRSV-specific antibody responses were detectable earlier in the BALF of animals infected with SU1-bel, compared with subtype 1 strains. PRRSV-specific Ab responses of animals infected with LV (■), 215-06 (▼) and SU1-bel (◆) were measured by ELISA in the BALF (A). An S/P ratio above 0.4 (dashed line) was considered to be a positive result. Data points show the mean S/P ratio for individual animals and bars display the mean S/P ratio for the group with error bars representing \pm SEM for $n=5$. S/P ratios from the SU1-bel infected group were correlated against viral copy numbers (B) and gross pathology score (C) the solid line shows linear regression and the dashed lines the 95% confidence interval. The Spearman r value and the p value for each data set is displayed on the graph.

The number of IFN- γ secreting cells in the BALF of pigs was determined by ELISpot assay. The SU1-bel group had significantly higher numbers of IFN- γ secreting cells compared with both the LV and control groups. Interestingly this response fell after stimulation with recall antigen, potentially because the very nature of the T cells in the BAL being effector cells, and

the time post infection may mean that upon isolation cells are still producing cytokines as they were *in vivo*. As IFN- γ is pro-inflammatory cytokine correlations with gross pathology were performed and show that there is a significant positive correlation between IFN- γ secreting cells and pathology in the SU1-bel group but not the LV group.

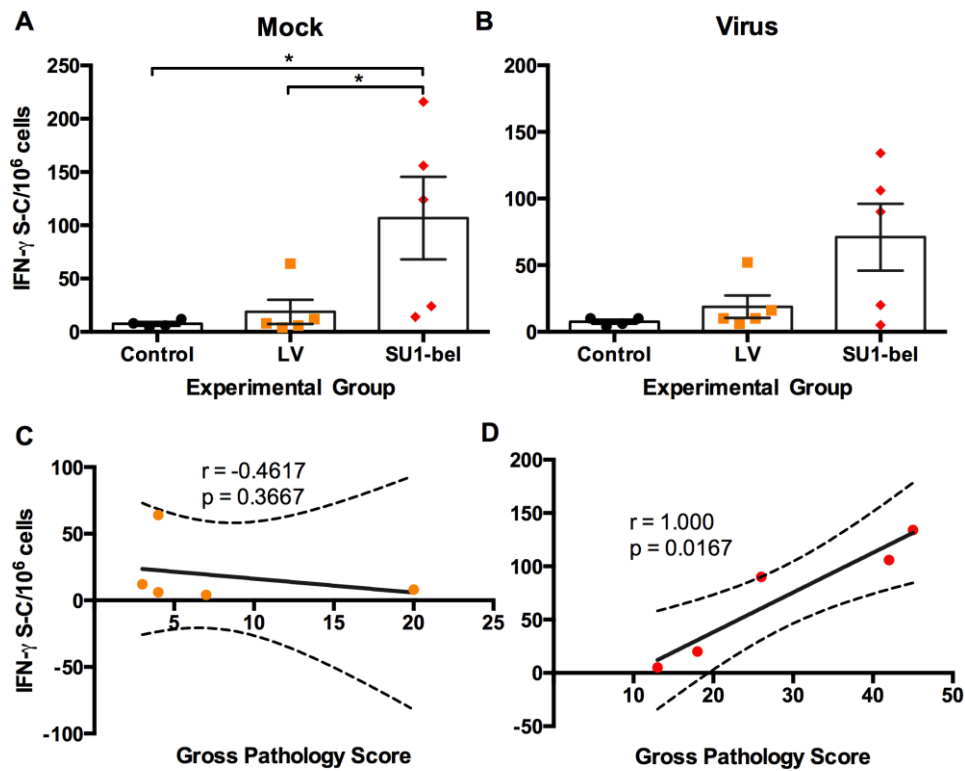


Figure 5.6 The number of IFN- γ secreting cells in the BALF was significantly higher in the SU1-bel group compared with both LV and control groups. The number of IFN- γ secreting cells in the BALF of animals infected with two strains of PRRSV; LV (■), SU1-bel (◆) and mock-infected controls (●), were determined by ELISpot in response to *ex vivo* mock stimulation with media (A) and virus stimulation (B). Data points show the individual values for each animal and bars display the mean number of IFN- γ secreting cells with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls). Significance is indicated by: * p < 0.05. The number of IFN- γ secreting cells was correlated with gross pathology scores in the LV (C) and SU1-bel (D) infected groups. Data points show absolute cell numbers plotted against gross pathology score of individuals, the solid line shows linear regression and the dashed lines the 95% confidence interval. The Spearman r value and the p value for each data set is displayed on the graph.

IFN- γ was also detected in BALF lymphocytes using multi-parameter flow cytometry, cells were surface stained for CD3, 4 and 8 for phenotyping and then intracellularly stained for IFN- γ (Fig. 5.14). Results show that both CD4 and CD8 T cells were positive for IFN- γ and that percentages of these cells were comparable for both phenotypes with both mock and

leukocyte activation cocktail (LAC) stimulation. In the case of CD4 cells this percentage was significantly higher in both the LV ($p = 0.0317$) and SU1-bel ($p = 0.0159$) infected groups compared with controls following mock stimulation with the SU1-bel group having the highest mean percentage. However, the values after *ex vivo* stimulation with LAC were not significantly different although both LV and SU1-bel animals had higher percentages of IFN- γ positive cells compared with controls. For CD8 cells this percentage was significantly higher in both the LV ($p = 0.0159$) and SU1-bel ($p = 0.0159$) infected groups compared with controls following mock stimulation. For LAC stimulated cells significant differences were observed between the groups with LV ($p = 0.0238$) and SU1-bel ($p = 0.0159$) both being significantly higher compared with controls. Although the percentages of IFN- γ positive cells were similar in both CD4 and CD8 T cells the absolute counts of these cell types were vastly different with a much higher number of CD8 cells being observed in both groups compared with CD4 cells. Meaning that there is a higher number of IFN- γ positive CD8 T cells compared to CD4.

