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Porcine Reproductive and Respiratory Syndrome Virus

$CD8^+$ T cell epitopes and recombination mechanisms

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submitted by

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Declaration

I declare that I followed the rules of good scientific practice in all aspects for this thesis.

Marlene Mötz, 2023

List of first-author publications

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2. Mötz, M.; Stadler, J.; Kreutzmann, H.; Ladinig, A.; Lamp, B.; Auer, A.; Riedel, C.; Rümenapf, T. A conserved stem-loop structure within ORF5 is a frequent recombination hotspot for Porcine Reproductive and Respiratory Syndrome Virus 1 (PRRSV-1) strains with a particular modified live virus (MLV) strain. *Viruses*. 2023; DOI: 10.3390/v15010258

List of co-authored publications (not included in this thesis)

1. Kiesler, A.; Seitz, K.; Schwarz, L.; Buczolich, K.; Petznek, H.; Sassu, E.; et al. Clinical and serological evaluation of LINDA virus infections in post-weaning piglets. *Viruses*. 2019; DOI: 10.3390/v11110975

2. Ahmadi, N.; Gausterer, J.C.; Honeder, C.; Mötz, M.; Schöpper, H.; Zhu, C.; et al. Long-term effects and potential limits of intratympanic dexamethasone-loaded hydrogels combined with dexamethasone-eluting cochlear electrodes in a low-insertion trauma Guinea pig model. *Hear Res*. 2019; DOI: 10.1016/j.heares.2019.107825

3. Seitz, K.; Kübber-Heiss, A.; Auer, A.; Dinhopl, N.; Posautz, A.; Mötz, M.; et al. Discovery of a phylogenetically distinct poxvirus in diseased Crocodilurus amazonicus. *Arch Virol*. 2021; DOI: 10.1007/s00705-021-04975-6

4. Kiesler, A.; Plankensteiner, J.; Schwarz, L.; Riedel, C.; Seitz, K.; Mötz, M.; et al. Prevalence of Linda virus neutralizing antibodies in the Austrian pig population. *Viruses*. 2021; DOI: 10.3390/v13061001

5. Feix, A.S.; Cruz-Bustos, T.; Ruttkowski, B.; Mötz, M.; Rümenapf, T.; Joachim, A. Progression of asexual to sexual stages of Cystoisospora suis in a host cell-free environment as a model for Coccidia. *Parasitology*. 2021; DOI: 10.1017/s0031182021001074

6. Chen, H.W.; Huber, V.; Szakmary-Braendle, K.; Seitz, K.; Moetz, M.; Ruemenapf, T.; et al. Viral Traits and Cellular Knock-Out Genotype Affect Dependence of BVDV on Bovine CD46. *Pathogens*. 2021; DOI: 10.3390/pathogens10121620

7. Kiesler, A.; Schwarz, L.; Riedel, C.; Högler, S.; Brunthaler, R.; Dimmel, K.; et al. New Emergence of the Novel Pestivirus Linda Virus in a Pig Farm in Carinthia, Austria. *Viruses*. 2022; DOI: 10.3390/v14020326

8. Zaruba, M.; Chen, H.W.; Pietsch, O.F.; Szakmary-Braendle, K.; Auer, A.; Mötz, M.; et al. ADAM17 Is an Essential Factor for the Infection of Bovine Cells with Pestiviruses. *Viruses*. 2022; DOI: 10.3390/v14020381

9. Stas, M.; Kreutzmann, H.; Stadler, J.; Sassu, E.L.; Mair, K.H.; Koch, M.; et al. Influence of PRRSV-1 vaccination and infection on mononuclear immune cells in the maternal-fetal interface. *Front Immunol*. 2022; DOI: 10.3389/fimmu.2022.1055048

List of abbreviations

β_2M	β_2-microglobulin
CD	Cluster of differentiation
CSFV	Classical Swine Fever Virus
ER	Endoplasmic reticulum
IFN	Interferon
ISAG	International Society for Animal Genetics
IUIS	International Union of Immunological Societies
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LV	Lelystad virus
MHC	Major histocompatibility complex
MLV	Modified life virus
NK cell	Natural killer cell
Nsp	Non-structural protein
nt	Nucleotide
ORF	Open reading frame
p.i.	Post infection
PAMP	Pathogen-associated molecular pattern
PCV-2	Porcine circovirus 2
PRR	Pattern recognition receptor
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RdRp	RNA-dependent RNA polymerase
RFS	Ribosomal frame shift
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
sgRNA	Subgenomic RNA
ssRNA	Single-stranded RNA
SLA	Swine leukocyte antigen
TAP	ATP-dependent transporters associated with antigens processing
T_{CM}	Central-memory T cell

TCR	T cell receptor
T_{EM}	Effector-memory T cell
TRS	Transcription regulatory sequence
UTR	Untranslated region

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1. Introduction

1.1. Porcine reproductive and respiratory syndrome virus (PRRSV)

PRRSV is the causative agent of the porcine reproductive and respiratory syndrome (PRRS) in domestic pigs worldwide. The virus is a member of the family *Arteriviridae*, order *Nidovirales*, genus Betaarterivirus, which is divided into the subgenera, *Eurpobartevirus* and *Ampobartevirus.* Each subgenus consists of one virus species: *Betaarterivirus suid 1*, or PRRSV-1, which is mainly prevalent in Europe, and *Betaarterivirus suid 2*, or PRRSV-2, which is predominant in North America and Asia. These two species show a high genetic variability, leading to the hypothesis, that they might evolved separately (Plagemann 2003). Furthermore, within these two PRRSV species, there are considerable amounts of diverse strains. The mutation rate of RNA viruses lies between $1*10^{-6}$ and $1*10^{-4}$ mutations per nucleotide site per year (Peck und Lauring 2018), resulting in a fast evolutionary rate. Apart from the relatively unstable RNA genome, PRRSV lacks RNA proofreading activity, unlike other *Nidovirales* members with larger genomes, like the *Coronaviridae* (Gorbalenya et al. 2006). This makes phylogenetic analyses very complex, but due to improving and more available sequencing techniques, sequences are being submitted to data banks more rapidly. Unfortunately, this high genetic diversity makes virus containment difficult, since vaccines are often not cross-protective against heterologous strains (Kim et al. 2015; Renukaradhya et al. 2015b). Subsequently, PRRSV causes a big financial burden on the swine industry worldwide (Neumann et al. 2005), caused by medical interventions and production losses. PRRS has first been observed in the early 1990s in North America (Collins et al. 1992) and Europe (Wensvoort 1993; Wensvoort et al. 1991), and was called ‘swine mystery disease’ or ‘blue-ear pig disease’. In 1997 a positive stranded RNA virus was determined as the common cause of the disease outbreaks, and was assigned to the family *Arteriviridae,* and together with the *Coronaviridae* to the new order *Nidovirales* (Cavanagh 1997). Since then PRRSV has been reported nearly worldwide, especially in countries with commercialized meat production of domestic pigs. Nevertheless, many aspects of viral pathogenesis, host interactions and immune responses are yet poorly understood. Subsequently, a great research network has been established, making PRRSV a frequent topic in veterinary sciences.

1.1.1. Genome and structure

PRRSV is an enveloped virus with a single-stranded RNA (ssRNA) genome of positive polarity. The viral capsid is between 45-60 nm in diameter (Dea et al. 1995) and the genome approximately 15.000 nucleotides (nt) long. The genomic RNA strand bears at least ten open reading frames (ORFs), which code for the viral structural proteins, needed for replication and proteolytic processing, and the non-structural proteins (nsps) of the viral envelope (figure 1).

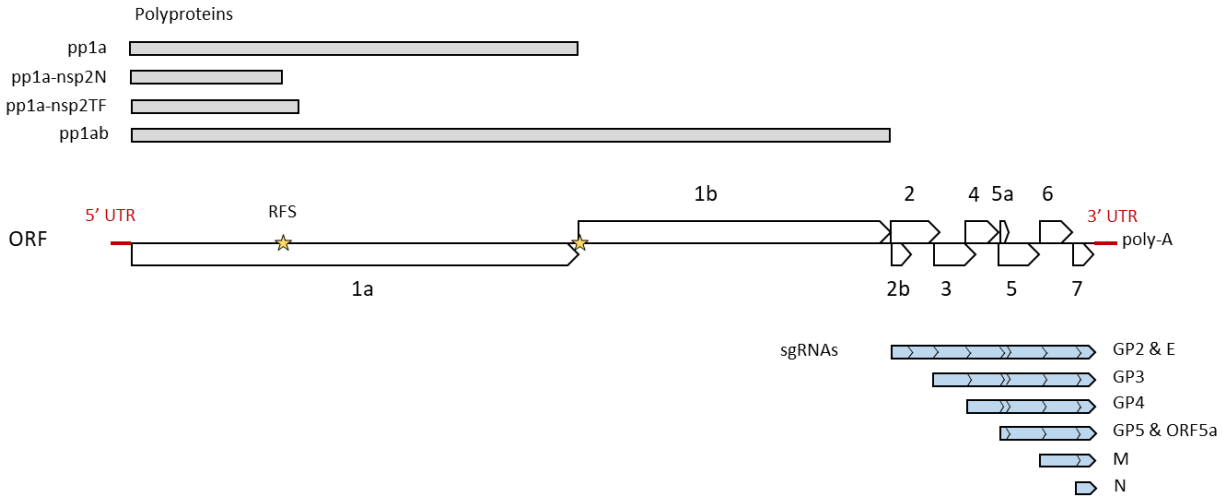

Figure 1. Depiction of the PRRSV genome and expression strategy of the ORFs; adapted from Matthew et al. (2015); ORF = open reading frame, UTR = untranslated region; RFS = ribosomal frameshift site; GP = glycoprotein, E = envelope, M = membrane, N = nucleocapsid.

The genome starts with a 5'cap structure followed by an untranslated region and ends with a 3' UTR and poly-A tail (Yun und Lee 2013). ORF1 encodes the virus' nsps, needed for replication and processing of the polyproteins. This reading frame possesses one translational start site, but two internal ribosomal frameshifts, resulting in the production of four distinct polyproteins: pp1a is generated from ORF1a, pp1ab from ORF1ab, pp1a-nsp2N by a -1 ribosomal frame shift (RFS), and pp1a-nspTF by a -2 RFS within ORF1a (Snijder und Meulenberg 1998; Fang et al. 2012). From these polyproteins at least 12 snps are generated by proteolytic processing (Music und Gagnon 2010). Nsp1, nsp2, nsp3, and nsp4 are viral proteases that are also involved in suppressing host IFN responses (Snijder et al. 2013; Boon et al. 1995). Nsp5 and nsp6 are transmembrane proteins (Boon et al. 1995), and together with nsp7 and nsp8 their functions are

not yet fully understood. Proteins encoded by ORF1b are nsp9, the RNA-dependent RNA polymerase (RdRp), nsp10, a helicase, nsp11, an IFN inhibitor, and nsp12, a mediator of subgenomic RNA (sgRNA) synthesis (Wang et al. 2019). ORF2-7 code for the structural proteins of the viral capsid. These are not directly translated, but generated through negative-stranded, polycistronic, sgRNA intermediates (van Marle et al. 1999b). Like all *Arteriviruses* sgRNAs transcription is initiated by base pairing of the body transcription regulatory sequences (TRSs) of the individual reading frames with the leader TRS of the 5' UTR (van Marle et al. 1999a). These TRSs are sequences of six nucleotides in length, and are essential to generate the nested, polyadenylated sgRNAs. The leader TRS is highly conserved among PRRSV-1 and PRRSV-2 strains (UUAACC) (Tan et al. 2001), whereas the body TRSs show variable sequences (Faaberg et al. 1998). All sgRNAs harbor the 5' leader TRS and are templates for the translation of viral proteins by host ribosomes. ORF2, ORF3, and ORF4 encode the glycoproteins GP2, GP3, and GP4, which form a trimeric protein complex, that is important for attachment to the cellular receptor CD163 and viral entry into the host cell (Wissink et al. 2005). Further, ORF5 encodes the major envelope glycoprotein GP5, which forms a dimeric complex with the membrane protein M, encoded by ORF6. This complex is a ligand for sialoadhesin (CD169), another cellular receptor of PRRSV (van Breedam et al. 2010). ORF7 encodes the N protein, which forms dimers to assemble a nucleocapsid around the genomic RNA. A depiction of the PRRSV genome can be found in figure 2. At last, there are two alternative reading frames: ORF2b codes for the envelope protein E (Wu et al. 2001), and ORF5a for a small unglycosylated protein (Johnson et al. 2011).

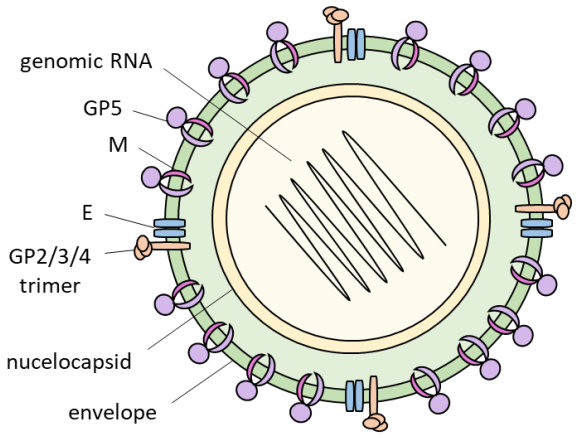

Figure 2. Structure of the PRRSV virion.

1.1.2. The PRRSV life cycle

Like with any other virus, the PRRSV life cycle starts with transmission from one host to another (see chapter 1.1.3.). This event is followed by the infiltration of susceptible target cells, which are primarily alveolar macrophages of the lungs. Two important cellular receptors are CD163 and CD169, which are expressed by cells of the monocyte/macrophage lineage, making the PRRSV cell tropism very specific. CD163 is the key entry mediator for PRRSV (Calvert et al. 2007). Macrophages from CD163 knock-out pigs have been shown to be resistant to infection (Burkard et al. 2017). CD169, also named Sialoadhesin or Siglec-1, is involved in (Vanderheijden et al. 2003), but not necessary for PRRSV infection (Prather et al. 2013). The uptake of the virions after receptor binding is mediated by clathrin-mediated endocytosis, and is dependent on a low pH (Nauwynck et al. 1999). After entering the host cell, the virions are uncoated to release their genomes into the cytoplasm, where RNA replication and translation of the viral proteins take place (Snijder et al. 2013), mediated by the RdRp, or nsp9. As discussed in chapter 1.1.1., the expression of viral proteins is mediated by direct translation and processing of the polyproteins of ORF1, and the generation of the ORF2-7 sgRNAs, for subsequent translation. Post-translational processing of the structural proteins occurs through the secretory pathway of the endoplasmic reticulum (ER) and the Golgi apparatus, where GP2-5 are N-glycosylated (Veit et al. 2014). Only the N protein remains in the cytoplasm, where it forms a nucleocapsid around the RNA genome (Spilman et al. 2009). The nucleocapsid obtains the viral envelope by budding from the Golgi apparatus. At last, the mature virions are released by the cell through exocytosis.

1.1.3. Pathogenesis

As discussed in chapter 1.1.1. and 1.1.2., PRRSV infects cells expressing the molecule CD163, or cells of the monocyte/macrophage lineage. Modes of transmission are through direct contact, artificial insemination, airborne transmission, vertical transmission, and the contact with contaminated items (Pileri und Mateu 2016). Clinical symptoms of infected animals are mild to acute respiratory disease, cyanosis of the tails, ear and vulva, fever, lethargy, and pulmonary lesions (Lunney et al. 2016). The severity of PRRS symptoms is very variable between different strains; some are considered highly pathogenic, whilst others are of intermediate or low

pathogenicity (Stadejek et al. 2017). Additionally, the clinical outcome of PRRS is often deteriorated by secondary infections, for example with Porcine circovirus 2 (PCV-2) (Martín-Valls et al. 2022), *Mycoplasma hyopneumoniae* (Thacker et al. 1999), *Bordetella bronchiseptica* (Brockmeier et al. 2001), Influenza (van Reeth et al. 1996), etc. Apart from respiratory disease, PRRSV can cause reproductive failure in pregnant sows. Vertical transmission usually occurs during the third trimester of gestation, when the fetuses become immunocompetent and start expressing CD163 and CD169 (Karniychuk and Nauwynck 2009). Transplacental infections of the fetuses can cause late term abortions, early farrowing, stillbirths, mummifications and the birth of weak, congenitally infected piglets (Mengeling et al. 1994). The reproductive form of PRRS causes the biggest production losses associated with PRRSV infections, and the prevention of transplacental infections is an important aspect of vaccine developments.

