CHARACTERISATION OF THE T CELL RESPONSE TO THE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND ITS APPLICATION TOWARDS THE DEVELOPMENT OF IMPROVED VACCINES

by

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A Thesis submitted in accordance with the requirements of the degree of Doctor of Philosophy in Microbial and Cellular Sciences

September 2014

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**ABSTRACT**

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important pig diseases worldwide. The causative PRRS virus (PRRSV) is rapidly evolving and there is an urgent need for the development of safer and more efficacious vaccines to improve PRRS control. Immunity to PRRSV is not well understood but there are data suggesting that virus-specific T cell IFN-γ responses play an important role. Therefore, this project focused on characterising the T cell response PRRSV and applied this knowledge to develop a novel vaccine strategy. The first part of the project aimed to identify the antigenic targets of the T cell response to PRRSV by utilising a proteome-wide synthetic peptide library and a cohort of PRRSV immune animals. The T cell IFN-γ response was directed at a range of viral proteins but the M and NSP5 proteins stood out as major antigens. Further experiments confirmed M and NSP5 as well conserved targets of in many instances dominant T cell responses. Characterisation of the responding T cell populations showed NSP5-specific responders to be CD8 T cells with a predominant CD44^{high}CD62L^{low}CD27^{low}CD25^{low} phenotype. The majority of cells were polyfunctional as assessed by co-expression of TNF-α and mobilisation of the cytotoxic degranulation marker CD107a. Both CD8 and CD4 T cells responded to M with a comparable phenotype to that observed for NSP-specific T cells. In addition, conserved antigenic regions of each protein were identified and specificity shown to associate with major histocompatibility complex haplotype, rather than PRRSV strain. Finally, a vaccine study was conducted using M and NSP5 proteins as T cell antigens formulated as a particulate vaccine with a molecular adjuvant. Vaccination primed antigen-specific CD4 but not CD8 T cell responses and did not confer significant protection of animals from viraemia upon challenge infection. Analysis of the lungs during the resolution of infection showed high levels of virus and M/NSP5 specific CD8 T cell IFN-γ responses, suggesting that vaccine priming of a CD8 T cell response is required for protection from PRRSV infection. It is hoped that this work will inform future PRRSV vaccine design, as well as contributing to the wider field of T cell vaccinology.
ACKNOWLEDGEMENTS

I would like to express my gratitude to the many that have helped me throughout the course of the PhD. To those who have given their time and expertise to assist me: Dr. Miriam Pedrera, Dr. Lucia Biffar, Dr. Rebecca Strong and Dr. Jean-Pierre Frossard. A big thank you to Melanie Eck, Dr. Nicolas Ruggli and Prof. Artur Summerfield, who made my time in Switzerland enjoyable and productive. I would also like to thank my supervisor at the University of Surrey, Dr. Graham Stewart for all his assistance. I would like to thank all of the other collaborators who helped with work on this project; namely Dr Sabine Essler, at the University of Veterinary Medicine Vienna, Dr Margarita Garcia-Duran, at Ingenasa, and Dr Sonia Zúñiga Lucas and Prof Luis Enjuanes, at the Centro Nacional de Biotecnología.

I am indebted to Dr. Adam Walters for introducing me to the world of particles, and to Dr. Satyranayra Somaravarapu from the UCL School of Pharmacy, who guided me through it. I am eternally grateful to all those I have worked with over the past four years; Dr. Victor Riitho, Dr. Helen Singleton, Dr. Sophie Morgan, Jenni Evans and Sarah McGowan; thank you for being so much more than just colleagues. Thank you to Bentley Crudgington for tea, biscuits and chauffeur services, amongst other things. A special thank you goes to Dr. Jane Edwards, for late night lab sessions and intellectual input/comedy, without whom I could not have reached this point. I would also like to acknowledge anyone who gave me a lift during the course of my project.

Finally I would like to thank my supervisor Dr. Simon Graham, for supervising and supporting me from the beginning until the very end. His guidance, patience and encouragement throughout the years have shaped both the project and my development, as a scientist and individual.

I would like to dedicate this thesis to my parents, who know why.
STATEMENT OF ORIGINALITY

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Helen Mokhtar

27th September 2014
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<tr>
<td>AAA</td>
<td>poly-adenylated</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
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<tr>
<td>bmDC</td>
<td>Bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell mediated immunity</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic T lymphocyte associated protein</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DCIR</td>
<td>Dendritic cell immunoreceptor</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
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<tr>
<td>DIVA</td>
<td>Differentiation of infected and vaccinated animals</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dpi</td>
<td>days post infection</td>
</tr>
<tr>
<td>E</td>
<td>PRRSV envelope protein</td>
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<tr>
<td>EAV</td>
<td>Equine arteritis virus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>ELISpot</td>
<td>Enzyme-linked immunosorbant spot assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HP-PRRSV</td>
<td>Highly pathogenic porcine reproductive and respiratory syndrome virus</td>
</tr>
<tr>
<td>HS</td>
<td>Heparin sulphate</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ISGF</td>
<td>Interferon stimulated gene factor</td>
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<tr>
<td>JAK-STAT</td>
<td>Janus kinase and signal transducer and activator of transcription</td>
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<tr>
<td>LAMP1</td>
<td>Lysosomal-associated membrane protein 1</td>
</tr>
<tr>
<td>LDV</td>
<td>Lactate dehydrogenase elevating virus</td>
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<tr>
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<td>Lipopolysaccharide</td>
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<td>PRRSV matrix protein</td>
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<td>Green African monkey kidney cell line</td>
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<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLV</td>
<td>Modified live vaccine</td>
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<tr>
<td>MoDC</td>
<td>Monocyte derived dendritic cell</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MVA</td>
<td>Modified Vaccinia Ankara</td>
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<td>PRRSV nucleoprotein</td>
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<td>Neutralising antibody</td>
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<td>NFκB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>NSP</td>
<td>Non-structural protein</td>
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<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PAM</td>
<td>Porcine alveolar macrophage</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PLGA</td>
<td>Poly-(lactic co-glycolic acid)</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptors</td>
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<tr>
<td>PRDC</td>
<td>Porcine respiratory disease complex</td>
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<td>PRRS</td>
<td>Porcine reproductive and respiratory syndrome</td>
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<td>PRRSV</td>
<td>Porcine reproductive and respiratory syndrome virus</td>
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<tr>
<td>PRV</td>
<td>Pseudorabies virus</td>
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<tr>
<td>RdRp</td>
<td>RNA dependant RNA polymerase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
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<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Secreting cells</td>
</tr>
<tr>
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<td>Standard error of the mean</td>
</tr>
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<td>Simian haemorrhagic fever virus</td>
</tr>
<tr>
<td>SLA</td>
<td>Swine leukocyte antigen</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% Tissue culture infective dose</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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Chapter I
General Introduction
Chapter 1. General Introduction

1.1. Porcine Reproductive and Respiratory Syndrome

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of the porcine reproductive and respiratory syndrome (PRRS), also historically called pig blue ear disease or swine infertility and respiratory syndrome. First described as ‘mystery swine disease’ in the late 1980’s and first isolated in 1991 [Wensvoort et al, 1991; Terpstra et al, 1991], PRRSV infection demonstrates a multifaceted clinical presentation, depending on the age of the pig. In sows, infection causes abortion, premature farrowing, stillborn piglets and mummified foetuses, as well as fever, lack of appetite and cyanosis of the mammary gland and ears (hence blue-ear pig disease) [Done et al, 1996; OIE Ad Hoc Group on PRRS, 2008]. Piglets that survive are often born both viraemic and seropositive for PRRSV and have low birth weights and vitality, difficulties breathing and suckling and present with oedema around the eyes [Mengeling et al, 1998; Nielsen et al, 2003; OIE Ad Hoc Group on PRRS, 2008]. In addition to the reproductive aspect of the disease seen in sows and gilts, PRRSV also plays an important role in the Porcine Respiratory Disease Complex (PRDC) [Thacker, 2001; Choi et al, 2003; Hansen et al, 2010]. Infection with PRRSV causes clinical respiratory signs, but most importantly it greatly enhances the pigs’ susceptibility to secondary viral and bacterial infections which define the PRDC. Subsequently the disease has a huge economic impact due to the increased mortality and reduced growth rate in growers and finishing pigs, as well as indirect costs such as treatment and control of outbreaks and disruption to breeding programmes.
The respiratory syndrome coupled with the reproductive problems make PRRSV one of the most economically significant swine pathogens in the world today. A 2005 study estimated that the annual economic impact of PRRSV in the US alone was $66.75 million in breeding pigs, and $493.57 million in growing pig populations, adding up to a loss of around $560 million each year [Neumann et al, 2005]. A more recent study recalculated this loss at over $600 million per annum [Holtkampt et al, 2011]. Estimates of the impact on the UK pig industry indicate that the cost of respiratory disease is £52,180 and reproductive disease is £93,590 per herd of 500 animals [Richardson, 2011]. Considering that an estimated 30% of herds are infected with PRRSV [source: BPEX: http://www.bpex.org.uk/R-and-D/R-and-D/PRRS.aspx] are there are around 5 million pigs on commercial farms in the UK [source: Defra: https://www.gov.uk/government/publications/farming-statistics-livestock-populations-at-1-december-2013-uk] the potential cost of PRRS to UK agriculture is not to be underestimated.

Since the simultaneous emergence of PRRSV in the US and Europe in the late 1980’s and early 1990’s, the virus spread rapidly throughout the continents and outbreaks of the disease were seen in most of the swine-producing areas of the world [Wensvoort et al., 1993]. Recent studies have put the seroprevalence in non-vaccinated herds in the UK to be between 35% [Velasova et al, 2012] and 40% [Evans et al, 2008]. Two distinct genotypes of the virus are generally observed, depending on the origin, and these are designated the European genotype (genotype 1) and the North American genotype (genotype 2). Both genotypes are found circulating globally and have distinctive clinical presentations and genetic and
antigenic characteristics. Within each of these two genotypes there also exists variability in nucleotide sequence, as well as a genetic shift, as demonstrated by the lack of historical field isolates circulating today [Murtagh et al, 2001].

PRRSV is currently endemic in most swine-producing countries since the virus can persist subclinically in pig populations and can still be shed when clinical signs are no longer observed. Acute outbreaks can also occur, usually when a novel serotype is introduced into a population, for example, in the case of the ‘pig high fever disease’ outbreak in China in 2006 [Tian et al, 2007].

1.2. Control of PRRS by biosecurity

To effectively contain PRRSV spread, it is important to understand the methods of transmission. The only other animal aside from pigs that have the potential to be productively infected with PRRSV is the wild boar, however infection of wild boar and other feral pigs is not considered to be significant in the context of PRRSV control due to the low prevalence of PRRSV in wild pig populations [Reiner et al, 2009]. PRRSV may be transmitted in semen and via items/equipment or personnel in contact with different herds [Robertson, 1992]. However, even with strict controls in place to prevent herd to herd spread, reinfection can still occur which suggests the possibility that the virus can be transmitted via aerosols [Dee et al, 2005; Torremorell et al, 1997]. Indeed, quantities of virus sufficient for productive infection have been detected as far as 9.1 km away from the source population [Cutler et al, 2011; Otake et al, 2010].
Although PRRSV has a prolonged persistence in the host, the virus is eventually cleared and this allows ‘herd closure’, a method of control in which a herd is vaccinated and then kept in isolation until the virus is deemed to be cleared from all animals (typically about 200 days). Any further outbreaks in the herd are caused by the introduction of a new strain of the virus. Physical control measures for keeping PRRSV outbreaks in check start with both active and passive surveillance, and any suspected cases are confirmed in the laboratory by immunohistochemistry and RT-PCR [OIE Ad Hoc Group on PRRS, 2008]. Upon confirmation of PRRSV infection, quarantine measures should be imposed and pig movement should not be allowed. Air filtration methods have also been suggested for preventing virus spread by aerosol [Alonso et al, 2013]. In theory these measures plus good biosecurity on farms (perimeter fences, only introducing pigs from PRRSV negative herds, minimal visitors, regular disinfection of equipment and vehicles etc.) should generally be sufficient to contain an outbreak. Zoning is also a useful way of preventing spread of PRRSV from an endemic area to a disease-free zone [Carlsson et al, 2009]. In reality however, these measures are not always enforceable, especially in areas that are made up of small-holdings and village farms rather than the larger commercial facilities.

1.3. Control of PRRS by vaccination

According to www.vetvac.org, an online database of commercially available livestock vaccines, there are currently 25 commercially available PRRSV vaccines; 15 live or live attenuated and 10 killed or inactivated vaccines. These are derived from both North American and European genotype viruses.
Modified live vaccines (MLV) can confer good protection from disease following challenge with a homologous strain of the virus but have shown limited efficacy upon challenge with heterologous strains [Park et al, 2012, Geldhof et al, 2012] and inability to completely control viraemia, even when challenged with a relatively homologous virus [Scortti et al, 2006b]. Poor protection is unsurprisingly also observed between American and European genotypes [Han et al, 2014], and whilst there is more protection conferred within the European genotype [Labarque et al, 2004], variable protection is seen even within the same virus cluster or subtype [Prieto et al, 2008]. Vaccination with MLV can cause long lasting viraemia and viral shedding and therefore the vaccine strain can be transmitted to unvaccinated pigs and across the placenta of pregnant sows leading to congenitally infected litters [Scortti et al, 2006a]. In the context of protection from emerging viruses, only partial protection was observed against a highly pathogenic European subtype 3 virus [Trus et al, 2014]. There are major safety concerns about the potential of the MLV to revert to virulence; vaccine related isolates have been shown to cause disease in experimentally infected pigs [Opriessnig et al, 2002] and vaccine strains have been linked to PRRSV outbreaks [Botner et al, 1999]. Emergence of a novel variant in China has been shown to be the result of recombination between the highly pathogenic PRRSV-2 strains and vaccine strains [Li et al, 2009a; Shi et al., 2013] and in the UK it has been demonstrated that there are circulating strains which have resulted from recombination events [Frossard et al, 2013]. Reversion of vaccine strains to virulence in the field has also been seen both with genotype 1 [Storgaard et al, 1999] and 2 viruses [Allende et al, 2000]. In addition there is a difficulty in distinguishing infected from MLV vaccinated animals (DIVA).
Inactivated/killed PRRSV vaccines are very safe but there is some debate over their efficacy. Inactivated vaccines generally afford little to no protection from heterologous challenge [Scortti et al, 2007, Zuckerman et al, 2007]. Induction of cell mediated immunity (CMI) after vaccination with inactivated vaccine in an oil adjuvant and challenge with a homologous virus strain has been reported [Piras et al, 2005] but little induction of neutralising antibody (NA) was seen by Vanhee et al, [2009] using the virus without adjuvant.

1.4. Molecular biology of PRRSV

PRRSV is a small enveloped virus with a positive sense single stranded RNA genome [Wensvoort et al, 1991; Benfield et al, 1992]. It has been classified as a member of the Arteriviridae family in the order Nidovirales due to its similarity with other members such as equine arteritis virus (EAV), simian haemorrhagic fever virus (SHFV) and lactate dehydrogenase-elevating virus (LDV). The virion itself is composed of a host derived lipid envelope measuring 50-72 nm in diameter encasing an isometric nucleocapsid of 20-30 nm diameter [Benfield et al, 1992].

The linear single stranded RNA genome of PRRSV is 15 kb in length and encodes at least 9 open reading frames (ORFs) [Meulenberg et al, 1995]. ORF 1a and 1b compose 75% of the genome and encode 15 non-structural proteins including the viral RNA dependent RNA polymerase (RdRp), replicase and protease. ORF1a overlaps ORF1b and the region contains a slippery sequence and pseudoknot structure so that ORF1b can be expressed by a ribosomal frame-shift mechanism [Brierley et al, 1989]. These ORFs encode two poly proteins (pp1a and 1b) that are
processed into non-structural proteins (NSPs) by the four protease domains encoded by the ORF1a. Figure 1.1 shows the organisation of the PRRSV genome.

**Figure 1.1 Schematic representation of the PRRSV genome.** The top panel shows the open reading frames (ORFs) of the genome from 5’ to 3’. The positive sense single stranded RNA genome has a 3’ polyadenylated tail (AAA), a 5’ cap and both ends have an untranslated region (UTR). The orange ORFs encode for the non-structural polyproteins which are then cleaved by viral proteases into the 14 non-structural proteins shown in the bottom panel (grey). The blue ORFs encode subgenomic mRNAs for the 7 structural proteins of PRRSV, including the E protein that is encoded by an alternative ORF inside ORF 2a and the most recently identified alternative ORFs; ORF5a and NSP2TF.

NSP1a consists of an N terminal zinc finger domain, a papain-like cysteine protease α domain and a C terminal extension. Besides its activity as an accessory protease, this protein acts as a regulator of subgenomic mRNA synthesis controlling expression of the structural protein genes [Nedailkova *et al.*, 2010]. NSP1a also possesses type I interferon (IFN) antagonist properties [Chen *et al.*, 2010a] acting through various mechanisms including, but probably not limited to, inhibition of the IFN and the JAK-STAT signalling pathways [Yoo *et al.*, 2010, Sun *et al.*, 2012].
affecting the interferon regulatory factor 3 (IRF3) and nuclear factor κB (NFκB) mediated IFN production pathways [Beura et al, 2012, Han et al, 2013] and reducing the activity of NFκB [Song et al, 2010, Subramanian et al, 2010]; and degrading CREB-binding protein (CBP) in a proteasome dependant manner in the nucleus by inhibiting its association with IRF3 (likely via a mediator) [Han et al, 2013, Kim et al, 2010]. NSP1b also exhibits IFN antagonist activity, again affecting the IRF3 and NFκB mediated pathways [Li et al, 2013, Subramanian et al, 2010], and also by degrading karyopherin-α1 (KPNA1) and blocking nuclear translocation of interferon stimulated gene factor 3 (ISGF3) [Patel et al, 2010, Wang et al, 2013b]. NSP1b also acts as an accessory protease via a papain-like cysteine protease β domain. NSP2 is the largest PRRSV protein at around 1000aa in length and is variable in length between strains. This transmembrane protein contains an N terminus cysteine protease domain as well as a deubiquitinating enzyme, and is involved in transmembrane modification [Sun et al, 2010]. NSP2 is also implicated in modulation of the type I IFN response by inhibiting NFκB signalling and deconjugating ISG15 [Sun et al, 2012]. Certain regions of NSP2 can induce strong antibody responses, although this area is hypervariable and subject to various insertions and deletions in different strains [Feng et al, 2008]. In EAV, NSP3 has a putatively essential role in remodelling the intracellular membrane and has been postulated to have the same function in PRRSV [Posthuma et al, 2008]. NSP4 is a 3C-like serine protease, which is the main protease involved in the cleavage of the viral polyprotein [Tian et al, 2009]; it also has an inhibitory effect on IFN-β transcription [Chen et al, 2014]. Little is known about the function of the NSP5 but it has been postulated to possess the same functions as NSP3 as it displays prominent hydrophobic domains [Posthuma et al, 2008, Fang and Snijder 2010]. There is
currently no function attributed to the proteins NSP6, NSP7α and β, NSP8 and NSP12, although the NSP7 has been found to be the target of a strong antibody response [Brown et al, 2009]. The RdRp or NSP9 is the catalytic subunit of the viral replication complex [den Boon et al, 1991, Beerens et al, 2007]. The NSP10 is a viral helicase containing an N-terminal zinc-binding domain with the ability to unzip dsRNA and DNA in a 5’ to 3’ direction [Seybert et al, 2005, Bautista et al, 2002]. NSP11 is an endoribonuclease that is only found in the Nidovirales with unknown cellular targets; it can also inhibit IFN-β and IRF-3 [Shi et al, 2011]. Recently a transframe protein ‘NSP2TF’ has been identified which is encoded by a transframe ORF of ORF1a via programmed ribosomal frameshifting [Fang et al, 2012]. This protein consists of two thirds of the NSP2 protein fused to the C-terminal region which is encoded by the transframe ORF and has been shown to be nonessential for replication but required for efficient growth rates [Fang et al, 2012].

ORFs 2-7 encode seven structural proteins via six nested subgenomic mRNAs (reviewed in Pasternk et al, 2006 and Sawicki et al, 2007), that form the nucleocapsid and the viral envelope (Table 1.2). The envelope is composed of five structural proteins, two major proteins; GP5 and the matrix protein (M), and three minor proteins; GP2, GP3 and GP4. Expression of all structural proteins is required for infectious virion formation [Wissink et al, 2005].
Table 1.2. The structural proteins of PRRSV

<table>
<thead>
<tr>
<th>ORF</th>
<th>Protein</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>2a</td>
<td>GP2</td>
<td>Minor envelope protein, essential for infectivity</td>
</tr>
<tr>
<td>2b</td>
<td>E</td>
<td>Non-glycosylated minor envelope protein, essential for infectivity, functions as an ion channel to facilitate release of the viral genome</td>
</tr>
<tr>
<td>3</td>
<td>GP3</td>
<td>Minor envelope protein, essential for infectivity</td>
</tr>
<tr>
<td>4</td>
<td>GP4</td>
<td>Minor envelope protein, essential for infectivity</td>
</tr>
<tr>
<td>5a</td>
<td>ORF5a</td>
<td>Unknown, but essential for viability</td>
</tr>
<tr>
<td>5</td>
<td>GP5</td>
<td>Major envelope protein, forms a disulphide linked heterodimer with M protein, essential for infectivity and virion formation</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>Non-glycosylated major membrane protein, possibly involved in virus assembly and budding, essential for infectivity and virion formation</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>Nucleocapsid protein, highly immunogenic, essential for infectivity and virion formation</td>
</tr>
</tbody>
</table>

[Dea et al., 2000; Meulenberg et al., 1994; Lee and Yoo, 2006; Wissink et al., 2005, Sun et al., 2013]

PRRSV N protein is abundantly expressed, making up between 20 and 40% of the virion and it interacts with the RNA genome to form the icosahedral virion core. It is also highly immunogenic but antibodies directed against N are non-neutralising [Murtaugh et al, 2002]. The M and GP5 proteins form disulphide linked
heterodimers in the endoplasmic reticulum (ER) of infected cells. Both proteins have short ectodomains exposed on the virion surface and large endodomains which have been hypothesised to interact with the nucleocapsid [Dokland, 2010]. M is the most well conserved structural protein of PRRSV whereas GP5 is poorly conserved between the PRRSV strains [Murtaugh et al, 2005] and has a hypervariable N-terminal region [Meng et al, 1995]. GP2 is a minor envelope protein and may interact with CD163 on the host cell surface [Das et al, 2010]. The hydrophobic E protein is an ion channel protein similar to the M2 protein of influenza A virus [Pinto et al, 1992] which facilitates the release of the viral genome into the endosome [Lee and Yoo, 2006]. GP3 is heavily glycosylated and forms multimeric complexes with GP2 and GP4 [Das et al, 2010; Wissink et al, 2005]. GP4 is another immunogenic protein which contains a variable neutralising epitope [Meulenberg et al, 1997] that is not cross-protective between strains [Vanhee et al, 2010]. A recently identified novel structural protein, designated ORF5a is expressed by an ORF that overlaps the 5’ end of ORF5 [Firth et al, 2011]. This protein is present in low levels in both infected cells and virions [Johnson et al, 2011] and it appears to be essential for virus viability [Sun et al, 2013].

1.5. The replication cycle of PRRSV

PRRSV displays a specific tropism for mononuclear phagocyte cells of the myeloid lineage, including macrophages and dendritic cells (DC), with the primary target cell generally considered to be the porcine alveolar macrophage (PAM) [Duan et al, 1997a, Duan et al, 1997b]. The host cell receptors necessary for PRRSV attachment and entry have been identified as CD169 (sialoadhesin) and CD163
Heparin sulphate (HS) serves as an initial binding receptor, as seen with many other viruses, with the M protein binding HS on the cell surface [Delputte et al, 2002] followed by CD169 mediated internalisation and uncoating of the genome. Viral replication occurs in the cytoplasm of the host cell [Benfield et al, 1992]. Initially, translation of ORF 1a/b occurs giving two large polypeptides that are cleaved by viral encoded proteinases into the viral replicase. These proteins then form a replication complex with the genome and synthesise a negative sense RNA (the antigenome) which is used as the template for genomic copies for the progeny virions. Subgenomic negative sense messenger RNAs are also transcribed in six nested sets which are then translated into the viral structural proteins [Meulenberg et al, 1993]. The membrane proteins localise to the endoplasmic reticulum while the genome is encapsulated by the nucleoproteins in the cytoplasm. The nucleocapsid then buds into the ER or the Golgi apparatus, where the virion acquires its lipid envelope, complete with glycoproteins correctly modified, and the progeny virion is then released from the cell via intracellular vesicles.

1.6. Innate immune responses to PRRSV infection

PRRSV infection elicits a remarkably weak innate immune response which is no doubt a key factor in the prolonged persistence of the virus in infected pigs. A varied induction of cytokines has been reported over the years, sometimes differing by cell type, virus strain or even different clones of the same isolate, as seen with interferon-α (IFN-α) [Lee et al, 2004; Chung et al, 2004; Wang et al, 2013a], interleukin 10 (IL-10) and tumour necrosis factor alpha (TNF-α) [Gimeno et al,
2011]. Interestingly, IFN-α induction upon PRRSV infection appears to be minimal [Van Reeth, et al, 1998; Loving et al, 2007] while the presence of IFN-α at the time of infection has a significant protective effect in vivo [Brockmeier et al, 2009; Albina et al, 1998]. As IFN-α secretion is a crucial factor in downstream immune responses, including NK cell activation, induction of monocyte to DC differentiation and maturation, activation of naive T cells and memory T cell proliferation, the PRRSV-specific down-regulation of the IFN-α response has important repercussions for the immunocompetency of the infected pig.

TNF-α is secreted by a range of leukocyte cell types including activated macrophages and it is a prototype proinflammatory cytokine inducing expression of other cytokines and inflammatory mediators. A number of studies have shown that PRRSV has the ability to impair TNF-α production both in the lung of the infected pig [van Reeth et al, 1999;] and at the cellular level in PAMs in vitro [Chiou et al, 2000; Thanawongnuwech et al, 2001]. Other studies have elaborated on this by showing that induction of TNF-α production upon PRRSV infection could vary depending on other factors such as virus strain [Hou et al, 2012] or pig breed. For example, there was a significantly reduced level of PRRSV replication seen in PAMs derived from Landrace pigs when compared to other breeds which was attributed to higher levels of TNF-α induction [Ait-Ali et al, 2007]. It is also of note that while TNF-α has a negative effect on PRRSV replication [Chang et al, 2005], the addition of TNF-α to cells already treated with IFN-α did not further reduce the ability of the virus to replicate [Lopez-Fuertes et al, 2000] showing that the two cytokines do not work synergistically to down-regulate viral replication.
Nuclear factor κB (NFκB) is a pleiotropic transcription factor important in activating many genes involved in the immune response including regulation of type-1 IFN and other cytokine production [Mogensen and Paludan, 2001]. NFκB activity can be induced by PRRSV infection by a mechanism that degrades IKB which would otherwise block the nuclear localisation signal of NFκB [Lee and Kleiboeker, 2005]. This shows that the NFκB signalling pathway is not affected by PRRSV infection and therefore the modulation of type-1 interferons occurs at the transcriptional or post-transcriptional level.

IL-10 is one of the most debated cytokines in the context of PRRSV immunomodulation. It is secreted by activated Th2 cells, B cells and macrophages and it has many functions. It is known to regulate cell mediated immune responses, including T cell, NK cell and macrophage activity, promote the proliferation and antibody production of B cells and inhibit production of proinflammatory cytokines, so has the ability to rescue cells from apoptosis [reviewed in Moore et al, 1993; Cohen et al, 1997]. It is hypothesised that induction of IL-10 would be an effective and simple mechanism by which PRRSV could control the host pro-inflammatory response, especially in the first two weeks post infection, but the experimental data are not conclusive. PRRSV infection has been reported to induce IL-10 gene expression in PBMC [Suradhat and Thanawongnuwech, 2003], monocytes [Charerntantanakul et al, 2006b] and PAMs [Suradhat et al, 2003] as well as increased IL-10 mRNA levels in the lungs of PRRSV infected pigs [Chung and Chae, 2003]. On the other hand, some studies have reported that there was no significant IL-10 production upon PRRSV infection of PAMs [Thanawongnuwech et al, 2001] or monocyte derived DCs (moDC) [Wang et al, 2007b].
A recent study by Gimeno et al, [2011] investigating the cytokine profiles of different antigen presenting cells infected with 39 different strains of PRRSV-1 yielded interesting results. TNF-α and IL-10 were both detected and not detected in all possible combinations depending on the virus strain and/or cell type, whereas IFN-α was not detected at all, and there was no correlation between the cytokine levels measured and the viral titre.