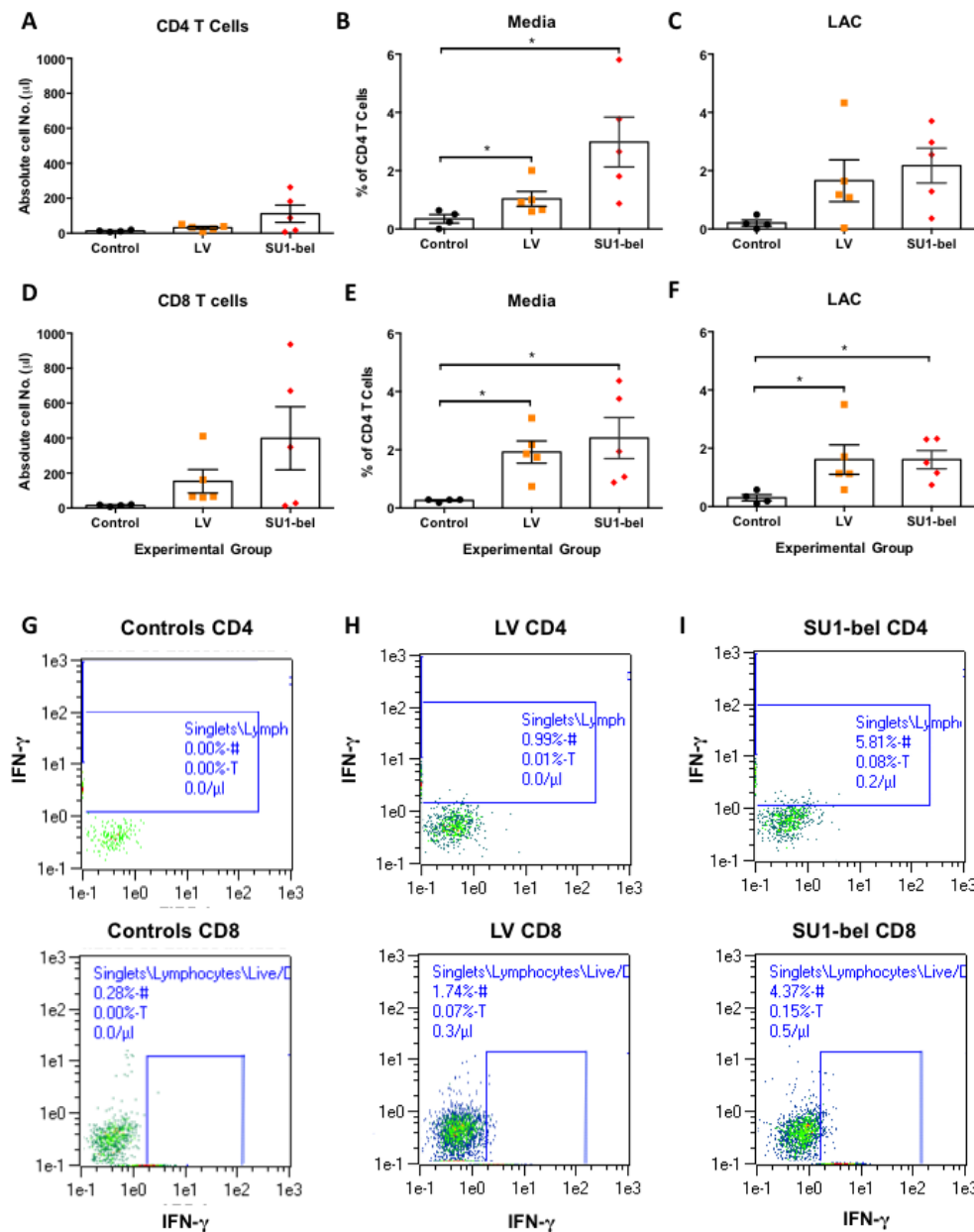


Figure 5.7 Percentage of IFN-gamma positive cells was significantly higher in the BALF of both LV and SU1-bel infected pigs. IFN- γ production of CD4 and CD8 T cells was determined using multi-parameter flow cytometry. The absolute number of CD4 (A) and CD8 (D) T cells is shown to allow comparison with percentage values. The percentage of CD4 and IFN- γ positive T cells in the BALF of pigs infected with LV (■), SU1-bel (◆) and mock-infected controls (●) after mock (B) and virus (C) stimulation. The percentage of CD8 and IFN- γ positive T cells in the BALF of pigs infected with LV (■), SU1-bel (◆) and mock-infected controls (●) after mock (E) and virus (F) stimulation. Data points show the individual values for each animal and bars display the mean number of IFN- γ secreting cells with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls). Plots show the fluorescence of IFN- γ positive CD4 and CD8 T cells of control (G), LV (H) and SU1-bel (I) infected BALF lymphocytes.

BALF cells were stimulated *ex vivo* with homologous virus and mock supernatant. A multi-analyte cytokine detection system was used to measure all cytokines in a single assay (Fig.5.8). TNF- α was only detected in virus-infected animals; the concentration of TNF- α was significantly higher in the LV and SU1-bel groups compared with controls, following mock stimulation. Virus stimulation of cells saw slightly lower concentrations of TNF- α compared with mock stimulation for both groups and neither was significantly higher than controls. Concentrations of IL-4 were highest in the BALF of LV-infected pigs being significantly higher than both controls and SU1-bel animals following mock stimulation. Trends were similar after virus stimulation with LV only being significantly increased above controls. The neutrophil attractant IL-8 was undetectable in control pigs after mock stimulation but was significantly higher in the SU1-bel group compared with controls and LV infected animals. IFN- α was detected in the BALF of all groups and was highest in the LV group, although no significant differences were found. IL-1 β concentrations in the LV group were comparable with controls after both mock and virus stimulation. The SU1-bel group displayed the highest mean concentration of IL-1 β after both mock and virus stimulation, however, this was not significantly different statistically. Mock stimulation of BALF cells did not produce any detectable level of IL-2 in any group. IL-2 was detectable in some animals from all three groups after virus stimulation although means did not differ between these groups. IL-6 production was variable after both mock and virus stimulation with means being comparable across the board. Mock stimulation of cells produced higher mean concentrations of IL-10 in the LV and SU1-bel groups compared with controls although not significantly so. Following stimulation with mock IL-10 increased slightly in the SU1-bel group compared with mock stimulated, but again no statistical differences were observed.