1.1.4. PRRSV immunity

It has been shown that PRRSV-specific innate and adaptive immune responses are highly dependent on the viral strain (Lunney et al, 2016). These differences can be observed by altering blood cell populations, humoral responses, viral load, and cytokine levels (Weesendorp et al. 2013). Immune responses towards PRRSV have been extensively studied, nevertheless, there are still many open questions to be answered.

1.1.4.1. Innate immunity

As a first line of defense, the innate immune system is critical to the outcome of an infection. At first, a virus has to pass anatomical structures, like the mucus of the lungs. Mucosal membranes do not only contain chemical barriers but also complement proteins and innate immune cells, like monocytes, macrophages, dendritic cells, natural killer (NK) cells, and granulocytes, ready to initiate immune cascades (Murphy et al. 2017). These innate cells are activated by the binding of pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs). When PRRSV antigens get recognized by PRRs, it can lead to the production of pro-inflammatory cytokines, interferon (IFN) responses (Chow et al. 2015), and NK-cell-mediated cytotoxicity (Cao et al. 2013). Unfortunately, PRRSV has the ability to

suppress important innate immune reactions, such as NK-cell responses (Dwivedi et al. 2011), cytokine signaling (van Reeth et al. 1999), and type-I IFN production (Sun et al. 2012). Therefore, the activation of the adaptive immune system, and the generation of a long-term memory are of great importance upon PRRSV infections.

1.1.4.2. Adaptive immunity

Adaptive immune responses are activated if the innate immune system is not able to clear the virus from the host. Viral proteins are processed by the cell and expressed on cell surface molecules, the major histocompatibility complexes (MHC), to activate T cell responses. T cells originate in the thymus and are distributed into secondary lymphoid organs through the bloodstream. Once they encounter a specific antigen, presented by MHC molecules of antigen-presenting cells, like macrophages or dendritic cells, they differentiate to become effector T cells. MHC class I (MHC-I)-bound antigens, or epitopes, which are generated by proteasomal degradation (see chapter 1.2.2.), are recognized by naive $CD8^+$ T cells. Differentiation of these cells into an effector phenotype changes the expression of cell surface markers, allowing them to migrate towards inflammatory sites, and to neutralize infected cells with cytotoxic granules, which gives them the name cytotoxic T cells. Antigens presented by MHC-II molecules stimulate naive $CD4^+$ T cells. This T cell subset can differentiate into a range of T helper (T_H) cells, namely T_H1, T_H2, T follicular helper (T_{FH}), and T_H17 cells, and are essential for generating a humoral immune response. Anti-PRRSV antibodies are already detected 7-9 days post infection (p.i.) (Lopez und Osorio 2004), but they are not able to neutralize the virus (Labarque et al. 2000; Yoon et al. 1994). Neutralizing antibodies often only occur at 28 days p.i. (Meier et al. 2000), whereas most of them are against the major structural proteins GP5, M and N (Loemba et al. 1996; Lopez und Osorio 2004). Since PRRSV antibody responses are often not sufficient to protect from (re)infections, and are barely able to effectively neutralize the virus, $CD8^+$ T cells are considered important correlates of protection. $CD8^+$ T cell expansion and differentiation is observed 4-5 weeks p.i. with PRRSV (Albina et al. 1998b; Kawashima et al. 1999). They are capable to identify infected cells via MHC-I-presented viral epitopes and eliminate them by inducing apoptosis, through the secretion of cytotoxic proteins like granzymes and granulysin. Furthermore, they produce IFN-γ to inhibit viral replication, activate

macrophages, and enhance MHC-I expression (Murphy et al. 2017). As the primary site of infection, PRRSV-specific $CD8^+$ T cell responses have been shown to be the strongest in the lung (Kick et al. 2019). Furthermore, the virus is highly susceptible to type I IFN responses (Albina et al. 1998a; Overend et al. 2007), which can be produced by $CD8^+$ T cells. Unfortunately, PRRSV has evolved to manipulate $CD8^+$ T cell and macrophage effector functions in many ways, like downregulating MHC-I expression (Kick et al. 2019; Du et al. 2016; Cao et al. 2016), altering IFN responses (Luo et al. 2008; Lee et al. 2004; Kim et al. 2010), and modulating transcription and protein expression in macrophages (García-Nicolás et al. 2014; Renson et al. 2017). What is specifically important in PRRSV immunology is the establishment of a long-term memory to prevent (re)infections. This protection is assured by memory T cells, which remain after most effector T cells have vanished after an infection. In general, there are two subsets of memory T cells: effector-memory T cells (T_{EM}) and central-memory T cells (T_{CM}). T_{EM} ensure swift effector functions, but a limited proliferation potential, and T_{CM} can quickly proliferate, but show a lower cytotoxic activity (Pennock et al. 2013). The establishment of a solid memory T cell response is crucial in PRRSV immunology to ensure protection after a vaccination or infection.

1.1.5. Vaccination

The search for a safe and cross-protective PRRSV vaccine is a relentless issue. Several modified life virus (MLV) vaccines are available and regularly used for prophylactic and metaphylactic purposes. Nevertheless, these vaccines are often not cross-protective against heterologous virus strains (Kim et al. 2015; Renukaradhya et al. 2015b), and are not completely safe, since they have the potential to revert to virulence (Charerntantanakul 2012) and recombine with other PRRSV strains (Li et al. 2009; Marton et al. 2019; Vandenbussche et al. 2021). The latter has been observed and described many times, and since sequencing methods are becoming more advanced and available, more recombinant PRRSV are appearing in genome data banks. This recombination potential is a big disadvantage of MLV vaccines, since some mosaic viruses have been the possible cause of severe PRRS outbreaks. A more detailed elaboration on recombination can be found in chapter 1.3.1. and 4.2. Due to the disadvantages of MLV vaccines, inactivated PRRSV vaccines have been intensively tested. Nevertheless, most

inactivated vaccines show limited protection from infection, vertical transmission and virus shedding (Kim et al. 2011; M Scortti, C Prieto, E Alvarez, I Simarro, J M Castro 2007; Nielsen et al. 1997). To overcome the problem of the missing cross-protection of PRRSV vaccines, the search for potent and conserved T cell epitopes is an important task. The administration of well-designed subunit vaccines would exclude the risk of recombination and the reversion to virulence, which makes them relatively safe. Several subunit vaccines have been tested, but they only provide weak, or partial protection against PRRSV-1 and PRRSV-2 strains (Renukaradhya et al. 2015a; Oh et al. 2019). The disadvantage of these vaccines is the need of an adjuvant, which can cause side-effects, and the absence of a long-lasting immunity. To date, no data on PRRSV mRNA vaccines has been published. The mRNA vaccine technology is relatively new, and has improved very quickly since the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic. Potent PRRSV antigens could be delivered with this system without the need of a live virus, making it a promising approach.

1.2. MHC-I presentation and the immunoproteasome

Immunopeptidomics, the research of the arrangement and dynamics of peptides presented by MHC molecules, has gained a lot of importance in the past decades. The search for immunogenic epitopes, with the potential to stimulate T cells, is especially important in the fields of vaccinology, cancer, transplantation biology, and autoimmune diseases. As already discussed in chapter 1.1.4.2., T cells require stimulation by peptide-loaded MHC molecules to differentiate, expand, and gain effector or memory phenotypes. Infected cells present foreign peptides, whereas naïve cells present self-peptides, to avoid T cell responses. Subsequently, these MHC-bound peptides are important mediators of immune responses and therefore a frequent focus of research. This thesis focuses on MHC-I molecules, since they are presenting antigens to $CD8^+$ T cells, which are important, and yet underestimated, correlates of protection upon PRRSV infection.

1.2.1. The major histocompatibility complex-I (MHC-I)

1.2.1.1. Structure and interaction with T cells

MHC-I molecules are large proteins that bind and display peptides on the surface of antigen-presenting cells. As discussed in chapter 1.2.2., the presented epitopes are generated by ubiquitin-mediated proteasomal degradation. The immunoglobulin-like MHC-I is a heterodimeric molecule. It consists of a membrane-spanning α-chain that is non-covalently linked to a β_2-microglobulin (β_2M) (Tysoe-Calnon et al. 1991). The α-chain harbors three domains (α_{1-3}), whilst β_2M consists of one domain only. A peptide-binding groove is formed by the α_1 and α_2 domains and is located at the top of the complex. This groove typically binds peptides between 8 and 12 amino acids in length with ionic interactions and hydrogen bonds (Murphy et al. 2017). Longer peptides are seldomly bound, since they are too large to fit the peptide-binding groove. In general, these peptides possess hydrophobic or basic amino acids anchor residues, interacting with the peptide groove (Murphy et al. 2017). Subsequently, not all proteasomal peptides are bound by MHC-I. Different allelic versions of MHC-I display different peptide-binding specificities, due to altering amino acid structures of the peptide-binding groove.

As already mentioned in chapter 1.1.4.2., MHC-I/peptide complexes are the key mediators for the activation of $CD8^+$ T cells. Antigen-presenting cells and $CD8^+$ T cells interact with two receptors on either side. The first interaction partners are the T cell receptor (TCR) and the α_1 and α_2 domains of the MHC-I/peptide complex (Garboczi et al. 1996). Due to the large repertoire of MHC-I bound peptides TCRs are also highly diverse. This diversity is achieved with the arbitrary rearrangements of TCR gene segments (Lefranc 2001). The second interaction partners are the heterodimeric CD8αβ receptor and the α_2 and α_3 domains of MHC-I (Albina 1997; Sun et al. 1995). Upon binding of the T cell to the antigen-presenting cell, signal cascades are initiated to activate transcription factors, metabolic activity, cell survival, adhesiveness and rearrangement of the cytoskeleton (Murphy et al. 2017).

1.2.1.2. SLA-I diversity

The swine leukocyte antigen (SLA) complex is a gene dense region located on chromosome 7 (Geffrotin et al. 1984). It consists of three clusters (SLAI-III) spanning the centromere. SLA-I, the focus of this thesis, harbors seven classical and three non-classical genes (Lunney et al. 2009). While most classical genes have been found to be pseudogenes (Renard et al. 2006), SLA-1, SLA-2 and SLA-3 are constitutively expressed. These specific genes code for the MHC-I α-chain and β_2M and show a high degree of genetic diversity. Subsequently, a nomenclature system was developed by the International Society for Animal Genetics (ISAG) and the International Union of Immunological Societies (IUIS) (Ho et al. 2009), which is elucidated in figure 4.

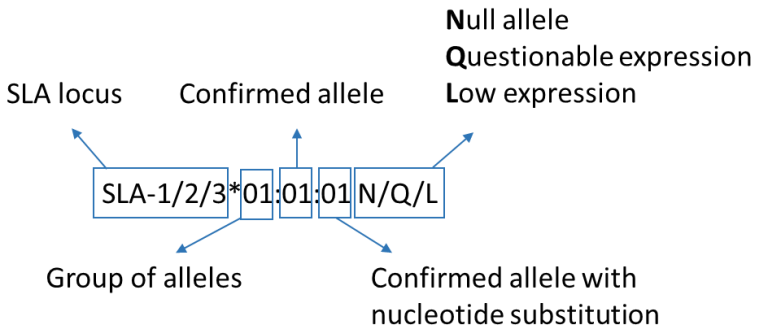

Figure 3. The nomenclature system of SLA-I alleles developed by the ISAG and IUIS.

Evaluation of SLA haplotypes can be done on a low-resolution level, with sequence specific PCRs, or on a high-resolution level, by sequencing of the specific genomic regions. Pig populations and breeds show different frequencies of SLA haplotypes (Hammer et al. 2021; Essler et al. 2013; Pedersen et al. 2014). This is an important aspect, since the high degree of SLA diversity impacts biomedical research (Hammer et al. 2020). More precisely, divergent haplotypes show different immune responses towards pathogens, vaccinations and allotransplantations. This enforces the importance of evaluating SLA haplotypes in immunologic research, to avoid biased data sets.

1.2.2. The immunoproteasome

Self and foreign proteins are constitutively being degraded and recycled by the cell. The degradation of cytosolic proteins is conducted by the 26S proteasome, a large protease complex with catalytic activity. It consists of the core particle, or 20S proteasome, with catalytic functions, and the regulatory particle, or 19S proteasome, with initiation functions flanking the core particle on either sides (Coux et al. 1996). Assembly of these subcomplexes is induced by IFN-γ (Tanaka und Kasahara 1998), forming a barrel-like structure. Dedicated cytosolic proteins are marked with polyubiquitin chains by E3 ligases and, subsequently, recognized by the 19S proteasomal subunit. Next, the targeted protein is unfolded and enters the catalytic center of the complex, to be hydrolyzed by the 20S core protein's catalytic core (Ferrington und Gregerson 2012). Peptide fragments are, however, not randomly generated, but preferentially cleaved after hydrophobic or basic amino acids (Murphy et al. 2017). The ATP-dependent transporters associated with antigens processing (TAP) proteins deliver the generated peptides into the ER. These ABC transporter proteins preferentially translocate peptides between 8 and 16 amino acids in length, restricting shorter or longer products to enter.

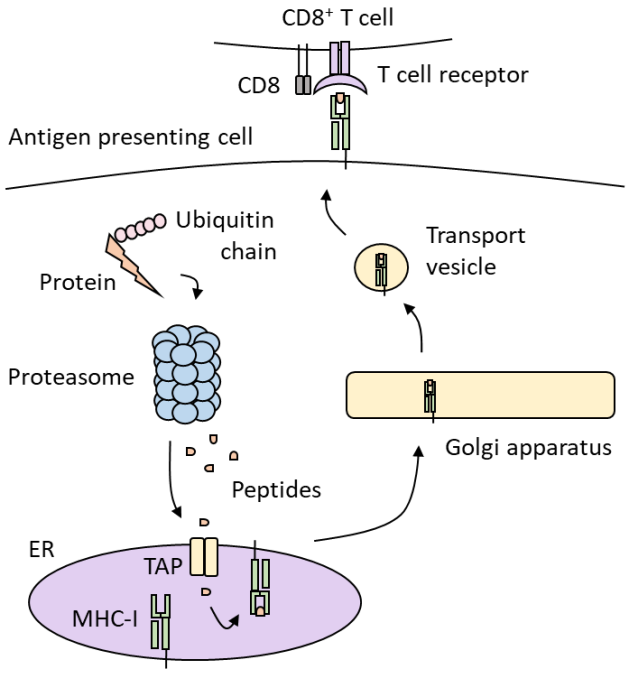

Figure 4. Ubiquitin-mediated proteasomal degradation of proteins and presentation of MHC-I epitopes to $CD8^+$ T cells.

Since these MHC-I presented peptides stimulate $CD8^+$ T cells, studies of these epitopes are important in the biomedical field. To date, the most conventional way of studying immunopeptidomes of viruses is with the generation of random, often overlapping, peptide libraries (Chung et al. 2016) or the use of MHC binding prediction softwares (Jurtz et al. 2017; Pan et al. 2019), to further stimulate T cells and measure their cytokine responses. Both methods have the limitation that it is unclear, whether the immunoproteasome will generate these hypothetical peptides *in vitro* or *in vivo*. Since the immunoproteasome follows a certain pattern of cleaving proteins, the direct isolation of MHC-I-bound peptides from cells, will provide naturally occurring peptides only.