PRRSV-associated immunomodulation of the innate immune response, especially of IFN-α, may have severe repercussions for the immune status of the infected animal and can pave the way for other viral and non-viral pathogens in the lungs, which would otherwise be controlled by induction of a normal immune function [Renukaradhya et al, 2010; Thanawongnuwech et al, 2000]. It could also cause disruption or failure of vaccines for diseases like pseudorabies virus [De Bruin et al, 2000] and CSFV [Li and Yang, 2003; Suradhat et al, 2006].

PRRSV also has the ability to modulate host cell receptors. Transfection of CD163 into non-PRRSV permissive cell lines allowed them to become infected by both genotype1 and -2 PRRSV [Lee et al, 2010] showing that CD163 expression is essential or sufficient to allow PRRSV infection. It is known that IL-10 can induce the expression of CD163 [Patton et al, 2009] indicating that PRRSV may enhance the availability of susceptible host cells through the induction of IL-10. Some strains of PRRSV have been shown to upregulate CD163 expression [Calvert et al, 2007; Van Gorp et al, 2008] however there was no correlation between the ability of the PRRSV strain to induce IL-10 and the upregulation of CD163 [Gimeno et al, 2011].

PRRSV has been shown to downregulate major histocompatibility molecules class I (MHC-I) and II (MHC-II) expression on dendritic cells (DC) and
subsequently PBMC cultured with PRRSV infected DCs were not efficiently stimulated suggesting that MHC downregulation affects the ability of the DC to present antigen [Park et al, 2008]. In the same study, TNF-α and IL-12 were seen to be induced suggesting that the downregulation of MHC-I/II could be responsible for the impaired response to PRRSV infection, rather than the lack of cytokines able to co-stimulate T cell responses. The costimulatory molecules CD80/86 which work in tandem to prime T cells have been shown to be downregulated in PRRSV infected monocyte derived DCs [Flores-Mendoza et al, 2008], however, contradictorily, expression on bone marrow derived DCs was upregulated [Peng et al, 2009] along with a simultaneous downregulation of MHC-I molecules [Chang et al, 2008].

Natural killer (NK) cells are cells of the innate immune system that can identify and kill infected cells via a complex network of activating or inhibitory signals [reviewed in Lanier, 2005]. Little is known about the interaction of PRRSV and NK cells but it has been shown that PRRSV-infected PAMs were reduced in their susceptibility to NK mediated lysis and NK cells incubated with PRRSV infected PAMs did not increase expression of the cytotoxic degranulation marker CD107a [Cao et al, 2013]. This impairment of NK cell responses could contribute to the failure of the innate immune response to effectively control PRRSV infections.
1.7. Adaptive immune responses to PRRSV infection

1.7.1. Antibody responses to PRRSV infection

The PRRSV-specific antibody (Ab) responses can be observed a week to ten days post infection [Loemba et al, 1996] but the antibodies found at this stage do not neutralise PRRSV infectivity [Yoon et al, 1994] and their development occurs in conjunction with B cell activation, especially in the tonsils [Lamontagne et al, 2001]. It typically takes at least four weeks for a neutralising antibody (NA) response to arise [Meier et al, 2000; Meier et al, 2003]. However, low levels of virus replication can be found in lungs and some lymphoid tissues, even in the presence of NA, suggesting that other immune responses, most likely T cell responses, are involved in the complete clearance of the virus [Labarque et al, 2000].

A study carried out using 8 isolates of varying pathogenicity observed that more virulent strains of PRRSV have higher levels of viral replication and elicit a more intense and rapid antibody response in vivo compared to more attenuated strains. The level of antibody response seems therefore to be linked to the levels of viral replication and potentially abundance of antigen, but it was also speculated that there may be antigenic differences between the strains tested [Johnson et al, 2004].

In addition to inconsistent induction of antibodies, isolates of PRRSV have also shown variable ability to facilitate antibody-dependant enhancement of infection (ADE) [Yoon et al, 1997] and this has been shown to be mediated by the FCγRIIb receptor in simian MARC-145 cells [Qiao et al, 2011]. Infection via this method has subsequently been shown to occur in PAMs and is associated with a down-regulation in the induction of key antiviral cytokines TNF-α and IFN-β [Bao et al, 2013].
1.7.2. T cell responses to PRRSV infection

Upon PRRSV infection, virus specific IFN-γ secreting T cells (SC), which are considered as important effector cells of anti-viral cell mediated immunity (CMI), exhibit a delayed response profile that can take up to four weeks to be detected, along with a measurable lymphoproliferative response [Bautista and Molitor, 1997; Lopez-Fuertez et al, 1999; Meier et al, 2003]. This belated response was observed upon infection with both genotype 1 and genotype 2 strains of PRRSV [Diaz et al, 2005; Meier et al, 2003].

Meier et al, [2003] demonstrated the gradual increase of the PRRSV specific IFN-γ response in parallel to the ‘two-step’ antibody response by inoculating 8 week old piglets with wild type virulent PRRSV. They found that an initial influx of non-neutralising antibodies was followed by the development of virus neutralising antibodies as the non-NA began to wane at around four weeks post-infection. The IFN-γ response showed the exact opposite temporal response; the initial observation of IFN-γ-SC occurring at 2-3 weeks post infection, then increasing gradually over time to reach a peak at the point when the experiment was terminated at 48 weeks post-infection. In addition, the differential induction of IFN-γ was measured in response to either a modified live PRRSV vaccine or a control modified live pseudorabies virus (PRV) vaccine and it was found that IFN-γ induction by the PRRSV vaccine strain was much lower than by the PR vaccine strain. Xiao et al, [2004] also observed a similar delayed and weak development of PRRSV-specific T cell response, with IFN-γ-SC not detected until at least 2 weeks post infection, which is also in keeping with the investigations of others [Yoon et al, 1994; Diaz et al, 2005].
IFN-γ is known to inhibit PRRSV replication in vitro [Bautista and Molitor, 1999; Rowland et al, 2001] and IFN-α is a major component contributing to the induction of T cells to become IFN-γ-SC [Cousens et al, 1999; Kadowaki et al, 2000]. Considering PRRSV has the ability to impair IFN-α production it is plausible that this could be an important factor in the initial weak IFN-γ response. Meier et al, [2003] suggested that the induction of just a few IFN-γ-SC could cause the subsequent induction of naive T cells into IFN-γ-SC via a positive feedback mechanism, leading to the gradual observed increase in IFN-γ-SC. A recent study showed that a highly pathogenic PRRSV-1 subgenotype 3 strain induced a much stronger IFN-γ response than classical strains and this T cell response was associated with enhanced clearance of the virus [Morgan et al, 2013, Weesendorp et al, 2013].

Regarding the phenotype of the cells mounting the T cell response, Bautista and Molitor [1997] found that it was CD4 T cells that were driving the proliferative response, however, Morgan et al, [2013] observed both CD4 and CD8 T cells produce IFN-γ over the course of infection.

Investigation into which PRRSV proteins contribute to the induction of CMI is important in expanding the current knowledge of T cell responses and how they are affected by different virus strains. Jeong et al, [2010] investigated the T cell responses induced by the PRRSV M or N proteins fused to GM-CSF and found that the M protein construct elicited higher levels of T cell proliferation and IFN-γ production than the N protein construct. In earlier studies, Bautista et al, [1998] also showed that the M protein was the strongest inducer of a lymphoproliferative response. This study also identified GP5 and N as inducers of proliferation and
another study identified GP3 as able to induce T cell proliferation in mice [Jiang et al, 2007].

Identification of T cell epitopes has been approached in several different ways to date. A study by Vashisht et al, [2008] synthesised the entire GP5 protein as a series of overlapping peptides and tested their ability to induce IFN-γ production in cells from pigs infected with North American strains of PRRSV. Two antigenic regions were identified using this method; GP5_{117-131} and GP5_{149-163}. Wang et al, [2010] also employed synthesis of overlapping peptides, this time of the M protein, to identify immunodominant T cell epitopes. They identified 4 peptides on the M protein that elicited a significant IFN-γ response and confirmed this by real-time PCR. Diaz et al, [2009] employed an in silico method to identify potential T cell epitopes on GP4, GP5 and the nucleocapsid protein (N) using the bioinformatic tools SYPEITHY matrix [http://www.syfpeithi.de] and the IEBD resource [http://www.iedb.org/]. Peptides identified in this way were then synthesised and their ability to stimulate IFN-γ production in cells from PRRSV vaccinated pigs was tested. Four epitopes were identified on the N protein, two on GP4 and one large immunodominant region on GP5. It was also concluded that N elicited the greatest T cell response followed by GP5 and then GP4. It is worthy of note that the epitopes identified here on GP5 corresponded to one of the epitopes identified by Vashisht et al, [2008].

Figure 1.2 shows a representation of the kinetics of antibody and T cell responses when pigs are infected with PRRSV [adapted from Lopez & Osario, 2004]. Viral load in tissues can be detected up to 5 months post infection whereas viraemia only lasts around one month. The IFN-γ SC response and the neutralising
antibody response are both delayed however the IFN-γ response gradually increases over time in contrast to the NA response which increases rapidly but then begins to decrease soon after.

Figure 1.2. Representation of the kinetics of the adaptive immune response in pigs following PRRSV infection. [adapted from Lopez & Osario, 2004].

1.7.3. γδ T cell responses to PRRSV infection

Like ruminants, it is known that pigs possess a large number of γδ T cells compared to humans and rodents and that these cells possess the characteristic of non-MHC restricted cytotoxic T lymphocytes [Tanaka et al, 1994] allowing their involvement in both innate and adaptive immune responses [Yang et al, 2000; Borghetti et al, 2006; Takamatsu et al, 2006]. In response to PRRSV infection, it has been shown by Olin et al, [2005] that the percentage of peripheral γδ T cells
increased and that they were able to produce IFN-γ until 10 weeks post infection. Upon restimulation with PRRSV, both CD4+ and γδ-TcR+ T lymphocytes from infected pigs produced IFN-γ, suggesting that γδ T cells are capable of demonstrating memory responses; however this data is not conclusive. Interestingly, Xiao et al, [2004] found that the levels of γδ T cells present in the lungs and various lymph tissues decreased upon PRRSV infection.

1.7.4. Regulatory T cell responses to PRRSV infection

Regulatory T cells (Tregs) have been implicated in the ability of PRRSV to persist and evade the host immune system. They are conventionally defined as CD4+FoxP3+CD25+, and in pigs can be described as CD8α−MHC-II− or CD8α+MHC-II−; corresponding to ‘natural’ or ‘induced’ Tregs, respectively [Kaser et al, 2008]. Yet again we come across contradictory evidence for the induction of Treg responses to PRRSV infection, but these differences may be genotype dependent. A 2009 study by Silva-Campa et al, showed that FoxP3+CD25+ Tregs could be induced by North American genotype PRRSV infected DCs. Another study in 2010 by Wongyanin et al, claimed that even the presence of PRRSV could increase the number of FoxP3+CD25+ Tregs in PBMC both in vitro and in vivo, and that when monocyte-derived DCs (MoDCs) were included in the culture, there was a significant additional expansion of FoxP3+CD25+ Tregs. This study also used North American genotype isolates. On the contrary, a study in 2010 by Silva-Campa et al, this time using several European genotype isolates showed that while PRRSV was able to infect and replicate in MoDCs, these cells were incapable of inducing Tregs.
1.8. Next generation vaccine development for PRRSV

In addition to vaccines derived from whole virus, there are a variety of other experimental approaches that have been and are currently being developed as next-generation PRRSV vaccines. Different molecular adjuvants including cytokines (IL-2, IL-4, IL-12, IFN-α and IFN-γ), pathogen-associated molecular patterns (PAMPs) such as the double-stranded RNA analogue poly(I:C), CpG oligodeoxynucleotides (CpG ODN), lipopolysaccharide (LPS) and other bacterial products, such as cholera toxin, have been investigated for their ability to enhance the efficacy of experimental PRRSV vaccines. Plasmid DNA vaccines have to date mainly focussed on ORFs 5 (GP5) and 7 (N protein), with GP5 expressing plasmid conferring limited protection from infection in pigs [Pirzadeh et al, 1998]. A neutralising antibody response was induced by NSP2, GP4 and GP5 in pigs vaccinated with ORF1, 4 or 5 [Barfoed et al, 2004] while the co-expression of GP5 and M increased the magnitude of T cell and Ab responses, when compared with GP5 or M alone [Jiang et al, 2006b]. Plasmids expressing cytokines such as IL-2, IL-4 and IFN-γ in combination with ORFs 5 and 7 have also been studied. IL-2 and IFN-γ were found to have a positive effect on virus specific T cell responses and reduction of viraemia in pigs, whereas IL-4 suppressed these responses [Romparto et al, 2006; Xue et al, 2004]. GP5 in combination with IL-18 enhanced T cell responses, but was not comparable to a live vaccine [Zhang et al, 2013]. Cytotoxic T lymphocyte-associated protein 4 (CTLA4) with GP5 could enhance both Ab and T cell responses in mice [Wang et al, 2013c] and a plasmid co-expressing swine ubiquitin and GP5 could improve the T cell but not the Ab response [Hou et al, 2008]. Various adenoviral vectored vaccines have been tested in pigs, including granulocyte macrophage-colony stimulating factor (GM-CSF)-GP5/3 [Wang et al., 2009], heat-shock protein 70 (HSP70)-GP5/3 [Li et
CD40 Ligand (CD40L)-GP5/3 [Cao et al, 2010], all of which induced T cell and Ab responses in pigs. Adenoviral vectored constructs such as GP5/M and GP5/3 or GP3/4/5 constructs have also been tested in mice, where they induced both T cell responses and antibody responses [Jiang et al, 2008, Jiang et al, 2006a]. In addition, an adenoviral vectored GP5/M construct induced T cell and NA in mice [Cai et al, 2010]. A Modified Vaccinia Ankara (MVA) vectored GP5/M construct induced T cell and Ab responses in mice [Zheng et al, 2007]. GP5 vectored in a pseudorabies virus (PRV) conferred protection from clinical signs in piglets without induction of anti-PRRSV Ab, suggesting a role for T cell responses [Qiu et al, 2005]. Baculovirus expressed GP2 and GP4 elicited high NA titres in mice [Karauppannan et al, 2013] whereas GP5 (with PCV2 capsid protein as a bivalent vaccine) and GP5/M constructs induced NA as well as T cell IFN-γ responses but had poor expression levels [Wang et al, 2007a; Xu et al, 2012]. Other subunit vaccines such as recombinant GP5 expressed in E. coli or in transgenic tobacco plants failed to provide protection and also exacerbated disease upon challenge (E. coli-expressed; Prieto et al, 2011) but could induce a gradual increase in Ab responses and a NA response after 4 doses of vaccine (tobacco plant-expressed; Chia et al, 2010). A recent study using the Nisin controlled expression system of Lactococcus lactis was employed to successfully induce produce M protein-specific mucosal T cell and Ab responses in mice [Wang et al, 2014]. Whilst this result and those from other studies are encouraging many have failed to translate successfully to the porcine system. In addition, the viral vectors used in a number of these studies pose their own issues such as the risk of recombination with wild type viruses and potential pre-existing host immunity rendering the vaccine inactive [Reviewed in Ura et al, 2014].
1.9. Vaccination approaches targeting T cell immunity

An effective anti-viral vaccine should induce a strong T cell response made up of both CD4+ helper T cells and CD8+ cytotoxic T cells. The induction of T cell responses depends on the presentation of antigen and associated co-stimulatory signals provided by an antigen presenting cell (APC). Therefore the focus of such a vaccine should be to target and activate the APC to induce the desired T cell response. Dendritic cells (DC) are ‘professional’ APC and are the only APCs to effectively activate naive T cells. While there are two major lineages of DC, myeloid DC (mDC) and plasmacytoid DC (pDC), it is mDC that are the true APCs, whereas the pDC function primarily through the action of the cytokines they secrete. Mature mDC secrete a chemokine called CCL22, which is a chemoattractant that recruits naive T cells. When a foreign agent is detected, the DC coordinate the host response, beginning with the immediate immune response by recruiting effector cells of the innate immune response. Memory T cells are then recruited, followed by naive T cells and B cells that are primed by the presentation of antigen [Piqueras et al, 2006]. At this stage, it is thought that Treg cells are attracted to keep the immune response ‘in check’. When a DC encounters an exogenous antigen, the antigen gets phagocytosed or endocytosed, processed by proteases and transported to the endosome, where fragments are loaded into the binding sites of MHC-II molecules. The MHC-II/antigen complexes are then transported to the cell surface where the antigen is presented to CD4+ T helper cells. On the other hand, when a DC encounters an endogenous antigen, such as a newly synthesised viral protein, the DC ubiquitinates the antigen before processing by the proteasome (which only recognises ubiquitinated proteins). The antigen is then processed into small peptides between 8 and 15aa long and transported via a transporter protein (transporter for
antigen processing; TAP) to the ER. Here the short peptides are degraded until they fit into the MHC-I binding groove and are then carried to the surface for presentation to CD8\(^+\) cytotoxic T cells. [Reviewed in Savina and Amigorena, 2007]. However, it is possible for exogenous antigen to be processed and presented via the MHC-I pathway, through a mechanism called cross-presentation. The finer details of this pathway are the subject of much study but two pathways are generally referred to; the ‘cytosolic’ and the ‘vacuolar’. Following the cytosolic pathway, internalised proteins access the cytosol and subsequently the proteasome, which then leads to MHC-I loading via TAP. In the vacuolar pathway, degradation and MHC-I loading occur in the phagosome. [Reviewed in Joffre et al, 2012]. Cross-presentation is important in the context of viral vaccines as stimulation of both CD4 and CD8 T cells is critical to achieve protection. In the porcine system the DC subsets and receptors expressed are not currently well defined and this could be a potential barrier to vaccine design.

Immunity can be conferred and can persist by vaccination with peptide epitopes fused to GM-CSF to target DCs. T cell immunity specific to the vaccine antigen was induced as evidenced by epitope spreading [Disis et al, 2002]. Delivery of genetic sequences encoding for single T cell epitopes (either p53 or HIVgp120) by particle bombardment mediated DNA transfer can also induce T cell immunity [Ciernick et al, 1996]. Approaches such as these have been employed for investigating the induction of a T cell immune response in a number of different infectious and non-infectious diseases, including HIV [Nchinda et al, 2009; Garcia et al, 2010] and other viral diseases such as hepatitis B [Chen et al, 2009], intracellular bacterial infections like tuberculosis [McShane et al, 2002] and cancers [Gilboa and
Vieweg, 2004; Mayordomo et al, 1995 and reviewed in Melief, 2008]. The potential of DC to coordinate such a complex immune response makes them ideal targets for vaccine development. Their ability to take up exogenous foreign proteins and present them via both of the MHC pathways, thus priming both CD4+ and CD8+ T cells can be used to illicit strong, long-term protection [Jung et al, 2002; Thery and Amigorena, 2001]. Currently, DC vaccines consist of DCs loaded with antigen *ex vivo* and then re-administered to the patient, with promising potential [Gatza, and Okada, 2002]; however, this approach is less than ideal for many reasons and methods must be developed to target DC *in vivo*. The maturation state of the DC *ex vivo* is a debated topic, as it has been suggested that fully matured DC when re-injected cannot return to the lymph node however reinjection of immature DC results in tolerance to the cells and inhibition of immune response [Shimizu et al, 2001]. Investigation into targeting antigens using ligands for specific surface receptors on DCs *in vivo* show encouraging results. Specific surface receptors such as DC-SIGN have been targeted, for example, inducing specific responses to HIV [Dai et al, 2009] and CD11c has also been targeted showing promise in preventing tumours in mice [Wei et al, 2009]. It has also been shown that targeting to the DCIR (a tyrosine based inhibitory motif containing DC immunoreceptor) initiates antigen specific CD8+ T cell immune responses [Klechevsky et al, 2010]. Trumpfheller et al, [2005] used a DEC-205 targeted approach to deliver HIV gag protein to DCs. In mice this approach induced high levels of IFN-γ and IL-2 producing CD4+ T cells. It also induced higher levels of CD4+ T cells than vaccination with plasmid DNA or recombinant adenovirus vectors.
1.10. Nanoparticulate vaccine formulations

Nanoparticles (NPs) are simply particles of a ‘nano’ size i.e. 1000nm or less. These can be formulated from numerous materials and have a diverse range of applications, such as in solar cells, silver NPs that kill bacteria in fabrics and iron NPs used to clean-up ground water. NPs are particularly interesting to the medical world due to their potential for specific design. This allows for various particle compositions and sizes, depending on the intended use. Because the NPs have a larger surface area to weight ratio than normal material, they have different electronic, optical and chemical reactivity properties when formed into NPs, making them highly reactive [reviewed in Discher and Eisenberg, 2002].

Molecules such as proteins or drugs can be encapsulated inside a biodegradable particle which allows for sustained release after administration [Song et al, 1997; Corrigan and Li, 2009]. In addition the particle may be magnetic or labelled or have antigens conjugated to their surface [Nobs et al, 2004; Aline et al, 2009; Park et al, 2010]. The particle itself can also trigger an immunogenic response which is useful in activating DC. Specific conjugates on the surface of the particle can further enhance the DC response, leading to T cell induction. DNA loaded NPs have also been used to deliver nucleic acid to various target sites, increasing the bioavailability and decreasing the degradation of the DNA [Bivas-Benita et al, 2004; Giger et al, 2010]. DC pulsed with nanoparticles containing hepatitis B surface antigen have been used as a vaccine and showed induction of antibodies in mice [Bharali et al, 2008].

The internalisation of the particle is a distinct advantage and if taken up by DC it allows the cargo of antigen to be potentially processed via the MHC-I or the
MHC-II pathway and present antigens leading to CD4$^+$ and CD8$^+$ T cell immune responses. Nanoparticles have great potential as vaccines due to their size and their capacity for conjugation to antigens, molecular adjuvants and to ligands for DC specific surface receptors. This allows the delivery of antigen and activation/maturation of the DC which induces both antibody and T cell responses; as well as the potential for sustained delivery. Indeed, nanoparticulate formulations have previously been used to successfully deliver antigen for cross-presentation [Hirosue et al., 2010] and sustained release of antigen has been achieved [Shen et al, 2006]. Nanoparticulate vaccines have also been employed in the context of PRRSV, with killed PRRSV encapsulated inside poly(lactic, glycolic acid) (PLGA) particles and administered intranasally to pigs resulting in enhanced Ab and T cell responses that are potentially cross-protective between strains [Dwivedi et al, 2013; Binjawadaqi et al, 2014].
1.11. Aims and Objectives

It is clear from previous work that the immunobiology of PRRSV infection is complex and there is a need for a better understanding to facilitate the development of new safe and efficacious PRRSV vaccines. There is evidence to suggest that T cell responses play an important role in protection against PRRSV [Zuckermann et al, 2007] and therefore this project focussed on improving our understanding of the PRRSV-specific T cell response. The hypothesis that PRRSV induces T cell responses that are crucial for protection from infection was explored through investigation into the kinetics and phenotype of the T cell response following PRRSV infection as well as the identification of proteins and peptides that are targets of the T cell response. Finally, the major PRRSV T cell antigens identified were evaluated for their vaccine potential using a novel particulate vaccine formulation.

The three specific objectives below are addressed in the following experimental results chapters:

1. To characterise the specificity of the T cell response to PRRSV utilising a proteome-wide synthetic peptide library (Chapter 3).
2. To further characterise the T cell response to PRRSV, including the specificity, kinetics and phenotype, and identify vaccine candidate antigens (Chapter 4).
3. To evaluate the vaccine potential of identified PRRSV T cell antigens using a rationally formulated particulate delivery system (Chapter 5).
Chapter II
Materials and Methods
Chapter 2. Materials and Methods

2.1. Ethics Statement

All work was approved by the AHVLA Ethics Committee and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under Project Licence numbers PPL 70/7057 and 70/7209. The experiments on pigs at the Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland, were performed in compliance with the Swiss Animal Protection Law and approved by the Animal Welfare Committee of the canton of Berne, Switzerland (authorization number BE89/11).

2.2. Virus Stocks

The PRRSV-1 subgenotype 1 MARC-145 cell adapted strain; Olot/91 [Plana et al, 1992, Plana-Durán, et al, 1997] was kindly provided by Dr Sonia Zúñiga Lucas and Prof Luis Enjuanes, Centro Nacional de Biotecnologia, Madrid, Spain, and propagated in MARC-145 cells. The PRRSV-1 subgenotype 3 virulent strain; SU1-Bel [Morgan et al, 2013] was isolated from material kindly provided by Dr Tomasz Stadejek, Warsaw University of Life Sciences, Poland; the prototype PRRSV-1 subgenotype 1 Lelystad strain (LV; Wensvoort et al, 1991) was kindly provided by Drs Eefke Weesendorp and Annemarie Rebel, Central Veterinary Institute, Lelystad, The Netherlands; and a PRRSV-1 subgenotype 1 UK isolate; 215-06 [Morgan et al, 2013], kindly provided by Dr Jean-Pierre Frossard, AHVLA Virology Department, were all propagated in porcine alveolar macrophages (PAMs; AHVLA Cell and Tissue Culture Unit). All four viruses were propagated in vitro by inoculation of T75
flasks containing sub-confluent MARC-145 cell or PAM monolayers. MARC-145
cells were maintained in Dulbecco’s Minimum Essential Medium (D-MEM)
(Invitrogen, Paisley, UK) supplemented with 100 IU/ml penicillin, 100 μg/ml
streptomycin (Invitrogen) and 10% FBS (Autogen Bioclear, Calne, UK) (cDMEM).
PAMs were maintained in RPMI-1640 medium (Invitrogen) supplemented as above
(cRPMI). Seventy two hours post-inoculation at 37°C in a 5% CO₂ humidified
atmosphere, the supernatant was collected, freeze/thawed twice at -80°C and
clarified by centrifugation at 524 x g for 10 minutes, aliquoted and stored at -80°C.
Mock virus supernatants were prepared in the same way but without inoculation of
virus. Virus titres were obtained by log₁₀ serial dilution of the virus suspension on
MARC-145 cell or PAM monolayers followed by immunoperoxidase (IPX) staining
to identify infection rates as described by Van der Linder et al, [2003]. Briefly, log₁₀
serial dilutions of virus were added to 95% confluent cells in octuplicate wells of a
96 well flat bottomed tissue-culture plate (Nunc, Fisher Scientific, Loughborough,
UK) and incubated for 72 hours. Cell monolayers were washed with 0.15 M NaCl,
air dried and then frozen at -80°C. A 1:100 dilution of the pan-PRRSV specific
monoclonal antibody SDOW17-A (Rural Technologies, Brookings, USA) diluted
1/100 in PBS supplemented with 0.01% Tween-80 (Sigma-Aldrich, Poole, UK) and
4% normal goat serum (Life Technologies) (IPX serum conjugate buffer) was added
to cells which were then incubated for 1 hour at 37°C. Cell monolayers were washed
3 times in PBS with 0.5% Tween-80 (IPX wash buffer) and polyclonal rabbit anti-
mouse horseradish peroxidase (HRP) conjugated secondary antibody was diluted
1:150 in IPX serum conjugate buffer and added to cells. After incubation for 1 hour
at 37°C, cells were again washed 3 times in IPX wash buffer and freshly prepared
carbazole substrate buffer (containing 0.06% 3 amino-9-ethylcarbazole, 0.01%
hydrogen peroxide, 0.08% glacial acetic acid in 0.04 M anhydrous sodium acetate; all Sigma-Aldrich) was added to the cells. Cells were incubated for 15 min at 37°C then examined under a light microscope and PRRSV-infected cells were observed by the presence of reddish brown cytosolic staining. Virus titres were calculated as log_{10}TCID_{50}/ml using the Spearman-Karber method [Drew, 2008].