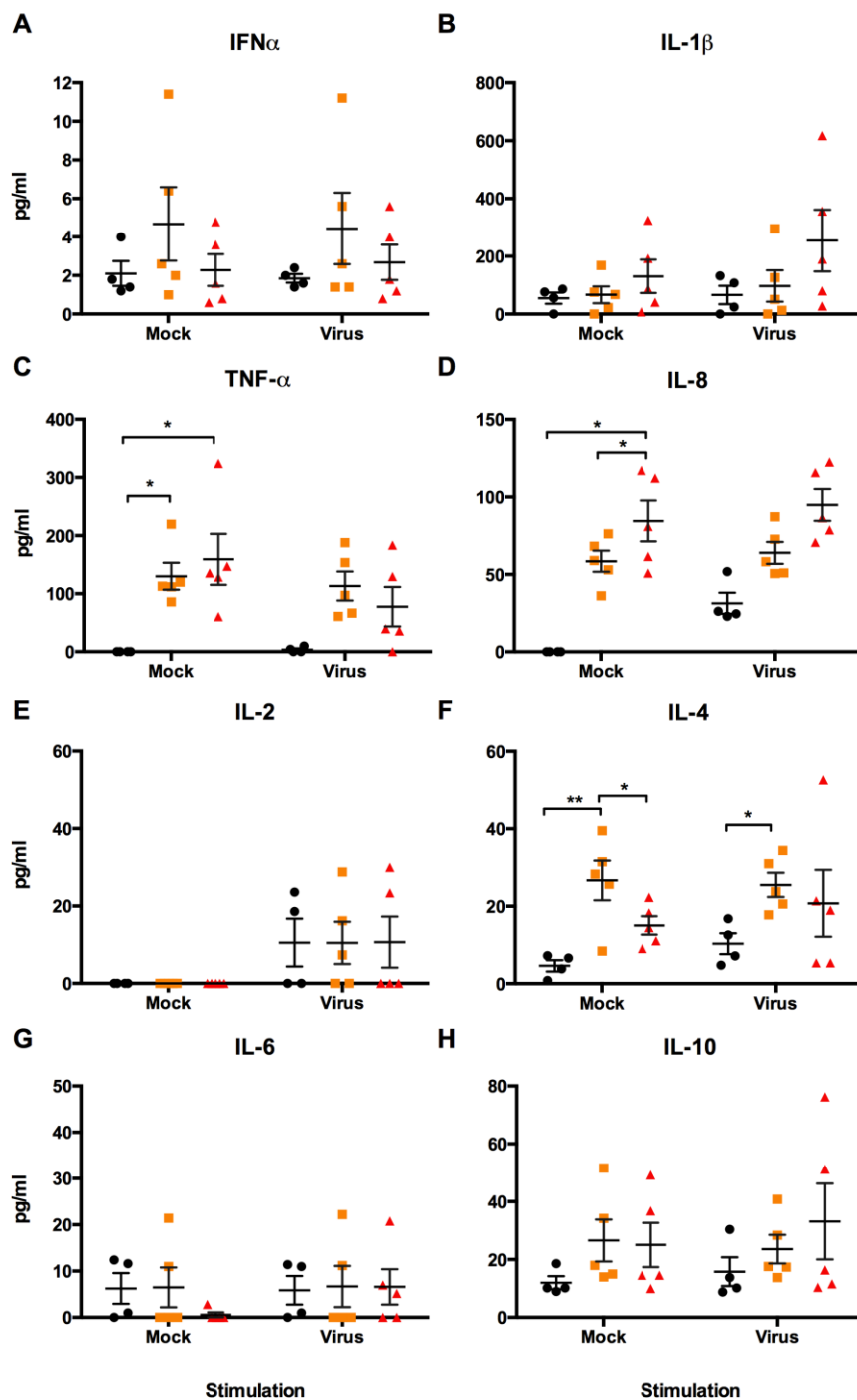


Figure 5.8 Cytokine responses of BALF cells after mock and ex vivo stimulations. BALF cells were mock stimulated and stimulated with homologous virus ex vivo and supernatants tested for cytokines using a multi-analyte assay system. BALF of pigs infected with LV (■), SU1-bel (◆) and mock-infected controls (●) were tested for the cytokines IFN- α (A), IL-1 β (B), TNF- α (C), IL-8 (D), IL-2 (E), IL-4 (F), IL-6 (G) and IL-10 (H). Data points show the individual values for each animal and bars display the mean cytokine concentration (pg/ml) with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls).

Cells from the mediastinal lymph nodes were stimulated *ex vivo* with homologous virus and mock supernatant. A multi-analyte cytokine detection system was used to measure all cytokines in a single assay (Fig.5.9). IFN- α concentration in the lymph node of Su1-bel infected pigs was significantly higher than both the LV group and controls. Following virus stimulation IFN- α concentration was only significantly higher compared with controls. IL-6 was produced in lymph node cells of both LV and SU1-bel animals and was significantly higher in the SU1-bel group compared with controls ($p = 0.0159$) after mock stimulation. Control animals had detectable levels of IL-6 after stimulation with virus. Only the SU1-bel group had detectable levels of both IL-8 and TNF- α after mock stimulation. and this increased after stimulation with homologous virus. In both cases responses were significantly higher in the Su1-bel group compared with LV and controls Interestingly, no IFN- γ could be detected in the lymph nodes of any of the pigs with either mock or virus stimulation. This is in stark contrast to the robust production of this cytokine observed in the BALF of infected pigs. IL-1 β concentrations in the LV and SU1-bel groups were highly variable, although the cytokine could not be detected in controls after either mock or virus stimulation a few animals from each group did have detectable levels.