1.3. Recombination

Viral recombination is the genetic cross-over of two or more different strains, after infecting the same host cell, and is an important process shaping viral evolution. Viruses with segmented RNA genomes, like *Orthomyxoviridae*, can reassort different genomes after infecting the same cell (Rabadan et al. 2008). This genetic shift, together with high mutation rates, leads to the fast evolution of Influenza A viruses (Lindstrom et al. 2004; Schweiger et al. 2006). RNA viruses with non-segmented genomes recombine by copy-choice replication. This mechanism relies on the RdRp's ability to switch templates during replication, causing a chimeric genome from two or more parental strains, with one or more cross-over sites (Hwang et al. 2001). Furthermore, the fidelity of the RdRp to the template (Fitzsimmons et al. 2018), RNA secondary structures (Carpenter et al. 1995; Nagy et al. 1999), host proteins (Prasanth et al. 2015), and bacterial co-infections (Erickson et al. 2018) can affect the recombination probability of positive-stranded RNA viruses (Wang et al. 2022a). Retroviral recombination relies on the same concept of template-switching during reverse transcription (Malim und Emerman 2001). Together with high mutation rates (see chapter 1.1.) recombination is a main contributor shaping the evolution of RNA viruses. Recombinant viruses have the potential to outcompete their parental strains by positive selection. These evolutionary advantages can be due to different cell and host tropisms, increased virulence, immune escape, and an overall increased fitness (Wang et al. 2022a). Nevertheless, these recombination events are random and can also result in less fitter chimeric strains that might be eliminated from the gene pool eventually.

1.3.1. Recombination of PRRSV

PRRSV has a high recombination potential, as it is often observed in positive-stranded ssRNA viruses (Patiño-Galindo et al. 2021). Due to constant improvements in sequencing technology and bioinformatics, the identification of PRRSV recombinants has become more frequent in the past decade (see chapter 4.2). To date, many recombinant PRRSV strains have been isolated and sequenced. These chimeric strains have been identified to be the cross-over results of two or more different field strains (Cavanagh 1997; Liu et al. 2018; Wang et al. 2020), field strains and vaccine strains (Li et al. 2009; Marton et al. 2019; Vandenbussche et al. 2021), or even two different vaccine strains (Kvisgaard et al. 2020). Even though these viruses are often not characterized concerning their pathogenicity and infectivity, many are considered concerning, after being isolated from pigs with mild to severe clinical symptoms (Kvisgaard et al. 2020, Liu et al. 2018). Since PRRSV is very prevalent in the commercial pig population, and MLV vaccines are regularly applied, the chance that two (or more) heterologous strains infect the same host is not as improbable. This is why closer PRRSV monitoring of pig populations, by virus isolation and whole-genome sequencing, is an important risk management tool. A more detailed elaboration of PRRSV recombinants can be found in chapter 3.2. and 4.2.

2. Aims

2.1. Identification of immunogenic MHC-I bound PRRSV peptides

Since PRRSV antibody responses after infection or vaccination are often not sufficient to neutralize the virus, $CD8^+$ T cells are considered important correlates of protection. The aim of the first part of this thesis was to develop a workflow for the isolation and identification of PRRSV-1-specific MHC-I bound peptides and explore their ability to stimulate $CD8^+$ T cells.

2.2. Recombination mechanisms of PRRSV

Recombination is a common process during PRRSV replication. Due to the isolation of three similar recombinant PRRSV-1 virus strains of PRRS-affected farms between 2018 and 2022, the aim of the second part of this thesis was to characterize these recombinants by describing and characterizing their clinical manifestation, whole-genome sequences, recombination hotspots, and possible recombination mechanism.

3. Manuscripts

3.1. Identification of MHC-I-Presented Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Peptides Reveals Immunogenic Epitopes within Several Non-Structural Proteins Recognized by $CD8^+$ T Cells

viruses

Article

Identification of MHC-I-Presented Porcine Respiratory and Reproductive Syndrome Virus (PRRSV) Peptides Reveals Immunogenic Epitopes within Several Non-Structural Proteins Recognized by CD8$^+$ T Cells

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Abstract: Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most relevant porcine pathogens worldwide. Active control of the disease relies on modified live virus vaccines (MLVs), as most inactivated vaccines provide very limited protection. Neutralizing antibodies occur late in infection; therefore, CD8$^+$ T cells are considered important correlates of protection and are a frequent focus of investigation. Our aim was to identify viral peptides naturally bound by the class I major histocompatibility complex (MHC-I) and to confirm their ability to stimulate CD8$^+$ T cells. For this purpose, we immunoprecipitated MHC-I/peptide complexes of PRRSV (strain AUT15-33) -infected cells (SLA-I Lr-Hp 35.0/24 mod) to isolate the viral epitopes and analyzed them with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Furthermore, we employed these identified peptides to stimulate peripheral blood mononuclear cells (PBMCs) of previously PRRSV-infected pigs and measured the PRRSV-specific CD8$^+$ T-cell response with an intracellular cytokine staining (ICS). Our data revealed that PRRSV non-structural proteins (NSPs), encoded in open reading frame 1a and 1b (ORF1), present the major source of MHC-I-presented peptides. Additionally, we show that our identified epitopes are able to trigger IFNγ responses *in vitro*. These findings are a basis for understanding the proteasomal degradation of PRRSV proteins, the cellular ability to display them via MHC-I, and their potential to restimulate CD8$^+$ T cells.

Keywords: porcine reproductive and respiratory syndrome virus; PRRSV; Arteriviridae; CD8$^+$ T cells; epitopes; swine leukocyte antigen class I; major histocompatibility complex class I; mass spectrometry; immunopeptidomics; restimulation; intracellular cytokine staining

1. Introduction

Porcine respiratory and reproductive syndrome virus (PRRSV) is an enveloped, single-stranded, positive-sense RNA virus within the family *Arteriviridae*, order *Nidovirales*. PRRSV belongs to the genus *Betaarterivirus* that is divided into two subgenera, *Eurpobartevirus* and *Ampobartevirus*. Each subgenus holds a single species: *Betaarterivirus suid 1*, i.e., PRRSV-1, which is mainly prevalent in Europe, and *Betaarterivirus suid 2*, i.e., PRRSV-2, which is

predominantly found in North America and Asia [1]. PRRSV strains show a high degree of genetic variation caused by high mutation rate [2,3], lack of RNA proofreading activity of the RNA-dependent RNA polymerase [4], and recombination events [5]. The virus infects cells of the monocyte/macrophage lineage and can be transmitted horizontally and vertically. PRRSV infection can cause a respiratory syndrome in nursery and fattening pigs, affecting lungs and airways, which is associated with fever and lethargy. A reproductive syndrome only occurs after PRRSV infection of pregnant sows, which can result in late-term abortions, mummification of fetuses, and the birth of weak, congenitally infected piglets [6]. The introduction of PRRSV into a farm is associated with high financial losses and makes PRRSV one of the most relevant pathogens in the swine industry [7]. Although the correlates of protection are not fully understood, modified live vaccines (MLVs) are widely used for prophylaxis and metaphylaxis, but they do bear certain limitations and risks, including limited protection from heterologous PRRSV strains [8] and the potential to revert to virulence [9]. Unfortunately, inactivated vaccines have low efficacy and show weak protection to heterologous challenge [10].

PRRSV manipulates the host immune responses at the humoral and cellular level, resulting in immunosuppression and secondary infections [11]. Affected animals display delayed neutralizing antibody responses [12,13] and disrupted IFN production [14], favoring the replication and spread of the virus. PRRSV additionally downregulates MHC-I expression in infected cells [15,16]. In pigs, MHC-I is a molecule encoded by three classical swine leukocyte antigen complex-I (SLA-I) genes (SLA-1/2/3) and is expressed on all nucleated cells [17]. These cell surface proteins bind and present antigens to $CD8^+$ T cells to cause cytotoxic responses. Furthermore, the generation of a $CD8^+$ T cell memory protects the host from recurring infections. In naïve cells, MHC-I presents endogenous, mostly cytosolic peptides, to signal that they are not infected. Most $CD8^+$ T cell epitopes are generated by the immunoproteasome, an ATP-dependent protease complex, which cleaves ubiquitinated proteins. Nevertheless, there are alternative antigen processing pathways, including autophagy [18] and protein cleavage by furin [19] and by signal peptide peptidases [20]. Classical proteasomal-derived peptides are bound and transported into the endoplasmic reticulum (ER) by the ATP-dependent protein complexes called transporters, which are associated with antigen processing-1 and -2 (TAP1/2). Inside the ER, peptides are loaded by the peptide loading complex (PLC) onto the immature MHC-I α-chain. This chain forms a peptide-binding cleft and has the ability to associate with peptides of most frequently nine amino acids (8–12) in length [21,22]. Longer peptides are rarely bound due to steric hindrance. Once the MHC-I molecule is loaded, it attains its mature conformation, dissociates from the PLC and is transported towards the cell surface for antigen presentation to $CD8^+$ T cells. These T lymphocytes recognize the antigen-presenting cells as infected and initiate cytolytic processes, including the production and secretion of cytotoxic granules and cytokines, such as IFNγ and TNFα.

$CD8^+$ T cells are considered important correlates of protection in PRRSV-infected pigs [23] since antibody responses are often not sufficient to clear the virus. To date, several studies have investigated the immunologic potential of randomly generated or predicted PRRSV-derived peptides [24–26]. However, it is not known whether these peptides are synthesized *in vivo*. The immunoproteasome generally cleaves proteins after hydrophobic and basic amino acids [21]. Therefore, it remains to be experimentally determined if certain predicted or randomly produced peptides are biosynthesized in the cell.

To clarify epitope specificity, our aim was to purify PRRSV-derived MHC-I bound peptide fragments from in vitro infected primary porcine alveolar macrophages (PAMs) to further determine peptide sequence identity by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Additionally, we confirmed the ability of these peptides to initiate a $CD8^+$ T-cell-specific cytokine response with an in vitro restimulation assay of PBMCs, isolated from PRRSV infected pigs, followed by an intracellular cytokine staining (ICS), and flow cytometry. With this approach, we provide a method for identifying $CD8^+$

T-cell epitopes of PRRSV-infected cells and report on the immunologic potential of peptides originating from the non-structural proteins encoded in open reading frame 1 (ORF1).

2. Materials and Methods

2.1. Porcine Alveolar Macrophages

PAMs were extracted by bronchoalveolar lavage (BAL) from euthanized, specifically PRRSV-free, and non-vaccinated pigs. Briefly, lungs were removed from the thorax and filled with 1–3 L of lukewarm and sterile PBS, the tissue was gently massaged, and BAL fluid was collected in glass bottles. Cells were washed three times with PBS and centrifuged with a Sorvall RC 26 Plus Centrifuge (Du Pont, Wilmington, NC, USA) at 300× *g* for 10 min at 4 °C. The cell pellet was resuspended in DMEM (Biowest, Nuaillé, France) and the cell number determined with a hemocytometer. Then, 1×10^8 cells were aliquoted in 1 mL fetal calf serum (FCS, Corning, New York, USA) + 10% DMSO (Carl Roth, Karlsruhe, Germany) and stored at −150 °C until further use. The viability of each batch of PAMs and the susceptibility towards PRRSV was assessed by titration.

2.2. Animals and PBMC Isolation

Heparinized whole blood was collected from twenty-three 4-month old piglets and two 12-month old gilts. The piglet cells were screened with a peptide pool, consisting of an equal amount of all synthetic peptides from Section 2.10, for an IFNγ response with an ICS. Subsequently, two batches of piglet PBMCs responded to the stimuli and were chosen for testing of the single peptides. Piglets were vaccinated once with Ingelvac PRRSFlex® EU (Boehringer Ingelheim Vetmedica GmbH, Rohrdorf, Germany) and 28 days later challenged with PRRSV strain AUT15-33 (GenBank: MT000052.1). Blood was collected 16 days post challenge. Gilts, which had been previously haplotyped and displayed a similar SLA-I background than the PAMs used for peptide isolation, were challenged with PRRSV strain AUT15-33, and three weeks post challenge, samples were obtained. PBMCs were isolated from heparinized whole blood following density gradient centrifugation (Pancoll human, density 1.077 g/mL, PAN-Biotech, Aidenbach, Germany), as previously described [27]. The recovered PBMCs were counted with a cell counter (XP-300 Haematology Analyser, Sysmex, Vienna, Austria) and cryopreserved in RPMI-1640 with stable glutamine with 100 IU/mL penicillin and 0.1 mg/mL streptomycin (all PAN-Biotech), 40% (*v*/*v*) heat-inactivated FCS (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA), and 10% DMSO (Hybri-Max™, Sigma-Aldrich, St. Louis, MO, USA) at −150 °C until further processing.

2.3. SLA-I Typing

PAMs used in this study were genotyped for their SLA class I haplotypes by running low-resolution PCR screening assays. Genomic DNA was isolated from 5×10^6 cells with a commercial kit following the manufacturer's instructions (E.Z.N.A. Tissue DNA Kit, Omega Bio-tek, Inc., Norcross, GA, USA). SLA-I low-resolution haplotypes (Lr-Hp) were identified by a sequence-specific primed PCR-based typing assay (PCR-SSP) to define the animals' SLA backgrounds at the allele-group level. SLA typing was performed by PCR-SSP with the complete set of typing primers specific for the allele groups of three SLA class I loci (SLA-1, SLA-2, and SLA-3). The criteria and nomenclature used for SLA-I haplotyping were based on those proposed by the international SLA Nomenclature Committee in the IPD-MHC database of suids (www.ebi.ac.uk/ipd/mhc/group/SLA, accessed on 26 August 2022) [22,28].

2.4. Virus

PRRSV-1 field isolate AUT15-33 was produced in PAMs. Cells were seeded in PAM medium (DMEM high glucose, Biowest; 10% FCS; 100 U/mL penicillin; 100 µg/mL streptomycin; 5 µg/mL chloramphenicol; 0.25 µg/mL amphotericin B) in a cell culture dish (Sarstedt, Nümbrecht, Germany) and inoculated with virus stock at a MOI of 0.1 for 1 h at room temperature (RT). The virus was removed and medium added to the cells. After 48 h,

supernatant was removed and virus titer determined on PAMs with a $TCID_{50}$ assay. Virus stocks were stored at −80 °C until further use.

2.5. Infection of PAMs

PAMs of high susceptibility were thawed at 37 °C and transferred into pre-warmed PAM medium. Four replicates with 5×10^8 cells each were seeded in PAM medium in 135 mm cell culture dishes. Additionally, four replicates of mock-infected cells were seeded in the same manner. One hour after seeding, medium was discarded and PRRSV added at a MOI of 0.1. After 1 h of incubation at RT, the supernatant was removed and fresh medium added to the cells. PAMs were incubated at 37 °C and 5% CO_2. Successful infection of cells was confirmed with an immunofluorescence staining. Briefly, cells were fixed with 4% paraformaldehyde (Carl Roth) for 20 min at 4 °C. Cells were permeabilized with 1% Triton X-100 (Carl Roth) for 5 min at RT. An in-house Cy3 labelled and produced anti-PRRSV-N monoclonal antibody (clone 810) was used to visualize infected cells.

2.6. Immunoprecipitation of the MHC-I/Peptide Complex

Eighteen hours post infection (p.i.), the medium was collected, and PAMs were scraped off in PBS and transferred to a 50 mL tube. Cells were washed three times at 300× *g* for 3 min at RT with a Sigma 3–10 centrifuge (Sigma, Osterode am Harz, Germany). The supernatant was discarded and the cell pellet frozen at −80 °C. For cell lysis, RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, protease inhibitor cocktail for tissue (Carl Roth), pH 7.5) was added to the frozen pellet and incubated over night at 4 °C. The cell lysate was centrifuged with a fixed-angle Mikro 20 centrifuge (Hettich, Kirchlengern, Germany) at 21.382× *g* for 20 min at 4 °C. The pellet was discarded and the supernatant stored at 4 °C until further use. Protein A/G magnetic beads (Thermo Fisher Scientific) were washed three times with RIPA buffer in a magnetic rack. For the precipitation of MHC-I/peptide complexes, the antibody PT85A was used as previously described [29]. PT85A and isotype control (Mouse IgG2a Isotype Control, Invitrogen, Waltham, MA, USA) were diluted in RIPA buffer and incubated with the magnetic beads for 1 h at 4 °C. Beads were washed three times with RIPA buffer and incubated with the centrifuged cell lysate for 1 h at 4 °C. The beads were washed three times with RIPA buffer and three times with IP wash buffer (150 mM NaCl, 50 mM Tris). At last, the MHC-I/peptide complex was eluted from the magnetic beads with 8 M Urea (BioUltra, ≥99%, Sigma Aldrich, St. Louis, MO, USA) at RT. Samples were stored at −20 °C until preparation for LC-MS/MS analysis.