2.3. PRRSV-1 peptide library

A synthetic overlapping peptide library of 1275 pentadecamer peptides offset by four amino acids was synthesised (JPT Peptide Technologies, Berlin, Germany) using the predicted amino acid sequences of the structural proteins of PRRSV-1 Olot/91 strain (GenBank Accession No.X92942.1) and the non-structural proteins of the closely related Lelystad strain (GenBank Accession No. AY588319.1) since the non-structural protein encoding open-reading frame sequences were not available for Olot/91 at this time. Lyophilised peptides were reconstituted in 10 mM N-2-Hydroxyethylpiperazine-N’-2-Ethanesulfonic Acid (HEPES) (Life Technologies) buffered 40% (v/v) acetonitrile at a concentration of 1.65 mg/ml. Peptides were combined into pools representing 19 proteins of PRRSV-1:

NSP1 – 96 peptides; NSP 2 – 269 peptides; NSP3 – 58 peptides; NSP4 – 51 peptides; NSP5 – 42 peptides; NSP6 – 4 peptides; NSP7 – 67 peptides; NSP8 – 12 peptides; RdRp – 173 peptides; helicase – 110 peptides; NSP11 – 56 peptides; NSP12– 37 peptides; GP2 – 60 peptides; E – 15 peptides; GP3 – 64 peptides; GP4 – 43 peptides; GP5 – 48 peptides; M – 41 peptides; and N – 30 peptides.
Analysis conducted during Animal Experiment 1 to identify antigenic peptides from within these pools involved testing individual peptides for GP5 and M, whereas peptides for NSP1 NSP2 and RdRp were tested in pools of 10 due to the large number of peptides. Once positive pools of 10 NSP1, NSP2 and RdRp peptides were identified, the constituent peptides were tested individually to identify the antigenic peptides. In Animal Experiment 3, antigenic M and NSP5 peptides were identified by screening peptides using a two-way matrix system as previously described [Franzoni et al, 2014].

2.4. Generation of experimental PRRSV vaccine formulations

2.4.1. Preparation of vaccine antigens

PRRSV-1 Olot/91 was grown up in bulk on MARC-145 cells in serum free RPMI-1640 then purified and concentrated using a 300 kDa MicroKros Filter Module (Spectrum Labs, Breda, The Netherlands). Virus was then inactivated by incubation with a 1:1000 dilution of β-propiolactone (Sigma-Aldrich) at 37°C for 2 hours with continuous shaking. The infective titre of the virus preparation before and after inactivation was determined by microtitration and IPX as described above.

Overlapping 20mer peptides, offset by 10 amino acids, encompassing the entire M and NSP5 proteins from PRRSV-1 Olot/91 strain were designed (Appendix B), synthesised (Mimotopes, Heswall, UK) and reconstituted in sterile DMSO at a concentration of 25 mg/ml.

A recombinant fusion protein EM4 that consisted of the portion of the PRRSV-1 GP5 ectodomain (MSSTYQYIYNLTICELNGTDWLSNHA) fused
directly to the C-terminal region of the PRRSV-1 Olot/91 M protein (MDAHVKSAAGLSIPASGNRAYAVRKPGLTSVNGTLVPGLRSL) was kindly provided by Dr Margarita Garcia-Duran, Ingenasa, Madrid, Spain. The protein was designed to express a potential neutralizing antibody epitope on GP5 [Ostrowski et al, 2002] and an immunogenic M protein antibody epitope, however, subsequent analysis showed that this fusion protein induced only non-neutralising anti-PRRSV antibodies when used to immunise pigs (Dr Margarita Garcia-Duran, personal communication). In brief, the EM4 protein was constructed by PCR amplification of the correspondent sequences from PRRSV-1 Olot/91 strain. The PCR product was cloned in the baculovirus vector pAcHLTA which has a His-tag for purification. Recombinant baculovirus were selected by plaque assay and grown to generate the appropriate virus stock. To produce the protein, Sf9 cells were infected at an MOI=2. Infected cells were collected by centrifugation, the cellular pellet was lysed and EM4 purified by ion metal affinity chromatography (IMAC) with Ni$^{2+}$ charged resin. The bound EM4 was eluted with imidazole and detected by Western blotting with anti-His antibody.

2.4.2. Formulation of vaccine antigens in chitosan particles

Particulate vaccine formulations were prepared with the help and supervision of Dr Satyranayrana Somaravarapu at the UCL School of Pharmacy, London. Three particulate formulations were prepared: (1) virus loaded particles (Virus-P), (2) peptide loaded particles (Peptide-P) and (3) control empty particles (Empty-P). For each vaccine dose of particles, 50mg of octanyl chitosan (Heppe Medical Chitosan GmbH, Halle Germany) was added to 715µl chloroform (Sigma-Aldrich) and
sonicated in a water bath until dissolved. The following components were then added to the chitosan in chloroform solution: For each vaccine dose of Virus-P: 50µg Adilipoline™ (Source Bioscience, Nottingham, UK), 200µl containing 10⁶ TCID₅₀ equivalent of β-propiolactone inactivated PRRSV-1 Olot/91 and 500µl 15% PVA. For each dose of Peptide-P: 50µg Adilipoline, 132µl of M and NSP5 peptide pool containing 100µg of each individual peptide, 500µl 15% PVA and 226µl sterile deionised water. For each dose of Empty-P: 600µl 15% PVA and 1.4ml sterile deionised water. The resultant mixtures were homogenised for 1 minute followed by probe sonication for 2 minutes (amplitude 17 microns). The mixtures were then added drop-wise to 20ml 1% PVA whilst homogenising for 4 minutes. The formulations were then set on magnetic stirring blocks in a fume hood and solvent was allowed to evaporate for 6 hours at room temperature. Prior to vaccination, the following was adsorbed onto the surface of the particles by adding drop wise whilst on a magnetic stirring block: To the Virus-P formulation - 50µg Adilipoline in 200µl sterile water/dose. To the Peptide-P formulation: 50µg Adilipoline in 200µl sterile water/dose and then subsequently, 200µg EM4 protein in 150µl sterile water/dose. All vaccine formulations were then made up to 4ml per dose with sterile water. Aliquots of each formulation were removed for quality control assessments.

2.4.3. Quality control of particulate vaccine formulations

2.4.3.1. Assessment of the physical properties of the particulate vaccine formulations

The surface charge of the particles was measured using a Zeta Sizer 3000 (Malvern Instruments, Malvern, UK). Particles were dispersed in distilled water and
analysed in a disposable zeta cell (Malvern Instruments) at 25°C. 20 readings of each sample were made in triplicate and each sample was measured in duplicate.

Particle morphology and size was assessed using scanning electron microscopy which was kindly performed by Mr Bill Cooley, AHVLA Pathology Department. In brief, particles were air dried onto poly-L-lysine coated coverslips (BD Biosciences) for 15 min at 37°C. The cover slip was then applied to double sided carbon (Agar Scientific, Stansted, UK) attached to a 13 mm aluminium stub (Agar Scientific) and sputter coated with gold to a thickness of 15 nm using an Emitech K550X Sputter Coater (Quorum Technologies Ltd, Ashford, UK). Particles were visualized using a Zeiss Evo LS10 scanning electron microscope (Zeiss, Welwyn Garden City, UK) at an accelerating voltage of 10-20 Kv.

2.4.3.2. Assessment of the antigen loading efficiency of particulate vaccine formulations

Aliquots of each formulation were centrifuged at 14000 rpm for 5 min and supernatants were harvested and stored at -80°C until analysed. ELISA and biochemical assay analyses of particle-free supernatants were conducted to determine the efficiency of virus or peptide encapsulation and coating with EM4 protein. Absorption of EM4 to particle surfaces was additionally assessed by flow cytometric staining and analyses of particles.
2.4.3.3. Assessment of EM4 in particle supernatants by ELISA

ELISA plates (MAXISorb, Nunc, Fisher Scientific) were coated (100 µl/well) overnight at 4°C with titrations of particle-free supernatants from Peptide- and Empty-P formulations in carbonate-bicarbonate buffer. A titration of recombinant EM4 protein (a kind gift from Dr Margarita Garcia-Duran, Ingenasa, Madrid, Spain) of known concentration was included as a standard. Plates were washed three times with 200 µl wash buffer (PBS, 0.05% Tween) and blocked by addition of 200 µl reagent diluent (PBS, 0.05% Tween, 5% BSA) for 1 hour at room temperature. 100 µl anti-EM4 mAb (EM11E10; a kind gift from Dr Margarita Garcia Duran, Ingenasa, Madrid Spain) diluted to 1 µg/ml in reagent diluent was then added at 100 µl/well and incubated for 1h at room temperature. After washing as before, 100 µl of rabbit-anti-mouse IgG-HRP antibody (Dako, Ely, UK), diluted 1 in 2000 in reagent diluent, was incubated for 1h at room temperature. Subsequently, plates were washed three times, followed by the addition of 100 µl of TMB substrate (Sigma-Aldrich). The substrate was incubated for a maximum of ten minutes in the dark before the reaction was stopped with 100 µl of 0.5 M H₂SO₄. The absorbance at 450 nm was finally read on a Victor⁴ microplate reader (Perkin Elmer, Seer Green, UK).

2.4.3.4. Assessment of PRRSV-1 in particle supernatants by ELISA

The ELISA was performed as described for the assessment of EM4 with the following modifications. Plates were coated with particle-free supernatants from Virus- and Empty-P formulations. For the standard, inactivated PRRSV Olot/91 at an equivalent titre of 10⁶.4⁵ TCID₅₀/ml was titrated in carbonate-bicarbonate buffer. Anti-PRRSV mAb clone 1AC7 (a kind gift from Dr Margarita Garcia Duran,
Ingenasa, Madrid Spain) was used as a primary antibody at a concentration of 10 µg/ml. The secondary rabbit-anti-mouse IgG-HRP antibody was diluted 1 in 2000 in reagent diluent.

2.4.3.5. Assessment of M and NSP5 peptides in particle supernatants by OPA assay

The biochemical fluorometric OPA assay was performed according to manufacturer’s instructions (Fluoraldehyde (o-phtalaldehyde, OPA) Reagent Solution, Thermo Scientific, UK) with minor changes described as follows. Particle-free supernatants from Peptide- and Empty-P formulations were serially diluted two-fold in water. A two fold serial dilution of the M and NSP5 peptide pool with a starting concentration of 500 µg/ml was used as the standard. 20 µl of samples and standards were mixed with 200 µl of Fluoraldehyde Reagent Solution in black 96 well microplates (OptiPlate; PerkinElmer) and the fluorescence was read immediately on a Victor4 microplate reader (Perkin Elmer) using excitation and emission wavelengths of 340 and 455 nm, respectively.

2.4.3.6. Assessment of EM4 binding to particles by flow cytometry

Particles were washed twice in PBS and then stained using an anti-EM4 mAb (E10C7) at 1 µg/ml or equivalent concentration of isotype control purified IgG2b antibody (AbD Serotec) by incubation for 30 min at 4°C. After two washes in particles were stained with anti-mouse IgG2-PE conjugated secondary antibody (BD Biosciences, Oxford, UK) and incubated as before. After a final two washes,
particles were analysed on a MACSQuant Analyzer flow cytometer (Miltenyi Biotec). Particles were gated on by assessing forward and side scatter measurements with logarithmic scaling. EM4 staining was assessed by setting a gate based on isotype control mAb background staining.

2.5. Experimental infection of pigs with PRRSV

The first study (Animal Experiment 1) was carried out at the Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland. Three specific pathogen free Large White pigs were rendered immune to PRRSV-1 Olot/91 by repeated experimental infection. The pigs were inoculated intranasally with $10^5$ TCID$_{50}$ (pigs 52 and 53) or $10^6$ TCID$_{50}$ (pig 54) of MARC-145 cell adapted Olot/91 PRRSV at 9 weeks, 18 weeks and 14 months of age. The third inoculation was administered two weeks prior to the analysis of T cell responses [Mokhtar et al, 2014].

The second study (Animal Experiment 2) was carried out at the AHVLA. Porcine circovirus type 2 (PCV-2) free Yorkshire cross Dutch Landrace pigs, were obtained from an isolated, high-health pig farm. Animals were randomly distributed so as to ensure an even genetic background amongst the groups (n=8). At seven weeks of age the pigs were inoculated intra-nasally with $10^5$ TCID$_{50}$ of LV or SU1-Bel PRRSV-1 in 1.5 ml of cell culture medium, with controls mock-infected with 1.5 ml of PAM cell supernatant diluted in RPMI [Morgan et al, 2013; Mokhtar et al, 2014].
The third study (Animal Experiment 3) was also carried out at the AHVLA. PCV-2 free, PRRSV antibody negative Large White/Landrace cross-bred pigs 12 weeks of age were inoculated intranasally with either MARC-145 cell lysate (mock) (n=2), $10^6$ TCID$_{50}$ PRRSV-1 Olot/91 (n=5) or $10^4$ TCID$_{50}$ PRRSV-1 SU1-Bel (n=5). Thirty five days post inoculation, pigs were inoculated again but with increased doses of the homologous virus; $10^7$ TCID$_{50}$ PRRSV-1 Olot/91 and $10^5$ TCID$_{50}$ PRRSV-1 SU1-Bel. Monitoring of clinical scores and rectal temperatures was performed daily from -2 day post-infection (dpi) until the end of the study, 60 dpi. Clinical parameters relevant for PRRSV infection were scored between 0 (normal) and 3 (severe), as previously described by Morgan et al, 2013 (Appendix A). These scores were added up to obtain a total score per pig and day. Heparinised blood and serum samples were taken at 0, 7, 14, 21, 30, 35, 42 and 50 dpi. Serum samples were stored at -80°C for later analysis to quantify virus and antibody levels, and heparinised blood was used immediately for isolation of peripheral blood mononuclear cells (PBMCs).

2.6. Experimental vaccination of pigs and challenge with PRRSV

Animal Experiment 4 was carried out at the AHVLA using 18 PRRSV antibody negative Large White/Landrace male piglets 8 weeks of age. Pigs were randomly assigned to three groups (n=6) which were vaccinated subcutaneously behind each ear with 2ml of either the (1) Virus-P, (2) Peptide-P or (3) Empty-P formulations prepared as described above. An identical boost vaccination was given on day 21 post-primary vaccination. Animals were challenged intranasally on day 42 post primary vaccination with $10^5$ TCID$_{50}$ of 215-06, a UK field PRRSV-1 strain.
Heparinised blood and serum samples were taken at -2, 0, 7, 14, 21, 28, 35, 42, 49, 56 and 61 days post-primary vaccination. Serum samples were stored at -80°C for later analysis to quantify antibody levels and for the quantification of PRRSV RNA. Heparinised blood was used immediately for isolation of peripheral blood mononuclear cells (PBMCs) for analysis of T cell responses. Monitoring of clinical scores and rectal temperatures was performed daily as described above from day -2 post-challenge until the termination of the study on day 21 post-challenge (day 63 post primary vaccination).

2.7. RNA isolation and PRRSV detection by quantitative RT-PCR

This analysis was kindly performed by Drs Miriam Pedrera and Jean-Pierre Frossard, AHVLA Virology Department. RNA was isolated from serum using the QIAamp Viral RNA Mini Kit (Qiagen, Crawley, UK) and PRRSV RNA was quantified by quantitative real-time reverse transcription PCR (qRT-PCR) as described Morgan et al, 2013, using the QuantiTect® Probe RT-PCR kit (Qiagen) according to the manufacturer’s instructions. Standard PRRSV RNA was synthesised by amplifying complementary DNA of the PRRSV ORF 7 region of both Olot-91, 215-06 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-Aldrich). The complete procedure was carried out as described by Morgan et al, 2013. Briefly, cDNA was amplified from 3 µl of RNA (extracted from infected cells using the QIAamp Viral RNA Mini Kit); for one reaction the PCR mastermix was
prepared with 3.2 µl 25 mM MgCl\textsubscript{2}, 5 µl 10 x buffer, 1 µl 10nM dNTPs, 0.4 µl Taq polymerase, 36.3 µl Milli-Q nuclease free water (all Promega) and 0.4 µl each of the ORF7 forward and reverse primers. The thermal profile used consisted in 3 cycles of 94°C 2 min, 46°C 1 min and 72°C 2 min followed by 35 cycles of 94°C 30 s, 53.4°C 1 min, 72°C 1 min and 72°C 5 min. The amplification product was visualised in a 2% agarose-Tris-acetate-EDTA (TAE) gel, DNA was purified from the gel using a QIAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions and quantified with the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific). ORF7 DNA was in vitro transcribed into RNA (Ambion® MEGAscript\textsuperscript{TM} T7 kit, Life Technologies) and purified (Ambion® MEGAClear\textsuperscript{TM} kit, Life Technologies) both according to the manufacturer’s instructions. The product was quantified and assessed for integrity using the 2100 Bioanalyzer (Agilent), and then serially diluted 10-fold in Milli-Q nuclease-free water and stored at -70°C. For detection of PRRSV RNA, 23 µl of PCR mastermix (12.5 µl Quantitect Mix, 0.25 µl RT enzyme, 0.5 µl each of the EU forward primer, EU reverse primer and the EU probe (EU forward primer: 5’ GAT GAC RTC CGG CAY C 3’; EU reverse primer: 5’ CAG TTG CTG CGC CTT GAT 3’ and EU probe: 5’ FAM-TCG AAT CGA TCC AGA CGG CTT-Tamra, R=A+G Y=C+T) and 8.75 µl Milli-Q nuclease free water) was added to 2 µl of viral RNA. The RT-PCR was performed on an Mx3000 Real Time PCR System (Agilent, Stockport, UK) using the PCR thermal profile detailed in Table 2.1.
Table 2.1. PRRSV-1 Quantitative RT-PCR thermal profile

<table>
<thead>
<tr>
<th>Segment</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>30 mins</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>15 mins</td>
<td>1</td>
</tr>
<tr>
<td>3-touchdown</td>
<td>94</td>
<td>20 sec</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>72↓*</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>94</td>
<td>20 sec</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>45 sec</td>
<td></td>
</tr>
</tbody>
</table>

*↓ indicates the touchdown section which is a decrease of 1°C in annealing temperature for each of the 17 cycles.

2.8. Detection of PRRSV-specific antibody responses

2.8.1. Detection of PRRSV-specific antibody responses by ELISA

PRRSV-specific antibody responses in serum samples from Animal Experiment 3 were determined using the HerdCheck PRRS X3 ELISA Kit (IDEXX Laboratories, Wetherby, UK), whereas, serum samples from Animal Experiment 4 were analysed using the INgezim PRRS 2.0 ELISA (Ingenasa). Both ELISA were conducted according to the manufacturer’s instructions.

2.8.2. Detection of PRRSV EM4-specific antibody responses by ELISA

Serum samples from Animal Experiment 4 were analysed for specificity to EM4 using an in-house developed ELISA. ELISA plates (MAXISorb, Nunc) were coated (100 µl/well) overnight at 4°C with anti-EM4 mAb (clone EM11E10; a kind
gift from Dr Margarita Garcia Duran, Ingenasa, Madrid, Spain) at a concentration of 1 µg/ml in carbonate-bicarbonate buffer (Sigma-Aldrich). The following day, plates were washed three times with 200 µl/well wash buffer (PBS, 0.05% Tween) and blocked for 1h in 200 µl reagent diluent (PBS, 0.05% Tween, 5% BSA (Sigma-Aldrich,). Subsequently, 100 µl serum, diluted 1 in 100 in reagent diluent was incubated for 1 h at RT. After three further washing steps, the secondary goat anti-swine IgG-HRP antibody (Jackson ImmunoResearch, Stratech Scientific Limited, Newmarket, UK) was incubated at a dilution of 1 in 20,000 for 1 h at RT. Three more washing steps followed, before 100 µl of TMB substrate (Sigma-Aldrich) was added to each well. After a maximum of ten minutes in the dark the reaction was by the addition of 100 µl 0.5 M H$_2$SO$_4$. The absorbance was then measured on a Victor$^4$ microplate reader (PerkinElmer) at 450 nm.

2.8.3. Detection of PRRSV-neutralizing antibody responses

This analysis was kindly performed by Drs Miriam Pedrera (Animal Experiment 3) and Lucia Biffar (Animal Experiment 4), AHVLA Virology Department. PRRSV neutralizing antibody (NA) titres (VNT) in serum samples were determined according to a previously described method [Weesendorp et al, 2013] with minor modifications. Sera were heat inactivated for 30 min at 56°C and duplicate serial 2-fold dilutions from 1/2 to 1/128 were then made in cRPMI. An equal volume of the homologous PRRSV strains containing 400 TCID$_{50}$ in cRPMI were added to each dilution and then incubated at 37°C for 60 min. Since the Olot-91 PRRSV strain is adapted to grow in MARC-145 cells and SU1-Bel PRRSV strain is adapted to grow in PAMs, both cell types were used to analyse sera from Animal
Experiment 3. MARC-145 cells (5x10³ cells/well) and PAMs (2x10⁵ cells/well) were cultured in cRPMI in 96 well plates with the virus-serum mixture for 3 days at 37°C, 5% CO₂. After 3 days, the cells were washed in 0.15 M NaCl solution, dried and frozen. The monolayers were IPX stained as described in Section 2.1. Each sample was analysed in duplicate and the mean titre was calculated; positive and negative sera were included and back titration of PRRSV was performed each time to confirm that the dose of virus used was within the accepted range for this assay, 80-8000 TCID₅₀/ml. NA titres were expressed as log₂ of the reciprocal of the serum dilution that fully neutralized the viral replication in 50% of the wells. Sera from Animal Experiment 4 were only analysed for their ability to neutralise PRRSV-1 Olot/91 infection of MARC-145 cells.

2.9. Isolation of peripheral blood mononuclear cells and magnetic bead based cell sorting to enrich and deplete cell populations

2.9.1. Isolation of peripheral blood mononuclear cells

Heparin blood was obtained from pigs by venopuncture of the external jugular vein and diluted 1:2 in HBSS (Invitrogen). 30 ml of blood was layered over 20 ml of room temperature 1.077 g/ml Histopaque (Sigma-Aldrich) and centrifuged for 25 min at 1000 x g at room temperature with the brakes off in a rotating bucket centrifuge (Beckman Coulter, High Wycombe, UK). Peripheral blood mononuclear cells (PBMC) were harvested from the interface and washed 3 times in cold sterile PBS (Invitrogen); spun for 5 min at 525 x g at 4°C. Cells were finally resuspended in cRPMI. Cell density was determined by analyzing 50 µl of cell suspension using a
MACSQuant Analyzer volumetric flow cytometer (Miltenyi Biotec, Bisley, UK) and gating on events with typical forward scatter (FSC) and side scatter (SSC) for PBMC.

2.9.2. Cryopreservation of peripheral blood mononuclear cells

PBMC were cryopreserved for future analyses by resuspending cells at a density of 1-2x10^7 cells/ml in cold 10% dimethylsulfoxide (DMSO) (Sigma-Aldrich) in FBS. Cells were dispensed into pre-cooled cryovials (maximum 3.6x10^7 cells/vial), which were placed into a pre-cooled Cryo 1°C Freezing Container (Nalgene, Fisher Scientific) containing 250 ml of 100% isopropyl alcohol (Sigma-Aldrich), which was transferred into a -80°C freezer for 24 hours. Cryotubes were then transferred directly into a liquid nitrogen storage container. To thaw cells, vials were transferred to a pre-heated 37°C water bath for rapid thawing, and washed in 50ml pre-warmed cRPMI. Cell density was then determined using volumetric flow cytometry, as described above, and density adjusted appropriately for downstream analysis.

2.9.3. Magnetic bead based depletion of CD4\(^+\) and CD8\(\beta^+\) T cells from peripheral blood mononuclear cells

In selected experiments, freshly isolated PBMC were incubated with 100 μl anti-porcine CD4 (clone 74-12-4; VMRD, Pullman, USA) or CD8-β mAb (clone PG1 64A; VMRD) in 1 ml PBS supplemented with 2% FBS (PBS/2% FBS) per 10^8 cells, for 15 min at room temperature. Cells were then washed twice in PBS/2% FBS
and then incubated with anti-mouse IgG microbeads (10 µl/10⁷ cells; Miltenyi Biotec) for 15 min at room temperature. The cells were then washed twice in PBS/2% FBS and resuspended in 1 ml PBS/2% FBS with 5 mM EDTA (MACS buffer) and an aliquot was removed for subsequent analysis of labelled cells pre-depletion. Cells were then applied to LD columns (Miltenyi Biotec) held in a MidiMACS magnet (Miltenyi Biotec), the flow-through and 2 x 3 ml washes with MACS buffer were collected, centrifuged, and cells resuspended in cRPMI. Two aliquots were removed; one for cell counting and one for post-depletion analysis. Pre- and post-depletion samples were analysed by staining with 5 µl anti-mouse IgG₂-PE conjugated secondary antibody (BD Biosciences, Oxford, UK) for 10 minutes at room temperature, washed in PBS and analysed by flow cytometry.

2.9.4. Isolation of monocytes and enrichment of dendritic cells from peripheral blood mononuclear cells

Porcine monocytes were isolated from PBMC by magnetic bead based sorting. In brief, PBMC were incubated with mouse anti-human CD14 coated microbeads (10 µl/10⁷ cells; Miltenyi Biotec) at room temperature for 15 min. Cells were passed through a 70 µm cell strainer (BD Biosciences) to remove any cell aggregates prior to being added to a LS column (as per manufacturer’s instructions) held on a MidiMACS magnet (both Miltenyi Biotec). The column was washed 3 times with 3 ml of MACS buffer and the CD14⁺ cells were eluted into 5 ml of cRPMI and subsequently counted on a volumetric flow cytometer and adjusted to the required density prior to use.
Dendritic cells (DCs) were enriched from PBMC by two rounds of magnetic bead-based cell sorting. PBMC were first depleted of CD14+ cells by incubation with mouse anti-human CD14 coated microbeads and washed as described above. Cells were resuspended at 2.5x10^8 cells/ml MACS buffer and applied to a LD column (1 ml per column, as per manufacturer’s instructions) as described above. The negative flow through and two washes of 1 ml MACS buffer was collected, washed with 50 ml PBS/2% FBS and then incubated with 10 μg/10^8 cells of mouse anti-porcine CD172a (clone 74-22-15A, Washington State University Monoclonal Antibody Center (WSUMAC), Pullman, USA) diluted in PBS/2% FBS to the volume of 10 times the volume of antibody to ensure adequate mixing of cells and antibody. After incubation at 4°C for 30 min, the cells were washed twice in PBS/2% FBS and further incubated with 10 μl/10^7 cells anti-mouse IgG-coated microbeads (Miltenyi Biotec) for 15 min at 4°C. After washing twice in PBS/2% FBS, CD172a+ cells were enriched using an LS column as described above. The column was washed 3 times with 3 ml of MACS buffer and the CD172a enriched cells were eluted into 5 ml of cRPMI, counted and adjusted to the required density prior to use.

2.10. Porcine IFN-γ ELISpot assay

ELISpot plates (96 well Multiscreen-IP Filter Plates; Millipore, Watford, UK) were prepared by pre-wetting each well with 15 μl of 35% ethanol for 1 min then washing 3 times with sterile PBS. The capture antibody (anti-porcine IFN-γ mAb, BD Biosciences) was prepared at 0.5 μg/ml in PBS and 50 μl added per well. The plates were then incubated at 4°C overnight. Capture antibody was then decanted and plates washed 3 times with unsupplemented RPMI-1640 medium.
Plates were blocked by addition of 100 µl/well cRPMI and incubation for 1 hr at 37°C. Freshly isolated PBMC or in selected experiments T cell subset depleted-PBMC were suspended at 1x10⁷ /ml (Animal Experiment 1) and 5x10⁶ /ml (Animal Experiments 2 and 3) in cRPMI and 100 µl of cells was added to each well. Experimental and control stimuli were added to each well and the total volume of each well was made up to 200 µl. Concanavalin A (ConA, Sigma-Aldrich) at 10 µg/ml was used as a positive control. Negative controls were: wells with media only, wells with cells and media or with Mock-virus supernatants prepared from cultures of uninfected MARC-145 cells or PAMs as described above. Viruses were diluted in cRPMI and added at a multiplicity of infection (MOI) of 0.1. Peptides were added at 1 µg/ml unless otherwise stated. All conditions were tested in triplicate. Plates were incubated at 37°C in a 5% CO₂ humidified atmosphere for 24 hours. Well contents were discarded and 100 µl of cold deionised water was added to each well and incubated at 4°C for 5 min. Wells were then washed 5 times with PBS with 0.05% Tween 20 (ELISpot Wash Buffer). Biotinylated anti-porcine IFN-γ mAb (BD Biosciences) was diluted to 0.05 µg/ml in ELISpot Wash Buffer with 1% FBS and added 50 µl/well. Plates were then incubated at 37°C for 2 hours or 4°C overnight. Plates were washed 3 times with ELISpot Wash Buffer and incubated with streptavidin-HRP (R&D Systems, Abingdon, UK; 0.5 µg/ml ELISpot Wash Buffer with 1% FBS; 50 µl/well) for 1hr at 37°C. Plates were washed 5 times and substrate was added; Animal Experiment 1 - DAB and Urea solution (Sigma-Aldrich) or Animal Experiments 2 and 3 - BCIP/NBT (R&D Systems) and incubated at room temperature in the dark until spots became visible, typically 15 – 60 min. The substrate was then discarded and the plates washed extensively with deionised water and left to dry in the dark. Spots were visualised using an automated ELISpot reader.
The unstimulated-corrected mean was obtained by calculating the mean IFN-γ SFC in the media- or mock-virus-stimulated wells, and subtracting this from each experimental value for each animal.