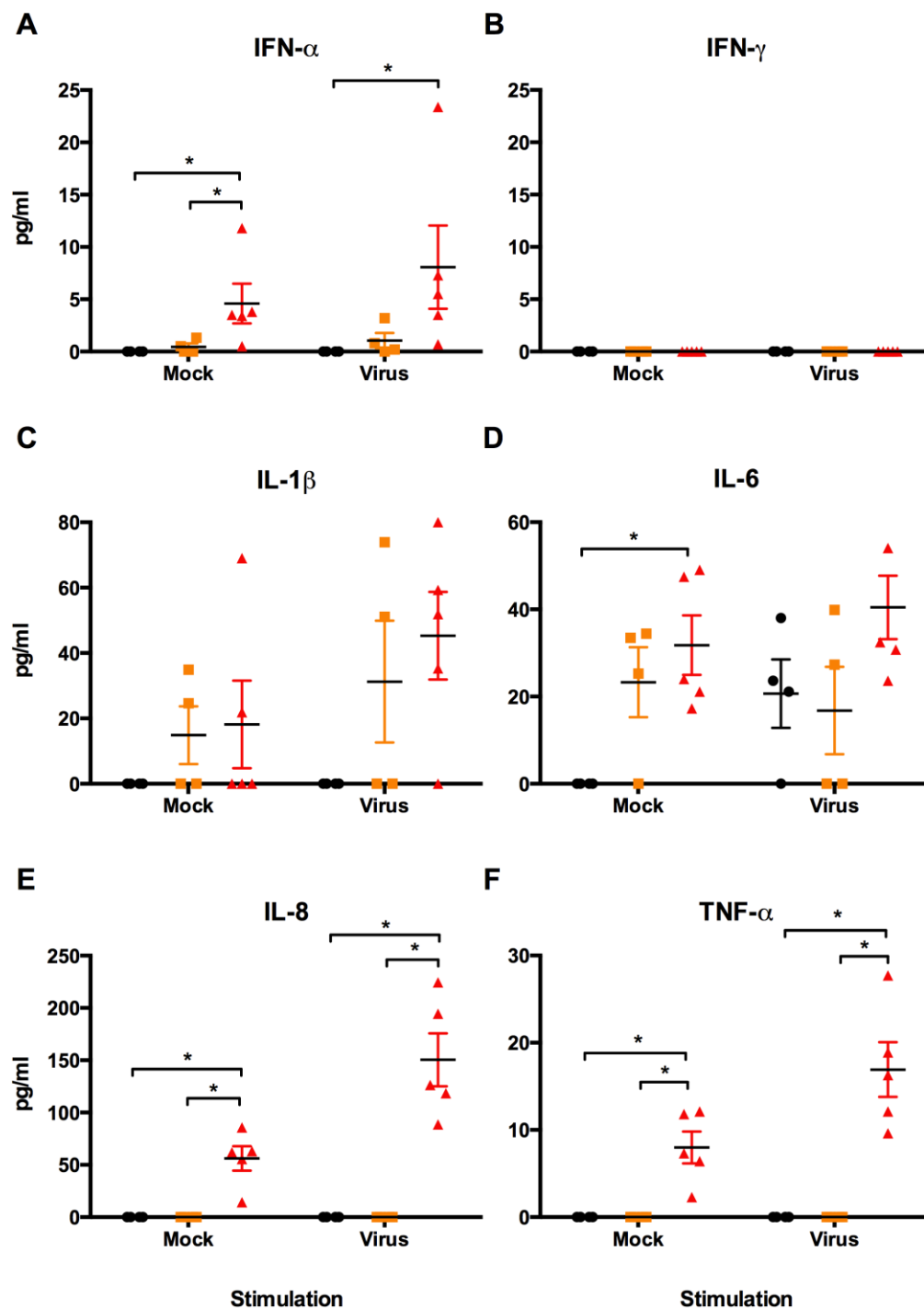


Figure 5.9 Cytokine responses of mediastinal lymph node cells after mock and virus *ex vivo* stimulation. Isolated cells from the mediastinal lymph nodes were stimulated with mock supernatant and homologous virus *ex vivo* and supernatants tested for cytokines using a multi-analyte assay system. BALF of pigs infected with LV (■), SU1-bel (◆) and mock-infected controls (●) were tested for the cytokines IFN- α (A), IFN- γ (B), IL-1 β (C), IL-6 (D), IL-8 (E) and TNF- α (F). Data points show the individual values for each animal and bars display the mean cytokine concentration with error bars representing \pm SEM for n=5 (infected groups) and n=4 (controls).

Infection of piglets with the Eastern European SU1-bel strain of PRRSV resulted in more severe clinical disease and pulmonary pathology compared with the subtype 1 strains LV and 215-06. Despite this, viral levels in both the serum and BALF of SU1-bel infected animals were not higher compared with the subtype 1 groups. Investigations into peripheral immune responses to the viruses revealed that the SU1-bel strain induced an earlier humoral and stronger cell-mediated defence. As the role of the immune system in causing disease pathology is well documented, local immune responses in the lungs of infected pigs are compared between groups and their relationship with pathology discussed. Due to the small group numbers and the large variation observed in the data sets this chapter focuses on 7 dpi, as this is the date that the greatest level of pathology was observed in the lungs.

Cell numbers in the BALF of PRRSV-infected pigs increased compared with controls, with this increase being greatest in the SU1-bel group. The percentage of lymphocytes in the BALF increased significantly above controls in the SU1-bel group. Interestingly infection with 215-06 had an opposite effect, with this group having a significantly lower percentage of lymphocytes compared with the rest of the three groups. Macrophages make up the major cell type in the BALF, during the process of phagocytosis, where macrophages take up and destroy pathogens and cell debris, FAS Ligand is released. This release of FAS Ligand has been shown to induce apoptosis of bystander leukocytes (Brown & Savill 1999). Previous studies have shown that infection with PRRSV can induce apoptosis of bystander lymphocytes in the lungs of infected pigs (Sirinarumitr et al. 1998; Labarque, Van Gucht, Nauwynck, Van Reeth & Pensaert 2003b). However, the 215-06 infected pigs also displayed the greatest increase in the absolute number of macrophages. As only $\gamma\delta$ -T cells showed a significant decrease in cell number below controls it is more likely that this decrease in lymphocyte percentage is due to an increase in the number of myeloid cells rather than the death of lymphocytes.

In the healthy pig alveolar macrophages make up the majority of cells in the alveolar space. As mentioned in section 1.6.1 these cells patrol the site of gas exchange for potential pathogens and foreign bodies and suppress over reactive inflammatory responses. Previous

studies have observed a fall in the number of alveolar macrophages following PRRSV infection (Sirinarumitr et al. 1998; Labarque et al. 2000), thought to be caused by the apoptotic effect of PRRSV infection (Sur et al. 1998; Choi & Chae 2002; Sirinarumitr et al. 1998). However, the alveolar macrophage populations in both the LV and 215-06 groups increased post-infection and the SU1-bel group remained comparable with controls. It is possible that alveolar macrophage numbers did increase between 3 and 7 dpi and apoptosis and/or killing of infected cells by the cytolytic immune response returned numbers back to control levels. Macrophages are known to have both proinflammatory and anti-inflammatory functions (Xu et al. 2013; Knapp et al. 2003), although convincing evidence of this in the pig is still to be found. So it is possible that the macrophages detected in the lungs of the 215-06 and LV groups are playing a role in suppressing inflammation in these animals, although, there was no negative correlation between macrophage numbers and pathology.

Monocytes, the precursors of macrophages, in the BALF of SU1-bel animals were significantly higher than controls, and these positively correlated with pathology. Monocytes in the BALF can have a direct inflammatory response through their production of reactive oxygen species (ROS) and proinflammatory cytokine production (Rosseau et al. 2000). It has also been shown that monocytes may contribute to pulmonary inflammation through enhancement of alveolar neutrophil, a proinflammatory cell type, recruitment via chemotactic protein-1 (CCL2) (Maus et al. 2003). This report corresponds with the finding that neutrophil numbers increased in the BALF of pigs infected with LV and SU1-bel. Both of which had a significant increase in monocyte population above controls. However, the inflammatory role of monocytes during PRRSV infection is brought into question by the high numbers found in the LV group, although these did not correlate with pathology as seen in the SU1-bel group. The LV animals did have the highest mean number of monocytes and yet had the lowest mean gross pathology score, despite the strong association between pulmonary monocytes and inflammation. It is possible that the immune response of these pigs possesses an immunosuppressive component that may not be present, or be at a lower level in the SU1-bel group. The influx of monocytes into the lungs of LV, 215-06 and SU1-bel infected pigs mirrors those results reported by (Labarque et al. 2000) who observed an influx of monocytes into the lungs of PRRSV infected pigs between five and 25 dpi.