2.7. Western Blot

For the Western blot analysis, the MHC-I/peptide complexes were isolated as described in Section 2.6. However, prior to cell lysis, PAMs were labelled with biotin (EZ-Link™ Sulfo-NHS-SS-Biotin, Thermo Fisher Scientific) for the detection of all cell surface proteins. Protein loading dye (6 M urea, 2% SDS, 10% glycerin, 0.01% bromophenol blue, 0.01% phenol red, 62.5 mM tris) was added to the samples and incubated for 5 min at 95 °C. Samples and a pre-stained protein ladder (New England BioLabs, Ipswich, MA, USA) were loaded on a 7.5% SDS gel and separated at 120 V. Proteins were blotted on a nitrocellulose membrane (BioTrace NT Nitrocellulose Transfer Membrane, Pall, New York, NY, USA) at 70 V for 1 h. The membrane was washed with PBS + 0.1% Tween20 (Carl Roth) and blocked with ROTI-Block (Carl Roth) for 1 h. Avidin horseradish peroxidase (HRP) (Thermo Fisher Scientific) was diluted in ROTI-Block (1:30.000), applied to the membrane, and incubated for 1 h. The membrane was washed three times for 10 min with PBS + 0.1% Tween20 and developed with the ECL Prime Western Blot detection reagent (GE Healthcare, Chicago, IL, USA), according to the manufacturer's instructions. Western blot was imaged with a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Sample Preparation and LC-MS/MS Analysis

Eluted peptides were reduced by incubation with a final concentration of 10 mM dithiothreitol at 56 °C for 1 h. After cooling down to RT, reduced cysteines were alkylated with iodoacetamide at a final concentration of 55 mM for 30 min in the dark. Urea content was diluted down to a concentration of 2 M prior to desalting and concentrating peptides via reversed-phase solid-phase extraction (SPE) using stage tips with two stacked C18 plugs (Empore™, MERCK KgaA, Darmstadt, Germany) [30]. Briefly, samples were acidified by addition of TFA to a final concentration of 1%. Stage tips were washed three times with 100% acetonitrile and equilibrated three times with stage tip buffer (0.4% formic acid, 2% TFA in water) before loading acidified peptide samples. Salts were removed by washing with 100 µL of 0.1% TFA and purified peptides eluted into a fresh HPLC vial with glass insert two times with 50 µL elution buffer (90% acetonitrile, 0.4% formic acid). Finally, eluted peptides were dried in a vacuum concentrator and reconstituted in 10 µL of 0.1% TFA. Label-free 1D-shotgun LC-MS/MS analysis was performed in a data-dependent acquisition (DDA) fashion on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000RSLC nano system (Thermo Fisher Scientific) via nanoflex ion source interface. Samples were loaded onto a trap column (Pepmap 100, 5 µm, 5 × 0.3 mm, Thermo Fisher Scientific) at 10 µL/min using 0.1% TFA. After loading, the trap column was switched in-line with a 50 cm, 75 µm inner diameter analytical column (packed in-house with ReproSil-Pur 120 C18-AQ, 3 µm, Dr. Maisch, Ammerbuch-Entringen, Germany) thermostatted at 50 °C. Mobile-phase A consisted of 0.4% formic acid in water and mobile-phase B of 0.4% formic acid in a mix of 90% acetonitrile and 10% water. The flow rate was set to 230 nL/min and peptides separated applying a 90 min gradient. MS scans were acquired at 300–1200 m/z in the Orbitrap at a resolution of 120,000 (at m/z 200) and an RF lens amplitude of 40%. AGC targeted 4×10^5 ions at maximum 100 ms. A TopN-dependent scan with a cycle time of 3 s set the acquisition of MS2 spectra in the Orbitrap at a resolution of 15,000 using a fixed first mass of m/z 120 and a quadrupole isolation window of 0.8 Da. Quadrupole isolation was enabled, and HCD was applied with a NCE of 30%. The AGC target was set to 1×10^4 with a maximum injection time of 150 ms. Peptide monoisotopic precursor selection (MIPS) was enabled for charge states 2–6 with an intensity threshold set to 5×10^4 and a dynamic exclusion of 20 sec. A single lock mass at m/z 445.120024 was employed [31]. XCalibur version 4.3.73.11 and Tune 3.4.3072.18 were used to operate the instrument.

2.9. Data Analysis and Peptide Sequence Identification

Peak lists obtained from MS/MS spectra were identified using X!Tandem (version X! Tandem Vengeance (2015.12.15.2)) and MS Amanda (version 2.0.0.17442). The search was conducted with SearchGUI (version v4.1.1) [32]. Protein identification was performed against a concatenated target/decoy version of the *NCBI Reference Sequences* (*RefSeq*) (0.5%) database considering the following species: AUT15-33 (8 target/16 decoy sequences), *Sus scrofa* (1431 target/2862 decoy sequences). Decoy sequences were created by reversion of target sequences. The following identification settings were used: unspecific cleavage; 10.0 ppm as MS1 and 0.02 Da as MS2 tolerances; fixed modifications: carbamidomethylation of C (+57.021464 Da), variable modifications: oxidation of M (+15.994915 Da). Peptides and proteins were inferred from the spectrum identification results using PeptideShaker (version 2.2.5) [33]. Peptide spectrum matches, peptides, and proteins were validated at a 1.0% false-discovery rate estimated using the decoy hit distribution. The sequence logo was created with IceLogo (version 1.3.8.) using the *Sus scrofa* reference proteome as consensus.

2.10. Synthetic Peptides

The LC-MS/MS-identified peptide sequences were sent to ProteoGenix (Schiltigheim, France) for peptide synthesis. Peptides were manufactured with >80% purity and quality-controlled with HPLC and MS. Synthetic peptides PepPRS02, -03, and -08 were reconstituted in one part DMSO (Carl Roth) and two parts H_2O under sterile conditions. The

remaining peptides were reconstituted in one part acetonitrile (≥99.9%, Sigma Aldrich) and two parts H_2O under sterile conditions. Peptides were stored at −80 °C.

2.11. In Vitro Stimulation of PBMCs

All steps were carried out under sterile conditions using a biosafety cabinet. PBMCs were defrosted in culture medium (RPMI 1640 with stable glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin (all PAN-Biotech), 10% FCS (GIBCO)) and centrifuged with a Heraeus Megafuge 40R (Thermo Fisher Scientific) at 400× *g* for 8 min at RT. Cells were counted with a Sysmex cell counter, and 5×10^5 cells per well, in eight replicates (a total of 4×10^6 cells per condition), were seeded into sterile 96-well round-bottom plates (Nerbe plus GmbH & Co. KG, Winsen, Germany). The plates were incubated at 37 °C and 5% CO_2 for a minimum of 6 h. Stimulation of PBMCs was carried out with 5 µg/mL peptide or DMSO/ACN as a medium control for 17 h at 37 °C and 5% CO_2. Four hours prior to harvesting cells, 1 µg/mL brefeldin A (BD GolgiPlug™, BD Biosciences, Franklin Lakes, NJ, USA) was added to inhibit cytokine secretion. A cocktail of phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Sigma Aldrich, St. Louis, MO, USA), ionomycin (500 ng/mL, Sigma-Aldrich), and brefeldin A was added as a positive control for cytokine production four hours before harvesting.

2.12. Intracellular Cytokine Staining

For the intracellular cytokine staining, PBMCs were harvested in PBS (without Ca^{2+} and Mg^{2+}, PAN-Biotech) with 3% (*v*/*v*) FCS (GIBCO) and washed twice. The cells were transferred into 96-well round-bottom microtiter plates (Greiner Bio-One, Frickenhausen, Germany) and stained in a four-step procedure. Primary antibody mix (Table 1) was added to the stimulated PBMCs and incubated for 20 min at 4 °C. Further, cells were washed twice in PBS for 4 min at 400× *g* and 4 °C. Secondary antibody (Table 1) and the Fixable Viability Dye eFluor 455UV (Thermo Fisher Scientific) were diluted in PBS and incubated with the cells for 20 min at 4 °C. Cells were washed twice as previously described. Next, cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Intracellular cytokines were stained with an antibody mix (Table 1) in permeabilization buffer for 20 min at 4 °C. Two final wash steps were executed before resuspending the PBMCs in permeabilization buffer. A fluorescence minus one (FMO) sample without anti-TNFα monoclonal antibody (mAb) was prepared as a background control.

Table 1. List of antibodies used in the ICS panel.

Marker	Clone	Isotype	Fluorophore	Labelling	Source
			Surface antigens		
CD8α	76-2-11	IgG2a	PerCPerfluor 710	Indirect [A]	In house
CD27	b30c7	IgG1	Alexa fluor 647	Direct	In house
CD8β	PPT23	IgG1	Alexa fluor 488	Direct	In house
			Intracellular antigens		
TNFα	Mab11	IgG1	Alexa fluor 700	Direct	Biolegend
IFNγ	P2G10	IgG1	PE	Direct	BD Biosciences

[A] Rat-anti-mouse anti-IgG2a-PerCPfluor710, eBiosscience.

2.13. Flow Cytometry

The CytoFLEX LX (Beckman Coulter GmbH, Krefeld, Germany) flow cytometer equipped with six lasers (355, 405, 488, 561, 638, and 808 nm) and a plate loader was used for the analysis of the stained samples. The VersaComp Antibody Capture kit (Beckman Coulter) was used to set-up single stains, following the manufacturer's instructions, in order to calculate the compensation values using the CytExpert software version 2.4 (Beckman Coulter). For all samples, 1×10^6 lymphocytes were recorded in total. Flow cytometry data were processed using FlowJo software version 10.8.1 (BD Biosciences).

3. Results

3.1. Isolation of MHC-I/Peptide Complexes by Immunoprecipitation

Biotinylated PAM lysates were immunoprecipitated with protein A/G beads linked to the anti-MHC-I antibody PT85A. Eluates were analyzed with an SDS-PAGE followed by a Western blot. Detection of biotin-labelled cell surface proteins with avidin-HRP confirmed the successful isolation of MHC-I α-chains at 45 kDa and β2-microglobulin molecules at 12 kDa, without major contaminations with other cellular proteins (Figure 1). The immunoprecipitation with a control IgG did not show isotype-specific binding towards proteins of the cell lysates. After the establishment of the immunoprecipitation protocol, samples for LC-MS/MS analysis were prepared without biotinylation to avoid analyte loss by additional washing steps.

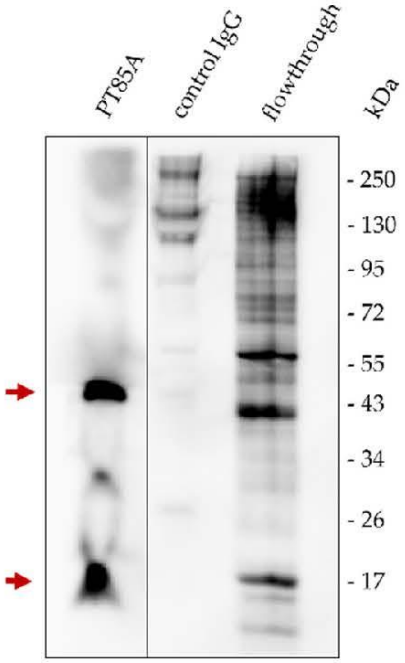

Figure 1. Western blot of isolated MHC-I molecules from porcine alveolar macrophages (PAMs). Cell surface proteins were immunoprecipitated with mAb PT85A linked to protein A/G magnetic beads. A mouse IgG2a isotype control was used to confirm the absence of isotype-specific binding. After running samples on a SDS-PAGE and blotting onto a nitrocellulose membrane, biotin was stained with avidin-HRP. The upper arrow indicates the MHC-I alpha chain at 45 kDa, which harbors the peptide binding groove, and the lower arrow β2-microglobulin at 12 kDa.

3.2. Peptides Originating from PRRSV Non-Structural Proteins Are Displayed by MHC-I

PAMs were infected with the PRRSV strain AUT15-33 at an MOI of 0.1. Additionally, another batch was mock-infected. From both naïve and infected cells, four technical replicates were produced. Since several studies, including our own unpublished results, demonstrate that MHC-I is downregulated upon PRRSV infection [15,16], cells were harvested at 18 h p.i. to ensure maximum sample yield. Successful infection of cells was confirmed in a small, separate cell culture dish by immunofluorescence (data not shown). After harvesting, the MHC-I/peptide complexes were isolated by immunoprecipitation. Eluted samples were analyzed by LC-MS/MS, and the obtained MS data searched against the AUT15-33 and *Sus scrofa* proteome. Database research of samples from naïve PAMs that were matched to the *Sus scrofa* proteome revealed a total of 2387 identified peptide groups, with peptides between 7 and 13 amino acids in length. The vast majority (63.64%) of those are nonamers (Figure 2a). These findings are in line with the literature, which demonstrates that most MHC-I bound peptides have a sequence length of nine amino acids [34]. Fur-

thermore, the epitope anchor residues are known to preferentially be hydrophobic or basic amino acids [21]. Our data confirms a conservation of hydrophobic amino acids at the anchor residues (position 2 and 9) of 9-mer peptides (Figure 2b).

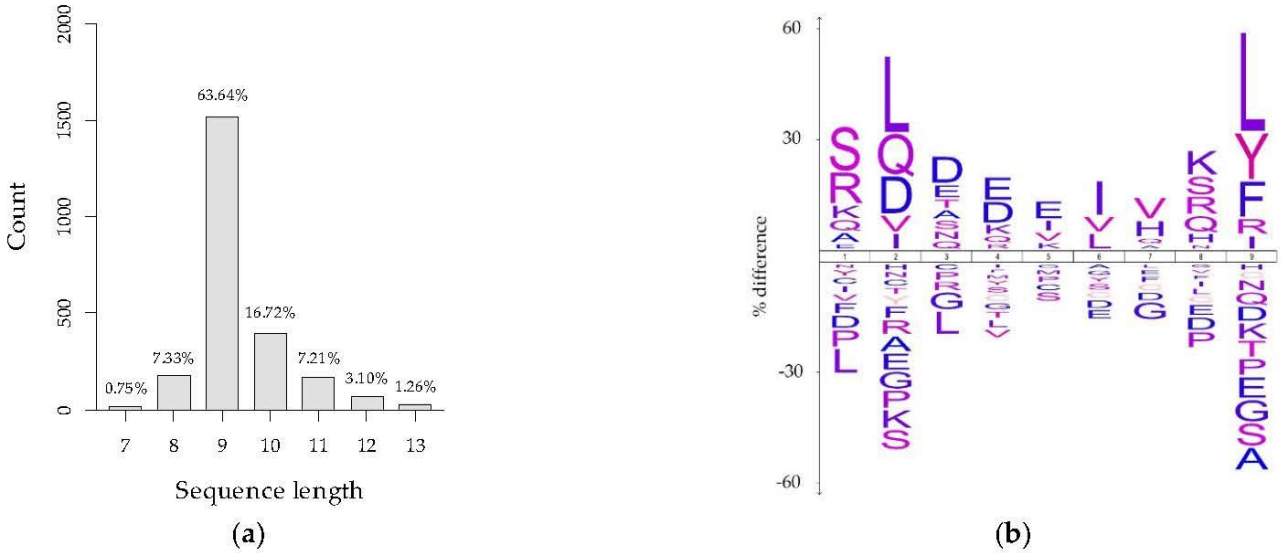

Figure 2. Length and sequence conservation of LC-MS/MS identified peptides from mock-infected PAMs searched against the *Sus scrofa* proteome. (**a**) Peptide sequence length (amino acids) of MHC-I-bound porcine peptides. Percentages of total isolated peptides are displayed above the bars. (**b**) Sequence logo of 9-mer peptides, depicting percent differences of amino acid frequencies from the *Sus scrofa* proteome as a reference.

Notably, the peptide spectrum matches obtained from MHC-I-bound peptide isolates of PRRSV infected PAMs—when searched against the AUT15-33 proteome—gave the most confident hits to proteins within ORF1, which codes for the virus' non-structural proteins (NSPs, Table 2, Figure 3). Seven peptides were matched to four of the viral proteases—NSP1α, NSP1β, NSP2, and NSP4. One peptide originates from the transmembrane protein NSP5 and one from NSP8. Four peptides were matched to NSP9, the viral RNA-dependent RNA polymerase. To confirm that the LC-MS/MS-obtained viral peptide sequences are also PRRSV-specific $CD8^+$ T-cell epitopes, an evaluation of their immunologic potential was conducted.