2.11. Assessment of antigen-specific cytokine responses from peripheral blood mononuclear cells

PBMC were stimulated with antigens or corresponding negative and positive controls as described above. Cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere for 18 hours. Cultures were mixed by repeat pipetting, centrifuged at 930 x g for 2 minutes at 4°C, and cell free-supernatants collected and stored at -80°C until analysed. Cytokines were quantified in culture supernatants using a commercial ELISA kits for porcine IL-2 (Life Technologies), IL-4 and IL-10 (both R&D Systems). ELISAs were carried out according to the manufacturers’ instructions and absorbance measurements were read using a Victor⁴ microplate reader (Perkin Elmer).

2.12. Multiparameter flow cytometric analysis of cytokine responses

2.12.1. Stimulation of peripheral blood mononuclear cells to assess antigen-specific T cell responses

Freshly isolated or previously cryopreserved PBMC were suspended in cRPMI at a density of 2x10⁷ /ml and seeded 50 µl/well in 96 well round bottom plates (Costar, Fisher Scientific). PBMC were then stimulated with 50 µl of either;
peptide pool or individual peptide (1 µg/ml, unless otherwise stated), PRRSV (MOI=0.1), or mock virus, Virus-, Peptide- or Empty-P formulations (1.5 x10⁶ particles/well), cRPMI or pokeweed mitogen (PWM; Sigma-Aldrich 5µg/ml) and incubated at 37°C in a 5% CO₂ humidified atmosphere. PBMC stimulated with peptides or media were incubated for 2 hours before addition of brefeldin A (0.1 µl/well) (GolgiPlug, BD Biosciences) and further incubation overnight. PBMC stimulated with PRRSV, mock virus or PWM were incubated overnight (18 hours) before addition of brefeldin A and further incubation of six hours. For detection of cytotoxic degranulation by surface detection of CD107a, monensin (0.067 µl/well; GolgiStop, BD Biosciences, Oxford, UK) and mouse anti-porcine CD107a-FITC (clone JM2E5) or mouse IgG1 isotype control-FITC mAbs (10 µl/well) (both AbD Serotec, Oxford, UK) were added upon addition of brefeldin A. After incubation, PBMC were washed in PBS ready for antibody staining. All washes were performed as follows: plates were centrifuged at 930 x g for 2 minutes at 4°C; supernatant was flicked off, cells mixed by vortex and either 200 µl/well PBS or Perm/Wash buffer (BD Biosciences) added as detailed below.

2.12.2. Flow cytometric staining of peripheral blood mononuclear cells to assess antigen-specific T cell responses

PBMC were stained for 30 min at 4°C with combinations of the following directly conjugated mAbs: CD4-PerCP-Cy5.5 (clone 74-12-4, BD Biosciences), CD8α-PE (clone 76-2-11, BD Biosciences) or CD8α-FITC (clone MIL12, AbD Serotec), CD44-eFluor450 (clone IM7, eBioscience, Hatfield, UK) or the following mAbs conjugated to fluorochromes using Zenon® Mouse IgG Labelling Kits (Life
Technologies): CD62L (clone CC32, AbD Serotec), CD27 (clone b30c7, kindly provided by Dr Wilhelm Gerner, University of Veterinary Medicine, Vienna, Austria; Reutner et al, 2012), CD25 (clone K231.3B2, AbD Serotec), as well as with the Near Infra-Red Fixable Live/Dead Viability Dye (Life Technologies) or the Zombie Near Infra-Red Fixable Viability Kit (Biolegend, London, UK). After incubation, PBMC were washed twice in PBS and then either resuspended in PBS 2% FBS with 0.09% sodium azide (FACS buffer) ready for flow cytometric analysis; or fixed and permeabilised by incubating in CytoFix/CytoPerm solution (100 µl/well; BD Biosciences) for 20 min at 4°C for intracellular staining. Fixed/permeabilised PBMC were washed twice in Perm/Wash Buffer (BD Biosciences) and then stained with the following directly conjugated mAbs: IFN-γ-Alexa Fluor 647 (clone CC302, AbD Serotec) and TNF-α-Brilliant Violet 421 (clone MAb11, Biolegend) or the respective isotype controls (Alexa Fluor 647 mouse IgG1 isotype control, AbD Serotec, and Brilliant Violet 421 mouse IgG1 isotype control, Biolegend) for 30 min at 4°C. PBMC were then washed twice in Perm/Wash Buffer and resuspended in 200 µl FACS Buffer per well ready for flow cytometric analysis.

2.12.3. Flow cytometric analysis of peripheral blood mononuclear cells to assess antigen-specific T cell responses

Flow cytometric analysis was performed either using a MACSQuant Analyser and the MACSQuantify analysis software (both Miltenyi Biotec), a CyAn ADP flow cytometer and the Dako Summit analysis software (both Beckman Coulter). Analyses of stained PBMC were performed by first excluding any doublets based on area and height measurements of forward scatter (FSC) and selection of live cells by gating on Live/Dead Fixable Stain negative events. Lymphocytes were
then selected based on their FSC and side scatter (SSC) properties. CD4^+CD8_{int} (CD4) and CD4^+CD8_{high} (CD8) T cell populations were gated upon and IFN-γ^+ cells within these populations were analysed for co-expression of cell surface markers or TNF-α. Gates were set based primarily on the corresponding isotype control and then adjusted using the relevant biological negative control (media or mock stimulated). The gating strategies are illustrated in Figures 2.1 and 2.2. The unstimulated-corrected mean was obtained by calculating the mean % of IFN-γ secreting cells in the media- or mock-virus-stimulated wells, and subtracting this from each experimental value for each animal.
Figure 2.1. Flow cytometric gating strategy used to interrogate cytokine responses of PBMC. Responses to PRRSV, proteins or peptides were assessed in freshly isolated or previously cryopreserved PBMC. Single cells were gated upon using FSC-A vs FSC-H and then lymphocytes were gated upon using typical FSC vs SSC properties (top panel). Live/Dead stain cells were assessed for CD4 and CD8α expression and the two populations CD4 (CD4+CD8αlow) and CD8 (CD4-CD8αhigh) T cells were assessed for IFN-γ and TNF-α expression. Gates were set using corresponding isotype controls and unstimulated biological controls.
Figure 2.2. Flow cytometric gating strategies used to interrogate expression of various T cell markers. CD4 or CD8 T cell populations were gated as shown in Figure 2.1 and assessed for expression of IFN-γ and either CD44, CD62L, CD27, CD25 or CD107a. Unstimulated shows cells incubated with media alone as a biological control, with the exception of CD107a which shows stimulated cells stained with IgG1-FITC isotype control. This figure shows CD8 T cells stimulated with either peptide (M and NSP5) or media.
2.12.4. Stimulation of mixed cultures of monocytes and dendritic cells to assess cytokine responses induced by pathogen-associated molecular patterns or vaccine formulations

Enriched blood DCs and purified monocytes were mixed at a ratio of 70:30 and suspended at a density of 4x10^6 cells/ml in cRPMI supplemented with 20 ng/ml of recombinant porcine IL-3 (a kind gift from Kirsten Morris, CSIRO Biosecurity Flagship, Australian Animal Health Laboratory, Geelong, Australia) to maintain pDC viability in vitro. Cells were seeded 50 µl/well in 96 well round bottom plates (Costar, Fisher Scientific) and stimulated with a panel of pathogen recognition receptor (PRR) agonists synthetic multi-TLR2 and -7 agonists CL513 (working concentration 10 µg/ml) and Adilipoline (working concentration 10 µg/ml; Invivogen); TLR-9 agonists CpG ODNs 21798 (class P; working concentration 2 µM; Miltenyi Biotec), 2216 (class A; working concentration 2 µM; Invivogen) and 2007 (class B; working concentration 2 µM; Invivogen); TLR-7 agonist imiquimod (working concentration 1 µg/ml; Invivogen), and TLR-3/RIG-I/MDA-5 agonist poly(I:C) (working concentration 10 µg/ml; Invivogen)) or with a titration of Virus-, Peptide- or Empty-P formulations, and incubated at 37°C in a 5% CO2 humidified atmosphere. Cells were incubated for 2 hours before addition of monensin (0.067 µl/well; GolgiStop, BD Biosciences) and further incubation overnight.
2.12.5. Flow cytometric staining of mixed cultures of monocytes and dendritic cells to assess cytokine responses induced by pathogen-associated molecular patterns or vaccine formulations

Cells were stained for 30 min at 4°C with combinations of the following directly conjugated mAbs: CD4-PerCP-Cy5.5 (clone 74-12-4, BD Biosciences), CD1-FITC (clone 76-7-4, Southern Biotec, Cambridge Bioscience, Cambridge, UK), CD14-PE-Texas Red (clone TuK4, Life Technologies), CD172a (clone 74-22-15A, WSUMAC) conjugated to PE using the Zenon® Mouse IgG2b Labelling Kit (Life Technologies), as well as with the Near Infra-Red Fixable Live/Dead Viability Dye (Life Technologies). After incubation, cells were washed twice in PBS and fixed and permeabilised using CytoFix/CytoPerm solution (BD Biosciences) as described above. After washing twice in Perm/Wash Buffer (BD Biosciences), cells were stained with the following mAbs: TNF-α-Pacific Blue (clone MAb11, Biolegend), IFN-α (clone K9, R&D Systems) conjugated to Alexa Fluor-647 using the Zenon® Mouse IgG1 Labelling Kit, and IL-12-biotin (clone 116211, R&D Systems) by incubation for 30 min at 4°C. After two further washes in Perm/Wash Buffer cells were incubated for 30 min at 4°C with streptavidin-PE-Cy7 (Biolegend). Cells were finally washed twice in Perm/Wash Buffer and resuspended in 200 µl FACS Buffer per well ready for flow cytometric analysis.
2.12.6. Flow cytometric analysis of mixed cultures of monocytes and dendritic cells to assess cytokine responses induced by pathogen-associated molecular patterns or vaccine formulations

Flow cytometric analysis was performed either using a CyAn ADP flow cytometer and the Dako Summit analysis software (both Beckman Coulter), or a BD LSRFortessa™ (BD Biosciences) and the Kaluza analysis software (Beckman Coulter). Analysis of enriched DCs and monocytes were performed again by excluding any doublets and selecting only live cells, as described above for PBMC. DCs were identified as possessing a CD14^-CD172a^int phenotype and monocytes as CD14^-CD172a^hi. DCs were further delineated based on CD1 and CD4 expression to reveal the two populations of myeloid DCs (mDCs), CD4^-CD1^- and CD4^-CD1^+, and plasmacytoid DCs (pDCs) CD4^+CD1^-. The cytokine secretion profile by each DC population was determined by gating on each population and assessing expression of IFN-α, TNFα and IL-12 as shown in Figure 2.3.
Figure 2.3. Flow cytometric gating strategy used to interrogate cytokine responses of antigen presenting cells. Responses were assessed in mixed cultures of enriched DCs and monocytes stimulated with PAMPs or vaccine formulations. Single cells were gated upon using FSC-A vs FSC-H and then cells with high forward scatter were gated. Cells that stained negative for Live/Dead stain (shown here against IL-12-PE-Cy7 for compensation reasons) were selected and CD14$^-$CD172a$^{high}$ cells monocytes and CD14$^+$CD172a$^{low}$ DCs gated, DCs were then further gated as CD1$^-$CD4$^+$ DCs, CD1$^-$CD4$^+$ mDCs and CD1$^+$CD4$^+$ mDCs. Each individual population was then assessed for expression of IL-12. IL-12 negative and positive populations were finally assessed for co-expression of TNF-α and IFN-α.
2.13. Assessment of cytokine responses of monocytes or enriched dendritic cell cultures to stimulation with vaccine formulations

Enriched blood DCs or purified monocytes were suspended at a density of 4x10^6 cells/ml in cRPMI, which was further supplemented for DC cultures with 20 ng/ml of recombinant porcine IL-3. Cells were seeded 50 µl/well in 96 well round bottom plates (Costar, Fisher Scientific) and titrations of Virus-, Peptide- or Empty-P formulations, diluted in 50 µl of cRPMI added. Addition of cRPMI or Adilipoline (10 µg/ml) was included as negative and positive controls respectively. Cultures were incubated at 37°C in a 5% CO₂ humidified atmosphere for 18 hours. Cultures were mixed by repeat pipetting, centrifuged at 930 x g for 2 minutes at 4°C, and cell free-supernatants collected and stored at -80°C until analysed. Cytokines were quantified in culture supernatants using commercial ELISA kits for porcine IL-8, IL-10 and IL-12 (all R&D Systems). Type I IFN bioactivity was quantified from culture supernatants using an Mx/CAT reporter gene assay originally developed for the quantification of bovine IFN-α/β [Fray et al, 2001]. Cultures were grown as above, but in DMEM medium (Life Technologies) supplemented with blasticidin (10 µg/ml; Life Technologies); and cell free supernatants were added to reporter bovine kidney cells (MDBK-t2) for 24 hours. A titration of recombinant porcine IFN-α (R&D Systems) was added as a standard. Lysates were prepared from the cultures and CAT enzyme measured by ELISA (Roche, Welwyn Garden City, UK). Absorbance measurements for all ELISAs were read using a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK) or Victor4 microplate reader (Perkin Elmer).
2.14. Analysis of T cell responses from the bronchoalveolar lavage fluid from vaccinated and challenged pigs.

At the termination of Animal Experiment 4 on day 21 post-challenge (day 63 post-primary vaccination), all animals were euthanized. Animals vaccinated with Peptide-P were euthanized by electrical stunning followed by exsanguination, however, it became apparent that this was resulting in oedema and hemorrhages in the lungs and so animals vaccinated with Virus- and Empty-P were euthanized by intravenous injection with pentobarbital. The left lung lobes were removed at the main bronchus. The bronchus end was washed with 70% ethanol to remove blood clots and clamps were used to secure the ends of the blood vessels. Both lobes of the lung were filled with 4 x 50ml aliquots of sterile PBS. After each addition of PBS, lungs were massaged gently 10-15 times and the bronchoalveolar lavage fluid (BALF) was collected into a sterile bottle. To determine the cellularity of the BALF an aliquot was analyzed directly on the volumetric MACSQuant Analyzer flow cytometer. To assess the T cell composition of the BALF a second aliquot was stained by incubation for 30 minute at 4°C with mAbs CD3-FITC (clone BB23-8E6-8C8), CD4-PerCP-Cy5.5 (clone 74-12-4) and CD8α-PE (clone 76-2-11) (all BD Biosciences). Cells were washed twice in PBS before being analysed by flow cytometry. To assess antigen-specific T cell responses, mononuclear cells were isolated by density gradient centrifugation (as described for PBMC isolation), washed with sterile PBS, counted and then stimulated with PRRSV-1 Olot/91 (MOI=0.1) or a pool of synthetic peptides representing the M and NSP5 proteins (each peptide used at a final concentration of 1µg/ml). Mock virus and cPRMI were again used as the respective negative controls. As described in Sections 2.12.1-3,
cells were incubated, before IFN-γ and TNF-α expression by CD4 and CD8 T cells was assessed by flow cytometry.

2.15. Sequence analysis of PRRSV

This analysis was kindly performed by Drs Jean-Pierre Frossard and Bhudipa Choudhury, AHVLA Virology Department, and Dr Richard Ellis, AHVLA Central Sequencing Unit. Total RNA was extracted from the vaccine strain Porcilis, 195-06 and SU1-Bel virus stocks following a previously described protocol [Rasmussen et al, 2010]. RNA was sequenced using the GS FLX system (454 Life Sciences, Burgess Hill, UK); reads were assembled with Newbler v.2.6 software and saved in Fasta format for downstream analysis. The sequences of the identified T cell antigenic peptides were aligned against the predicted sequences from the virus genomes and an additional 12 PRRSV isolates (GenBank accession numbers shown in parentheses): Genotype 1 subtype 1 – Olot/91 (X92942), H2 (AY035938, JN862511, KJ769653, KJ769654 and KJ769655), Lelystad (AY588319), Porcilis (KF991509), Cresa3266 (JF276434), 195-05 (KJ769656, JN862404, KJ769657, KJ769658 and KJ769659), Cresa3249 (JF276433), Cresa3267 (JF276435), Cresa3256 (JF276432), Cresa3262 (JF276431) Cresa2982 (JF276430) and 07V063 (GU737264); Genotype 1 subtype 3 - Lena (JF802085) and SU1-Bel (KJ769660, KJ769661, KJ769662, KJ769663 and KJ769664); and Genotype 2 - VR2332 (EF536003), using the clustal W algorithm on MegAlign (DNASTar Lasergene 9 Core Suite, Madison, USA).
2.16. MHC haplotype determination by low-resolution PCR-based analysis

This analysis was kindly performed by Dr Sabine Essler, University of Veterinary Medicine Vienna, Austria. Genomic DNA was extracted from PBMC using a QIAamp DNA extraction mini kit, according to the manufacturer’s protocols (Qiagen, Crawley, UK) and genotyped for their swine leukocyte antigen (SLA) class I and II haplotypes by running low-resolution PCR screening assays (PCR-SSP) as previously described [Essler et al, 2013]. In brief, this method relies on 47 discriminatory PCR primer pairs, which are designed to amplify the SLA class I alleles by groups that have similar sequence motifs and amplify products of 114–316 bp. This method is used to type alleles at the three classical SLA class I loci (SLA-1, SLA-3 and SLA-2). All typing primers were previously validated and were optimized to be used at the same annealing temperature (65°C) and concentration (5 pmol/each). Typing of each animal included a negative control without DNA to check for reagent contamination, and was electrophoresed in a standard 96-well format. Result interpretation is based on the presence of a smaller PCR product than the positive control in each lane [Ho et al, 2009].

2.17. Statistical analysis

GraphPad Prism 6.01 (GraphPad Software, La Jolla, USA) was used for graphical and statistical analysis of data sets. Analysis of variance (ANOVA) was employed to analyse fixed effects on different traits with various post-hoc tests as detailed in figure legends. A p value < 0.05 was considered statistically significant.
Chapter III

Proteome-wide screening of the porcine reproductive and respiratory syndrome virus reveals a range of T cell antigen reactivity
Chapter 3. Proteome-wide screening of the porcine reproductive and respiratory syndrome virus reveals a range of T cell antigen reactivity

This work has in part been published in H. Mokhtar, M. Eck, S.B. Morgan, S.E. Essler, J.P. Frossard, N. Ruggli, S.P. Graham. 2014. Proteome-wide screening of the European porcine reproductive and respiratory syndrome virus reveals a broad range of porcine T cell antigen reactivity; Vaccine doi: 10.1016/j.vaccine.2014.04.054 and is reproduced with permission from Elsevier Limited, license number 3443860057893.

3.1. Rationale

The economic impact of PRRSV and the threat of emerging highly pathogenic strains necessitate the development of improved vaccines. Immunity to PRRSV remains relatively poorly understood, although there are data suggesting that virus-specific T cell IFN-γ responses play an important role in clearance of and protection from the virus. In light of this, identification of the PRRSV proteins that induce a strong T cell response is paramount to allow for their incorporation into next generation vaccines. Recent efforts to elucidate the specificity of T cell responses to PRRSV have led to the identification of antigenic regions/epitopes on the structural proteins M, N, GP3, GP4, GP5 [Wang et al, 2011, Diaz et al, 2009, Vashisht et al, 2008, Bautista et al, 1999] and the non-structural proteins NSP2,
NSP5, NSP9 (the viral RNA-dependent RNA polymerase, RdRp) and NSP10 [Parida et al, 2012, Burgara-Estrella, et al, 2013]. However, studies to date have focussed their analysis on pre-defined PRRSV antigens or used predictive bioinformatics to pre-screen putative antigenic peptides in silico, and in most instances the phenotypes of the responding T cell populations were not discerned. In contrast, this study aimed for a comprehensive analysis of the antigen specificity of the T cell response induced by PRRSV-1 infection and to ascribe a phenotype to cells responding to defined antigenic peptides using a synthetic peptide library spanning the PRRSV proteome. T cell reactivity was initially investigated in a small cohort of pigs following experimental infection with an attenuated PRRSV-1 strain, Olot/91. The pattern of T cell antigen reactivity was then assessed in groups of pigs experimentally infected with the related prototype PRRSV-1 strain Lelystad, and a divergent pathogenic genotype 1 subtype 3 strain, SU1-Bel, in an attempt to identify conserved T cell antigens.

3.2. Results

3.2.1. Summary

Work presented in this chapter aimed to better characterise the T cell response to PRRSV-1 by utilising a synthetic peptide library spanning the proteome and a small cohort of pigs rendered immune to PRRSV-1 Olot/91 by repeated experimental infection. Using an IFN-γ ELISpot assay as a read-out, 9 antigenic regions on 5 of the viral proteins were identified and the corresponding responder T cell phenotype determined. The diversity of the IFN-γ response to the PRRSV
proteins suggests that antigenic regions are scattered throughout the proteome and no one single antigen dominates the T cell response. To address the identification of well-conserved T cell antigens, groups of pigs infected with a closely related avirulent PRRSV-1 strain (Lelystad) and a divergent virulent subtype 3 strain (SU1-Bel) were subsequently screened. Whilst T cell responses from both groups were observed against many of the antigens identified in the first study, animals infected with the SU1-Bel strain showed the greatest response against peptides representing NSP5. The proteome-wide peptide library screening method used here, as well as the antigens identified, warrant further evaluation in the context of next generation vaccine development.

### 3.2.2. Screening of the proteome-wide synthetic peptide library with pigs immune to PRRSV-1 Olot/91

PBMC from three PRRSV-1 Olot/91 immune pigs were stimulated in vitro with peptide pools representing 19 proteins of PRRSV-1 and responses measured by IFN-\(\gamma\) ELISpot assay (Figure 3.1). Results showed that all animals mounted significant IFN-\(\gamma\) responses to the structural proteins M, N and GP3. Pig 52 only responded to these proteins, whereas pigs 53 and 54 also showed significant responses to other structural proteins, GP4 and GP5, as well as non-structural proteins NSP1, NSP2 and the viral polymerase RdRp (NSP9). In addition, pig 54, which interestingly had initially been infected with the higher doses of PRRSV Olot/91, showed reactivity to NSP7, NSP11 and GP2. Due to the large numbers of PBMC required, it was not possible to pursue the further examination of all positive peptide pools and therefore based on these results as well as the current literature,
GP5 and M were selected as representative structural proteins and NSP1, NSP2 and RdRp selected as representative non-structural proteins. For confirmation and mapping of the T cell responses to specific antigenic regions, overlapping peptides representing GP5 (Figure 3.2) and M (Figure 3.3) were screened individually, and peptides making up the NSP1 (Figure 3.4), NSP2 (Figure 3.5) and RdRp (Figure 3.6) protein pools were screened in pools of 10 due to their long length. Peptides that stimulated significant IFN-γ responses compared to unstimulated controls in two or more pigs were identified (Table 3.1) and their recognition confirmed by titration of peptides using a 10-fold dilution series starting at 1μg/ml (Figure 3.7).
Figure 3.1. T cells from PRRSV-1 Olot/91 immune pigs display broad profiles of antigen reactivity. PBMC from three PRRSV-1 Olot/91-immune pigs were stimulated in vitro with synthetic peptides pooled to represent 19 PRRSV-I proteins and IFN-γ secreting cells enumerated by ELISpot assay. Representative data of one of two experiments are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10⁶ PBMC for triplicate cultures. Error bars represent SEM. Values for each peptide pool-stimulated condition were compared to the corresponding unstimulated control using a one-way ANOVA followed by a Dunnett’s Multiple Comparison Test; ***p<0.001, **p<0.01, *p<0.05.
Figure 3.2. Identification of individual antigenic peptides from PRRSV GP5. PBMC isolated from three PRRSV-1 immune pigs were stimulated in vitro with individual overlapping 15mer peptides comprising the GP5 protein. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10^6 PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
Figure 3.3. Identification of individual antigenic peptides from PRRSV M protein. PBMC isolated from three PRRSV-1 immune pigs were stimulated in vitro with individual overlapping 15mer peptides comprising the M protein. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10^6 PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
Figure 3.4. Mapping of antigenic peptides from PRRSV NSP1. PBMC isolated from three PRRSV-1 immune pigs were stimulated in vitro with pools of ten 15mer peptides comprising the NSP1 protein. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10⁶ PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
Figure 3.5. Mapping of antigenic peptides from PRRSV NSP2. PBMC isolated from three PRRSV-1 immune pigs were stimulated *in vitro* with pools of ten 15mer peptides comprising the NSP1β protein. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10⁶ PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
Figure 3.6. Mapping of antigenic peptides from PRRSV RNA dependant RNA polymerase (RdRp). PBMC isolated from three PRRSV-1 immune pigs were stimulated in vitro with pools of ten 15mer peptides comprising the RdRp protein. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10⁶ PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
Figure 3.7. Identification of PRRSV-1 antigenic peptides recognised by primed T cells. Ten-fold dilutions of putative antigenic peptides were screened using PBMC from three PRRSV-1 Olot/91 immune pigs and T cell responses enumerated by IFN-γ ELISpot assay. Pools of 10 peptides from NSP1β, NSP2 and RdRp, previously identified to elicit an IFN-γ response were tested, whereas for GP5 and M, individual putative antigenic peptides were evaluated. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10^6 PBMC for triplicate cultures and error bars represent SEM.
Table 3.1. Summary of individual peptides, representing GP5 and M, or peptide pools, representing NSP1β, NSP2 and RdRp, able to induce IFN-γ release from PBMC from Olot/91 immune pigs

<table>
<thead>
<tr>
<th>Protein</th>
<th>IFN-γ inducing peptides/peptide pools</th>
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<tr>
<td>GP5</td>
<td>38, 39</td>
</tr>
<tr>
<td>M</td>
<td>4, 5, 16, 24, 38, 40, 41</td>
</tr>
<tr>
<td>NSP1</td>
<td>Pool 1, pool 4, pool 9</td>
</tr>
<tr>
<td>NSP2</td>
<td>Pool 3, pool 7</td>
</tr>
<tr>
<td>RdRp</td>
<td>Pool 5</td>
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Pig 52 showed statistically significant IFN-γ responses to NSP1β peptide pools 1 and 4, and peptides GP5_{61-75} (peptide 38; FDWAVETFVLYPVAT), M_{13-27} (peptide 4; AAQKLVLAFSITYTP) and the overlapping peptides M_{159-173} (peptide 40) and M_{161-175} (peptide 41), suggesting that the antigenic region lay within the consensus sequence of RAVKRGVVNLVKY. This pig also showed reactivity to NSP2 peptide pool 3 and to a lesser degree pool 7, although not deemed statistically significant. Pig 54 showed a similar response with an additional reactivity detected against RdRp peptide pool 5. Pig 53 showed a significant response to NSP1 peptide pool 1, RdRp peptide pool 5 and peptide GP5_{61-75} (peptide 38).