Neutrophils are a major player in acute inflammation and the innate immune response. Although largely associated with bacterial infections the role of these cells during viral infection is beginning to come to the fore. They are the first cells to be recruited to the site of infection where they have a direct antimicrobial function through production of ROS, antimicrobial proteases and extracellular traps (Fuchs et al. 2007; Hoshino et al. 2008). Unfortunately the side effect of these responses is inflammation, and although inflammation is required to induce downstream responses, if gone unchecked, they can have disastrous consequences for the host. Studies in mice have shown that depletion of neutrophils prior to infection with influenza leads to increased mortality (Tumpey et al. 2005). A recent study has also reported that influenza antigen displayed on neutrophils in the lung are able to perform as an antigen presenting cell for effector cytolytic CD8 T cells (Hufford et al. 2012). Neutrophil numbers in the BALF of PRRSV infected pigs in this study increased significantly above controls, which had a negligible neutrophil population, in both the LV and 215-06 infected groups, and were highest in the SU1-bel group. Neutrophil numbers also correlated with pathology in the SU1-bel group, but not the LV. Interestingly the 215-06 did not display and increase in neutrophils as has been reported for PRRSV (Labarque et al. 2000) despite this group having the second greatest mean gross pathology score. Macrophages are known to phagocytose apoptotic neutrophils and release the immunosuppressive cytokine transforming growth factor (TGF)- β , which can inhibit pro-inflammatory mediators (Elbim et al. 2009). This may explain why the 215-06 group had fewer neutrophils, as it had the highest number of alveolar macrophages and the SU1-bel group, which was abundant with neutrophils, had the smallest population of alveolar macrophages. Both the LV and the SU1-bel groups had high concentrations of the neutrophil chemoattractant IL-8 produced by BALF cells, probably leading to the huge influx of these cells into the lung. Unfortunately this data is not available for the 215-06 group as it was decided to progress with cytokine analysis on only those groups displaying the lowest and greatest levels of gross pathology for comparison.

CD4⁺CD8⁺ DP T cells are a subtype of T cell that were once thought to be purely antigen-specific memory cells found in the periphery and lymph (OBER et al. 1998; Periwal & Cebra 1999). However a study by (Nascimbeni 2004) has described the presence of these cells in

tissues at the site of infection, and that these cells may actually have an effector function. DP T cells were increased in the BALF of both LV and SU1-bel infected animals above controls and 215-06 infected pigs. In the case of the SU1-bel group this cell type population positively correlated with gross pathology. However, a study performed by (Z. Xiao et al. 2004) found that PRRSV infection did not have an effect upon the population of DP T cells in the lung. Although a study performed with porcine respiratory coronavirus did find that DP T cell populations did increase post-infection and contributed to the severity of pulmonary inflammation (Renukaradhya et al. 2010). However, the LV group had a significantly higher number of DP T cells compared with the 215-06 group, which showed greater pathology, so these cells alone are likely not the main culprit for the pulmonary damage.

A similar picture can be observed in the population cytotoxic T cells, with numbers increasing above controls in both 215-06 and SU1-bel infected groups but not in the 215-06 group. Again these cells positively correlated with pathology gross pathology in the SU1-bel group only. This influx of cytotoxic T cells has also been observed in the BALF of PRRSV infected pigs in during previous studies (Samsom et al. 2000; Labarque et al. 2000). Cytotoxic T cells remove virus through a direct cytolytic effect upon infected cells through release of cytotoxic granules and FAS ligand interactions (Price et al. 2005). This response and consequently cellular death may contribute to the pathological changes observed in the lung post-infection. In fact studies have shown that although cytotoxic T cells are effective at removing virus they also contribute significantly to pulmonary pathology during the course of infection (Cannon et al. 1988; H. D. Chen et al. 2001).

Tregs are a specialised immunomodulatory lymphocyte distinguished by their expression of the fork head box protein (Foxp3). Tregs suppress the immune response through a number of pathways including the production of immunosuppressive cytokines such as IL-10 and TGF- β , a direct cytolytic effect on effector cells through perforin production and inhibiting the antigen presentation ability of dendritic cells (Vignali et al. 2008). This study describes, for the first time, the increase in Tregs in the BALF of pigs after infection with PRRSV. Results showed an increase in the Treg population, as a percentage of CD4 T cells, in both the LV and SU1-bel infected groups, but not the 215-06 group. Interestingly the population in the SU1-bel group

inversely correlated with pathology. (Wongyanin et al. 2010) showed that Tregs could be induced *in vitro* by culturing peripheral blood mononuclear cells in the presence of virus, leading to a significant increase of these cells in the cultures, and that infection of pigs with PRRSV led to a significant increase of Tregs in the peripheral blood. Another study by (Silva-Campa et al. 2012) found that Treg populations increased *in vivo* after infection with PRRSV observing significant increase in cells in both the mediastinal lymph node and tonsils alongside an increase in TGF- β . The LV group had a significantly higher proportion of Tregs compared with the 215-06 group, perhaps explaining why this group presented with lesser pathology. Tregs are known to produce the immunosuppressive cytokine IL-10, which dampens immune responses by inhibiting proinflammatory cytokines, expression of class II major histocompatibility complex (MHC) and inhibiting T cell proliferation (Isomaki et al. 1996). Interestingly IL-10 concentrations in the BALF increased above controls, although not significant statistically IL-10 levels did inversely correlate with pathology in the SU1-bel group only as did Treg population. It is possible that the variation in both pathology and local immune response of the three pigs of the SU1-bel group with that consistently had poorer immune responses and less pathology was due to the action of Tregs. However, this area requires further study, perhaps by enriching this cell type and performing further functional assays, as IL-10 is produced by a number of cells types. An induction of Tregs after PRRSV infection has been suggested as a mechanism for the increased susceptibility to secondary infection often observed in the field. However, none of the pigs with increased numbers of these cells showed a poorer response to ADV vaccination. This may be because Tregs play more of a role in controlling the immune response, and preventing it becoming inflammatory rather than suppressing enough to lead to secondary disease. We did not see any difference in the response to ADV vaccination in PRRSV infected pigs compared with mock-infected controls. This may be part of the experimental design, as seen in this data the response to PRRSV infection happens much more at the local site of infection and so muscular administration of the vaccine may have been suboptimal, however there was no way of knowing what effects local administration of the ADV vaccine may have had, and what effect it may have had upon the infection data. Future work should further explore the effect of PRRS infection on immunosuppression but select a respiratory pathogen that can be given intra-nasally.

PRRSV-specific antibody was not detected in the BALF of either the LV or 215-06 infected animals at 7 dpi, but was detected in four out of five animals in the SU1-bel group. The SU1-bel group also had the highest mean number of B cells in the BALF although this was not statistically significant. It has long been suggested that PRRSV may utilise antibody dependant enhancement for entering the alveolar macrophage, where non-neutralising antibodies coat the virus, which is then phagocytosed by the macrophage as part of its anti-microbial action (YOON et al. 1996; Qiao et al. 2011). A study by (YOON et al. 1996) reported that the ADE of PRRSV may be variable between different PRRSV isolates, culture of PRRSV with non-neutralizing antibodies enhanced PRRSV replication *in vitro* and addition of antibody to neutralise these antibodies reduced virus yields. However, the antibodies tested in this study did not show any correlation with viral copies in the BALF of SU1-bel pigs, and the lower viral load in the BALF of these pigs compare with others does not suggest that ADE is playing a role in this case. Interestingly antibody did correlate with gross pathology in the SU1-bel group. Antibodies can contribute to cellular damage through the deposition of antigen-antibody complexes in the tissues and by activating complement, a branch of the innate immune system, which if left unchecked, causes substantial inflammation (Polack et al. 2002). A study by (S. Xiao, Mo, et al. 2010b) observed an up-regulation of complement cascade genes during highly pathogenic PRRSV infection which lead to severe tissue damage. Histological examination of lung tissue for complement and antibody complexes would help determine if this mechanism is having a significant role in pathology during PRRSV infection.