Table 2. Overview of the most confident hits of mass spectra obtained from the analysis of MHC-I-bound peptides of PRRSV-infected PAMs matched with the PRRSV genome.

Peptide	Sequence	PRRSV Protein Origin	Length (Amino Acids)
1	SVVFPLARM	NSP1α	9
2	LVKVAEVLYR	NSP1α	10
3	RLQINGIR	NSP1β	8
4	LDKMWDRV	NSP2	8
5	LALEQRQL	NSP2	8
6	VISESGDLI	NSP4	9
7	DIKLSPAII	NSP4	9
8	SQALSTYCF	NSP5	9
9	VEKLKRII	NSP8	8
10	QGFVLPGVL	NSP9	9
11	GRCLEADL	NSP9	8
12	LLEIQPML	NSP9	8
13	VITDKPSFL	NSP9	9

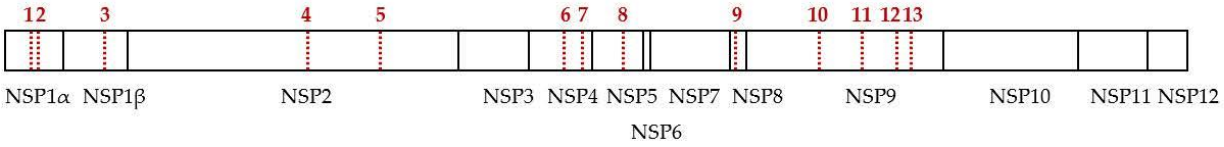

Figure 3. Position of identified MHC-I-bound peptides (dashed red lines) from infected PAMs within the PRRSV ORF1 polyprotein. Numbers above the red dashed lines correspond with Table 2. NSP, non-structural protein.

3.3. MHC-I-Bound PRRSV Peptides Elicit a CD8⁺ T-Cell-Specific IFNγ Response

The thirteen most confident hits for PRRSV ORF1-derived peptides and two *Sus scrofa*-derived peptides (C1: ELNDRFANY, C2: KLRDLEDSL) were synthesized and their immunogenic potential assessed with an in vitro restimulation of PBMCs, followed by an ICS. We preferred an ICS over the classical ELISPOT assay due to its lower background, higher sensitivity, and ability to sort for $CD8^+$ T-cell specifically. PBMCs from our biobank of 23 randomly chosen piglets were screened with a pool of 13 LC-MS/MS-identified PRRSV peptides (data not shown). Our screening revealed two responders among the piglets, displaying an elevated IFNγ production compared to the negative controls. To shed light on the piglets' MHC-I background, the responder PBMCs and the PAMs used for the isolation of MHC-I peptides were SLA-I haplotyped (Table 3). The low-resolution haplotypes (Lr-Hp) revealed that all PBMCs used in this study share a same allele with the PAMs. Further, the four batches of PBMCs were used to assess the immunologic potential of the 13 individual LC-MS/MS-identified PRRSV peptides (P1–P13), the peptide pool, and two endogenous porcine peptides (C1 and C2) in an in vitro restimulation assay. Additionally, a negative control (ACN/DMSO) for negative background activation and a positive or activation control (PMA/ionomycin) were employed. Cytokine production by $CD8^+$ T cells and CD27 expression in response to the different stimuli were evaluated via ICS, followed by a flow cytometric analysis. The gating strategy to assess PRRSV-peptide-specific $CD8^+$ cytokine responses and CD27 expression is described in Figure 4.

Table 3. SLA-I low-resolution haplotypes (Lr-Hp) of PAMs used for peptide isolation and PBMCs for in vitro restimulation.

Pig	SLA-1	SLA-3	SLA-2	Lr-Hp
PAM [1]	12XX, 13XX	05XX	10XX	35.0
	07XX, 08XX	04XX	06XX	24.0 mod
Gilt16 [2]	12XX, 13XX	05XX	10XX	35.0
	02XX, 18:01	01XX	11XX	57.0
Gilt24 [2]	12XX, 13XX	05XX	10XX	35.0
	02XX, 07XX	04XX	02XX	2.0
Piglet55 [2]	13XX	04XX	06XX	24.0 mod
	08XX	05XX	blank	49.0
Piglet62 [2]	blank	04XX	06XX	24.0
	11:03	05XX	16:02	59.0

[1] PAMs used for isolation of MHC-I peptides; [2] PBMCs used for restimulation with synthetic peptides. mod, modified.

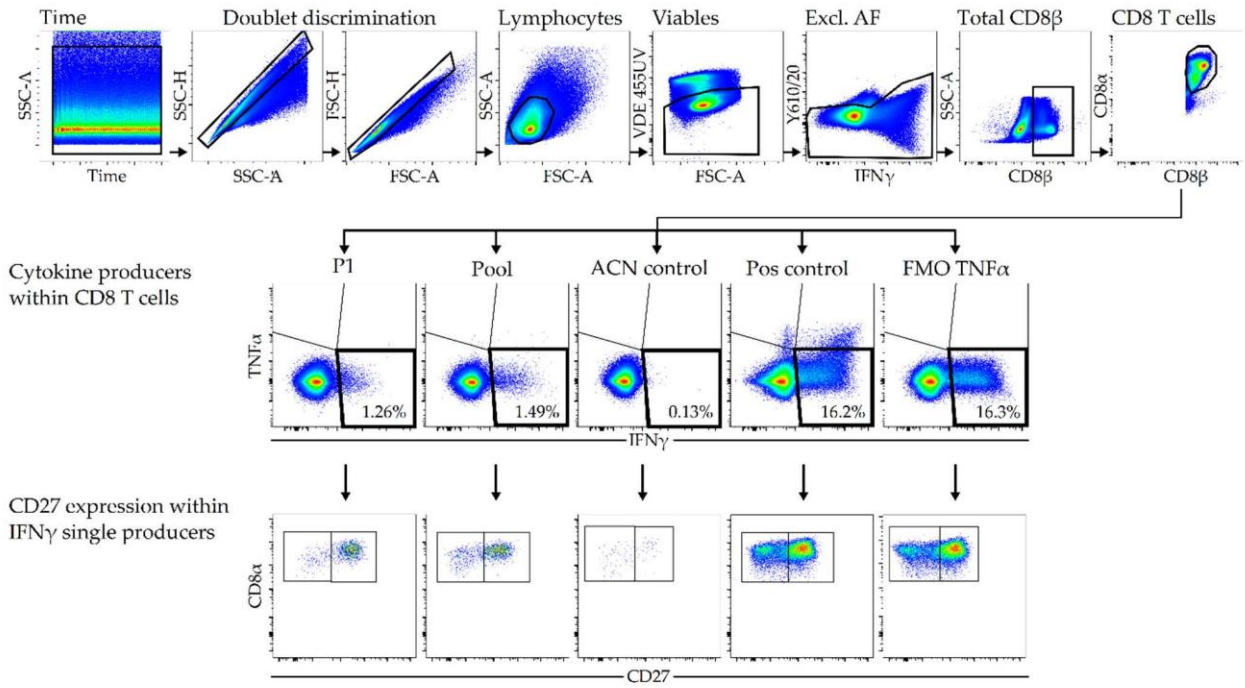

Figure 4. Gating strategy to assess PRRSV-peptide-specific CD8$^+$ T-cell responses. Thawed porcine PBMCs were stimulated in vitro with the identified PRRSV-peptides (5 µg/mL), ACN/DMSO, or PMA/ionomycin. Cells were harvested, stained in a four-step procedure and analyzed by flow cytometry. For all samples, a time gate and a double-doublet discrimination (SSC-H vs. SSC-A and FSC-H vs. FSC-A) was applied. Lymphocytes were gated based on their light scatter properties (FSC-A vs. SSC-A); viable cells were selected based on their staining with the Fixable Viability Dye eFluor 455UV and the use of an empty channel—bandpass filter Y610/20—allowed for the exclusion of cells with autofluorescent signal. Thereafter, total CD8$^+$ T cells were gated on and were further analyzed for their co-expression of CD8β. These cells were analyzed for their expression of IFNγ and TNFα. The middle panel shows the cytokine production in response to peptide 1 (P1), peptide pool, negative control, positive control, and FMO control. The lower panel shows CD27 expression of IFNγ-producing CD8$^+$ T cells in response to the stimuli. Representative pseudocolor plots from one animal are shown.

Our analysis revealed that the peptide pool, consisting of equal amounts of all 13 identified MHC-I-bound PRRSV peptides, triggered a CD8$^+$ T-cell-specific IFNγ response in all four animals, which was 5.4 to 10.5 times higher than the negative controls (Figure 5). Between 0.69% and 1.49% of the total CD8$^+$ T-cell population produced antigen-specific IFNγ after restimulation with the peptide pool. There was no notable rise in TNFα or IFNγ/TNFα co-producing CD8$^+$ cells. Notably, peptide 1 (P1) showed the strongest IFNγ response in all animals, which was between 0.36% and 1.26% of the total CD8$^+$ T-cell population. MHC-I-bound porcine peptides (C1 and C2) isolated from naïve cells did not trigger the production of IFNγ, confirming their endogenous properties. Additionally, we employed a staining for CD27, a marker for naïve T cells. During differentiation towards an effector (memory) phenotype, CD27 expression is depleted [34]. In our study, the restimulation with the peptide pool and the single peptides exhibited a higher frequency of naïve or CD27$^+$CD8$^+$ T cells compared to the positive control, PMA/ionomycin, which is a potent stimulus (Figure 4). Between 31.7% and 70.1% of the PMA/ionomycin-stimulated cells, between 43.4% and 73.0% of the cells stimulated with the peptide pool, and between 60.7% and 88.9% of the P1-restimulated cells belonged to this naïve population. The amount of CD27$^-$ CD8$^+$ cells, which represent a terminally differentiated T-cell phenotype, is the lowest in PBMCs restimulated with P1 compared to the restimulation with the peptide pool

and the positive controls. This lets us conclude that the individual peptides are weaker stimulants for T-cell differentiation than pooled peptides.

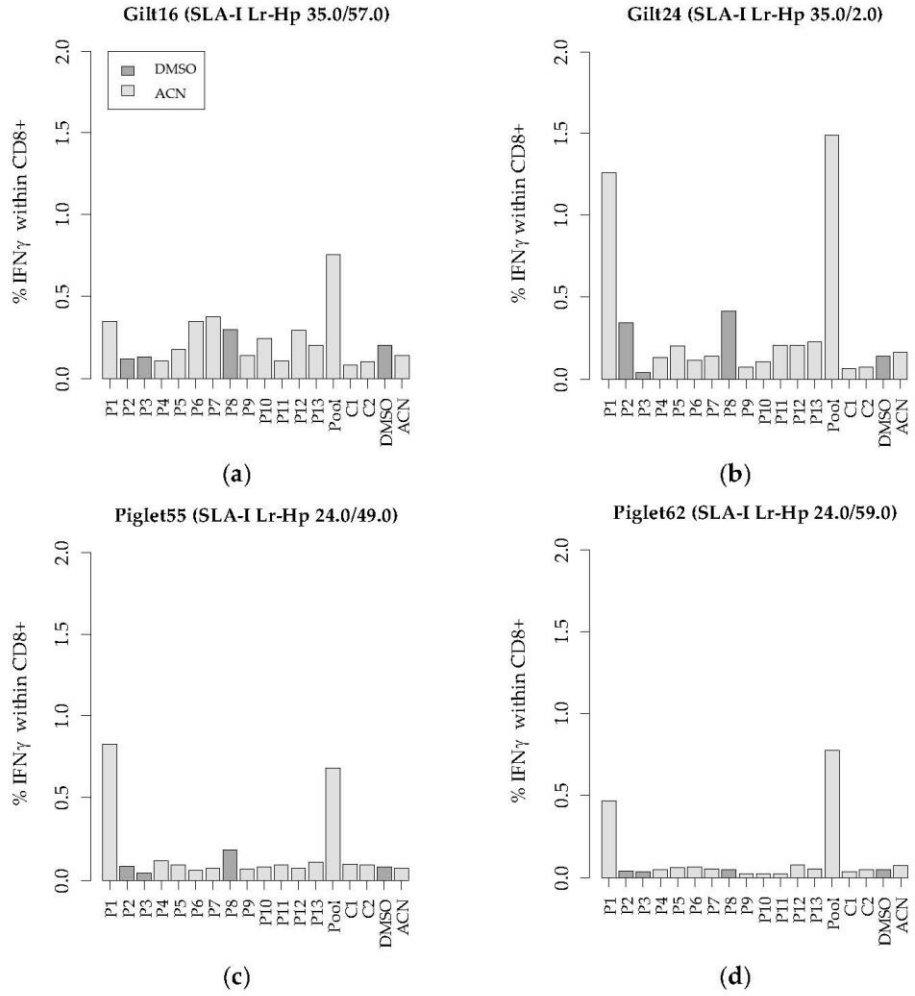

Figure 5. IFNγ-producing cells within the total CD8$^+$ T-cell population after restimulation of PBMCs with MHC-I-bound porcine and PRRSV-derived peptides. PBMCs were isolated from two gilts (**a**,**b**) and two piglets (**c**,**d**). P, PRRSV-derived peptides; C, control peptides from naïve cells; DMSO and CAN, medium control; Pool, pool of all 13 PRRSV peptides. SLA-I low-resolution haplotypes (Lr-Hp) of the donor animals are indicated above each graph.

4. Discussion

CD8$^+$ T-cell responses are important correlates of protection in PRRSV-infected animals due to their ability to identify and eliminate infected cells. It has been shown that PRRSV-induced neutralizing antibody responses are delayed [13], strain specific [35,36], and often not fully capable of neutralizing the virus [36]. Therefore, the study of the activation of CD8$^+$ T-cell effector responses is a crucial task in understanding host responses after PRRSV infections. Additionally, these findings are important for optimizing vaccinations and therapeutic efforts. To understand which viral antigens contribute to eliciting CD8$^+$ T-cell responses, immunogenic MHC-I-bound peptides have to be identified.

To date, several studies have been investigating PRRSV epitopes by cross-presentation of either predicted or randomly produced viral peptides or peptide libraries. Wang et al. designed overlapping peptides from PRRSV membrane proteins and identified four immunodominant epitopes. Since all our ICS analyzed PRRSV-specific, MHC-I-bound peptides derived from NSPs, there is no overlap of peptide sequences with this study. Pan et al. [24]

predicted nine PRRSV epitopes with NetMHCpan 4.0 [37], of which three were derived from NSPs. One of those peptides elicited an IFNγ response in their analysis but was also not identified in our screen. Chung et al. [38] created an overlapping peptide library of all PRRSV ORFs and evaluated their immunogenic potential by restimulating PBMCs. They identified immunogenic peptides within NSP1α, NSP2, and NSP4 using an ELISPOT assay. One 20-mer within NSP4 is covering the sequence of our identified peptide 7. Another immunogenic 20-mer is partly overlapping with our PRRSV peptides 1 and 2. Parida et al. [39] investigated the stimulation potential of PBMCs with overlapping peptides from PRRSV NSP9 and NSP10 using a proliferation assay in combination with an ELISPOT. They were able to identify immunogenic peptides, but none of them overlap with our analyzed peptide sequences. In addition, Burgara-Estrella et al. [40] and Mokhtar et al. [41] screened PRRSV peptide libraries by ELISPOT, but we could not find an overlap of peptide sequences with the epitopes that we used for our in vitro stimulation experiments. The varying SLA-I backgrounds of the pigs used in the different studies might be an explanation for the non-overlapping peptide sequences. Furthermore, the diversity of identified $CD8^+$ T-cell-stimulating epitopes in the literature lets us conclude that there is a much broader PRRSV epitope repertoire than already reported. Nevertheless, the predicted or randomly generated epitopes from these studies are hypothetical, and it is not clear whether they are actually generated *in vivo*. The immunoproteasome has certain patterns of hydrolyzing proteins [21,42], and therefore, only a limited number of these peptides might naturally occur.