The pools of ten peptides from NSP1, NSP2 and RdRp that elicited a significant IFN-γ response were subsequently screened individually to attribute the response to specific antigenic peptides (Figure 3.8). Rather unexpectedly, the responding peptides in NSP1 pool 1 were different for each pig; NSP1_{149-163} (peptide 5; DQPFPGATHVLTNSP) in pig 53, NSP1_{69-83} (peptide 3; ECTPSGCCWLSAVFP) in pig 52 and NSP1_{129-143} (peptide 1; YPITGPVPGMGLFAN) in pig 54. In NSP1 pool 4, the responding peptide was NSP1_{349-363} (peptide 10;
DDDVTGFVRLTLSR), and in NSP2 pools 3 and 7 were NSP2\textsubscript{293-407} (peptide 4; AATTLVREQTPDNP) and NSP2\textsubscript{725-739} (peptide 5; QRLMTWVEVFSHLP), respectively. Two non-overlapping peptides from the RdRp pool 5 gave a positive response: RdRp\textsubscript{142-156} (peptide 4; HGAGNMGVDGSIWDF) and RdRp\textsubscript{262-276} (peptide 5; YSVMEYLDSPDTPF).
Figure 3.8. Resolution of individual antigenic peptides from NSP1 peptide pool 1. PBMC isolated from the three PRRSV-1 immune pigs were stimulated in vitro with each individual peptide that comprised the identified antigenic NSP1 peptide pool 1. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10^6 PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
Figure 3.9. Resolution of individual antigenic peptides from NSP1, NSP2 and RdRp pools of ten peptides. PBMC isolated from the three PRRSV-1 immune pigs were stimulated in vitro with each individual peptide that comprised the identified antigenic pool 4 of NSP1, pools 3 and 7 of NSP2 and pool 5 of the RdRp. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC) /10^6 PBMC for triplicate cultures and error bars represent SEM. Values for each peptide stimulated condition were compared to the corresponding unstimulated control using a one way ANOVA followed by a Dunnett’s Multiple Comparison Test; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.
3.2.3. Assessment of the phenotype of T cells responding to identified PRRSV-1 antigenic peptides

In order to determine the phenotype of the T cells responding to the identified individual or pools containing antigenic peptides, PBMCs were depleted of either CD4⁺ or CD8β⁺ T cells and then stimulated with antigenic or irrelevant control peptides or pools (Figure 3.10). Responses were considered to be from CD4 or CD8 T cells when a statistically significant decrease in the response to peptide was observed upon depletion. Although IFN-γ responses were waning in magnitude by this time-point post-infection, it was still discernible for at least one of the responder pigs that CD4 T cells were responsible for responses to peptides GP5₆₁‐₇₅ and M₁₃‐₂₇ and to the NSP2 peptide pools, whereas, CD8 T cells responded to NSP1 peptide pool 1, RdRp peptide pool 5 and the overlapping peptides M₁₅₉‐₁₇₃ and M₁₆₁‐₁₇₅.
Figure 3.10. Elucidation of the phenotype of PRRSV-1 antigenic peptide-specific T cells. Antigenic peptides were used to stimulate PBMC (grey bars) or PBMC depleted of CD4+ (black bars) or CD8β+ (white bars) T cells from three PRRSV-1 Olot/91 immune pigs and remaining IFN-γ secreting T cells enumerated by ELISpot assay. Pools of 10 peptides from NSP1, NSP2 and RdRp, previously identified to elicit an IFN-γ response were tested, whereas for GP5 and M, individual antigenic peptides were evaluated. Data are presented as the unstimulated-corrected mean IFN-γ spot forming cells (SFC)/10^6 PBMC for triplicate cultures. Error bars represent SEM and asterisks denote statistical significance (p<0.05) of responses compared to the other depleted PBMC population.
3.2.4. Determination of porcine MHC (SLA) haplotypes and assessment of association with T cell specificity.

In order to determine whether the T cell responses to particular peptides could be attributed to a certain haplotype, animals were MHC typed. All three animals were heterozygous and a number of haplotypes were shared: MHC class I – Pig 52 (Lr-27.0/24.0), Pig 53 (Lr-05.0/24.0), Pig 54 (Lr-05.0/24.0) and MHC class II - Pig 52 (Lr-0.23/0.14), Pig 53 (Lr-0.27/0.02) and Pig 54 (Lr-0.23/0.02). It is therefore possible that the common MHC class I haplotype 05.0, shared by pigs 53 and 54, could restrict the CD8 T cell response to RdRp_{142-156} and RdRp_{262-276} (Figure 3.9). Despite pigs 53 and 54 being MHC class I haplomatched only the latter responded, at the point of testing, to the overlapping M peptides and since the response was also observed with pig 52 (Figure 3.10), it may only be speculated that this response was restricted by Lr-24 in 2/3 animals. With regards to the CD4 responses, the MHC class II haplotype 0.23, shared by pigs 52 and 54, could restrict the CD4 T cell responses to and M_{13-27}. Since CD4 T cell responses to GP5_{61-75} were detected in all pigs, it suggests that this peptide may be presented by MHC class II molecules present in more than one haplotype (Figure 3.10). However, the testing of additional MHC typed animals is required to confirm these associations.
3.2.5. Assessment of sequence conservation of identified antigenic peptides amongst PRRSV strains.

The level of amino acid sequence conservation of the antigenic regions was investigated amongst 14 PRRSV-1 strains, two of which were divergent subtype 3 strains, SU1-Bel and Lena, and the PRRSV-2 reference strain, VR2332 (Table 3.2 and 3.3). The analysis revealed that the NSP1β149-163 and M antigenic peptides were highly conserved amongst PRRSV-1 isolates and only two or three amino acid substitutions were seen in VR2332. NSP1349-363, the two RdRp antigenic regions and GP561-75 were also well conserved within genotype 1 strains, but less well conserved in PRRSV-2. NSP169-83 and NSP1129-143 were not well conserved, even within PRRSV-1 strains however the most variable sequences were found within the NSP2 protein. Conservation of antigen sequence between and within genotypes is an important consideration when evaluating antigens for incorporation into potential PRRSV vaccines. The analysis shows that, while certain antigens may induce a superior cellular immune response, e.g. GP5, these may not be the best antigens to generate an immune response against the repertoire of PRRSV strains in the field. Further studies could be conducted to assess whether the mutations in these T cell antigenic peptides would have a functional consequence in terms of MHC binding and T cell recognition.
Table 3.2. Assessment of the conservation of identified T cell antigenic regions of the non-structural proteins among different PRRSV-1 isolates and the prototype PRRSV-2 strain

<table>
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<tr>
<th>PRRSV Strain</th>
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<th>NSP1 69-83</th>
<th>NSP1 126-143</th>
<th>NSP1 149-163</th>
<th>NSP1 349-363</th>
<th>NSP2 393-407</th>
<th>NSP2 725-739</th>
<th>RdRp142-156</th>
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Table 3.3. Assessment of the conservation of identified T cell antigenic regions of the structural proteins among different PRRSV-1 isolates and the prototype PRRSV-2 strain

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3.2.6. Assessment of T cell antigen reactivity in pigs experimentally infected with the prototype PRRSV-1 strain Lelystad or the divergent SU1-Bel strain.

The peptide library screening was expanded to assess the T cell antigen specificity from pigs infected with two different PRRSV-1 strains; Lelystad serving as a highly conserved strain (overall 95.2% nucleotide identity with Olot/91) and the pathogenic SU1-Bel as a divergent strain (overall 76.4% nucleotide identity with Olot/91). Stimulation of PBMC, isolated on day 28 post-infection, with the pools representing the 19 PRRSV proteins showed that irrespective of the inoculation strain the response profile was broadly similar to the previous experiment (Figures...
3.11 and 3.12). All but one of the animals (7/8) infected with the Lelystad strain mounted a significant IFN-γ response to NSP1 and 2, whereas only 2/8 and 1/8 showed a significant response to M and GP5, respectively (Figure 3.11). A significant proportion of pigs infected with the divergent SU1-Bel strain responded to these four antigens (NSP1 – 3/8, NSP2 – 6/8, M and GP5 – both 4/8) (Figure 3.12). In spite of these pigs being tested for T cell reactivity after a single infection, in contrast to the animals in the previous experiment, which were tested after repeated inoculation, a very similar profile of T cell reactivity was observed. These results do not show any obvious focussing of the immune response towards a particular protein, however, further in depth phenotyping of the responding T cell populations in each case may reveal differences. Interestingly, the non-structural protein 5 (NSP5) elicited a strong IFN-γ response in 6/8 SU1-Bel-infected pigs (Figure 3.12) compared to the insignificant response seen in all but one of the pigs infected with the Lelystad strain (Figure 3.11). Comparison of the deduced NSP5 amino acid sequences from SU1-Bel and Lelystad showed these proteins to be well conserved sharing 88.8% identity. Comparison of Olot/91 NSP5 showed 97.6% and 89% identity with the Lelystad and SU1-Bel sequences, respectively (Figure 3.13). In addition, amongst the pigs infected with Lelystad strain, one pig responded to GP2; as well as 2/8 pigs showing a response to NSP3 and one pig to NSP8. Amongst the pigs infected with the SU1-Bel strain, 4/8 pigs additionally showed a significant response to GP2, 2/8 pigs showed significant responses to the N protein and viral polymerase (RdRp), and single pigs responded to NSP7 and helicase.
Figure 3.11. Recognition of PRRSV-1 proteins by T cells from pigs experimentally infected with the closely related PRRSV Lelystad strain. PBMC from pigs experimentally infected with PRRSV-1 Lelystad (n=8) were stimulated in vitro with synthetic peptides pooled to represent 19 PRRSV proteins and IFN-γ secreting cells enumerated by ELISpot assay. Data are presented as the mean unstimulated-corrected IFN-γ spot forming cells (SFC)/10⁶ PBMC (triplicate cultures) for each animal and error bars show the SEM. Values for each peptide pool-stimulated condition were compared to the corresponding unstimulated control using a one-way ANOVA followed by a Dunnett’s Multiple Comparison Test; ***p<0.001, **p<0.01, *p<0.05.
Figure 3.12. Recognition of PRRSV-1 proteins by T cells from pigs experimentally infected with the divergent PRRSV SU1-Bel strain. PBMC from pigs experimentally infected with PRRSV-1 SU1-Bel (n=8) were stimulated in vitro with synthetic peptides pooled to represent the PRRSV proteins and IFN-γ secreting cells enumerated by ELISpot assay. Data are presented as the mean unstimulated-corrected IFN-γ spot forming cells (SFC)/10^6 PBMC (triplicate cultures) for each animal and error bars show the SEM. Values for each peptide pool-stimulated condition were compared to the corresponding unstimulated control using a one-way ANOVA followed by a Dunnett’s Multiple Comparison Test; ***p<0.001, **p<0.01, *p<0.05.
Figure 3.13. Assessment of the conservation of the predicted amino acid sequences of NSP5 between Lelystad, Olot/91 and SU1-Bel strains of PRRSV-1.

4.3. Discussion

The work presented in this chapter represents the first comprehensive approach to mapping of the specificity of the T cell response to PRRSV. Using T cells from pigs immune to PRRSV-1 Olot/91, it was possible to screen a proteome-wide synthetic peptide library to identify antigenic peptides recognised by CD4 or CD8 T cells. Screening of peptide pools representing 19 PRRSV proteins showed that antigenic peptides were scattered throughout the proteome and no one protein stood out that was recognised by all animals. An apparently immunodominant CD4 T cell epitope that was recognised by Pig 52 and 54 was readily defined on the M protein (M13-27), but no one immunodominant epitope was identified for Pig 53. Resolution of peptide pools was possible only through a two-step process of screening the individual peptides at a single concentration followed by titration of peptides to remove ‘false positive’ peptides. On reflection it may have been more effective to have screened the single peptides at a lower concentration to reduce the non-specific reactivity against a number of peptides. Peptides which showed significant reactivity following titration were then subjected to screening with...
PBMC depleted of either CD4$^+$ or CD8$\beta^+$ cells by magnetic sorting. This approach had the advantage that the cells utilised in the assay had not been labelled with magnetic beads. The resulting data showed that removal of either T cell population ablated the response to a particular peptide suggesting that the depleted population contained the peptide-specific T cells. An alternative strategy to magnetic sorting of T cell populations would be to use intracellular cytokine staining and flow cytometry, which would have the advantage of allowing assessment of multiple parameters. The antigenic sequences identified in this study showed no overlap with those previously mapped [Diaz et al, 2009, Vashisht et al, 2008, Bautista et al, 1999, Parida et al, 2012, Burgara-Estrella et al, 2013] with the exception of M$\_13-27$, which showed significant overlap with an antigenic peptide identified in a highly pathogenic PRRSV-2 strain [Wang et al, 2011]. The different MHC haplotypes of the animals studied most likely accounts for the multitude of antigens identified in different studies as it is known that the porcine MHC is highly polymorphic [Lunney et al, 2008]. Algorithms developed to predict peptide binding to human MHC molecules, SYFPEITHI [http://www.syfpeithi.de] and PropPred [www.imtech.res.in/raghava/propred], have been used to pre-screen PRRSV sequences for potential T cell epitopes [Diaz et al, 2009, Burgara-Estrella et al, 2013]. The NetMHCpan prediction server [www.cbs.dtu.dk/services/NetMHCpan/] has recently been expanded to predict peptide binding to a broad range of porcine MHC class I molecules and was used to successfully identify a CD8 T cell epitope on foot-and-mouth disease virus [Hoof et al, 2009, Patch et al, 2011]. To further test this immunoinformatics approach, we examined the ability of the NetMHCpan to predict the binding of the identified CD8 T cell antigenic peptides to the MHC class I alleles potentially expressed by the responder pigs. The consensus antigenic peptide
M\textsubscript{161-173} (RAVKRGVVNLVKY) was predicted to bind strongly to allele SLA-3*0401 and the RdRp\textsubscript{262-276} peptide was predicted to bind strongly to 2 alleles (SLA-1*0401 and SLA-1*0801). However, neither the NSP1\textsubscript{149-163} nor the RdRp\textsubscript{142-156} peptides were predicted to bind any of the potential alleles, suggesting that these algorithms would currently fail to predict all antigenic peptides from PRRSV.

While the peptide library approach was intended to be a comprehensive analysis of the antigen-specificity of PRRSV-specific T cells, it has recently come to light that PRRSV expresses an additional two proteins, ORF5a [Johnson et al, 2011] and NSP2TF [Fang et al, 2012], which are both expressed by a ribosomal frame shift mechanism. The ORF5a protein is of potential interest as it is a structural protein encoded by an alternative ORF within the immunogenic GP5 genome region, even though a non-protective non-neutralising antibody response was induced upon immunisation of pigs with the ORF5a protein [Robinson et al, 2013]. The NSP2TF is derived from an alternative coding region of NSP2, another immunogenic protein [Chen et al, 2010b]. Future analysis of PRRSV-specific T cell specificity should include both ORF5a and NSP2TF.

In conclusion, this study has shown that PRRSV infection induces a T cell response with a broad-range of antigen specificities. The in-depth study of the three Olot/91 inoculated pigs revealed CD8 T cell responses to the M protein, the viral polymerase and NSP1\textbeta, and CD4 T cell responses to NSP1\textbeta, NSP2, GP5 and M proteins. The results of the experiments described here validate the use of this proteome-wide screening approach for the comprehensive identification of PRRSV-specific T cell epitopes. Both M and GP5 have been described in the literature as vaccine candidate antigens, and their identification by this study serves to confirm
both the approach and the importance of these proteins in future vaccine development. However, the study also highlights the potential of non-structural proteins, most notably NSP1, NSP2 and NSP5, as additional vaccine candidate antigens. Further deployment of this proteome-wide library screening approach in future larger studies will help further identify, characterise and prioritise PRRSV T cell antigens to aid next generation vaccine development.
Chapter IV

Detailed characterization of the T cell responses to two major PRRSV antigens
Chapter 4. Detailed characterisation of the T cell response to two major PRRSV antigens

4.1. Rationale

The experiments described in Chapter 3 served as a starting point for the characterisation of the T cell responses to PRRSV utilising the synthetic peptide library approach. It is well known that both neutralising antibodies (NA) and T cell responses are vital for resolution of many viral infections, however in the case of PRRSV, there are varying and sometimes conflicting reports as to the contributions of each response. Studies have shown clearance of viral RNA in the absence of NA suggesting that T cell responses play an important part in protective immunity to PRRSV [Kim et al, 2007, Zuckermann et al, 2007]. A better understanding of the kinetics of infection and immunological correlates of protection may help tease apart the contribution of immune responses to PRRSV and subsequently inform rational vaccine design. Whilst not the only measurable indicator of T cell responses, IFN-\( \gamma \) is considered a suitable parameter to quantify virus-specific T cell responses, as well as being a potential correlate of protection from PRRSV [Lowe et al, 2005, Charerntantanakul et al, 2006a]. Chapter 3 described a broad range of IFN-\( \gamma \) reactivity to the PRRSV proteins, most notably the M protein, as well as the viral polymerase, NSPs 1, 2 and 5, and GP5; all of which have been previously identified in the literature [Wang et al, 2011, Diaz et al., 2009, Vashisht et al, 2008, Bautista et al., 1999, Parida et al, 2012, Burgara-Estrella, et al, 2013]. The objective of the study reported in this Chapter was to utilise the proteome-wide synthetic peptide screening method validated in Chapter 3 to further characterise T cell responses to PRRSV in terms of specificity, phenotype and function. To address this, both the
conventional Olot/91 and divergent pathogenic SU1-Bel PRRSV strains were used in an experimental infection and re-challenge model. T cell reactivity was monitored longitudinally and antigen reactivity assessed after each infection by screening of the PRRSV peptide library. Two antigens that were strongly recognised by both groups of animals were selected for further characterisation. In addition to defining and characterising antigenic peptides, flow cytometric antibody panels were deployed to quantitatively and qualitatively assess the antigen-specific T cell responses.

4.2. Results

4.2.1. Summary

Groups of pigs (n=5) were infected with either an attenuated PRRSV-1 subtype-1 strain (Olot/91) or a pathogenic subtype-3 strain (SU1-bel) and then challenged after 35 days with the homologous virus. PBMC were stimulated with homologous PRRSV ex vivo and IFN-γ responses assessed by ELISpot assay or multi-parameter flow cytometry. Virus-specific CD4 and CD8 T cell IFN-γ responses were detected from day 14 post-infection and peaked at 35 days post-infection. PBMC were stimulated ex vivo with pools of synthetic peptides representing 19 PRRSV proteins. In both groups, significant T cell reactivity was observed against the structural M protein and the non-structural protein 5 (NSP5), which were significantly boosted following the re-challenge infection, and these two antigens were selected for further study. CD4 T cell responses were identified in 3/10 animals and were directed against a single antigenic region on the M protein. CD8 T cell responses were directed against three antigenic regions on NSP5 and one
epitope on the M protein, with animals showing distinct reactivity profiles associated with specific MHC haplotypes. Further characterisation of IFN-γ producing M and NSP5-specific CD4 and CD8 T cells showed them to predominantly express the phenotype CD44\textsuperscript{high}CD62L\textsuperscript{low}CD27\textsuperscript{low}CD25\textsuperscript{−} which suggests a mixed population of effector and effector memory T cells. Significantly, many of these cells were dual-functional with co-expression of TNF-α and appeared to have a cytotoxic function as assessed by surface mobilisation of the degranulation marker CD107a. Overall, this data has shown that M and NSP5 represent well-conserved targets of primarily cytotoxic CD8 T cell responses from PRRSV-immune pigs and warrant further evaluation as vaccine candidate antigens.

4.2.2. Outcome of experimental infection and subsequent re-challenge with PRRSV-1 Olot/91 or SU1-Bel

Groups of 5 pigs were inoculated intranasally with 10\textsuperscript{6} TCID\textsubscript{50} of the attenuated PRRSV-I subtype 1 strain Olot/91 and 5 pigs were inoculated with a lower dose of 10\textsuperscript{4} TCID\textsubscript{50} of the divergent subtype 3 PRRSV strain with the aim of infecting animals but with a reduced likelihood of severe clinical disease. On day 35 post-infection, all animals were challenged by inoculation of the ten-fold higher dose of homologous virus that was used in the primary infection (Olot/91: 10\textsuperscript{7} TCID\textsubscript{50}; SU1-Bel: 10\textsuperscript{5} TCID\textsubscript{50}). As predicted both groups showed mild clinical scores throughout the experiment (Figure 4.1A), with the SU1-Bel group displaying greater clinical scores than the Olot/91 group, despite the lower inoculation dose (statistically significant on 2, 6, 7, 9, 10, 17 and 18 days post-infection; dpi). In the SU1-Bel group, clinical scores peaked around 7 dpi (becoming statistically different
to pre-infection scores from 2 dpi) and slightly later (between days 10-20 dpi) for the Olot/91 group (not statistically significant). Importantly there was no increase in clinical scores upon re-challenge at 35 dpi in the Olot/91 group and only mild clinical scores observed on 37 and 38 dpi in the SU1-Bel group. Quantitative RT-PCR analyses of serum samples showed low (statistically insignificant) viral copy numbers in the Olot/91 group, peaking on 14 dpi and resolving completely by 30 dpi (Figure 4.1B). Statistically significant viral copies were seen on 7 dpi in the SU1-Bel group but these were rapidly cleared by 14 dpi. There was also no detectable viraemia upon re-challenge, which taken together with the clinical scores suggests that the initial infection affords a high-degree of protection against re-infection.
Figure 4.1. Outcome of infection and subsequent re-challenge infection with an attenuated PRRSV-1 strain (Olot/91) or a virulent sub-genotype 3 strain (SU1-Bel). Pigs were experimentally infected intranasally with either PRRSV-1 Olot/91 (closed circles; n=5) or SU1-Bel (open circles; n=5) strains on day 0 and day 35 post infection (marked with ↓). Clinical signs were scored daily (A) and viral copy number in serum was assessed on days 0, 7, 14, 21, 30, 35, 42, 50 and 56 (B). Results are expressed as the mean data for each group, error bars represent the standard error of the mean (SEM) and values were compared to the corresponding value on day 0 using a two-way analysis of variance (ANOVA) followed by a Bonferroni’s multiple comparison test; **** p < 0.0001.
4.2.3. Association of neutralising antibody and T cell responses with clearance of PRRSV-1 infection and immunity to re-challenge

In the PRRSV-1 Olot/91 infected group, NA were detectable from 14 dpi and levels steadily increased to a statistically significant peak at 30 dpi, upon which levels began to wane (Figure 4.2A). However there appeared to be a boosting effect upon re-challenge at 35 dpi. Conversely in the PRRSV-1 SU1-Bel infected group, no neutralising antibody titres were measurable until after the re-challenge (42 dpi, Figure 4.2B) and the resolution of the viraemia associated with the primary infection.

Assessment of PRRSV-specific T cell IFN-γ responses was conducted by flow cytometry following ex vivo stimulation of PBMC with the homologous virus. Both groups showed a higher magnitude of IFN-γ expressing CD4 (CD4⁺CD8αlow) T cells in response to homologous virus stimulation than CD8 (CD4⁻CD8αhigh) T cells (Figure 4.2 C and D). The Olot/91 group showed an increase in CD4 T cell responses from 14 dpi, and compared to 0 dpi these responses were statistically significant (p=0.05) from 30 dpi onwards and began to wane by 56 dpi. The SU1-Bel group displayed a higher overall magnitude of PRRSV-specific CD4 T cell responses (statistically significant on 21 dpi; p = 0.0001) which were statistically significant (p = 0.01) by 14 dpi, reaching a peak at 21 dpi (p = 0.0001). There was a slight rise in IFN-γ CD4 T cell responses after re-challenge (statistically significant at 50 dpi; p = 0.01). There were no statistically significant increases over time in CD8 T cell responses in either group. Analysis of T cell IFN-γ responses of individual animals to homologous virus confirmed the finding that the response measured was dominated by CD4 T cells in all animals (Figure 4.3). Statistically significant CD4 T cell responses were seen as early as 14 dpi (Olot/91 infected pig 87; p = 0.0001, and SU1-Bel infected pigs 72, 73, 88, 89 and 01; p ≤ 0.05) and

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reached a peak at various different time points post-infection (30 dpi - pigs 70, 71 and 86; 35 dpi - pigs 00 and 73). Some animals showed evidence of boosting of T cell IFN-γ responses after re-infection (SU1-Bel group: pigs 72, 88 and 01). Certain animals also displayed a statistically significant CD8 T cell IFN-γ response (Olot/91 group: pigs 70; days 14, 35, 42 and 51 dpi; p ≤ 0.05, and pig 87; days 14 and 51; p ≤ 0.01). There were no statistically significant CD8 T cell responses to virus re-stimulation in any animal from the SU1-Bel infected group.
Figure 4.2. Assessment of the association between neutralising antibody and T cell responses and the clearance of PRRSV viraemia following experimental infection and re-challenge with Olot/91 and SU1-Bel. Serum was collected weekly from pigs and neutralising antibody (NA) titres were assessed by testing the ability of the serum to neutralise homologous virus using an in house serum neutralisation assay (A). PRRSV NA titre is plotted against the left y-axis and the PRRSV viraemia as assessed by qRT-PCR is plotted against the right y-axis. PBMC were collected weekly from pigs and stimulated ex vivo with the homologous virus or a mock supernatant. IFN-γ expression by CD4⁺CD8αlow (CD4) and CD4⁺CD8αhigh (CD8) T cells was assessed by flow cytometry using the gating strategy detailed in Figure 2.1 (B). The mean % of mock-corrected IFN-γ⁺ T cells is plotted against the left y-axis and PRRSV viraemia data are again plotted on the right y-axis. Data is presented as the mean for each group and error bars show the SEM. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.3. Assessment of the longitudinal PRRSV-specific IFN-γ T cell responses over the course of infection and re-challenge with Olot/91 and SU1-Bel strains of PRRSV-1. PBMC were collected weekly from pigs and stimulated ex vivo with the homologous virus or a mock supernatant. IFN-γ expression by CD4⁺CD8αlow (CD4; open circles) and CD4⁺CD8αhigh (CD8; closed circles) T cells was assessed by flow cytometry. The mean % of mock-corrected IFN-γ⁺ T cells from duplicate cultures are presented and error bars show the SEM. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
4.2.4. Screening of the PRRSV-1 proteome-wide synthetic peptide library to identify T cell antigens

The synthetic peptide library composed of overlapping 15mers spanning the PRRSV-1 proteome were pooled to represent their respective proteins and their recognition by PBMC were assessed using an ex vivo IFN-γ ELISpot assay, as in Chapter 3. Figures 4.4 and 4.5 show the IFN-γ responses to each protein after infection (21 dpi) and re-challenge (51 dpi and 21 days post-re-challenge), for the Olot/91 and SU1-Bel groups, respectively. Pigs in the Olot/91 group displayed in general a greater magnitude of response to peptide pools after re-challenge as well as an increase in the number of pigs mounting significant responses to certain proteins (Figure 4.4). The most prominent response was to the M protein, which all pigs showed a statistically significant response to after both infection and re-challenge. Peptides representing NSP5 also induced significant IFN-γ responses, with 80% (4/5) of pigs responding after infection and all pigs then responding after re-challenge. Other proteins of note especially given their identification in the studies reported in Chapter 3 were NSP2 (80% responding after infection and after re-challenge), RdRp (40% then 40%), helicase (60% then 60%) and GP5 (40% then 60%).

Similar to the Olot/91 group, the SU1-Bel group also displayed greater responses after re-challenge (Figure 4.5). The magnitude of the response to the NSP5 after re-challenge was the most striking observation in all animals except pig 89 which did not react to NSP5 peptides. Interestingly, pig 88 did not mount a significant response to NSP5 after infection but this pig’s response to NSP5 after re-challenge infection had the greatest magnitude of all pigs in this group. In addition to
significant responses to NSP5 after both infections (40% responding after infection; 80% responding after re-challenge infection), significant responses of note were also observed against NSP2 (80% then 60%), GP5 (40% then 60%) and the M protein (80% then 100%).