Cytokine responses in the BALF and mediastinal lymph nodes of pigs were investigated as these biochemical play a pivotal role in the pathology of viral disease. Interestingly the best responses were seen in BALF cells that were stimulated with mock compared with homologous virus stimulation. This is most likely because, unlike stimulation of PBMCs where a memory response is being recalled, the cells found in the BALF are effector cells, so are still producing cytokines when they are isolated from the lung. As PRRSV primarily replicates in the BALF these cells are also still being stimulated by the viral particles that are still present in the cell sample. IFN- γ is an anti-viral cytokine which inhibits viral replication by stimulating the production of PKR a protease which is activated by dsRNA, an intermediate during PRRSV replication, and disrupts viral protein synthesis (Goodbourn et al. 2012). IFN- γ is produced

primarily by NK cells during the innate response, and then by T helper 1 cells and cytotoxic T cells during the adaptive phase (He et al. 2004). IFN- γ has cytolytic effects by activating the Stat-1/IRF-1 cellular pathway in target cells which induces apoptosis (Bernabei et al. 2001), it also activates the antimicrobial process of respiratory burst, the production of nitric and up-regulation of lysozymal enzymes (Decker et al. 2002). Both of these mechanisms are important in anti-viral defence, but they can also contribute significantly to inflammation. Numbers of IFN- γ secreting cells in the BALF was determined using an ELISpot assay, results show a significant increase in the number of cells in the SU1-bel group above both controls and LV infected pigs. This also positively correlated with gross pathology in the SU1-bel group suggesting that IFN- γ may indeed be having a proinflammatory effect. The IFN- γ response was further dissected by intracellular staining of lymphocytes to try and determine whether the response came primarily from CD4 or cytotoxic T cells. The results show that similar percentages of both CD4 and cytotoxic T cells were positive for IFN- γ , however the absolute number of cytotoxic T cells in the BALF was drastically higher than CD4 cells, so in reality the majority of the IFN- γ in the BALF came from cytotoxic T cells rather than CD4. This finding is mirrored in a study on hepatitis B viral infection which reported that cytotoxic T cells appeared to be the main effector cell during infection, and had a significant impact upon pathogenesis (Thimme et al. 2003). IFN- γ is the hallmark of a Th1 response, the significantly higher concentration of IL-4 in the BALF of LV pigs compared with SU1-bel indicates that there may be a Th1/2 imbalance. The Th1 response is associated with a proinflammatory cell-mediated response, whereas the Th2 is anti-inflammatory humoral response, when in balance these keep one another in check, however a skew towards a Th1 response and poor Th2 response can lead to severe inflammation (Muller et al. 1998). It is likely that this imbalance is contributing to the pathology seen in the SU1-bel infected animals.

The type I IFN α cytokine is the hallmark of the innate anti-viral response (Pfeffer et al. 1998), it triggers the expression of hundreds of different genes, which in turn induce a number of antiviral mechanisms, such as RNA degradation, and inhibition of translation and virus trafficking (Y. Sun et al. 2012). IFN α was significantly increased in the mediastinal lymph nodes of pigs infected with the SU1-bel strain compared with controls and the LV group. This is despite common opinion that PRRSV does not induce an IFN α response (Calzada-Nova et

al. 2011; Patel et al. 2010; Van Reeth et al. 1999). No differences were observed between the groups in the BALF even though (Chung et al. 2004) has reported a significant induction of IFN- α producing cells in the lungs of PRRSV infected pigs. Perhaps by this time point this early cytokine response is no longer found in the lung and the only indication that it existed lies within the draining lymph nodes.

IL-1 and TNF- α are among some of the first cytokines to be produced after viral infection. Both have proinflammatory effects and play a role in the recruitment of immune cells to the site of infection (Sergeie et al. 2007). In this study TNF- α was significantly higher in the BALF of both SU1-bel and LV infected animals compared with controls, although it could only be detected in the lymph nodes of the SU1-bel infected pigs, perhaps because lower cell concentrations had to be used for this assay due to the very small size of the lymph nodes and subsequent difficulty in isolating sufficient cell numbers. IL-1 concentrations were higher in the SU1-bel infected group compared with controls and the Su1-bel group, although this was not statistically significant the cytokine did show a positive correlation with pathology. Both IL-1 and TNF- α production have been reported in the BALF of PRRSV infected pigs albeit it at relatively low levels, and in a model which induced only mild disease severity (Van Gucht et al. 2003). These results partly explain the influx of cells into the lung after infection with PRRSV, although both cytokines have proinflammatory effects, as the LV group displayed only minimal pathology it is likely that a number of proinflammatory cytokines combined with poor immunomodulation, is the cause of the greater pathology seen in the SU1-bel group.

This chapter continues the observation that immune responses are stronger in the SU1-bel group compared with LV and 215-06 infected animals, which were observed in the periphery. The SU1-bel group shows a continuous trend towards a more proinflammatory response. Correlation does not imply causation, however, due to the large variation and small group number in the SU1-bel group correlations with pathology were used in this chapter to provide an indication of how the data would look if three pigs had not displayed lower pathology scores than the others. This data illustrates that it is not merely one factor of the immune response that is responsible for the pathology observed, rather a combination of an influx of

inflammatory cells into the lung, production of proinflammatory cytokines and finally a reduced immunosuppressive response as suggested by Treg populations and IL-10 concentrations.

Chapter VI

Discussion

This study set out to explore the pathogenicity of, and immune responses to, a strain of European subtype three PRRSV (SU1-bel), and compare this to the reference strain LV and the British field strain 215-06. This is the first time that both the 215-06 and SU1-bel strains have been used for experimental infection of pigs. Studies have been performed that compare a subtype three strain to other European isolates (Karniychuk, Geldhof, Vanhee, Doorsselaere, Saveleva & Nauwynck 2010b; Weesendorp et al. 2012) but this work further dissects the local immune response to these viruses and discuss their link with pathology. The previous study performed on the subtype three strain Lena (Karniychuk, Geldhof, Vanhee, Doorsselaere, Saveleva & Nauwynck 2010b) had already reported that these viruses may have greater pathological effect compared with subtype one strains more commonly found in Western Europe. As the general result of infection with subtype one strains is mild clinical signs and minimal tissue pathology the importance of this subtype and its possible introduction to the large swine herds of Western Europe make these strains important areas of research. Due to the exceptional ability of viruses to mutate it is natural that strains of the same virus might induce differential disease processes, and this has been shown for a number of viruses, including Influenza (Garigliany et al. 2010; Summers et al. 1984; Yun et al. 2008).