With our study, which is based on methods from a pioneer study of the 1990s [43], we provide, to our knowledge, the first sequences of MHC-I-bound peptides that have been directly isolated from cells infected with PRRSV *in vitro*. These peptides are products of ubiquitin-mediated degradation of the immunoproteasome and presented by MHC-I molecules on the cell surface of PAMs. After isolation of the MHC-I/peptide complexes by immunoprecipitation, we analyzed the bound peptides with LC-MS/MS. Our data confirm the successful isolation of porcine endogenous and PRRSV MHC-I-bound peptides. The LC-MS/MS turned out to be the bottleneck of the study since immunopeptidomic analyses are not well-established in most mass spectrometry facilities. The vast majority of peptide sequences from naïve cells, obtained by peptide-spectrum matching with the *Sus scrofa* genome, were 9-mers, which possess hydrophobic MHC-I anchor residues. Furthermore, databank research with the PRRSV proteome of LC-MS/MS-obtained mass spectra from infected cells revealed that the most confident sequences originate from several NSPs of ORF1. These proteins are located in the cytoplasm and are plausible targets for ubiquitin-mediated proteasomal degradation. Interestingly, no confident peptide-spectrum matches were obtained for the structural proteins encoded by ORF 2–7 although they are present in a several-fold molar excess over ORF1-encoded proteins. This is plausible for the four glycoproteins, as they reside in the secretory pathway. However, we would have expected peptides derived from the strongly expressed ORF7 encoded nucleocapsid (N) protein, which is cytosolic. Our data represent only a preliminary glance at PRRSV-derived MHC-I bound peptides, and further in-depth studies have to be conducted to validate and expand these data. A caveat of this study is the heterogeneity of the SLA-class I haplotypes that diverge between peptide isolation and challenge models. There is a large number of known SLA-I haplotypes with altering frequencies in the pig population, and an inbred line would be favorable. Unfortunately, for our study, we did not have access to inbred pigs. Nevertheless, the gain of the genetic heterogeneity shows that our peptides might have the potential of stimulating different SLA-I haplotypes.

To assess whether the determined peptides from ORF1 are able to trigger an IFNγ response of $CD8^+$ T cells, we investigated the immunologic responses towards these potential epitopes in a small-scale pilot study. IFNγ production is an important correlate of protection after PRRSV infection to regulate antiviral immune responses [22,44]. In order to investigate whether our LC-MS/MS-identified peptides possess the ability to trigger a PRRSV-specific IFNγ $CD8^+$ T-cell response, we restimulated PBMCs, which

were isolated from PRRSV-challenged pigs, in an in vitro assay. To detect and measure cytokine production, we performed an intracellular cytokine staining (ICS) followed by flow cytometry. The use of an IFNγ ELISPOT was omitted because of its poorer sensitivity and the inability to assess cytokine production at the single-cell level. Our experiments revealed the presence of IFNγ-producing $CD8^+$ T cells upon restimulation with PRRSV peptides. Additionally, the ICS panel included a CD27 staining. Within the porcine $CD8^+$ T-cell population, CD27 expression has previously been described as a marker for the naïve phenotype and a lack of CD27 expression as an effector (memory) phenotype [34]. Our results show a lower amount of $CD8^+CD27^-$ T cells upon restimulation with the PRRSV single peptides or peptide pool compared to the positive control. This means there is a larger population of naïve than terminally differentiated $CD8^+$ T cells upon stimulation with PRRSV-specific MHC-I peptides compared to the positive control. We hypothesize that this could be due to the short incubation time of the PBMCs with the peptides and/or the relatively weak restimulation potential of our PRRSV-derived single peptides in comparison to the positive control, which is a strong stimulus.

All gilts and piglets, which were the donors of the PBMCs used for our in vitro restimulation experiments, possessed a similar SLA-I background as the PAMs used for the isolation of the MHC-I-bound PRRSV-specific peptides. A model using inbred pigs would be the ideal setting, but due to limited access and animal welfare reasons, cells for our studies were used from pre-existing cell banks. All of the cells used in this study share at least one Lr-Hp but have a different second set of SLA-I alleles. Therefore, we can hypothesize that the immunogenic PRRSV-derived peptides are not necessarily confined to a specific SLA-I haplotype but might have the potential of stimulating others, too. More research is needed to verify this hypothesis.

One limitation in the development of cross-protective PRRSV vaccines is the high mutation rate and occurrence of highly divergent strains in the field. Nevertheless, the viral NSPs have functional constraints limiting variability, while the structural proteins show a lower degree of conservation. Consequently, it can be expected that $CD8^+$ T-cell-stimulating epitopes derived from NSPs possess the potential to generate immune responses to multiple PRRSV strains. Our identified epitopes are especially conserved among PRRSV strains used for MLV vaccines, PRRSV-1 strains, and also partly in PRRSV-2 isolates (Figure 6). This could be of importance for the redesign of existing vaccines to make them more efficient and cross-protective against heterologous PRRSV strains. Furthermore, our preliminarily identified PRRSV epitopes could be first candidates for being included in novel, rationally designed mRNA vaccines or vector vaccines.

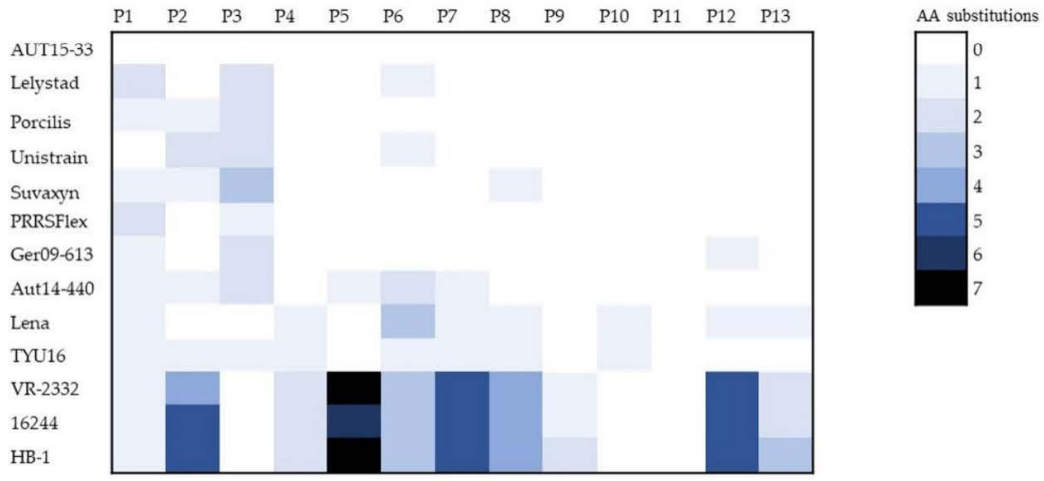

Figure 6. Heat map illustrating conservation of isolated AUT15-33 peptides compared to other PRRSV vaccine strains (94881: Ingelvac PRRSFLEX EU; DV: Porcilis PRRS, MSD; Amervac: Unistrain PRRS, HIPRA; 96V198: Suvaxyn PRRS MLV, Zoetis), PRRSV-1, and PRRSV-2 (VR-2332, 16244, HB-1) isolates. Numbers of amino acid substitutions are coded according to the color key. P, peptide; AA, amino acid.

Our LC-MS/MS- and ICS-based identification method is a straightforward way to identify additional PRRSV-specific $CD8^+$ T-cell epitopes. To explore the PRRSV immunopeptidome further, follow-up studies investigating different SLA-I haplotypes and different PRRSV strains have to be conducted to gain a better insight into proteasomal processing, MHC-I presentation, and $CD8^+$ T-cell restimulation in PRRSV-infected animals. Additionally, this identification method can be applied to investigate the immunopeptidome of other intracellular pathogens.

5. Conclusions

We have established a method for identifying MHC-I-displayed $CD8^+$ T-cell epitopes from PRRSV-infected PAMs with an LC-MS/MS-based assay. Further, we confirmed the immunogenicity of some identified peptides by an in vitro PBMC restimulation assay, ICS staining, and flow cytometry measurement. Peptides originating from several PRRSV NSPs were shown to elicit an IFNγ response and are strong candidates for immunization efforts. Further $CD8^+$ T-cell epitopes from multiple PRRSV strains and cells with different SLA-I backgrounds must be identified and their immunologic potential investigated in the future to deepen the understanding of ubiquitin-mediated proteasomal degradation and presentation of PRRSV protein-derived peptides to $CD8^+$ T cells.

Author Contributions: Conceptualization, T.R.; methodology, T.R., M.M., M.R.S., S.E.H., F.F., A.L., A.C.M., C.R. and A.S.; software, M.M.; validation, M.M., M.R.S. and S.E.H.; formal analysis, M.M. and M.R.S.; investigation, M.M., M.R.S., S.E.H., T.D., F.F., A.K., K.S., C.R. and T.R.; resources, M.R.S., S.E.H., A.L. and A.C.M.; data curation, M.M., M.R.S., S.E.H., F.F. and A.C.M.; writing—original draft preparation, M.M.; writing—review and editing, M.R.S., S.E.H., T.D., F.F., A.K., K.S., A.L., A.C.M., C.R., A.S. and T.R.; visualization, M.M. and M.R.S.; supervision, T.R., A.S. and C.R.; project administration, T.R.; funding acquisition, T.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Isolation of cells from donor animals was approved by the institutional ethics committee of the University of Veterinary Medicine Vienna and the national authority according to the Law for Animal Experiments, Austrian Tierversuchsgesetz (BMWFW-2021-0.117.108), approval code 180/12/2020 from 10 December 2020.

Informed Consent Statement: Not applicable.

Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [45] partner repository with the dataset identifier PXD035499 and 10.6019/PXD035499.

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Conflicts of Interest: The authors declare no conflict of interest.

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3.2. A conserved stem-loop structure within ORF5 is a frequent recombination hotspot for Porcine Reproductive and Respiratory Syndrome Virus 1 (PRRSV-1) with a particular modified live virus (MLV) strain

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A Conserved Stem-Loop Structure within ORF5 Is a Frequent Recombination Hotspot for Porcine Reproductive and Respiratory Syndrome Virus 1 (PRRSV-1) with a Particular Modified Live Virus (MLV) Strain

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Abstract: The emergence of recombinant PRRSV strains has been observed for more than a decade. These recombinant viruses are characterized by a genome that contains genetic material from at least two different parental strains. Due to the advanced sequencing techniques and a growing number of data bank entries, the role of PRRSV recombinants has become increasingly important since they are sometimes associated with clinical outbreaks. Chimeric viruses observed more recently are products of PRRSV wild-type and vaccine strains. Here, we report on three PRRSV-1 isolates from geographically distant farms with differing clinical manifestations. A sequencing and recombination analysis revealed that these strains are crossovers between different wild-type strains and the same modified live virus vaccine strain. Interestingly, the recombination breakpoint of all analyzed isolates appears at the beginning of open reading frame 5 (ORF5). RNA structure predictions indicate a conserved stem loop in close proximity to the recombination hotspot, which is a plausible cause of a polymerase template switch during RNA replication. Further research into the mechanisms of the stem loop is needed to help understand the PRRSV recombination process and the role of MLVs as parental strains.

Keywords: porcine reproductive and respiratory syndrome virus; PRRSV; Arteriviridae; recombination; recombinant virus; modified live virus vaccine; ORF5; stem loop

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded, positive sense RNA virus within the family *Arteriviridae*, order *Nidovirales*, genus *Betaarterivirus*, which is further divided into the subgenera *Eurpobartevirus* and *Ampobartevirus*. Each subgenus holds one species, namely *Betaarterivirus suid 1*, or PRRSV-1, and *Betaarterivirus suid 2*, or PRRSV-2. These two species are of striking genetic divergence, indicating that they might have evolved separately [1]. Furthermore, nucleotide sequences of different strains within a species show high variations caused by high mutation rates [2], missing proofreading activity of the RNA-

dependent RNA polymerase (RdRp) [3], and frequent recombination of different virus strains [4]. The PRRSV genome harbors at least ten open reading frames (ORFs), of which ORF1 codes for non-structural proteins and ORFs 2–7 code for the structural proteins of the viral envelope, membrane, and nucleocapsid [5]. Clinical signs of infected animals include respiratory disorders, reproductive symptoms in sows, and the birth of weak, congenitally infected piglets [6]. A PRRSV introduction into a farm frequently causes high financial losses and makes it one of the most relevant porcine pathogens worldwide [7]. Modified live virus (MLV) vaccines are so far the only effective, commercially available, and widely used practical option to successfully combat PRRSV. However, they bear the risks of reverting to virulence [8] and recombining with wild-type strains [9–11]. Recombination is a common phenomenon in viruses, which requires the co-infection of one cell with two or more strains. The mechanisms of recombination differ according to the structure of the viral genome. DNA viruses tend to recombine after a double strand breaks and repairs [12] as observed in eukaryotes during homologous recombination. Segmented RNA viruses can reassort their genes after infecting the same cell as seen in *Orthomyxoviridae* [13]. Non-segmented RNA viruses, however, use a different strategy, called copy-choice replication. This phenomenon occurs during RNA replication, as the viral RdRp switches templates, resulting in a chimeric genome of two or more progenitors [14]. The same mechanism is found in retroviruses during reverse transcription [15]. Although recombination events have been associated with increased virulence [16], immune evasion, or drug resistance [15], it remains unclear whether these incidences are random or follow a strategy to modify the virus' fitness.

To date, several PRRSV recombinants have been isolated and characterized. These chimeric viruses are the result of the recombination of either different wild-type strains [17,18], wild-type and vaccine strains [9–11], or even two divergent vaccine strains [19]. Thus, the administration of PRRSV MLV vaccines, especially during an acute infection event, bears the potential of recombination with wild-type strains, and their subsequent distribution and establishment in the field. Subsequently, active characterization of circulating virus strains is an important, but not established, surveillance method for PRRSV containment.

Here we report on three PRRSV-1 isolates from Austria and Germany originating from the recombination of a wild-type strain and a specific MLV vaccine strain. Interestingly, although the strains show the highest homology to three different wild-type strains from ORF1–4, the genomic breakpoint occurs at the same location within ORF5. Hereby, we present the clinical outcome, phylogenetics, and possible recombination mechanism of these novel isolates. We hypothesize that the wild-type strains recombine with the Ingelvac PRRSFLEX® EU (Boehringer Ingelheim Vetmedica GmbH, Rohrdorf, Germany)vaccine strain within ORF5. Furthermore, we propose a conserved stem-loop structure after ORF5a to be the driving force of recombination. Finally, we would like to see more detailed research into the mechanism of the putative ORF5 stem loop and further search for similar chimeric viruses. We also would like to highlight the potential of recombination by administering PRRSV MLV vaccines to the domestic pig population.

2. Materials and Methods

2.1. Cells

Porcine alveolar macrophages (PAMs) were isolated by bronchoalveolar lavage (BAL) from euthanized pigs that tested free of PRRSV RNA with an RT-qPCR. First, the lungs and trachea were removed without damaging the tissue, to avoid the presence of erythrocytes in the BAL fluid. Then, sterile phosphate-buffered saline (PBS) was poured through the trachea into the lungs. After gently massaging the organ, the BAL fluid was poured into a glass bottle and kept on ice until further processing. Next, the fluid was centrifuged, and the pellet was washed 3 × with PBS. Finally, the cell number was determined, and batches of 1×10^8 cells were frozen in FCS (FCS, Corning, NY, USA) +

10% DMSO (Carl Roth, Austria) at −150 °C until further use. African green monkey cells (MARC-145) were obtained from the Friedrich-Loeffler-Institute in Germany.

2.2. Virus Isolation by Cell Culture

Serum samples were briefly centrifuged at 10,000 × *g* and 30 µL were utilized to infect 2×10^6 PAMs and 5×10^5 MARC-145 cells in 24-well plates. An aliquot of the PAMs from the corresponding donor animal was previously tested for high susceptibility toward PRRSV. Infection was assessed after 48 h using immunofluorescence. Supernatants were collected and stored at 4 °C until further use, and cells were fixed with 4% PFA (Carl Roth, Germany) for 20 min at 4 °C. After permeabilization of cell membranes with 1% Triton-X 100 (Carl Roth, Karlsruhe, Germany) for 5 min at RT, cells were inoculated with an in-house-produced mAb anti-PRRSV N (clone P10/b1) for 1 h. A Cy3-linked (Dianova, Hamburg, Germany) detection serum was added to visualize PRRSV-infected cells. Cell supernatants were used for further virus passaging and confirmation of the immunofluorescence results by RT-PCR.