Considering both groups together, the frequency of T cell IFN-γ responder animals to the most dominant antigens after both infection and re-challenge were: M protein - 90% responding after infection and 100% responding after re-challenge; NSP5 - 60% responding after infection and 90% responding after re-challenge; NSP2 - 80% responding after infection and 70% responding after re-challenge; and GP5 - 40% responding after infection and 60% responding after re-challenge.
Figure 4.4. Recognition of PRRSV-1 proteins by T cells from pigs experimentally infected with the Olot/91 strain. PBMC from pigs experimentally infected with PRRSV-1 Olot/91 (n = 5) were isolated on day 21 post-infection and day 51 post-infection (16 days post-rechallenge), and stimulated in vitro with synthetic peptides pooled to represent 19 PRRSV-1 proteins. IFN-γ secreting cells were enumerated by ELISpot assay. Data are presented as the mean unstimulated-corrected IFN-γ spot forming cells (SFC)/5x10^6 PBMC (triplicate cultures) for each animal and error bars show the SEM. Values for each peptide pool-stimulated condition were compared to the corresponding unstimulated control using a one-way ANOVA followed by a Bonferroni’s multiple comparison test; **** p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.
Figure 4.5. Recognition of PRRSV-1 proteins by T cells from pigs experimentally infected with the SU1-Bel strain. PBMC from pigs experimentally infected with PRRSV-1 SU1-Bel \( (n = 5) \) were isolated on day 21 post-infection and day 51 post-infection (16 days post-rechallenge), and stimulated in vitro with synthetic peptides pooled to represent 19 PRRSV-1 proteins. IFN-\( \gamma \) secreting cells were enumerated by ELISpot assay. Data are presented as the mean unstimulated-corrected IFN-\( \gamma \) spot forming cells (SFC)/5x10^6 PBMC (triplicate cultures) for each animal and error bars show the SEM. Values for each peptide pool-stimulated condition were compared to the corresponding unstimulated control using a one-way ANOVA followed by a Bonferroni’s multiple comparison test; **** \( p < 0.0001 \), *** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \).
Whilst IFN-γ is a good indicator of anti-viral type 1 T cell (Th1) responses, there are other cytokines that could provide additional information on the responses induced by each viral protein. IL-2 is another marker of the type 1 response, inducing proliferation and maintenance of antigen specific T cells whose expression is associated with the presence of polyfunctional T cells. IL-10 is a pleiotropic cytokine that can have immunosuppressive effects on type 1 T cells. IL-4 is an autoregulatory cytokine which drives the type 2 CD4 T cell (Th2) response and inhibits the induction of the Th1 response. To assess the ability of peptides representing PRRSV proteins to induce these cytokines, ELISAs were performed on culture supernatants of PBMC stimulated with the peptide pools after re-challenge as above (Figure 4.6). One pig was chosen as a representative of each group and cell free supernatants were assessed for presence of IL-2, IL-4 and IL-10 (IFN-γ ELISpot data is shown in the top panel for direct comparison). IL-2 and IL4 expression was low in both pigs, but statistically significant IL-2 responses were detected against NSP2 (SU1-Bel infected pig 01) and NSP4 (Olot/91 infected pig 86) and IL-4 responses to NSP3 and E (pig 01) and GP2 and E (pig 86). Pig 01 mounted a significant IL-10 response to more viral proteins (NSP2, RdRp and helicase) than pig 86 whose only significant IL-10 response was to RdRp. Based on these results and the results of the IFN-γ ELISpot it was decided to take the M protein and NSP5 forward for further analysis due to their ability to induce consistent and/or high levels of IFN-γ in a large number of animals from both groups. NSP2 was considered as another potential T cell antigen however its comparatively large size (269 15mer peptides), coupled with the induction of IL-10 seen above and the hypervariable regions and deletions described in different strains made it less attractive for further testing and evaluation as a vaccine candidate antigen.
Figure 4.6. Cytokine secretion by PBMC upon stimulation with PRRSV-1 protein pools. PBMC were isolated from two representative pigs; one experimentally infected with Olot/91 (pig 86) and one experimentally infected with SU1-Bel (pig 01) and stimulated ex vivo with synthetic peptides pooled to represent 19 PRRSV-1 proteins. IFN-γ secreting cells were enumerated by ELISpot assay. Levels of IL-2, IL-4 and IL-10 in cell supernatants after 24 hours were measured using commercial ELISA kits. Data shown is the mean unstimulated mean corrected values for triplicate cultures and error bars show the SEM. Values were compared to the unstimulated control using a one way ANOVA followed by a Bonferroni’s multiple comparisons test; **** p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.
4.2.5. In-depth characterisation of the IFN-γ T cell response to PRRSV-1 M and NSP5 proteins

4.2.5.1. Longitudinal assessment of M and NSP5-specific T cell responses over the course of PRRSV-1 infection and re-challenge

The T cell responses to peptide pools representing M and NSP5 proteins were measured longitudinally over the time-course of infection/re-challenge using previously cryopreserved cells and flow cytometry. The IFN-γ response to the M protein differed significantly between individual animals both in terms of magnitude and phenotype (Figure 4.7). The frequency of M-specific IFN-γ secreting CD8 T cells observed in pigs 86, 87 and 00, all infected with Olot/91, were of an order of magnitude greater than the other animals and are therefore presented with a different y-axis scaling (Figure 4.7). These 3 animals all mounted a significant CD8 T cell response to the M protein, as did the remaining animals in the Olot/91 group; pigs 71 and 70. These animals however also mounted a CD4 T cell response greater in magnitude than their CD8 T cell response. Within the SU1-Bel group, pig 72 also mounted a CD4 T cell response to the M protein. Pigs 73, 89 and 01 did not mount a significant response to the M protein at any time point tested with the preserved cells. The earliest statistically significant T cell response was seen on 7 dpi in pigs 71 (CD8) and 72 (CD4) and significant responses continued until 51 dpi (pig 70 and 72 - CD4; pig 87 and 00 - CD8). Unexpectedly, no boosting of T cell responses was observed subsequent to re-challenge infection, with the exception of pig 72 which showed a slight increase in the proportion of IFN-γ secreting CD4 T cells.

As with the response to the M protein, there was a variation in the magnitude and kinetics of T cell IFN-γ responses to the NSP5 protein between individual
animals (Figure 4.8). Compared to M-specific responses, the IFN-γ response to NSP5 was in the majority of animals greater and in all cases was exclusively from the CD8 T cells. As had been shown in the IFN-γ ELISpot assay data, all pigs responded to NSP5 with the exception of pig 89. Pigs 70, 71 and 86 (all infected with Olot/91) presented with exceptionally strong responses to NSP5, peaking at a frequency of 1:10 CD8 T cells expressing IFN-γ in response to NSP5. As before, these animals are shown with a different y-axis scaling in Figure 4.8. Responses tended to peak between days 21-35 after infection and there was no evidence of boosting after re-infection.
Figure 4.7. Assessment of the longitudinal M protein-specific IFN-γ T cell responses over the course of infection and re-challenge with Olot/91 and SU1-Bel strains of PRRSV-1. PBMC were collected weekly from pigs and stimulated *ex vivo* with a synthetic peptide pool representing the M protein or medium alone. IFN-γ expression by CD4^+^CD8α^low^ (CD4; open circles) and CD4^+^CD8α^high^ (CD8; closed circles) T cells was assessed by flow cytometry. The mean % of unstimulated-corrected IFN-γ^+^ T cells from duplicate cultures are presented and error bars show the SEM. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.8. Assessment of the longitudinal NSP5-specific IFN-γ T cell responses over the course of infection and re-challenge with Olot/91 and SU1-Bel strains of PRRSV-1. PBMC were collected weekly from pigs and stimulated *ex vivo* with a synthetic peptide pool representing NSP5 or medium alone. IFN-γ expression by CD4\(^+\)CD8\(_{\text{low}}\) (CD4; open circles) and CD4\(^+\)CD8\(_{\text{high}}\) (CD8; closed circles) T cells was assessed by flow cytometry. The mean % of unstimulated-corrected IFN-γ\(^+\) T cells from duplicate cultures are presented and error bars show the SEM. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
4.2.5.3. Assessment of the phenotype and polyfunctionality of PRRSV-1 M and NSP5-specific T cell responses

PBMC isolated from 30 dpi were stimulated with either, the homologous virus, M or NSP5 peptide pools in order to determine the specific phenotype of antigen-specific T cells. After stimulation, cells were stained with the surface markers CD4, CD8α, CD62L; also known as L-selectin, a lymph node homing receptor typically expressed on naïve T cells that have not yet encountered antigen; CD44; a lymphocyte adhesion and homing molecule; CD25; the receptor for IL-2 and CD27; part of the TNF receptor superfamily. Antigen specific co-expression of IFN-γ and each marker was assessed after exclusion of non-responding pigs based on Figures 4.7 and 4.8. Statistically significant phenotypes were identified through the use of a two-way ANOVA with Bonferroni’s post hoc test. Figures 4.9 and 4.10 show that the majority of IFN-γ positive cells express high levels of CD44 and low levels of CD62L, regardless of CD4/CD8 phenotype or stimulus (with the exception of virus specific CD8 T cells which show equal proportions of both CD44^{high} and CD44^{low} cells, possibly due to the very low frequency of stimulated IFN-γ expressing CD8 T cells. IFN-γ expressing T cells also predominantly showed a CD27^{low} phenotype, with the majority of the remaining IFN-γ^{+} T cells falling into the CD27^{-} gate (Figure 4.11). Antigen-specific T cells were either CD25^{+} or IFNγ^{+} with the exception of M-specific CD8 and virus-specific CD4 T cells which showed small double positive populations (Figure 4.12). T cells that were positive for the cytotoxic degranulation marker CD107a were also positive for IFN-γ and the highest proportions were seen in the M and NSP5 stimulated T cells, whereas virus
stimulated cells showed a higher proportion of both CD4 and CD8 IFN-γ single positive cells (Figure 4.13). M specific CD4 and NSP5-specific CD8 T cells showed higher proportions of IFN-γ⁺TNF-α⁺ (double positive) cells, whereas M specific CD8 T cells showed more TFN-α single positive T cells (Figure 4.14). Both virus specific CD4 and CD8 T cells had higher proportions of IFN-γ single positive cells although all antigen specific cells had significant proportions of double positive T cells.
**Figure 4.9. Assessment of the expression of CD44 and IFN-γ on antigen-specific T cells.** PBMC were isolated from previously identified T cell responder pigs on day 30 post-infection and stimulated with either synthetic peptides representing M or NSP5 proteins or the homologous virus. The expression of CD44 on IFN-γ+ CD4 and CD8 T cells was assessed by flow cytometry as shown by representative dot plots. The mean % of unstimulated-corrected CD44^{high} and CD44^{low} IFN-γ+ T cells from duplicate cultures are presented for individual animals, and error bars show the SEM. The mean relative proportions of CD44 expression on IFN-γ+ T cells from all animals are additionally presented. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.10. Assessment of the expression of CD62L and IFN-γ on antigen-specific T cells. PBMC were isolated from previously identified T cell responder pigs on day 30 post-infection and stimulated with either synthetic peptides representing M or NSP5 proteins or the homologous virus. The expression of CD62L on IFN-γ⁺ CD4 and CD8 T cells was assessed by flow cytometry as shown by representative dot plots. The mean % of unstimulated-corrected CD62L_{high} and CD62L_{low} IFN-γ⁺ T cells from duplicate cultures are presented for individual animals and error bars show the SEM. The mean relative proportions of CD62L expression on IFN-γ⁺ T cells from all animals are additionally presented. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.11. Assessment of the expression of CD27 and IFN-γ on antigen-specific T cells. PBMC were isolated from previously identified T cell responder pigs on day 30 post-infection and stimulated with either synthetic peptides representing M or NSP5 proteins or the homologous virus. The expression of CD27 on IFN-γ⁺ CD4 and CD8 T cells was assessed by flow cytometry as shown by representative dot plots. The mean % of unstimulated-corrected CD27high, CD27low and CD27⁻ IFN-γ⁺ T cells from duplicate cultures are presented for individual animals and error bars show the SEM. The mean relative proportions of CD27 expression on IFN-γ⁺ T cells from all animals are additionally presented. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.12. Assessment of the expression of CD25 and IFN-γ by antigen-specific T cells. PBMC were isolated from previously identified T cell responder pigs on day 30 post-infection and stimulated with either synthetic peptides representing M or NSP5 proteins or the homologous virus. The expression of CD25 and IFN-γ by CD4 and CD8 T cells was assessed by flow cytometry as shown by representative dot plots. The mean % of unstimulated-corrected CD25^{IFN-γ+}, CD25^{IFN-γ+} and CD25^{IFN-γ-} T cells from duplicate cultures are presented for individual animals and error bars show the SEM. The mean relative proportions of CD25 and IFN-γ expressing T cells from all animals are additionally presented. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.13. Assessment of CD107a mobilisation on IFN-γ expressing antigen-specific T cells. PBMC were isolated from previously identified T cell responder pigs on day 30 post-infection and stimulated with either synthetic peptides representing M or NSP5 proteins or the homologous virus. The surface expression of CD107a on IFN-γ+ CD4 and CD8 T cells was assessed by flow cytometry as shown by representative dot plots. The mean % of unstimulated-corrected CD107a+ and CD107a- IFN-γ+ T cells from duplicate cultures are presented for individual animals and error bars show the SEM. The mean relative proportion of surface CD107a on IFN-γ+ T cells from all animals is additionally presented. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
Figure 4.14. Assessment of co-expression of TNF-α and IFN-γ by antigen-specific T cells. PBMC were isolated from previously identified T cell responder pigs on day 30 post-infection and stimulated with either synthetic peptides representing M or NSP5 proteins or the homologous virus. The expression of TNF-α and IFN-γ by CD4 and CD8 T cells was assessed by flow cytometry as shown by representative dot plots. The mean % of unstimulated-corrected TNF-α+IFN-γ+, TNF-α+IFN-γ+ and TNF-α+IFN-γ+ T cells from duplicate cultures are presented for individual animals and error bars show the SEM. The mean relative proportions of TNF-α and IFN-γ expressing T cells from all animals are additionally presented. Cells used in this experiment were previously cryopreserved and subsequently thawed for analysis.
4.2.5.4. Mapping of T cell antigenic peptides and epitopes on PRRSV-1 M and NSP5 proteins

Identification of the antigenic peptide targets of M and NSP5 specific T cell responses was assessed using a two-way matrix pooling system to screen the individual 15mers. The matrices were designed horizontally vs. vertically so that each peptide was uniquely present in 2 defined pools. An illustration of how peptides were combined into matrix pools and how the CD8 T cell IFN-γ responses to these pools enabled the identification of putative antigenic peptides is shown in Table 4.1, using CD8 T cell responses of pig 86 to NSP5 peptides as an example. The results of the matrix pool screens are shown in Tables 4.2 and 4.3. PBMC from 21 or 30 dpi were stimulated with each matrix pool and IFN-γ responses were measured by flow cytometry. As observed before, the M matrix pools gave a diverse response profile and pools that induced a significant IFN-γ response and their corresponding putative antigenic peptides are summarised in Table 4.2.
Table 4.1 Example of the two-way matrix-pooling system used to identify T cell reactive peptides from pools representing PRRSV-1 M and NSP5 proteins.

<table>
<thead>
<tr>
<th>NSP5 Matrix Pools</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
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<td>2</td>
<td>3</td>
<td>4</td>
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</tr>
<tr>
<td>I</td>
<td>13</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>J</td>
<td>19</td>
<td>20</td>
<td>21</td>
<td>22</td>
<td>23</td>
<td>24</td>
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<tr>
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<tr>
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<td>37</td>
<td>38</td>
<td>39</td>
<td>40</td>
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</tr>
</tbody>
</table>

*The 40 overlapping 15mer peptides (#1-40) representing PRRSV NSP5 were pooled using a matrix system so that each peptide was uniquely represented in two matrix-pools (Pools A-M).

*Matrix pools A, B and M stimulated significant CD8 T cell reactivity from pig 86 and so overlapping peptides #37 and #38 were identified as putative antigenic peptides for further testing.
Table 4.2. Summary of the M matrix pools that induced a significant IFN-γ T cell response and the corresponding putative antigenic peptides.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Positive matrix pool</th>
<th>Corresponding antigenic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>A B C H</td>
<td>7 8 9</td>
</tr>
<tr>
<td>71</td>
<td>A B C H</td>
<td>7 8 9</td>
</tr>
<tr>
<td>86</td>
<td>A B E F I J L</td>
<td>13 14 17 18 19 20 23 24 31 32 35 36</td>
</tr>
<tr>
<td>87</td>
<td>A B E F I J L</td>
<td>13 14 17 18 19 20 23 24 31 32 35 36</td>
</tr>
<tr>
<td>00</td>
<td>A B E F I J L</td>
<td>13 14 17 18 19 20 23 24 31 32 35 36</td>
</tr>
<tr>
<td>72</td>
<td>A B C F H J</td>
<td>7 8 9 12 19 20 24</td>
</tr>
<tr>
<td>73</td>
<td>A B E I J</td>
<td>13 14 17 19 20 23</td>
</tr>
<tr>
<td>88</td>
<td>A B H</td>
<td>7 8</td>
</tr>
<tr>
<td>89</td>
<td>E F I</td>
<td>17 18</td>
</tr>
<tr>
<td>01</td>
<td>A B D F H I J</td>
<td>7 8 10 12 13 14 16 18 19 20 22 24</td>
</tr>
</tbody>
</table>
Table 4.3. Summary of the NSP5 matrix pools that induced a significant CD8 IFN-γ T cell response and the corresponding putative antigenic peptides.

<table>
<thead>
<tr>
<th>Pig</th>
<th>Positive matrix pool</th>
<th>Corresponding antigenic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>C D E F G M</td>
<td>3 4 5 6 39 40</td>
</tr>
<tr>
<td>71</td>
<td>C D E G M</td>
<td>3 4 5 39 40</td>
</tr>
<tr>
<td>86</td>
<td>A B M</td>
<td>37 38</td>
</tr>
<tr>
<td>87</td>
<td>A B M</td>
<td>37 38</td>
</tr>
<tr>
<td>00</td>
<td>A B M</td>
<td>37 38</td>
</tr>
<tr>
<td>72</td>
<td>C D G M</td>
<td>3 4 39 40</td>
</tr>
<tr>
<td>73</td>
<td>C D M</td>
<td>39 40</td>
</tr>
<tr>
<td>88</td>
<td>A C D G M</td>
<td>1 3 4 37 39 40</td>
</tr>
<tr>
<td>89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>01</td>
<td>C D M</td>
<td>39 40</td>
</tr>
</tbody>
</table>

CD8 T cell responses to NSP5 were either to pools A, B and M (pigs 86, 87 and 00) or pools C, D and M (pigs 70, 71, 72, 73, 88 and 01) which corresponded to putative over-lapping antigenic peptides 37 and 38 (pools A, B, M) or 39 and 40 (pools C, D, M). In addition, pigs 70, 71 and 88 responded to pools E and G, adding the putative antigenic peptides 3, 4 and 5 to their profile (pig 70 also responded to pool F; peptide 6). As expected pig 89 did not mount a significant response to any NSP5 matrix pools.

Putative antigenic peptides were then screened individually on PBMC from each pig to confirm their ability to induce a T cell IFN-γ response. Responses to the M peptides was from both CD4 and CD8 T cells, with pigs 70, 71, 72 and 88
presenting a significant CD4 T cell response to peptide #8; M\textsubscript{29-43} (MIYALKVSRGRLLGL). Pig 71 also responded to peptide #9; M\textsubscript{33-47} (LKVSRRGRLLGLHIL). Pigs 86 and 87 gave a CD8 T cell response to peptide 13; M\textsubscript{49-63} (FLNCSFTFGYMTYVR), (Figure 4.15). As expected, responses to NSP5 were exclusively from CD8 T cells (Figure 4.16). Pigs 70, 71 and 88 showed a statistically significant response to peptide 4; NSP5\textsubscript{13-27} (FLLWRMMGHAWTPIV).

In addition, these pigs, as well as pig 72, 73, and pig 01, showed a significant response to peptides 39 and 40; consensus sequence: NSP5\textsubscript{156-167} (DGSFSSAFLRY). Pigs 86, 87 and 00 responded to peptides 37 and 38; consensus sequence: NSP5\textsubscript{149-159} (LHNMLVGDGSF) (Figure 4.16). A summary of identified antigenic peptides with their amino acid position and sequences is shown in Table 4.4.
Figure 4.15. Assessment of T cell reactivity against putative antigenic peptides from the M protein. PBMC were isolated from pigs on day 58 post infection and stimulated ex vivo with putative antigenic peptides identified from the matrix pool screen and IFN-γ expression by either CD4 (open bars) or CD8 (closed bars) T cells was assessed by flow cytometry. Data shown are the mean of duplicate cultures after correction with the unstimulated mean and error bars show the SEM. Values were compared to the unstimulated control using a one way ANOVA followed by a Bonferroni’s multiple comparisons test; **** p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.
Figure 4.16. Assessment of T cell reactivity against putative antigenic peptides from NSP5. PBMC were isolated from pigs on day 58 post infection and stimulated ex vivo with putative antigenic peptides identified from the matrix pool screen and IFN-γ expression by CD8 T cells was assessed by flow cytometry. Data shown are the mean of duplicate cultures after correction with the unstimulated mean and error bars show the SEM. Values were compared to the unstimulated control using a one way ANOVA followed by a Bonferroni’s multiple comparisons test; **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. 
Table 4.4. Summary of antigenic peptides identified on M and NSP5, with the corresponding amino acid position and sequence.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide #</th>
<th>Peptide position on protein</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>8</td>
<td>29-43</td>
<td>MIYALKVSRRGLGLLGL</td>
</tr>
<tr>
<td>M</td>
<td>9</td>
<td>33-47</td>
<td>LKVSRRGLLGLLHIL</td>
</tr>
<tr>
<td>M</td>
<td>13</td>
<td>49-63</td>
<td>FLNCSFTFGYMTYVR</td>
</tr>
<tr>
<td>NSP5</td>
<td>4</td>
<td>13-27</td>
<td>FLLWRMMGHAWTPIV</td>
</tr>
<tr>
<td>NSP5</td>
<td>37</td>
<td>145-159</td>
<td>KYRCLHNMLVGDGSF</td>
</tr>
<tr>
<td>NSP5</td>
<td>38</td>
<td>149-163</td>
<td>LHNMLVGDGSFSSAF</td>
</tr>
<tr>
<td>NSP5</td>
<td>39</td>
<td>153-167</td>
<td>LVGDGSFSSAFFLRY</td>
</tr>
<tr>
<td>NSP5</td>
<td>40</td>
<td>156-170</td>
<td>DGSFSSAFFLRYFAE</td>
</tr>
</tbody>
</table>

The minimal length peptides, which most likely represent the natural epitopes required to induce an IFN-γ response from immunodominant NSP5-specific CD8 T cells were investigated. The 11mer consensus sequences of the overlapping antigenic peptides from NSP5 (NSP5156-167 - DGSFSSAFFLRY and NSP5145-159 - LHNMLVGDGSF) and all of the possible derived 10mer, 9mer and 8mer sequences were synthesised (Table 4.5) and titrations were used to stimulate PBMC from pig 71 and 86, as representative animals, based on their previous response profiles (Figure 4.17). Of the peptides representing the NSP5 39/40 consensus sequence (NSP5156-167 - DGSFSSAFFLRY), the 8mer peptide GSFSSAFF induced the most sustained CD8 T cell IFN-γ response suggestive of it being the minimal length antigenic peptide (Figure 4.17). The shortest derivative of NSP5 37/38 consensus peptide (NSP5145-159 – LHNMLVGDGSF) to induce the most sustained CD8 T cell response was the 10mer HNMLVGDGSF (Figure 4.17). Due to practical limitations this analysis was not attempted for the other antigenic regions on NSP5 or M protein.
Table 4.5 Minimal length peptides from NSP5 37/38 and NSP5 39/40 tested on PBMC from PRRSV-immune pigs.

<table>
<thead>
<tr>
<th>NSP5 37/38</th>
<th>Amino acid position</th>
<th>Amino acid sequence</th>
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<tbody>
<tr>
<td></td>
<td>145-159</td>
<td>KYRCLHNMLVGDGSF</td>
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<td>149-158</td>
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<th>Amino acid position</th>
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</tr>
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<td>160-167</td>
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Identification of minimal length peptides that are recognised by NSP5 specific CD8 cells. PBMC from pigs 71 and 86 were stimulated *ex vivo* with selected antigenic NSP5 overlapping peptides and peptides representing the consensus 11mer sequence and their truncated derivatives in a log_{10} dilution series. IFN-γ expression by CD8 T cells was assessed by flow cytometry. The mean % of unstimulated-corrected IFN-γ⁺ T cells from duplicate cultures is presented and error bars show the SEM. For clarity, peptides that failed to evoke a significant response are not presented on the plots.
4.2.5.5. Assessment of sequence conservation of identified antigenic M and NSP5 regions amongst PRRSV strains and T cell recognition of variant peptide sequences

The level of antigenic amino acid sequence conservation both within genotype 1 PRRSV strains and with the prototype genotype 2 virus was investigated, as in Chapter 3, to help further evaluate the identified antigens for potential vaccine application. M and NSP5 sequences used for comparison are shown in Table 4.4; identified variant sequences were then synthesised and tested for their ability to induce T cell responses in PBMC from representative pigs 86 and 71 as before (Figure 4.18). The CD4 T cell antigenic region of M was well conserved with only 1 variant, a related substitution of a histidine for an arginine at position 37, which still induced CD4 T cell responses (although not deemed statistically significant) (Figure 4.18). The NSP5\textsubscript{13-27} antigenic region had 4 potential amino acid substitutions, all but one of which still induced a statistically significant CD8 T cell IFN-γ response. The individual 15mers that made up NSP5\textsubscript{145-159} were less well conserved however; all but one of their 6 variants induced a statistically significant IFN-γ response (although 4 of these lay outside the consensus sequence of the two peptides and therefore probably had no effect on the antigenic region). Whilst there were only 2 amino acid substitutions in the NSP5\textsubscript{153-170} region, these removed the peptides ability to induce an IFN-γ response (Figure 4.18). This is encouraging as clearly some variants retained their ability to induce an IFN-γ response, but also in some cases confirms that the antigenic region lies in the consensus sequence of two overlapping peptides.
Table 4.6. Assessment of the conservation of identified T cell antigenic regions among different PRRSV-1 isolates and the prototype PRRSV-2 strain. The overlapping antigenic peptides NSP5 37-40 (NSP5_{149-170}) are shown as one antigenic region.

<table>
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<th>NSP5_{149-170}</th>
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135
Figure 4.18. Effect of amino acid substitutions in the sequences of identified M and NSP5 antigenic peptides on T cell reactivity. Variant peptides were designed and synthesised based on the amino acid substitutions observed in different PRRSV isolates (Table 4.4). PBMC were isolated from pigs 71 and 86 and stimulated ex vivo with selected original and variant peptides. IFN-γ expression by CD4 T cells to M29-43 (pig 71) and CD8 T cells to NSP513-27 (pig 71), NSP5145-159 (pig 86) and NSP5153-170 (pig 71) peptides were assessed by flow cytometry. Data shown are the mean of duplicate cultures after correction with the unstimulated mean and error bars show the SEM. Values were compared to the unstimulated control using a one way ANOVA followed by a Bonferroni’s multiple comparisons test; **** p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.
4.2.5.6. Potential association of M and NSP5 specific T cell responses with defined MHC haplotypes.

As in Chapter 3, animals were MHC typed in order to determine whether T cell responses to identified peptides could be attributed to specific MHC class I and II haplotypes (Tables 4.5 and 4.6). With regards to the MHC (SLA) class I, pigs 70, 71, 72 and 88 were haploidentical heterozygotes, expressing haplotypes Lr-22.0 and Lr-35.0. Pig 86, 87 and 89 also shared the haplotype Lr-35.0 with the other animals, as well as haplotype Lr-38.0 between themselves. This could suggest that the antigenic region NSP5_{156-167} recognised by pigs 70, 71, 72, 73, 88 and 01 is restricted by the SLA-I haplotype Lr-22.0. It is not possible to suggest a specific haplotype restriction for the antigenic region NSP5_{145-159} as the responding pig 00 does not share either of its haplotypes with any of the other responding pigs. However, it could be speculated that this region is restricted by more than one haplotype, potentially Lr-38.0 (taking into account the non-responding pig 89) and Lr-35.0 (in the absence of Lr-22.0). The antigenic region NSP5_{13-27} could potentially be recognised by multiple haplotypes. Only pigs 86 and 87 respond to the peptide M_{49-63} and this could therefore be restricted by the Lr-38.0 (again taking into account the non-responding pig 89). The pigs that responded to the CD4 T cell antigenic peptide M_{29-43} (pigs 70, 71, 72 and 88) were haploidentical, sharing both SLA-II haplotypes Lr-0.01 and 0.15b, this combination appearing exclusively in these animals suggesting restriction by one of these two haplotypes.
Table 4.7. Porcine MHC (SLA) class I low-resolution (Lr) haplotypes of PRRSV infected pigs and CD8 T cell reactivity against identified antigenic peptides.

<table>
<thead>
<tr>
<th>Pig</th>
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Table 4.8. Porcine MHC (SLA) class II low-resolution (Lr) haplotypes of PRRSV infected pigs and CD4 T cell reactivity against identified antigenic peptides.