This study found that experimental infection with SU1-bel also induced a much more significant pathogenesis, with pigs displaying fever, greater clinical signs and more severe pathology, particularly in the lungs. However, unlike the study by Karniychuck et al (2010) the Su1-bel group did not have the highest viral loads, in fact viral loads were lower than the LV group in both the serum and lung, and viral loads in the lung did not correlate with pathology. This may seem an unusual finding but a similar phenomenon has been reported in mice infected with influenza, where increased pathology appeared to be linked with the immune response rather than enhanced viral loads (Marcelin et al. 2011). Alongside lower viral loads a higher concentration of the anti-viral cytokine IFN- γ was detected from cells isolated from the BALF of SU1-bel infected pigs, as also seen in the blood, this possibly explains why the viral loads in the BALF of the SU1-bel group had significantly lower levels of virus compared with the LV and 215-06 animals. However, this response is a double-edged sword as although IFN- γ is

indeed an anti-viral cytokine it is also pro-inflammatory in nature and in all likelihood is contributing strongly to the pathology observed in this group. In fact the importance of IFN- γ in pathology has already been acknowledged during SARSCoV infection where the cytokine has been shown to induce Fas-mediated apoptosis of alveolar epithelial cells and inhibit their proliferation (THERON et al. 2005). A similar observation was reported during experimental infection of IFN knockout mice with respiratory syncytial virus, this study showed that although IFN- γ was required for complete virus clearance it also had a pathogenic role as respiratory signs were linked with IFN- γ levels in the lung and led to airway obstruction which was less severe in knock out mice (van Schaik et al. 2000).

Anti-PRRSV antibodies appeared in both the blood and the BALF earlier in the SU1-bel group compared with the LV and 215-06 infected animals. Although no neutralizing antibodies were detected in the serum or the BALF of any pigs. Antibody dependant enhancement has been argued in the case of PRRSV (YOON et al. 1996) it has also been reported for a model of respiratory syncytial virus in monkeys (Ponnuraj et al. 2003). However, viral load data from both the serum and BALF of these PRRSV infected pigs do not support this idea, perhaps because of strain variation. It is possible though that these enhanced antibody responses do contribute to the faster clearance of virus from both the serum and BALF alongside IFN- γ levels. As has already been discussed it is possible that antibody complex deposition and activation of complement by these early appearance antibodies may contribute to the pathology observed in the SU1-bel pigs, particularly as antibody positively correlated with lung pathology in this group. Testing the tissue samples for immune complexes and complement activation would be useful in determining the role, if any, this humoral response has in pathology.

Alveolar macrophage populations increased in the BALF of LV and 215-06 infected animals, however the population in the SU1-bel group remained comparable with controls. Macrophages are major producers of pro-inflammatory cytokines, indeed pigs depleted of macrophages prior to infection with swine influenza had significantly lower levels of TNF- α and IFN- γ (Kim et al. 2008). Cytokine analysis showed that the comparatively low numbers of macrophages in the SU1-bel group did not effect cytokine production with this group having

the highest levels of both TNF- α and IFN- γ in the BALF. Possible explanations for this lower population of macrophages include apoptosis of the cells induced by enhanced viral replication (Sur et al. 1998) or destruction of virus infected cells by the cytolytic immune response. As viral loads do not support the idea of enhanced viral replication in the SU1-bel group, it appears more likely that the difference may be caused by the cytolytic effect caused by the great influx of IFN- γ producing cytotoxic T cells that were observed in the BALF of SU1-bel infected pigs, as described for human immunodeficiency virus infection (Autran et al. 1990). Monocytes the precursor cells to macrophages were, on the other hand, highest in the SU1-bel group. This observation may have come from a requirement for these cells to replenish the macrophage pool in SU1-bel pigs. Whatever the reason, the pro-inflammatory nature of this cell type (Rosseau et al. 2000) is likely to have had a role in the pathology observed in this group. Further work is required to elucidate on the identity of these cells, staining to determine whether the macrophages present are resident or infiltrating cells (Davidson et al. 2005) may help elucidate on the fate of resident macrophages, and apoptosis virus combined staining of tissues or cells would clarify if macrophages are indeed being killed by the virus or the immune response.

Despite their association with bacterial infections, PRRSV appeared to induce the infiltration of neutrophils into the alveolar spaces. Neutrophils are well known for their pro-inflammatory properties and a number of studies have described their role in inflammation of the lung during virus infection. During SARSCoV infection monocytes produce IL-8 which attract neutrophils to the lungs (Yen et al. 2006), neutrophils can then strongly induce neutrophil extracellular traps, as shown during a study on influenza with neutrophils co-incubated with infected alveolar epithelial cells, leading to tissue damage (Narasaraju et al. 2011). There is another side to neutrophils though, (Fujisawa 2001) used mice with neutrophilic leucocytosis to show that neutrophils can have an inhibitory effect on viral replication. In this study the phagocytosis by macrophages was suppressed and *in vitro* the neutrophils were able to inhibit viral replication. Also neutrophil depleted mice infected with influenza virus displayed enhanced virus replication and severe pulmonary inflammation, indicating that even though neutrophils have such a deleterious inflammatory effect, if numbers fall too low it can have disastrous consequences for disease progression (Tate et al. 2009). The general picture of neutrophils

and their role in pulmonary disease corresponds with the findings that SU1-bel pigs had high concentrations of IL-8 and subsequent infiltration of neutrophils into the alveolar spaces.

The cytokine IL-1 is a potent attractor of lymphocytes to the site of infection; IL-1 levels were highest in the SU1-bel group and positively correlated with gross pathology. This is likely to have induced the mainly lymphocytic infiltration into the alveolar spaces of SU1-bel infected animals. Both double positive and cytotoxic T cells saw an increase in population, although cytotoxic T cells much more so, and both populations positively correlated with gross pathology. Not much information is available about the pro-inflammatory effects of DP T cells, although it is known that they can produce IFN- γ (Meier et al. 2003). Some DP T cells did produce IFN- γ as can be seen in the intracellular staining of cells isolated from the BAL. DP T cells have also been shown to produce IL-10 in response to infection with swine influenza and also demonstrated virus specific proliferation (Platt et al. 2011). It is unlikely that DP T cells produced the IL-10 observed in this study as they showed opposite correlations with pathology, meaning that animals with the highest numbers of DP T cells also had the lowest concentrations of IL-10 in the BALF. Although DP T cells did increase in the BALF of SU1-bel animals, numbers do not seem high enough for the virus to have induced a significant proliferation. The increase in the number of cytotoxic T cells in the BALF of SU1-bel infected animals was much more marked than that of DP T cells. Intracellular staining also showed that there were much larger numbers of these cells producing IFN- γ . Cytotoxic T cell responses are essential in anti-viral immunity as they lyse virus-infected cells preventing viral replication, and the IFN- γ they produce has potent anti-viral effects. Peripheral cytotoxic T cells have been shown to have a specific response to SARSCoV, moreover these cells paralleled the number of IFN- γ secreting cells (Y.-D. Wang & W. F. Chen 2004). Influenza infected mice given an anti- IFN- γ treatment displayed reduced viral pathology (Caruso et al. 1998) further strengthens the argument that IFN- γ production in the SU1-bel pigs may be one of the main players in the orchestration of pulmonary pathology. Cytotoxic T cells have also been found to be important in establishing immunity to influenza virus but also lead to the possible potentiation of lethal pathology (Moskophidis & Kioussis 1998).