2.3. RT-PCR

Organ samples were homogenized in sterile PBS for 3 min at 30 oscillations/sec with a Tissue Lyser II (Qiagen, Hilden, Germany) and stainless-steel beads. Samples were centrifuged for 3 min at 13,000 rpm. Organ supernatants and serum samples were used for extracting nucleic acids with a QIAamp Viral RNA Mini QIAcube Kit in a QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracts were used to detect PRRSV with an ORF7-specific RT-PCR as previously described [20].

2.4. Virus Propagation and RNA Extraction

Supernatants of infected cells (described in 2.2.) were used to infect more PAMs to produce a sufficient amount of virus for RNA extraction. Virus of the second passage on 5×10^7 cells provided a total of 10^7 $TCID_{50}$ with titers of $2 \times 10^5/mL$. 50 mL of each preparation was cleared by centrifugation at 10,000× *g* with a Fibrelite 14.2 rotor for 5 min and filtration through a 0.45 µm sterile filter (Pall, Port Washington, NY, USA). After that, the virus was concentrated in a Beckman Ti55.2 rotor (Beckman Coulter, Vienna, Austria) at 35,000 rpm for 2 h. Next, the pelleted material was resuspended overnight in 500 µL phosphate-buffered saline at 4 °C, and the insoluble debris was removed in a microcentrifuge at 10,000× *g* for 1 min. The supernatant was pelleted at 45,000 rpm in a TLA45 rotor at 4 °C for 1 h. The sediment was resuspended in 50 µL dH_2O and lysed in 600 µL lysis solution of RNeasy kit (Qiagen, Hilden, Germany). All further steps were carried out according to the manufacturer's instructions.

2.5. cDNA Preparation and Sequencing

Purified RNA samples were subjected to Sanger and Next Generation Sequencing (NGS). For Sanger sequencing, RNA was primed with oligonucleotide T20, and cDNA was produced with MuLV Reverse transcriptase (NEB, Ipswitch, MA, USA) at 42° for 2 h. The cDNA was purified with the NEB Monarch kit and used as a template for a series of 15 overlapping PCR fragments with primer pairs PRS328–345 (Appendix A, Table A1). PCR fragments were submitted to Sanger sequencing (Eurofins) using primers from either end. Using this technique, sequences were assembled to about 98%. For NGS, 1 µg of RNA was sent on dry ice to Clontech for 75-nucleotide (nt) paired-end Illumina sequencing with 2×10^6 reads. NGS data were aligned to the preliminary Sanger sequence to yield a composite sequence that immediately showed all ORFs. The 5'ends were determined by 5' RACEas reported earlier [20]. The Basic Local Alignment Search Tool (BLAST) of the National Library of Medicine (NIH) and CLC Genomics Workbench 22 (Qiagen, Hilden, Germany) were used for sequence alignments.

2.6. Recombination Analysis and RNA Structure Predictions

The recombination analysis was performed with SimPlot version 3.5.1. [21] and RDP5 version 5.29 [22]. For similarity and recombination analysis, sequence alignments were performed using CLC Genomics Workbench 22 (Qiagen, Hilden, Germany), with a gap open cost of 10.0 and a gap extension cost of 1.0. An alignment of the recombinant strains and a vaccine strain was imported into SimPlot to perform a Kimura 2-parameter test, with Ingelvac PRRSFLEX® EU (GenBank: KT988004) as a query sequence, 200 bp steps, and a transition/transversion (Ts/Tv) ratio of 2,0. For recombination analysis, sequence alignments were imported to RDP5. A full exploratory recombination scan using the RBG, GENECoNV, Bootscan, MaxChi, Chimera, SiScan, and 3Seq programs was performed using default parameters. RNA secondary structure predictions were made with LocARNA version 1.9.1 linking Vienna RNA package 2.3.2 [23–25].

3. Results

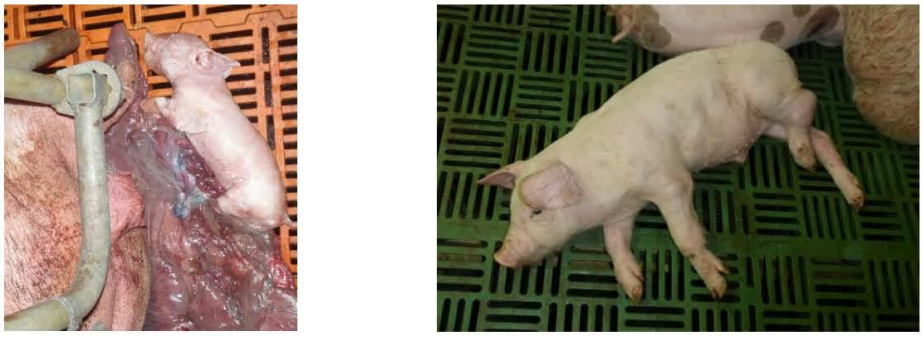
3.1. Isolation and Sequencing of Chimeric PRRSV Strains

During clinical outbreaks or routine surveillance of pig herds in Germany and Austria, three PRRSV isolates were collected that exhibited a suspiciously high homology to the Ingelvac PRRSFLEX® EU upon ORF5 sequencing. The acronyms of the viral strains indicate the nation, year of isolation, and entry number in our files. To exclude the possibility of assessing a mixed virus population, PRRSV was isolated from the clinical samples on PAMs. In parallel, MARC-145 cells were inoculated with the clinical samples. GER18-258, AUT20-1664, and AUT22-97 could be isolated from PAMS showing a cytopathic effect and a positive immunofluorescence signal upon staining with a mAb anti-PRRSV N. None of the samples led to a productive infection of MARC-145 cells, indicating the absence of attenuated MLV vaccine or PRRSV-2 strains. Only a small number of PRRSV-1 strains replicate in monkey cells without prior adaptation. So far, we isolated only one virus showing this phenotype [26].

The determination of the genomic sequences was achieved by Sanger sequencing of PCR products and a 5'-RACE. Isolate GER18-258 harbors 15,088 nt, isolate AUT20-1664 15,030 nt, and isolate AUT22-97 15,073 nt in total. A BLAST analysis of the full genome sequences revealed an 89% homology of GER18-258 to isolate GER09-613 (KT344816.1), 88% homology of AUT20-1664 to Lelystad virus (NC_043487.1), and 93% homology of AUT22-97 to isolate AUT15-33 (MT000052.1). Together with the immunofluorescence results, this confirmed that all three isolates are Betaarterivirus suid 1, or PRRSV-1, strains. To assess the origin and potential pathogenicity of these isolates, veterinarians handling the farms were contacted and a clinical report was compiled.

3.2. Anamnesis and Clinical Findings in the Affected Farms

GER18-258 was derived from a farrow-to-finish farm in Southern Germany. The sow and nursery units were known to be free of PRRSV for more than ten years, while no PRRSV vaccines were applied. Reproductive disorders characterized by stillborn piglets (5%, Figure 1a) and weak born piglets (20 %) occurred. Approximately 30% of the sows in the farrowing unit were off feed and showed fever. In the affected batch, 70% of the suckling piglets died prior to weaning, and in the two consecutive farrowing batches the preweaning mortality was 50% and 30%. Based on clinical examination, approximately 30% of the nursery pigs and 40% of the fattening pigs exhibited the following symptoms: coughing, sneezing, increased respiratory rates, dyspnea, and conjunctivitis. In addition, swollen joints (Figure 1b) were noticed in individual nursery and fattening pigs. All-cause mortality increased from 3% to 5.5% in the nursery and from 2.5% to 5% in the fattening unit. PRRSV was detected in the lung and lymph nodes of six necropsied piglets with a commercial RT-PCR kit. Bacterial isolation from the lung tissue revealed growth of *Streptococcus suis* and *Staphylococcus aureus*. *Streptococcus suis* was also found in the swollen joints of the nursery pigs.



(**a**) (**b**)

Figure 1. PRRS affected pigs on the farm of isolate GER18-258. (**a**) Stillborn piglet (**b**) Pig with swollen joints

AUT20-1664 was obtained from an Austrian nursery unit with pigs from two different sow farms. Sows from farm A were vaccinated against PRRSV (ReproCyc® PRRS EU, Boehringer Ingelheim Vetmedica GmbH, Germany) every three months after the introduction of a new wild-type virus strain led to a severe PRRS outbreak in 2015. Piglets from this herd were vaccinated against PRRSV (Ingelvac PRRSFLEX® EU, Boehringer Ingelheim Vetmedica GmbH, Germany) at three weeks of age. Piglets from farm B were vaccinated against PRRSV with the same vaccine when entering the nursery unit at the age of approximately 6.5 weeks. About 1% of the piglets exhibited respiratory symptoms and delayed growth. Diagnostic investigations of such runt pigs revealed mixed infections of PRRSV, PCV2 (no further genotyping done), and Influenza A virus (swine H1N1 of avian origin). During the bacteriologic investigation of lung samples, *Streptococcus suis*, *Pasteurella multocida,* and *Mycoplasma hyorhinis* could be isolated.

AUT22-97 was isolated in 2022 in an Austrian piglet-producing farm after facing respiratory distress and increased mortality in the nursery unit. Prior to PRRSV detection, the PRRSV status of the farm was unknown, and no PRRSV vaccine was administered. The clinical signs started in the rearing period with respiratory distress, wasting, and increased mortality rates of up to 10%. PRRSV antibodies of 10-week-old piglets were tested with positive results in 10/10 samples. A PRRSV-1 ORF1 RT-qPCR was performed in pools of five with positive results. Besides PRRSV, an infection with *Actinobacillus pleuropneumoniae* could be confirmed in the lung tissue of affected pigs. In addition, four sows aborted while clinical signs occurred in the nursery unit.

More detailed clinical reports can be found in the Supplementary File S1.

3.3. PRRSV Isolates Are Recombinant Viruses Harboring ORF5–3′ Sequences from a Particular Modified Life Vaccine Strain

To assess the genomic structure of the chimeric isolates, we conducted a full genome similarity analysis of the Ingelvac PRRSFLEX® EU sequence to our recombinant isolates (Figure 2). This revealed a breakpoint of all isolates upstream of ORF5a. From this position onwards, the 3'-end matched the vaccine strain, while the 55'-end matched other PRRSV-1 strain sequences. Hence, we concluded that only a single crossover occurred.

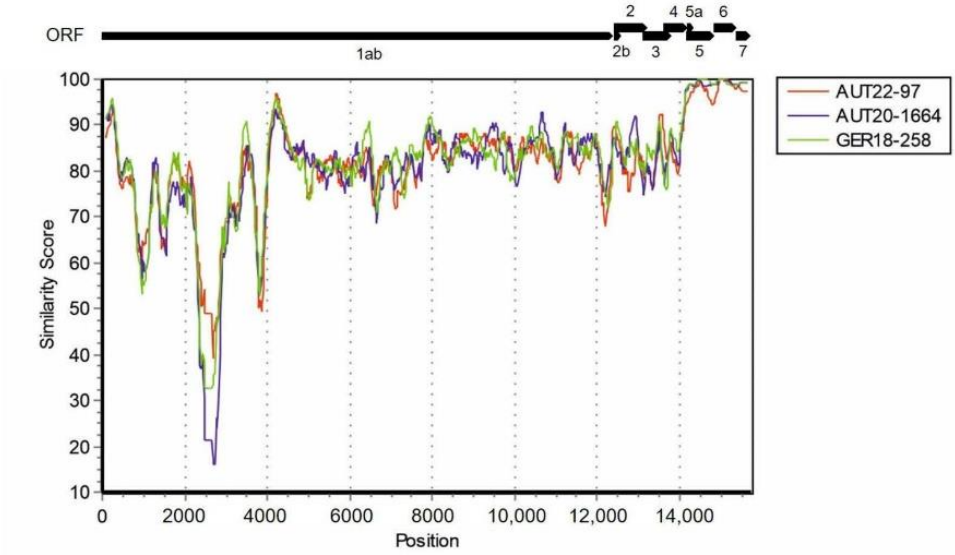
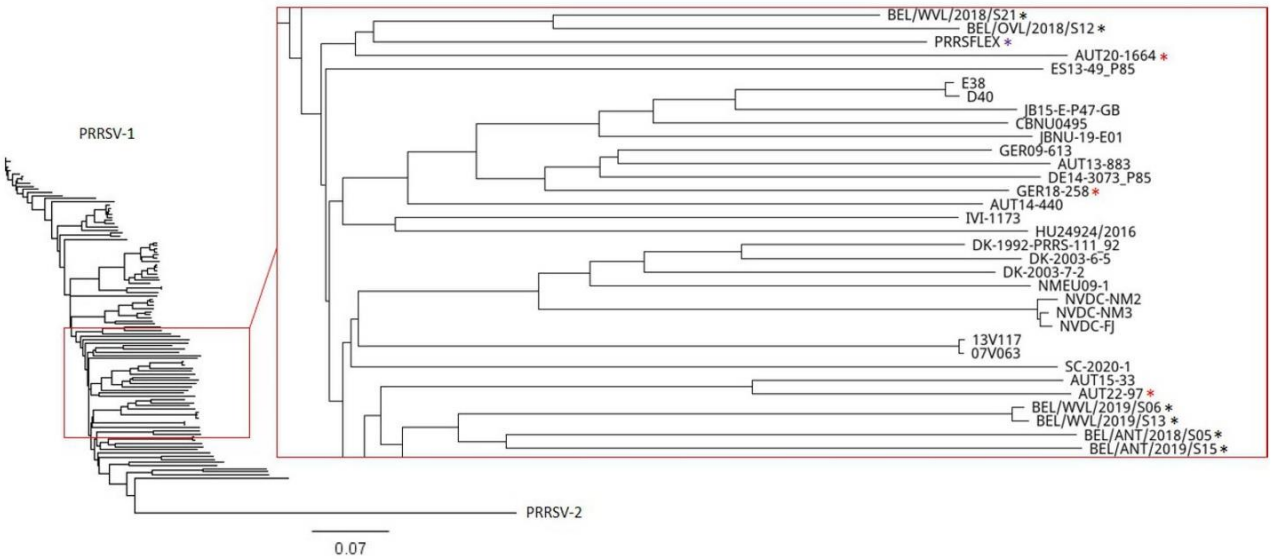
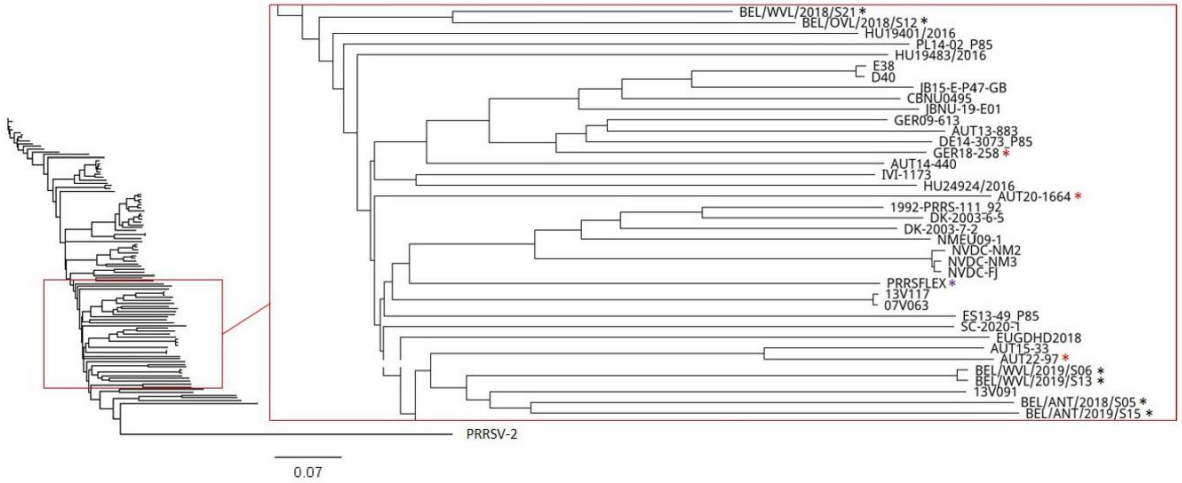


Figure 2. Similarity plot of three PRRSV isolates compared to MLV vaccine PRRSFLEX. A Kimura 2-parameter test with a transition/transversion (Ts/Tv) ratio of 2.0 was performed with the PRRSFLEX sequence as a query. The scheme above the plot shows the positions of the PRRSV open reading frames (ORFs).