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4.3 Discussion

IFN-γ secreting T cell responses appear important, if not essential for resolution of viraemia, in accordance with the published literature [Zuckermann et al, 2007]. This study showed, at least in the context of PRRSV SU1-Bel infection, that viraemia was cleared before induction of neutralising antibodies suggesting that T cell responses could be sufficient to resolve PRRSV infection in certain strains. This data supports the results of an earlier study that assessed the immune responses induced by infection with SU1-Bel [Morgan et al, 2013]. The ability of PRRSV infection to prime both CD4 and CD8 T cells was in accordance with the literature, also agreeing with the observed stronger CD4 T cell response following in vitro stimulation with PRRSV [Piras et al, 2005]. However, comparison with the frequencies of PRRSV peptide specific CD4 and CD8 T cells revealed that assessment of PRRSV-specific CD8 T cell responses by stimulation with live virus greatly underestimates the true response. It may be speculated that a low level of productive PRRSV infection occurs within PBMC and this limits the availability of NSPs and trafficking of PRRSV peptides into the ‘direct’ or cytosolic MHC class I processing and presentation pathway.

Strong T cell responses were directed against the M and NSP5 proteins in pigs infected with both Olot/91 and SU1-Bel strains. These proteins have been identified previously in the literature [Wang et al, 2011, Bautista et al, 1999, Burgara-Estrella et al, 2013] but the phenotype of the responding T cells had not yet been defined. Indeed, the ability of NSP5 to induce IFN-γ has only been reported in one other study to date, and none of the identified antigenic peptides matched those identified here [Burgara-Estrella et al, 2013]. The antigenic region ‘M6’ identified as
a T cell epitope of a highly pathogenic PRRSV-2 strain by Wang and colleagues [2011] contains the peptide M$_{29}$-$M_{43}$ identified here as a CD4 T cell epitope, which is an encouraging finding in the context of a potential pan- or at least broadly cross-reactive PRRSV vaccine.

The M protein was recognised by both memory CD4 (CD4$^+$CD8$\alpha_{low}$) and CD8 (CD4$^-$CD8$^{high}$) T cells [Gerner et al, 2009] whereas the NSP5 was the target of a strong CD8 T cell response. An antigen specific CD8 T cell response is important for the control of viral replication, especially in chronic or persistent viral infections, but the virus specific CD4 T cell response observed here (Figure 4.3) and in Chapter 3 suggests that both may be important for the clearance of PRRSV-1. In addition, neither of these proteins induced significant amounts of the immunosuppressive cytokine IL-10 or the Th2 response driving cytokine IL-4.

Despite the inter-animal variability, further in-depth phenotyping of the responding CD4 and CD8 T cells defined them as having the predominant phenotype CD4$^{high}$CD62L$^{low}$CD27$^{low/-}$CD25$^-$/+ T cells that also displayed a marker of cytotoxic degranulation (CD107a) and produced TNF-\(\alpha\). Based on studies in humans and mice the high expression of cell adhesion molecule CD44 and low expression of peripheral lymph node homing receptor, CD62L putatively identifies these responding T cells as effector or effector memory T cells [Wiesel et al, 2009]. Swine workshop cluster 2 (SWC2) has recently been identified as an orthologue of human CD27 [Reutner et al, 2012], costimulation of which is important for survival and proliferation of activated T cells [Mikkelsen, et al, 2011, Hendriks et al, 2003]. In porcine PBMC, both CD27- and CD27+ T cell populations have been distinguished suggesting that CD27 expression could define effector (T$_{EM}$) and central memory
(T_{CM}) T cell populations again as described for humans and mice. The IFN-γ^+ T cells studied here showing low or no expression of CD27 would classify them as T_{EM} cells rather than T_{CM} cells based on the human system [Kobiyashi et al., 2006, Tomiyama et al., 2004]. Porcine effector memory cells as defined by lack of or low CD27 expression have recently been described in the context of classical swine fever vaccination [Franzoni et al, 2013]. However, it has also recently been reported that both CD27^+ and CD27^- CD4 T cells respond to recall stimulation with PRRSV or swine influenza virus [Reutner et al, 2013].

CD25 is the IL-2 receptor molecule for the cytokine IL-2, which induces proliferation and differentiation of activated T cells and is expressed on the surface of activated lymphocytes but is then lost on memory cells [Saalmuller et al, 2002]. Co-expression of CD25 and IFN-γ was not observed, with the exception of virus-specific CD4 T cells, which indicates that the majority of the cells present were IFN-γ secreting memory T cells or a small proportion of CD25 expressing activated T helper cells. It may only be speculated that the increased proportion of virus specific CD4 IFN-γ^+CD25^+ cells could be cells that are becoming additionally reactivated due to the cytokine milieu triggered by the live virus infection.

Dual functional T cells as defined by the simultaneous secretion of IFN-γ and TNF-α have been correlated with the quality and robustness of the T cell response [Seder et al, 2008] and the ability of the M and NSP5 proteins to stimulate these cells further supports the use of these two antigens in future vaccine development. CD107a or LAMP1 mobilisation is considered a marker for cytotoxic degranulation [Betts et al, 2003] and the majority of the M and NSP5 specific T cells were shown to express both IFN-γ and CD107a. PRRSV stimulated T cells also exhibited the
ability to mobilise CD107a in conjunction with IFN-γ but displayed a larger proportion of IFN-γ single positive cells. CD107a expression has been described on porcine CD8 T cells previously [Franzoni et al, 2013] but its expression on porcine CD4 T cells has rarely been reported although CD4 cytotoxic activity has been described in the context of African Swine fever infection (however, these cells also expressed CD8) [Denyer et al, 2006], as well as extensively in humans and mice [reviewed in Marshall and Swain, 2011]. Due to limitations on cell numbers and availability of anti-porcine antibodies it was not possible to conduct further characterisation of the responding T cell subsets, however in the future further analysis should be conducted. Areas of interest include additional memory markers such as CCR7 and isoforms of CD45, as well as secretion of the cytokine IL-2. Further elucidation of porcine T cell memory markers and phenotypes may be valuable tools in the quest to better dissect T cell responses, identify immunological correlates/surrogates of protection and aid vaccine development for PRRSV and other pathogens.

The epitopes/antigenic peptides on each antigen that the individual pigs responded to appeared dependent on the MHC haplotypes of the pigs rather than the virus strain they were infected with and certain antigenic regions could potentially be attributed to a specific haplotype. This could be an important finding if vaccination in combination with selective breeding for disease resistance was to be considered in the control of PRRS. Analysis of the sequence conservation of these antigenic peptides showed them to be highly conserved amongst PRRSV-1 strains and in some instances with the PRRSV-2 prototype strain. Pigs could still respond to antigenic peptides with certain amino acid substitutions identified in divergent strains (for
example in NSP5\textsubscript{153-170}), which is encouraging for vaccine applications when considering diverse PRRSV strains in the field [Reviewed in Meng, 2000].

In conclusion this chapter has identified the M and NSP5 proteins as important targets of the T cell response to PRRSV and characterised the responding cells as well as identifying specific antigenic regions. Whilst the specific antigenic regions may differ from pig to pig, at the very least these two proteins as a whole should be evaluated further for their potential inclusion in next generation vaccines for the control of PRRSV.
Chapter V

Assessment of the immunogenicity and efficacy of an experimental particulate vaccine formulation of PRRSV or defined T cell antigens
Chapter 5. Assessment of the immunogenicity and efficacy of an experimental particulate vaccine formulation of PRRSV or defined T cell antigens

5.1. Rationale

There is a requirement for the development of vaccines which induce strong cellular immune responses to enhance control of PRRSV and also other important viral pathogens. Various vaccine delivery systems are being developed and evaluated to induce T cell mediated immunity however one strategy which possesses a number of desirable features is the use of nanoparticles to deliver vaccine antigen directly to dendritic cells (DCs). The previous Chapters have shown that resolution of PRRSV infection is associated with the induction of T cell responses. T cells target a range of viral antigens; however the M and NSP5 proteins have been identified as having particular vaccine potential since they bear multiple CD8 T cell epitopes, and in the case of M also bears CD4 T cell epitopes, as well as being well conserved amongst PRRSV isolates [Mokhtar et al, 2014]. This Chapter describes a study to assess the immunogenicity and efficacy of nanoparticulate delivery of PRRSV antigens.

In collaboration with the UCL School of Pharmacy, London, adjuvanted vaccine formulations of inactivated PRRSV (as a crude and diverse mixture of antigens, which may be recognised by both B cells and T cells) or peptides representing the M and NSP5 proteins (T cell antigens) and a recombinant PRRSV GP5-M fusion protein (EM4) which does not induce neutralising antibodies in pigs (Dr Margarita Garcia-Duran, personal communication) were prepared. Hydrophobic
chitosan-based nanoparticles were selected for this study. Chitosan and its derivatives are abundantly expressed biological polysaccharides which may be formed into nanoparticles [Reviewed in Jayakumar et al, 2010]. Chitosan may offer an inherent adjuvant effect, through binding the innate immune sensor, TLR4, and has been shown to bind specifically to mannose receptors expressed on DCs [Villiers et al, 2009]. It has also been shown that targeting multiple TLRs results in enhanced vaccine immunogenicity and efficacy [Querec et al, 2006; Kasturi et al, 2011] and therefore a number of pathogen-associated molecular patterns (PAMPs) were screened to identify those that exerted potent and complimentary immunostimulatory effects on porcine DCs, for inclusion as a molecular adjuvant. Following *in vitro* characterisation of these formulations, groups of pigs were vaccinated and then challenged by inoculation of a field strain of PRRSV. Over the course of vaccination and subsequent challenge infection, the induction and kinetics of antigen-specific antibody and T cell responses were monitored in an attempt to associate immune responses with the degree of protection conferred.

5.2. Results

5.2.1. Summary

Three particulate vaccine formulations were prepared using hydrophobic chitosan: (1) Encapsulation of β-propiolactone inactivated PRRSV and encapsulation and coating with the multi-TLR2 and TLR7 agonist Adilipoline (Virus-P); (2) Encapsulation of synthetic M and NSP5 peptides, coating with a recombinant M-GP5 fusion protein (EM4) (Peptide-P) and inclusion of Adilipoline as before (Peptide-P); and (3) negative control empty particles (Empty-P).
Characterisation of the formulations showed highly efficient antigen encapsulation into and absorption onto the surface of the particles, which were found to be in the low µm rather than nm size range and differed significantly in their surface charges dependant on their composition. The formulations were additionally screened on porcine leukocytes to assess the activation of DCs and monocytes, and the presentation of antigens to T cells. Both washed and unwashed particles bearing Adilipoline induced strong IL-12 and type I IFN responses from DCs and IL-8 responses from both DCs and monocytes. All three particle formulations induced TNF-α responses from DC subsets and monocytes, suggesting responses induced by the chitosan polymer. Both the Virus-P and Peptide-P formulations resulted in the stimulation of CD4 and CD8 T cells from PRRSV-immune pigs, with the greatest responses being detected in the CD4 T cell population.

Vaccination with these particulate formulations induced antigen-specific antibody responses, which were most pronounced following booster immunisation. M and NSP5-specific CD4, but not CD8, T cell IFN-γ reactivity was measurable following the booster immunisation in animals vaccinated with Peptide-P. Upon challenge, CD4 and CD8 T cell reactivity was detected in all groups, with the greatest responses being detected in the Peptide-P vaccinated group. Assessment of protection against PRRSV challenge infection revealed that only a minority of vaccinated pigs (2/8 from the Peptide-P group and 1/8 from the Virus-P group) showed some evidence of enhanced viral clearance. Assessment of T cell responses in the lungs, at the point of resolution of the challenge infection, showed strong M and NSP5-specific T cell responses, which were almost exclusively from the CD8 T cell population. It can therefore be concluded that this study has provided further evidence for the role of M and NSP5-specific T cells, and CD8 T cells in particular,
in the immune response against PRRSV. However, further work is required to improve the immunogenicity of these nanoparticle formulations with a particular emphasis on enhancing the cross-presentation and activation of CD8 T cells.

5.2.2 Design and quality control of particulate vaccines

A panel of pathogen recognition receptor (PRR) agonists (synthetic multi-TLR2 and -7 agonists CL513 and Adilipoline – both; TLR-9 agonists CpG ODNs 21798 (class P), 2216 (class A) and 2007 (class B); TLR-7 agonist imiquimod, and TLR-3/RIG-I/MDA-5 agonist poly(I:C)) were tested for their ability to induce type 1 T cell-driving cytokines, IL-12, TNF-α and IFN-α, by porcine plasmacytoid dendritic cells (pDCs) using flow cytometry (Figure 5.1). Both CpG ODN 2007 and poly(I:C) induced relatively poor cytokine responses. The other PAMPs induced significant cytokine responses, with many pDCs co-expressing all three cytokines. However, the multi-TLR ligand Adilipoline sustained statistically significant numbers of triple-cytokine expressing pDC down to a dilution of 1:100, and retained ability to induce statistically significant numbers of IL-12 producing pDCs at a dilution of 1:1000. Adilipoline was therefore selected for inclusion as a molecular adjuvant that would complement the TLR4 agonist properties of chitosan.
Figure 5.1. Screening of pathogen-associated molecular patterns for the capacity to stimulate type 1 T cell directing cytokine responses from porcine plasmacytoid DCs. Enriched blood DC cultures were stimulated with titrations of a panel of pathogen-associated molecular patterns (PAMPs): TLR-2/7 agonists CL531 and Adilipoline; TLR-9 agonists CpG ODNs 21798 (class P), 2216 (class A) and 2007 (class B); TLR-7 agonist imiquimod, and TLR-3/RIG-I/MDA-5 agonist poly(I:C). Expression of TNF-α, IL-12 and IFN-α by plasmacytoid dendritic cells (pDCs) was assessed by flow cytometry as described in Chapter 2.12.5-6.
Based on the data presented in Chapters 3 and 4, as well as the assessment of immunostimulatory properties of Adilipoline described above, chitosan-based particulate formulations were prepared as illustrated in Figure 5.2. The first formulation was made by encapsulating β-propiolactone inactivated PRRSV-1 Olot/91 (Virus-P). The inactivated virus served as a crude and diverse mixture of antigens, which may be recognised by both B cells (antibody) and T cells. This formulation was adjuvanted by the inclusion of Adilipoline both within and coated to the surface of the particles. The second formulation was similarly adjuvanted but instead of virus the particles encapsulated synthetic peptides representing the M and NSP5 proteins, as T cell antigens (Peptide-P). The peptides were designed as overlapping 20mers offset by 10 amino acids spanning the M and the NSP5 protein sequences from PRRSV-1 Olot/91 (Appendix B). These particles were additionally coated with a recombinant M/GP5 fusion protein fragment (EM4) consisting of the main GP5 ectodomain followed by the C-terminal portion of M protein that was known to possess a non-neutralising antibody epitope. The third formulation, which served as the negative control, consisted of empty chitosan particles (Empty-P). Virus-P contained 100 µg total of Adilipoline and 10⁶ TCID₅₀ inactivated PRRSV-1 Olot/91 per dose. Peptide-P also contained 100 µg total of Adilipoline, as well as 100 µg of each peptide and 200 µg EM4 protein per dose.
Figure 5.2. Schematic representation of the three hydrophobic chitosan based particles that were formulated and evaluated for immunogenicity and efficacy in pigs.
5.2.2. Quality control and *in vitro* assessment of particulate vaccine formulations

The vaccine particle preparations were examined by scanning electron microscopy (SEM) to assess whether each formulation of particles were in the nanometre size range (Figure 5.3A). Particles displayed a spherical morphology and were sized at typically less than 5µm in diameter, ranging up to about 20 µm, although the Virus-P tended to be larger. The Virus-P also appeared to collapse more easily, possibly due to the volume of antigen that was encapsulated (Figure 5.3A). Preparations were also assessed for charge and this analysis showed that the three particle formulations differed significantly in their surface charges, which most likely reflected their composition (Figure 5.3B). The Empty-P were positively charged, and the Peptide-P were even more so, whereas the Virus-P were negatively charged.
Figure 5.3. Assessment of the morphology, size and charge of particulate vaccine formulations. Representative SEM micrographs of chitosan particles (Batch 2 – boost preparation). Scale bars indicate that the diameter of the majority of the particles were in the low micrometre range. The surface charge of the three particle formulations prepared for the prime (Batch 1) and booster (Batch 2) doses were assessed using the zeta potential using a Zetasizer Nano instrument. Results presented are the mean of two measurements of the same sample and error bars represent SEM.
ELISA analyses were conducted to determine the efficiency of virus or peptide encapsulation and coating with EM4 protein (Figure 5.4). This was assessed by quantifying antigen in particle-free supernatants. Inactivated PRRSV Olot/91 was evaluated using a novel antigen capture ELISA that was established and could detect less than $10^4$ TCID$_{50}$ PRRSV. Based on the starting concentration of $2.5 \times 10^5$ PRRSV TCID$_{50}$/ml in the formulation and a residual concentration of approximately $1 \times 10^4$ TCID$_{50}$/ml in the supernatant, the encapsulation efficiency of virus into the nanoparticles was calculated to be around 96% efficient (Figure 5.4). The adsorption of EM4 protein onto the Peptide-P surface was similarly estimated by quantifying its presence in supernatants using a specific antigen-capture ELISA. Based on a starting concentration of 50µg/ml of EM4 in the formulation and a residual concentration of 0.28 µg/ml in the supernatant, the adsorption efficiency was estimated to be over 99% efficient (Figure 5.4). The coating of EM4 onto the surface of Peptide-P was additionally assessed by flow cytometry and showed that ~80% of Peptide-P had detectable levels of EM bound to their surface (Figure 5.5). Given the high efficiency of EM4-coating, a fluorometric-based biochemical assay was conducted to assess the encapsulation of M and NSP5 peptides. Based on the starting total peptide concentration of 825 µg/ml in the formulation and a maximal residual concentration of 30 µg/ml in the supernatant, the encapsulation of peptide was estimated to be at least 98% efficient (Figure 5.4). Supernatant from Empty-P showed a comparable fluorescence to that of one of the Peptide-P batches, which could be due to free amine groups on the chitosan giving artifactual results. Assessment of Adilipoline incorporation was not possible by ELISA and the complex nature of the compound meant it was not readily amenable to assessment by other means e.g. HPLC.
Figure 5.4. Assessment of PRRSV, EM4 and peptide incorporation into particle formulations. The presence of inactivated PRRSV-1 Olot/91 (Virus) and EM4 protein in particle-free supernatants were assessed by antigen-capture ELISAs. The presence of M and NSP5 peptides in particle-free supernatants was assessed by fluorometric OPA assay. Representative standard curves for each assay are presented, as are the mean triplicate values of titrations of particle supernatants, and error bars represent SEM. ‘Prime’ = vaccine batch 1; ‘Boost’ = vaccine batch 2.
Figure 5.5. Assessment of EM4 coating of chitosan particle formulations by flow cytometry. The absorption of EM4 protein onto the surface of chitosan particles was assessed by staining particles with a specific mAb and visualisation by staining with a fluorochrome labelled secondary antibody. Representative dot plots are shown of the forward and side scatter properties of each of the three formulations prepared for the booster immunisation and their staining following incubation with isotype control or EM4 specific mAbs (A). The percentage of cells falling into the positive staining gate is graphically presented in panel B.
The ability of the vaccine formulations to deliver antigens into MHC processing and presentation pathways was assessed \textit{in vitro} by measuring the stimulation of T cell cytokine responses from PBMC isolated from PRRSV-immune pigs (pigs 71 and 86 from Animal Experiment 3, Chapter 4). Both the Virus-P and Peptide-P formulations resulted in the stimulation of CD4 and CD8 T cells, with the greatest IFN-\(\gamma\) and TNF-\(\alpha\) responses being detected in the CD4 T cell population (Figure 5.6). The greatest proportion of IFN-\(\gamma^+\)TNF-\(\alpha^+\) CD4 T cells was induced by washed and unwashed Virus-P, however the greatest IFN-\(\gamma^+\)/TNF-\(\alpha^+\) CD8 T cell response was induced by washed and unwashed Peptide-P in both pigs. Cytokine production induced by all Virus- and Peptide-P preparations from CD4 T cells were statistically significant, as was cytokine from CD8 T cells induced by washed Peptide-P in pig 71 and both washed and unwashed Peptide-P in pig 86. This data suggested that uptake of the antigen-loaded particles by antigen-presenting cells led to the processing and presentation of antigen by both MHC class I and II molecules.
Figure 5.6. *In vitro* stimulation of peripheral blood mononuclear cells with vaccine particle formulations stimulates CD4 and CD8 T cell responses. PBMC from two pigs immune to PRRSV Olot/91 (71 and 86) were stimulated with each of the three particulate vaccine formulations (Peptide-, Virus- or Empty-P), which for Peptide- and Virus-P were additionally tested with (Washed) and without (Unwashed) washing to potentially remove any unbound antigen. IFN-γ and TNF-α co-expression by CD4 and CD8 T cells was then assessed by flow cytometry. The mean data for triplicate cultures are presented and error bars represent SEM.
The vaccine particle formulations were additionally screened *in vitro* to assess the activation of blood DCs and monocytes and further characterise their immunostimulatory properties. Cultures of enriched blood DCs, which included both pDC and myeloid DC (mDC) subsets, and monocytes were cultured in the presence of titrations of particle formulations, which for Peptide- and Virus-P were additionally tested with (washed) and without (unwashed) pre-washing to potentially remove any unbound Adilipoline. No stimulation and addition of soluble Adilipoline were included as negative and positive controls, respectively. After incubation, the presence of IL-8, IL-10, IL12 and type I IFN in culture supernatants were assessed by cytokine ELISA (Figure 5.7). Statistically significant (p = 0.0001) IL-8 responses were induced by both the washed and unwashed adjuvanted particles at all particle doses tested. The highest concentration of Empty-P induced an IL-8 response from monocytes but this was not detectable in DC cultures. No IL-10 was detected in supernatants from DC cultures and only the higher concentrations of Virus-P induced a statistically significant (p = 0.0001) monocyte IL-10 response. Whilst not detected in monocyte cultures, high levels (statistically significant; p = 0.0001) of IL-12 and type I IFN were detected in DC cultures, induced by both Virus- and Peptide-P, irrespective of washing, suggesting that particle-associated Adilipoline was stimulating these responses.
**Figure 5.7. Assessment of the cytokine response profiles from porcine DCs and monocytes pulsed with vaccine particle formulations.** Cultures of enriched blood DCs and purified monocytes from naïve pigs were cultured for 18 hours in the presence of titrations of particle formulations: particle:cell ratios of 10:1 – blue bars; 3:1 – orange bars; 1:1 – green bars; and 0.4:1 – red bars). Both Peptide- and Virus-P were tested with (washed) and without (unwashed) washing to remove unbound Adilipoline. No stimulation and soluble Adilipoline were included as negative and positive controls, respectively. After incubation, the levels of IL-8, IL-10, IL-12 and type I IFN were quantified in cell-free culture supernatants by cytokine ELISA. The results presented are the mean unstimulated corrected values from triplicate cultures and error bars represent SEM.
Cytokine responses were further assessed by flow cytometric analysis of mixed cultures of enriched blood DCs and monocytes (Figure 5.8) Low percentages of monocytes produced IL-12 in response to all of the P preparations (only unwashed particles statistically significant; \( p = 0.01 \); compared to empty particles at the two highest doses) but up to 50% of monocytes produced TNF-\( \alpha \) with the various preparations; the most being induced by washed Peptide-P (statistically significant; \( p = 0.05 \); until the lowest dose). High percentages (60-80%) of pDCs produced TNF-\( \alpha \) in response to stimulation with the highest dose of particles (all preparations statistically significant; \( p = 0.05 \); at the highest dose), however lower percentages of pDCs produced IL-12 upon stimulation (washed particles only statistically significant; \( p = 0.0001 \); at the highest dose). The preparations that induced the greatest percentages of responding cells were the washed Peptide-P (around 80% at the highest input of NPs) and to a lesser extent, the Virus-P. CD1\(^+\) mDCs produced very little IL-12 upon stimulation but the preparations did stimulate the cells to produce TNF-\( \alpha \) (all preparations statistically significant; \( p = 0.0001 \)). Adjuvanted particles induced IL-12 expression by CD1\(^-\) mDCs, albeit at low levels (statistically significant; \( p = 0.0001 \); at the highest dose for both virus-P and unwashed peptide-P; and at the second highest dose for washed peptide-P) and induced a more pronounced TNF-\( \alpha \) response (statistically significant; \( p \leq 0.05 \); for all preps until the second lowest dose). It appeared that the chitosan was inducing the TNF-\( \alpha \) production in the monocytes and the pDCs as evidenced by the lack of statistical significance in between the different preparations.
Figure 5.8. Assessment of the induction of monocyte and DC subset cytokine responses following in vitro stimulation with vaccine particle formulations. Mixed cultures of enriched blood DCs and monocytes from a PRRSV-naïve pig were cultured for 18 hours in the presence of titrations of particle formulations, which for Peptide- and Virus-P were additionally tested with (Washed) and without (Unwashed) washing to potentially remove any unbound Adilipoline. Unstimulated and soluble Adilipoline were included as negative and positive controls, respectively. After, incubation the expression of TNF-α and IL-12 was assessed by flow cytometry. The mean unstimulated-corrected data for triplicate cultures are presented and error bars represent SEM.
5.2.3 Assessment of immune responses and protection following experimental vaccination in pigs

Groups of pigs (n=6) were vaccinated twice with each of the vaccine particle formulations (Virus-P, Peptide-P and Empty-P) with a three week interval between prime and booster inoculations. After a further three weeks, all animals were challenged with a UK field strain of PRRSV-1 (215-06) and assessed for protection. Over the duration of the experiment, the induction of PRRSV-, M- and NSP5-specific T cell and antibody responses were monitored and protection assessed by measuring viraemia by quantitative RT-PCR.