The apparent imbalance in the Th1/Th2 response that was observed in this study is difficult to discuss as the theory has its limitations, such as whether two cytokines are enough to determine a true imbalance. However Th1/Th2 imbalance and a skew towards the inflammatory, cell mediated Th1 response has been suggested to be one of the immunopathology mechanisms responsible for the pathology observed during SARSCoV infection (WONG et al. 2004). The data in this study definitely shows a preference of the immune response against SU1-bel to the inflammatory Th1 response compared to the LV group, which had higher levels of IL-4, the main Th2 cytokine. The pro-inflammatory cytokine TNF- α is also a potent exogenous pyrogen. TNF- α production was high in the SU1-bel infected animals, and this links in with the fever that was observed in this group. The up regulation of TNF- α in SARSCoV infected patients is thought to play a hugely important role in both the inflammation and high fevers that are often associated with disease (W. Wang et al. 2007a).

Despite the mixed messages from the research community as to whether PRRSV infection is able to induce Tregs with reports that Tregs can be induced in vitro with Northern European viruses (Silva-Campa et al. 2012; Wongyanin et al. 2010) but not with European strains (Silva-Campa et al. 2010). This study is the first to report the presence of Tregs in the BALF of PRRSV infected pigs, the SU1-bel strain seems to have more in common with the North American genotype rather than the European in regards to Treg induction. Tregs induced by influenza virus were able to suppress CD4 and CD8 T cell proliferation in a murine model of influenza infection (Betts et al. 2012). This observation may explain why those pigs that had the highest numbers of Tregs in the BALF also had the lowest numbers of other lymphocytes. In the case of respiratory syncytial virus Tregs have been shown to modulate immunopathology, Treg depleted mice had an increased frequency of virus-specific CD8 cells co-producing IFN- γ and TNF- α (Fulton et al. 2010). Alongside this induction of Tregs, the SU1-bel group also had higher concentrations of the anti-inflammatory cytokine IL-10. IL-10 is produced primarily by monocytes but also by Tregs. This data suggests that the IL-10 produced in SU1-bel infected animals is more likely sourced from Tregs, as monocyte populations were highest in those animals with the greatest pathology suggesting they may have more of a pro-inflammatory role. Treg depleted mice infected with respiratory syncytial virus did not produce any detectable levels of IL-10 (Loebbermann et al. 2012) again

strengthening the idea that the main source of IL-10 in respiratory virus infection may in fact be Tregs. As the LV group also displayed an increase in Treg population it may be that this cell subset is the mechanism keeping pathology to minimum despite the presence of pro-inflammatory cells and cytokines. However, the LV group did not have an increase in IL-10 as was seen in the SU1-bel infected animals. It could be that these cells are relying on their immunosuppressive mechanism of granzyme B production (Loebbermann et al. 2012) to modulate responses.

Overall it appears clear that infection with the SU1-bel strain of PRRSV has a very different clinical and pathological result compared to subtype one strains. In many respects this virus seems to behave more like a Northern European strain with more pronounced clinical signs and pathology. As viral loads were lower in this group and yet immune responses stronger it is, in all likelihood, the immune response that is to blame for these differences. It is difficult to pin point an individual aspect of the immune response that may be responsible, although at this point IFN- γ production by cytotoxic T cells is looking most likely. In more likelihood it is rather a combination of different inflammatory components of the immune system coupled with a poor regulation by the immunomodulatory branches, which is to blame. However there is a benefit to this type of response, those animals in the SU1-bel group did show the fastest clearance of virus from the serum and those which survived until the end of the study displayed a remarkable recovery with clinical signs disappearing and negligible pathology detected in the tissues. As this is the second of the subtype three PRRSV viruses to be identified as ‘highly pathogenic’ it gives cause for concern over the possible introduction of these strains into Western Europe. Particularly as the results of infection in the field are likely to be more severe due to the exposure to environmental pathogens that were not present in the present controlled environment. This scary thought and the impact that the introduction of these viruses may have upon the European pork industry makes these viruses an important focus for research.

Further work on dissecting the mechanisms of immunopathology during PRRSV infection is required, not only for the welfare of the animals but so those aspects of the immune system which are giving rise to more efficient viral clearance can be exploited for veterinary treatment,

A vaccine which provides as strong protection from virus infection but without the inflammatory side effects would be invaluable to the pork industry, and downstream the consumers whose never quenching thirst for value for money meat may become a more realistic idea. One of the main limitations of this study can be found in animals. Despite starting the experiment with a total of 96 pigs, the staggered post-mortem dates and large variability in data sets made finding statistical differences between data sets difficult, despite clear trends for the SU1-bel pigs to suffer most from clinical disease, pathology and have the greatest immune responses. Any future studies would have to carefully plan the number of groups, and post-mortem dates to get the best quality data possible. In an ideal world pigs would be lavaged alive at daily time points so that the onset of pathology can be properly mapped, however, this would pose particular ethics issues which would have to be considered. This method would allow data to be compared between time points in the same animal which would be particularly helpful based on the variation observed in this study. As the onset of inflammation in the lung is such an expansive subject area the aspects of the immune system to be investigated should be divided up. A number of questions remained unanswered about SU1-bel and complement activation, macrophage apoptosis and there are many more cytokines, chemokines and even adhesion molecules that all play a major role in inflammatory responses. When studying innate immunity it would also be interesting to investigate the role of pulmonary epithelial cells in pathology as they really are the first line of defence in the lung and are even capable of producing cytokines and chemokines. This would have to be done by growing these cells *in vitro* and comparing responses after stimulation with different virus strains. This study clearly showed an important role of adaptive immunity in late stage acute inflammation, of most interest would be to isolate the cells from the lungs and then sort them into individual populations. Luckily with broncho-alveolar lavage yields very high cell numbers, even in very small pigs, so the inefficient nature of most cell sorting techniques does not matter as much as when sorting cells from say the lymph nodes. It would also be interesting to compare not only subtype one and three, but also North American strains in the same study.

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