Each vaccine dose was delivered in a volume of 4ml, with 2ml inoculated subcutaneously behind each ear. After both prime and boost inoculations, mild local reactions were observed in the groups vaccinated with the Virus- and Peptide-P, suggesting that the presence of Adilipoline and/or antigens was inducing local inflammatory immune responses (Figure 5.9).
Figure 5.9. Illustrative photographs of the transient swelling and erythema of the skin at sites local to the injection of antigen-loaded, Adilipoline-adjuvanted chitosan particles. Photographs were taken 24-hours post-inoculation with either Virus-P (pig P14-7512) or Peptide-P (pig P14-7507) are shown. Swellings and mild and erythema as indicated by red arrows were observed after both prime and booster inoculations with both of the Adilipoline-adjuvanted particles and had diffused by 48-72 hours post-inoculation. No swelling was observed in the animals inoculated with the empty particles.
Over the course of vaccination and challenge, antigen-specific antibody (Ab) and T cell responses were assessed primarily by ELISA and flow cytometry, respectively. Assessment of PRRSV-specific Ab using a commercial nucleoprotein based ELISA showed that after the booster immunization, specific Ab was detected in the Virus-P group, albeit at low levels (not statistically significant compared to pre-immunization), which were rapidly boosted (statistically significant; \( p = 0.001 \); compared to pre-immunization on day 28 post-immunization and thereafter) following challenge infection (Figure 5.10). Comparable statistically significant (\( p = 0.001 \)) PRRSV-specific Ab responses were detected in the Peptide- and Empty-P groups only from day 56 post-immunization (14 days post-challenge). Assessment of EM4-specific Ab showed low levels (not statistically significant compared to pre-immunization) in the Peptide-P group, which were significantly boosted, becoming statistically significant (\( p = 0.001 \)) following the second immunization (day 28 post-immunization; Figure 5.10). As expected for this subdominant epitope there was little evidence of further boosting upon the challenge infection and levels remained low in the Virus- and Empty-P groups. Assessment of serum samples from the Virus-P group for their capacity to neutralize infection with PRRSV-1 Olot/91 was carried out using a MARC-145 cell based neutralization assay. The results showed that only 2 of the six animals had a detectable virus-neutralizing antibody titre calculated to be 1:4 (pig 12) and 1:11 (pig 15).
Figure 5.10. Assessment of PRRSV-specific antibody responses following vaccination and challenge. Longitudinal serum samples were analysed for PRRSV specific antibody using a commercial PRRSV N-protein based ELISA and assessed for EM4-specificity using an in-house ELISA. Data presented are the mean ELISA OD values for each group (n=6) and error bars represent SEM.
Following *in vitro* stimulation of PBMC samples with PRRSV-1 Olot/91 or M and NSP5 peptides, IFN-γ and TNF-α co-expression by CD4 and CD8 T cells was assessed by flow cytometry. Single cytokine producing cells were also assessed however there were very low levels of single IFN-γ⁺ cells and most IFN-γ⁺ cells were also positive for TNF-α and therefore only dual-positive cells were used for further analysis. M and NSP5-specific CD4, but not CD8, T cell reactivity was measurable but not statistically significant following the booster immunisation in animals vaccinated with the Peptide-P but not in the Virus- or Empty-P groups (Figure 5.11). Upon challenge, CD4 and CD8 T cell reactivity was detected in all groups; CD4 responses were statistically significant on day 56 after immunisation when stimulated with NSP5 (in the peptide-P group; \( p = 0.001 \)) and stimulated with M and virus (in all groups; \( p \leq 0.05 \)). CD8 responses were statistically significant (\( p \leq 0.01 \)) on day 56 post-immunization when stimulated with NSP5, M (virus-P and blank-P groups), and stimulated with virus (in all groups). The greatest magnitude of response was CD4 T cell responses detected in the Peptide-P group in response to stimulation with M. No statistically significant responses from CD4 T cells (compared to before vaccination) were observed until day 49 post-immunization (7 days post-challenge (Peptide-P vaccinated group, all stimulations). Assessment of PRRSV, M and NSP5 specific IL-2, IL-4 and IL-10 responses was performed on days 35 (day 14 post-boost) and 56 (day 14 post-challenge) post-immunization. These cytokines were selected as markers to further assess type 1 responses (IL-2), potential type 2 responses (IL-4) and potential Treg responses (IL-10). No IL-4 in response to virus or peptide or IL-2 and IL-10 in response to virus stimulation was detected (Data not shown). In contrast statistically significant IL-2 responses from PBMC from the Peptide-P vaccinated group was measured at both time-points (day
35, both NSP5 and M stimulated; p = 0.01; day 56 M stimulated; p = 0.05), and statistically significant IL-10 responses from the same group in both NSP5 and M stimulated on day 35, which were absent from both the Virus- and Empty-P groups; p = 0.05, (Figure 5.12). IL-2 is presented as OD rather than concentration due to technical difficulties in reading the standard curve.
Figure 5.11. Longitudinal T cell responses to ex vivo stimulation with M, NSP5 or whole virus. Peripheral blood mononuclear cells (PBMC) collected over the course of vaccination and challenge were stimulated with peptides representing the PRRSV M and NSP5 proteins or PRRSV-1 Olot/91. IFN-γ and TNF-α co-expression by CD4 (open circles) and CD8 T cells (closed circles) was assessed using flow cytometry. Data represent the mean background corrected response for each group ± SEM.
Figure 5.12. Assessment of PRRSV M and NSP5 specific IL-2 and IL-10 responses post-vaccination and challenge. On days 35 (day 14 post-boost) and 56 (day 14 post-challenge) PBMC were stimulated with pools of synthetic peptides representing PRRSV M and NSP5 proteins and the secretion of IL-2 and IL-10 into culture supernatants were assessed after 24 hours by ELISA. The mean unstimulated-corrected data for each group are presented and error bars represent SEM. Asterisks denote statistical significance when compared to empty-P as analyzed by a two-way analysis of variance (ANOVA) followed by a Dunnett’s multiple comparison test *p<0.05, **p<0.01 ***p<0.001.
Three weeks after the booster immunisation, all pigs were challenged intranasally with $10^5$ TCID$_{50}$ of a UK field stain PRRSV-1 215-06 [Morgan et al, 2013]. Animals were scored for PRRS-related clinical signs post-challenge (Appendix A) and transient pyrexia and mild clinical signs were observed on day 4 post-infection in a proportion of animals from each experimental group; Virus-P group: 3/6 animals, Peptide-P group: 4/6 animals and Empty-P group: 5/6 animals (Figure 5.13).

![Figure 5.13. Assessment of clinical signs following PRRSV challenge infection.](image)

Rectal temperatures and clinical signs were scored on a daily basis and the summed clinical scores for each experimental group are presented.
To assess whether these immune responses translated into a degree of protection against the challenge PRRSV infection, quantitative (q)RT-PCR was conducted on serum samples to assess the RNAemia (Figure 5.14). All animals regardless of vaccination group showed significant levels of PRRSV RNA in sera on day 7 post-challenge. These levels were sustained on day 14 post-challenge for all animals except 2 Peptide-P and 1 Virus-P vaccinated animals that had reduced levels. By day 19, all animals, bar two Virus-P vaccinees, had negligible amounts of viral RNA remaining.

![Figure 5.14](image-url)

**Figure 5.14.** Assessment of PRRSV viraemia post-challenge by intranasal inoculation with the UK field PRRSV-1 isolate 215-06. Serum samples were collected post-challenge and PRRSV RNA assessed by quantitative RT-PCR. Data points represent the mean values for individual animals with lines representing the mean values for each group.
Upon termination of the experiment, day 21 post-challenge, post-mortem examinations were conducted on all animals. Gross lung pathology was only identified for a single animal from each of the Virus- and Empty-P groups. Lungs were lavaged, cellularity and composition determined, and T cell responses measured following stimulation with PRRSV or a pool of M/NSP5 peptides. For technical reasons it was not possible to collect lavage fluid from all animals and since responses at this late-stage post-challenge did not differ significantly between the groups the compiled results from all animals are presented in Figure 5.15. The results showed that there was a significant T cell population in the lavage fluid with a greater number of CD8 compared to CD4 T cells. Assessment of antigen-specific responses showed a high number of PRRSV and M/NSP5 specific T cells in the lavage fluid, which were almost exclusively CD8 T cells. There was a statistically significant association between the numbers of PRRSV-specific CD8 T cells in the lungs and the virus load of the animals, as assessed by RNAemia, with greater numbers of cells being present in those animals that appear to still be actively clearing the infection.
Figure 5.15. Assessment of specific T cell IFN-γ responses in the lung following PRRSV challenge infection. On day 21 post-challenge, lungs were lavaged post-mortem and the numbers and phenotype of T cells calculated by volumetric flow cytometry (A). Isolated cells were stimulated with PRRSV or a pool of M and NSP5 peptides. IFN-γ and TNF-α expression by CD4 and CD8 T cells was assessed by flow cytometry and the results expressed as both relative proportions (B) and the estimated absolute cell numbers (C). The association between antigen-specific CD8 T cells and the virus load of the animals as assessed by RNAemia is presented (D). Data points represent the mean background corrected response for individual animals, irrespective of vaccination group, with bars representing the mean and SEM.
5.3. Discussion

The results presented in this Chapter have shown that hydrophobic chitosan may be used to generate particulate formulations using either whole PRRSV or defined PRRSV antigens. Co-formulation with the multi-PRR ligand Adilipoline further enhanced the immunostimulatory nature of these particles in vitro and potentially in vivo. However, despite this encouraging initial data, vaccination with these formulations failed to induce strong antigen-specific T cell responses, which are believed to be required for the effective control of PRRSV infection. It is worthy to note that the Peptide-P formulation appeared to prime CD4 as opposed to CD8 T cell responses. Review of the initial in vitro data showed that the formulations were capable of directing peptides into the MHC class I pathway but the resultant CD8 T cell responses were lower than expected based on earlier results presented in Chapter 4. Tests also need to be performed to demonstrate that the vaccine formulations do not stimulate T cells from naïve animals. The data from the lung lavages following the resolution of the PRRSV infection was striking and would further suggest that it is a lack of CD8 T cell response that may underlie the poor vaccine efficacy observed. On-going analysis is being conducted on additional blood samples to further assess whether enhanced control of PRRSV was evident at least in a small proportion of vaccinated animals and whether they displayed distinct immunological responses. In addition lung biopsies were collected at day 21 post challenge and qRT-PCR will be conducted to assess PRRSV load at this time-point. It was shown that in vitro the vaccine formulations did result in cross-presentation of antigen to CD8 T cell, albeit with a lower efficiency than to CD4 T cells. Formulations also
induced significant IL-12 and IFN-α, which are both reported to drive cross-presentation and induction of CD8 T cell responses. The majority of the IL-12 response was produced by pDCs, with only a small response being detected by CD1mDC. Recent data has shown CD1mDC to be orthologous to the CD8α-like mDC population defined in humans and mice (J. Edwards and S. Graham, unpublished data). CD8α-like mDC express IL-12 [Hochrein et al, 2001] and are highly efficient at cross-presenting exogenous antigen to CD8+ T cells [Heath et al, 2004]. Recent data from mice depleted of CD8α+ mDCs has shown that this population is essential for the induction of CD8+ T cell responses against RNA viruses and intracellular bacteria [Shortman and Heath, 2010]. Further work should therefore address how efficiently chitosan particles are taken up and processed by CD1mDC, since enhancing the targeting and activation of this DC subset could be crucial to enhancing CD8 T cell induction in vivo.

Interestingly, vaccination with peptide loaded particles induced an M– and NSP5-specific IL-10 response from PBMC. Whilst the cellular source of this cytokine was not discerned, due to a lack of monoclonal antibodies compatible with flow cytometry, it is tempting to speculate that immunisation had induced, in addition to CD4+ Th1 cells, a regulatory T cell response. IL-10 production from regulatory T cells can play an important role in defining the magnitude and quality of Th1 responses and has been shown in a mouse model to determine vaccine efficacy against leishmaniasis [Stober et al, 2005]. It therefore appears beneficial to investigate further whether the vaccine formulations are indeed inducing Treg responses and whether the adoption of alternative antigen delivery systems may alter the induction of IL-10 responses and their impact on protective Th1 responses [Darrah et al, 2010].
Another factor which needs to be considered in the future is the route of inoculation. While the subcutaneous delivery ensured efficient delivery of the vaccine \textit{in vivo}, recent studies have shown that intranasal delivery of an adjuvanted PLGA based nanoparticulate formulation of PRRSV antigens can protect against challenge infection [Binjawadagi \textit{et al}, 2014a, 2014b]. The inherent mucoadhesive properties of hydrophobic chitosan [Lehr \textit{et al}, 1992] makes this a viable proposal for investigating the induction of mucosal immunity which may be required for protection against PRRSV and intranasal immunisation should be considered in future work. In summary this experiment has shown promising initial data and has laid the groundwork for further optimisation of particulate vaccine formulations for the induction of T cell responses to PRRSV and other porcine pathogens.
Chapter VI

General Discussion
Chapter 6. General Discussion

6.1. Summary

PRRSV causes an ever increasing financial burden to pork industries worldwide and is a major threat to both animal welfare and food-security. Due to the replication cycle being prone to mutation and recombination events, PRRSV is a rapidly evolving virus. This is most dramatically illustrated by the emergence of highly pathogenic variants in South East Asia and Eastern Europe and is occurring despite the availability and use of a range of killed and modified live vaccines. Moreover, modified live vaccines have been responsible for the spread of PRRSV around the globe, with the introduction of vaccine-derived genotype 2 viruses into both European and Asian pig populations. Thus, safe and efficacious vaccines are urgently sought to improve PRRS control strategies. The development of such vaccines is however hindered by a lack of understanding of the immunological basis of immunity to PRRSV as well as the diversity of the pathogen. A large proportion of the research conducted to date has focussed on the antibody response to PRRSV, be it neutralising or non-neutralising, however this response has been deemed to be insufficient or potentially unnecessary for clearance of viraemia. It is now generally believed that T cell responses are crucial in providing immunity to PRRSV and therefore the research presented here addresses the hypothesis that identification of the targets of T cell responses within PRRSV would aid the design of more effective vaccines.
Chapters 3 and 4 describe the first use of a synthetic proteome-wide peptide library for identification of T cell antigens and epitopes of PRRSV-1. The library was screened on animals in three different experiments that were infected with three different strains of PRRSV-1, including the divergent subtype 3 strain SU1-Bel. Whilst in some cases different antigenic epitopes were identified, the M protein stood out as being the most consistently immunogenic protein recognised by the majority of animals. In addition NSP5 was found to be a strong inducer of T cell responses in two of the animal experiments. T cell responses to these two proteins were further defined in Chapter 4 and their efficacy in a vaccine formulation was tested in vivo in Chapter 5. The experimental vaccine induced T cell responses upon ex vivo restimulation; however these responses were mainly from CD4 T cells. Data from the earlier experiments and analysis of T cell responses in the lungs of vaccinated and control animals suggested that CD8 T cells are important in protection from infection. These observations may explain why the vaccine formulation failed to confer the expected protection against PRRSV challenge infection.

6.2. Discussion

Whilst the M and NSP5 proteins were identified as major targets of T cell responses in both Chapters 3 and 4, animals from the different experiments responded to a diverse set of proteins, as well as different epitopes within these proteins. This can be attributed to the different MHC haplotypes of the pigs involved and the highly polymorphic nature of the porcine MHC (SLA). In addition, the pigs used were not of the same breed (Large White, Dutch Landrace or a cross-breed of the two) and therefore may have been another contributory factor to explain the
variation in the specificity of T cell responses. In spite of the different pig breeds and PRRSV-1 strains used, it is however encouraging that common T cell antigens were identified. Any potential vaccine would need to be applicable for a wide range of commercial pig breeds representing an array of MHC haplotypes, but it is reassuring to note that all animals vaccinated with M and NSP5 mounted specific CD4 T cell responses to both antigens and CD8 T cell responses were additionally detected post-challenge. Determining T cell antigens and potentially epitopes recognised in the context of a wide variety of pig breeds/MHC haplotypes and a diverse range of PRRSV-1 and -2 strains would provide further rationale for a successful and commercially viable PRRSV vaccine. The increasing threat of emerging highly pathogenic PRRSV-1 [Karniychuk et al, 2010; Weesendorp et al, 2013; Morgan et al, 2013] and -2 strains [Tian et al, 2007] as well as emergence of novel PRRSV-1 strains [Chen et al, 2011] means that these strains should also be included in efforts to identify well conserved targets for both T cell and neutralising antibody responses.

Identification of important T cell epitopes allows for the design of MHC-tetramers; these are complexes of four MHC molecules loaded with peptide and fluorescently labelled, and are extremely useful tools in directly assessing antigen-specific responses. These can be used to confirm and quantify the presence of MHC restricted antigen specific T cells [Meidenbauer et al, 2003], as well as to analyse the frequency of antigen specific T cell in circulation over the course of infection, an approach that has already been used in pigs in the context of foot and mouth disease virus [Patch et al., 2010]. MHC tetramers can also be used in combination with functional assays, such as assessment of antigen specific T cell proliferation and phenotype. In addition, MHC tetramers have the potential to be used for adoptive T
cell transfer, allowing selection and expansion of peptide specific T cells [Knabel et al, 2002] for re-introduction into animals. Immunising a naïve animal by adoptive transfer of peptide specific T cells followed by challenge would confirm whether the cells mediate protection. This type of analysis has rarely been conducted in veterinary species; however McKeever et al, [1994] showed that naïve calves immunised with *Theileria parva* specific CD8 T cells were protected from challenge. Adoptive transfer in pigs is a realistic proposal due to the availability of several in-bred MHC homozygous lines of pigs.

As the lungs are the primary site of PRRSV infection and therefore the early immune response, further work comprising an in-depth analysis of the immune response in the lung over the course of infection should be conducted. In Chapter 5, it was striking to note the magnitude of PRRSV and M/NSP5 specific CD8 T cell responses at the point of resolution of challenge infection. The experimental design for such a study would be more challenging than longitudinally analysing T cell responses in peripheral blood. However, a time-course tissue-harvest design, perhaps using inbred MHC-matched lines of pigs, to improve inter-animal variability, could be adopted. Whilst it would need to be considered from both an ethical and practical stand-point there would also be an alternative possibility of sequentially lavaging lungs from live pigs under anaesthesia.

The main marker of T cell responses utilised in this project was IFN-γ and in later experiments in tandem with TNF-α. This stemmed from emerging data that enhanced viral control associated with multi-cytokine secreting, so called ‘polyfunctional’ T cells [Koup and Douek 2011]. The three cytokines that are often considered in this context are IFN-γ, TNF-α and IL-2. It would have therefore been
informative to test the IL-2 expression by antigen-specific T cells, especially as it has been shown to enhance PRRSV specific T cell responses [Romparto et al, 2006]. Unfortunately intracellular staining with anti-IL-2 mAbs proved technically problematic and future work should aim to perfect the application of this mAbs flow cytometry method to aid further understanding of the potential role of polyfuctional anti-viral T cell responses.

Screening of vaccine induced T cell responses for induction of IL-4 and IL-10 as markers of an undesirable immune response was conducted as a limited aspect of this study. It may be possible that particular viral proteins have an inherent propensity for inducing immunosuppressive or Th2 responses, as has been described for the induction of Treg responses by PRRSV-2 N protein [Wongyanin et al, 2012]. The nature of the vaccine formulation or adjuvant can play a major role in determining the cytokine bias of the T cell response, via its effect on DCs. In this study, chitosan particles were selected to encapsulate the protein or peptide. Encapsulated ovalbumin induced higher levels of both IgG1 and IgG2a in mice compared to soluble antigen [Uto et al, 2007] indicating the induction of a balanced Th1/Th2 response. Furthermore, it has been reported that nanoparticle complexes (200-600nm) rather than micro particles (2-8µm) have been associated with bias towards a Th1 response [Gutierro et al, 2002]. The particulate formulations prepared in this study were larger than had been anticipated and were in the low µm rather than nm range. It is therefore possible that the larger particle size may have contributed to the relatively weak induction of type 1 T cell responses.

Whilst far from optimal, the particulate vaccine formulation described in Chapter 5 demonstrated that it was possible to induce both Ab and CD4 T cell
immune responses using protein fragments and synthetic peptides representing M and NSP5. The approach of incorporating peptides into the particulate formulation was driven out of difficulties to express either the full-length M or NSP5 proteins in bacterial or insect cell systems, attributed to the multiple hydrophobic trans-membrane domains present in both proteins. However, peptide vaccines have several advantages over protein; in addition to the relative ease, production can be well controlled and the product can be of a high purity. Both CD4 and CD8 T cell epitopes can be strung together, regions of the protein that may have an immunosuppressive activity can be excluded and the well conserved regions can be utilised. In addition, a study by Zhang and colleagues compared the processing of long peptides versus intact protein and found that whilst the protein was only processed via the endosome, the long peptides were processed via both the endosome and the cytosol, leading to better cross presentation [Zhang et al, 2009]. This enhancement of presentation by MHC-I molecules has also recently been corroborated by Rosalia et al, [2013].

Chitosan was selected as the polymer core of the particulate vaccines due to its inherent immunostimulatory properties [Zhu et al, 2007, Zaharoff et al, 2007]. It was recently reported that formulation of an adenoviral vector with chitosan can actually have a negative effect on the induction of CD8 T cells which was attributed to an inhibition of APC activation [Lemke et al, 2011]. However, this may simply be due to the chitosan binding to the virus and interfering with the required expression of the vaccine antigen. Nevertheless, if chitosan may also impair the MHC class I presentation of antigen, delivered as protein or peptide, then another polymer, such as poly-(lactic-co-glycolic acid) (PLGA) could be a better choice for further
evaluation. PLGA is a synthetic biodegradable polymer which does not have any inherent immunostimulatory properties and is currently FDA approved as a drug delivery system [Danhier et al, 2012].

Another strategy that may enhance vaccine potency and is amenable to particulate formulations is the addition of DC targeting moieties [Kreutz et al, 2013]. In order to improve the prospects of porcine DC specific targeting a better characterisation of porcine DC subsets is required. This could enable delivery of antigen into the appropriate cross presentation pathway by selectively targeting the CD8α-like DC population [Joffre et al, 2012]. Current knowledge of porcine blood DC subsets demonstrates the existence of two mDC populations alongside pDCs [Summerfield et al, 2003]. CD1+ mDCs have recently been shown to be CD8α-like, secreting IL-12 and type I interferon in response to poly(I:C) and CpG ODN and best able to cross present viral antigen to CD8+ T cells (J. Edwards and S. Graham, unpublished data). Thus the CD1+ mDC represents a model cell population to assess targeting of antigen and the consequences on cross-presentation.

The TLR2/7 ligand Adilipoline was selected as the molecular adjuvant for the vaccine formulation due to its ability to sustain a cytokine response from pDCs, however if the target cell population is CD1+ mDCs, then further assessment of molecular adjuvants should be carried out with this in mind. Enhancement of cross-presentation has been shown in CD8α+ DCs in mice by triggering TLR7, generating a strong cytotoxic T cell response [Crespo et al, 2014] but synergistic combinations of TLR ligands should be considered, as it has been shown that stimulation of TLRs3 and 9 can also induce strong cytotoxic T cell responses [Schwarz et al, 2003; Datta et
al, 2003]. Targeting these TLRs has also been shown to improve the quality of the CD8 T cell response as evaluated by polyfunctional T cells [Kwissa et al, 2007].

The route of delivery is another variable that should be considered in the development of PRRSV vaccines and future studies should compare mucosal versus parenteral routes of inoculation. While intranasal delivery of vaccines is less invasive and thus is being promoted for human vaccines, there are concerns whether this practical benefit holds true for vaccinating livestock. What is perhaps more relevant to livestock is assessing whether intranasal delivery results in the induction of more protective mucosal immune responses. There is a growing body of data to suggest that targeting DCs at mucosal sites is crucial to programme mucosa-homing T cell responses as illustrated by Mikhak et al, [2013] and Sandoval et al, [2013].

Alternatives to particulate delivery should also be considered for induction of CD8 T cell responses. The use of alternate prime/boost vaccination regimens employing recombinant replication-deficient adenovirus or MVA vectors are the subject intense investigation for the development of vaccines to induce CD8 T cell responses against a range of pathogens including HIV [Ratto-Kim et al, 2012], Plasmodium spp. [Reyes-Sandoval et al, 2011] and Mycobacterium tuberculosis [You et al, 2012]. A recent study in a mouse model showed that the greatest CD8 T-cell IFN-γ response in BALF was obtained following intranasal boosting after intramuscular priming, which translated into a degree of protection against heterotypic influenza virus challenge infection [Lambe et al, 2013]. Another viral vector vaccine delivery system that warrants consideration for induction of T cell immunity is the use of cytomegaloviruses (CMV). It has recently been shown in the primate/SIV model, that a Rhesus monkey CMV vector induced potent T effector
memory responses that resulted in an unprecedented degree of protection against viral challenge delivered at mucosal sites [Hansen et al, 2009; Hansen et al, 2011]. Porcine CMV was originally identified in 1955 the causative agent of inclusion body rhinitis [Done 1955]. In addition to causing rhinitis and generalized infection in newborn piglets, in utero infection with porcine CMV can cause foetal death. Like other CMV, porcine CMV infection may become latent and are rarely eliminated from the host; as such, porcine CMV infections have high prevalence in swine herds around the world. Despite this, most published research on porcine CMV has been in the context of xenotransplantation and the potential risk it may pose to humans [Gollackner et al, 2003] rather than from a veterinary perspective. The complete sequence of the porcine CMV genome has recently been published which shows it more closely related to members of the Roseolovirus genus than with other cytomegaloviruses [Gu et al, 2014]. Nevertheless, this data provides a basis to explore as to whether this virus can be engineered as a vaccine delivery vector [Ranasinghe and Walker 2013].
6.3. CONCLUSION

Overall, this study has provided data that has improved our understanding of the T cell responses induced by PRRSV-1 infection, which appear to play a key role in providing protection against reinfection. Using a synthetic peptide library spanning the PRRSV proteome, the NSP5 and M proteins were identified as the two well conserved antigens targeted by both CD4 helper and CD8 cytotoxic T cells. The vaccine potential of these antigens was assessed using a novel particulate formulation. Unfortunately, this vaccine induced CD4 but failed to prime a CD8 T cell response which likely was responsible for the lack of efficacy observed against PRRSV challenge infection. It is hoped that this data will aid development of the next generation of PRRS vaccines that are urgently required to improve control of this disease.
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## APPENDICES

### Appendix A. PRRS clinical scoring system

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Behaviour</strong></td>
<td>Attentive (curious, stands up immediately)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Slightly reduced (Stands up hesitantly but without help)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Tired, gets up only when forced to, lies down again</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Dormant, will not stand up</td>
<td>3</td>
</tr>
<tr>
<td><strong>Breathing</strong></td>
<td>Frequency 10-15/min, barely visible chest movement</td>
<td>0</td>
</tr>
<tr>
<td>(Judged before approaching pig)</td>
<td>Frequency &gt;20/min distinct chest and abdominal movement</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Frequency &gt;30/min, breathing through open mouth</td>
<td>3</td>
</tr>
<tr>
<td><strong>Body tension</strong></td>
<td>Relaxed, straight back</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Stiffness and bent back while standing up, afterwards normal</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Bent back and stiff walking remains</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Cramps</td>
<td>3</td>
</tr>
<tr>
<td><strong>Body shape</strong></td>
<td>Full stomach, &quot;round&quot; body</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Empty stomach, thinned body muscles</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Emaciated, backbone and ribs visible, head size too big compared to body size</td>
<td>3</td>
</tr>
<tr>
<td><strong>Nasal discharge</strong></td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Present and clear</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Present and discouloured</td>
<td>2</td>
</tr>
<tr>
<td><strong>Sneezing</strong></td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild (few incidences 1-5 observation duration)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Severe (more frequent incidences, &gt;5 observation duration)</td>
<td>2</td>
</tr>
<tr>
<td><strong>Coughing</strong></td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild (few incidences as 1-5 observation duration)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Severe (more frequent incidences &gt;5 observation duration)</td>
<td>2</td>
</tr>
<tr>
<td><strong>Skin changes</strong></td>
<td>Evenly light pink, hair coat flat</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Reddening/purpling of skin</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ear necrosis</td>
<td>3</td>
</tr>
<tr>
<td><strong>Eyes/conjunctiva</strong></td>
<td>Light pink</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Reddened, clear secretion</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Highly inflammatied, turbid secretion</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Highly inflammatied, turbid purulent secretion, accentuated blood vessels</td>
<td>3</td>
</tr>
<tr>
<td><strong>Defecation</strong></td>
<td>Soft faeces, normal amount</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Reduced amount of faeces, dry</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Only small amount of dry, fibrin-covered faeces, or diarrhoea</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No faeces, mucus in rectum or watery diarrhoea</td>
<td>3</td>
</tr>
<tr>
<td><strong>Appetite/leftovers at feeding</strong></td>
<td>Greedy, hungry, trough empty, clean</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Eats slowly when fed, trough almost empty, almost no left overs</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Does not eat when fed but tastes food, Food only partially eaten.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Does not eat at all shows no interest for food, trough still full, nothing eaten</td>
<td>3</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td>37 C-39 C</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>39 C-40 C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>40 C-41 C</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt; 41 C</td>
<td>3</td>
</tr>
</tbody>
</table>

**Other** | Note any additional observations |

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Appendix B. M and NSP5 peptide sequences synthesised for vaccine formulation

PRRSV-1 Olot/91 M Peptides

MGSLDDFCNDSTAAQKLVLAS
STAAQKLVLAFSITYTPIMI
FSITYTPIMIYALKVSRSRGL
YALKVSRSRGLLGLLHILIFL
LGLLHILIFLNCSFTFGYMT
NCSTFTFGYMTYVRFQSTNRV
YVRFQSTNRVALTLGAVVAL
ALTLGAVALLWGVYDFSFTES
LWGVTYDFSFTESWKFVTSRCL
WKFVTSRCLCLGLGRRLYLA
CLGLGRRLYLPAAHHESAAG
PAAHHESAAGLHSIPASGNR
LHSIPASGNRAYAVRKPGLT
AYAVRKPGTLTSVNGTLVPGL
SVNGTLVPGLRLSLVLGGKRA
RSLVLGGKRAVRGCVNLVVK
VLGGKRAVRGCVNLVKYGR

PRRSV-1 Olot/91 NSP5 Peptides

GGLSTVQLLCVFFLLWRMMG
VFFLLWRMMGHAWTPIVAVG
HAWTPIVAVGFFLLNEILPA
FFLLNEILPAVLVRAVFSFA
VLVRAVFSFALFVLAWATPW
LFVLAWATPWSAQVLMLRLL
SAQVLMLRLLTASLNKNKL
TASLNKNKLINALGALGVV
LAFYALGGVGLAAEIGTFA
GLAAEIGTFAGRLSLSQAL
GRLSLSQALSTYCFPRVLL
STYCFPRVLLAMTSCVPTII
AMTSCVPTIIIGGLHTLGV
IGGLHTLGVILWLFKYRCLH
LWLFKYRCLHNMLVGDGSFS
NMLVGDGSFSASSFLRYFAE