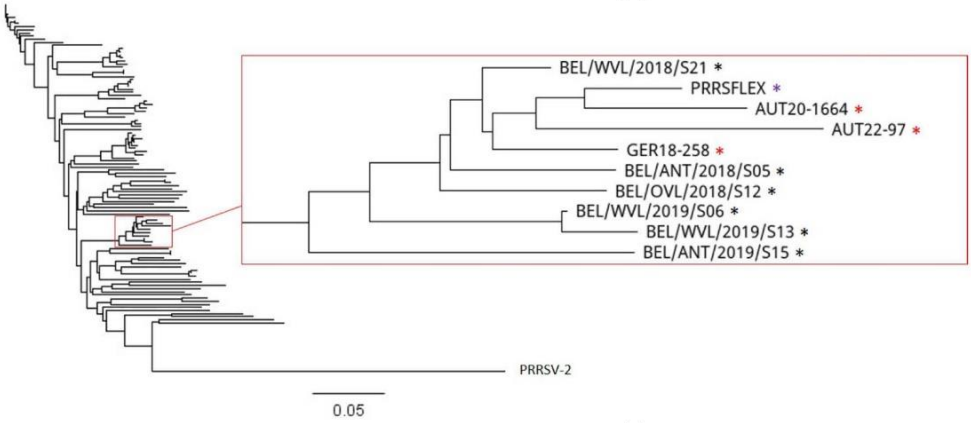
A phylogenetic tree of a whole genome sequence alignment including the most common PRRSV-1, PRRSV-2, and PRRSV vaccine strains is shown in Figure 3a. A tree of the ORF1–4 (Figure 3b) disclosed only a clustering of isolate GER18-258 with the German isolates GER09-613 and DE14-3073_P85 and Austrian isolate AUT13-883. Isolate AUT20-1664, which showed the highest percent identity of only 88.3% with the Lelystad virus upon BLAST search, does not cluster with any given strain in our ORF1–4 phylogenetic tree. The ORF1-4 region of isolate AUT22-97 clustered with Austrian isolate AUT15-33. Repeating the same phylogenetic analysis with ORF5–7 (Figure 3c) revealed a clustering of all three isolates with the virus strain used for the Ingelvac PRRSFLEX® EU vaccine (GenBank 94881) and six Belgian recombinant viruses described by Vandenbussche et al. [11]. These Belgian strains were identified as recombinant viruses between different wild-type strains and the Ingelvac PRRSFLEX® EU vaccine strain with a recombination breakpoint within ORF5.



(**a**)



(**b**)



(**c**)

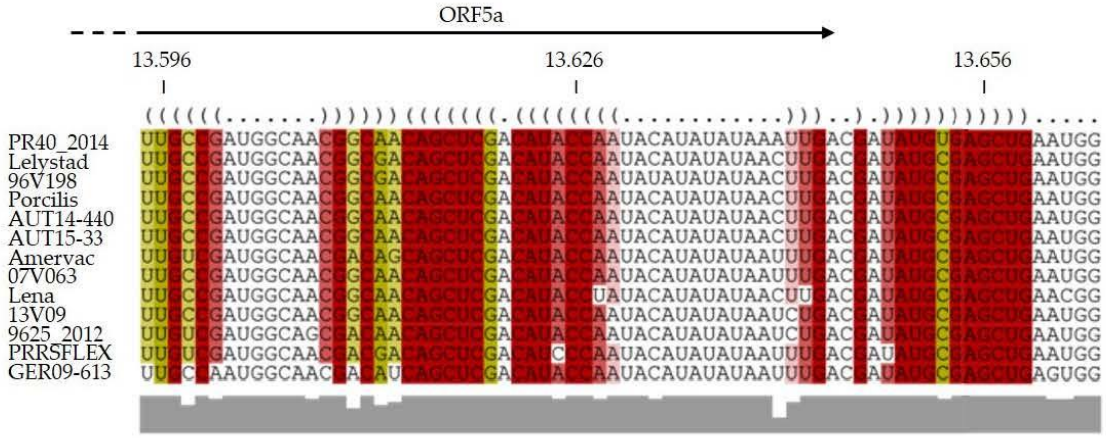
Figure 3. Phylogenetic trees of common PRRSV strains (Supplementary File S2) and our recombinant isolates (GER18-258, AUT20-1664, AUT22-97). The neighbor-joining method was used to generate trees of (**a**) whole genomes, (**b**) ORF1–4, and (**c**) ORF5–7. Individual PRRSV-2 strains are not shown, since our recombinants cluster is within PRRSV-1. Nucleotide distance was measured with the Jukes–Cantor model, and bootstrap analysis was performed with 100 replicates. Red asterisks

mark our recombinant isolates, purple asterisk the Ingelvac PRRSFLEX® EU strain, and black asterisks recombinant Ingelvac PRRSFLEX® EU isolates described by Vandenbussche et al. [11].

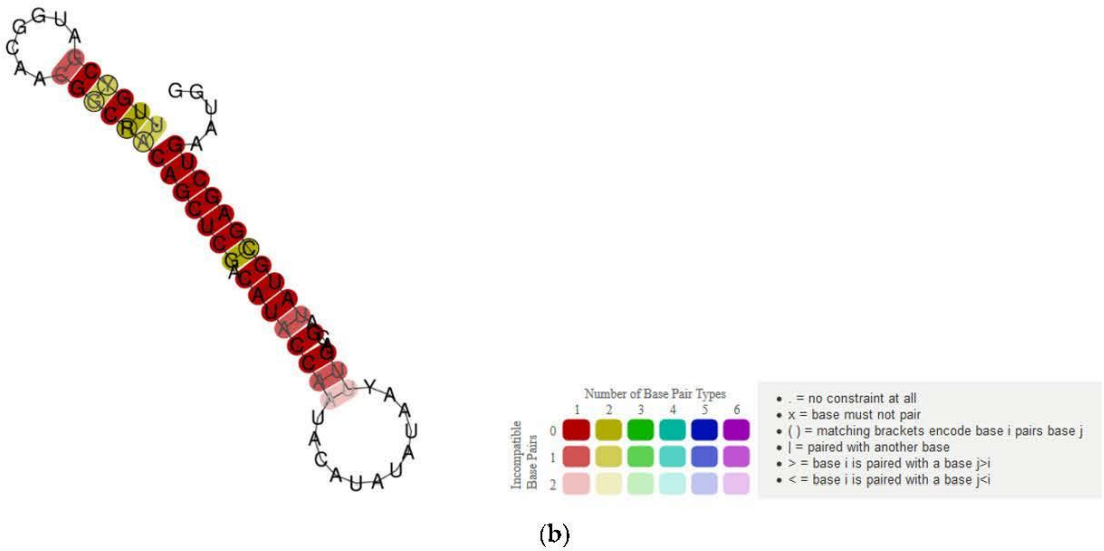
Next, we conducted a more thorough recombination analysis with the program RDP5, using six different recombination detection methods. This analysis revealed that GER18-258 is a derivative of strain GER09-613 as the major parent (88.9% similarity) and PRRSFLEX as the minor parent (99.4% similarity). The recombination breakpoint is predicted to be between nt 13,551 and 13,660. For isolate AUT20-1664, the analysis revealed an unknown major parent and PRRSFLEX as the minor parent (99.0% similarity), with a recombination breakpoint between nt 13,497 and 13,641. For AUT22-97, the program predicted AUT15-33 to be the major parent (92.5% similarity) and PRRSFLEX to be the minor parent (97.8% similarity), with the breakpoint between nt 13,510 and 13,616. Altogether, these findings let us conclude that isolate GER18-258 is most likely a recombinant of a distant parent of strain GER09-613 and the PRRSFLEX MLV vaccine, isolate AUT20-1664 a recombinant of a yet unassigned wild-type strain and the PRRSFLEX MLV vaccine, and AUT22-97 a recombinant between an AUT15-33 derivative and the PRRSFLEX MLV.

3.4. RNA Structure Predictions Reveal a Conserved Stem Loop Upstream of ORF5a

The recombination analysis indicated that all three PRRSV isolates recombined within the same narrow genomic region with the same MLV vaccine strain. Therefore, we began to investigate the RNA secondary structure. Stem-loop structures are known to cause the RdRp to halt or become dislocated during replication [27]. We used the online tool LocARNA to upload an alignment of common PRRSV-1 vaccine and wild-type strains for RNA folding predictions (Figure 4a). The output revealed the presence of a stem loop upstream of ORF5a conserved in all given sequences (Figure 4b). Interestingly, this stem loop is conserved among a broad range of PRRSV-1, but not within PRRSV-2 strains. Since the minor parent of our three recombinant viruses, the PRRSFLEX MLV strain, also harbors this conserved stem loop, it is plausible that the RdRp has switched templates at this location in all three independent recombination events.



(**a**)



(**b**)

Figure 4. Predicted RNA folding of PRRSV sequence alignments. (**a**) Alignment and (**b**) predicted RNA folding of putative stem loop within ORF5 of several PRRSV-1 vaccines (Porcilis® PRRS, Amervac® PRRS, Ingelvac PRRSFLEX® EU) and wild-type strains. Figures were created with LocARNA version 1.9.1.

4. Discussion

A search utilizing keywords "PRRSV" [and] "recombination" yields more than 600 hits in PubMed®. Initially considered a rare event, recombination now appears to be a regular and, within the species *Betaarterivirus suid 1*, clinically relevant phenomenon. Attention was drawn to chimeric virus strains with the advent of severe clinical cases in China [28] and Denmark [19,29]. The latter case shook the field as two harmless vaccine strains had apparently recombined to create a virulent genotype.

Here, we report on three different recombinant PRRSV-1 strains from geographically distant farms in Germany and Austria, isolated between 2018 and 2022. GER18-258 was identified in a farrow-to-finish farm in southern Germany after pigs exhibited clinical signs, such as respiratory distress, conjunctivitis, swollen joints, stillbirths, the birth of weak piglets, and increased pre-weaning mortality. AUT20-1664 was responsible for less severe clinical signs, mainly retarded growth and respiratory signs in piglets. AUT22-97 was detected in animals with respiratory distress, wasting, and increased mortality. In all three farms, co-infections with common respiratory bacteria or other viruses were detected. These often occur in PRRS outbreaks and can exacerbate the clinical outcome [30], as it was observed on the farm of isolate GER18-258 where *Streptococcus suis* caused swollen joints in nursery pigs.

Initially, only part of the viral genomes was sequenced in all three cases. Routine detection and sequencing of PRRSV nucleic acid is usually achieved with an ORF5 or ORF7-specific RT-PCR. Considering isolate GER18-258, the initial sequencing of ORF5 led to the assumption that the vaccine strain was circulating in the herd. Only the full-genome sequencing revealed a possible crossover between a wild-type and a vaccine strain. This suggests that exclusive partial sequencing covering only one PRRSV ORF may lead to wrong assumptions regarding the present PRRSV isolate.

Recombination of PRRSV strains is facilitated by the ability of the RdRp to switch RNA templates during replication. One requirement for this copy-choice replication is that two (or more) strains infect the same host and the same cell. This scenario is not as far-fetched since it is not unusual that more than one PRRSV strain is present in a herd, and that PRRSV MLV vaccines are administered to infected pig herds as a metaphylactic measure. The Committee for Medicinal Products (CVMP) of the European Medicines Agency (EMA) outlined that the benefits of PRRSV MLV vaccines continue to outweigh

their risks. The farm on which AUT20-1664 was isolated did indeed administer the Ingelvac PRRSFLEX® EU vaccine prior to the isolation of the recombinant strain. Interestingly, on the farms where GER18-258 and AUT22-97 were isolated, no pigs were vaccinated with this particular product. Therefore, the option that the recombinant virus strains did not originate on these farms, but were introduced from another farm of unknown origin, has to be considered. This finding underlines the importance of accurate compliance with biosafety protocols on livestock farms.

The recombination analysis confirmed our suspicion that the MLV Ingelvac PRRSFLEX® EU is a recombination partner of all three isolates. While GER09-613 was determined as the major parent of isolate GER18-258, no major parent could be identified for isolate AUT20-1664, and AUT15-33 was predicted to be the major parent for isolate AUT22-97. For all three recombinants, the analysis revealed a recombination breakpoint at the beginning of ORF5 with Ingelvac PRRSFLEX® EU as the minor parent. Despite the MLV vaccine part, the isolates retained their wild-type character and could be passaged on PAMs, but not on MARC-145 cells. Vandenbussche et al. [11] discovered similar PRRSV strains in 2021. The authors isolated 124 Belgian PRRSV-1 strains from pig sera to perform whole-genome sequencing. Eleven of those turned out to be vaccine virus recombinants, of which four displayed a recombination breakpoint with the Ingelvac PRRSFLEX® EU strain within ORF5. These Belgian recombinant strains clustered with our recombinant isolates in the phylogenetic tree of Figure 3c. Since several PRRSV-1 isolates recombined at approximately the same location, we hypothesize that the RdRp did not randomly switch templates but used defined breakpoints or areas.

However, we should not ignore the shaping power of evolution. Therefore, it is also necessary to ask what makes this genomic region so attractive for recombination and what evolutionary advantage the recombinants might carry. The PRRSV genome harbors a capped 5'-UTR with conserved RNA secondary structures, playing important roles in RNA replication and transcription [31–33]. Deletions in the 5'-UTR have resulted in reduced or absent replication and infectivity [34], leading to the conclusion that the stem loops within the 5'-UTR are involved in the RdRp-dependent replication process. Chang et al. [35] showed that the 5' leader transcription regulatory sequence (TRS) of bovine coronavirus is located in the loop of a cloverleaf-like RNA structure, making it accessible for the RdRp. All *Arterivirus* 5' leader TRSs are linked to the body TRSs in front of each individual ORF on the intermediate negative strand RNA [36]. The leader TRS base pairs with the body TRSs to initiate transcription of the subgenomic RNAs (sgRNAs). Subsequently, all polycistronic sgRNAs contain the 5' leader sequence. These nested sgRNAs are templates for the structural proteins of the viral envelope, membrane protein, and nucleocapsid protein. RNA secondary structure predictions of several PRRSV-1 wild-type and vaccine strains let us hypothesize that ORF5 contains a conserved stem loop upstream of our isolates' predicted recombination breakpoints. This loop could be the driving force for the RdRp to switch templates and produce a chimeric genome. While the stem-loop structure is a plausible localization for recombination events, it is most likely not its main purpose, since conserved stem-loop sequences often have regulatory functions [27]. Nevertheless, this hypothesis has to be explored by investigating stem-loop structure and function in more detail.

The question whether the recombination between field and vaccine strains occurs by chance or has a selective advantage remains unsolved. The most recent isolate, AUT22-97, derives from AUT15-33 that emerged seven years ago as an outbreak with severe reproductive losses and clinical signs in nursery pigs on Austrian farms [37]. AUT15-33 is characterized extensively and shows both considerable virulence in reproductive as well as respiratory challenge trials [20,38]. We frequently find descendants of AUT15-33 in current clinical samples from Austria indicating high competitiveness in the field. Hence, it is surprising that an already vital wild-type strain is outcompeted in the field by a recombinant equipped with MLV PRRSFLEX® EU ORF5–7 sequences. The high number of alto-

gether seven cases, including the Belgian chimeric viruses, indicates that there is something special to the recombined elements of Ingelvac PRRSFLEX® EU. A simple immunological escape is unlikely as the immune response towards the vaccine strain, at least regarding ORF5–ORF7, is mostly unaffected. Future experiments, including reverse genetics of cloned AUT22-97 and AUT15-33 and infection trials to compare the clinical outcome of wild-type and chimeric viruses, will address this important question.

5. Conclusions

We report on three recombinant PRRSV strains isolated from geographically distant farms suffering from mild to severe clinical cases of reproductive or respiratory disorders. NGS and recombination analyses confirmed the parental strains of isolate GER18-258 to be GER09-613 from ORF1–4 and the MLV Ingelvac PRRSFLEX® EU from ORF5–7. Isolate AUT20-1664 is a chimeric virus of an unknown strain providing ORF1–4 and the MLV Ingelvac PRRSFLEX® EU contributing to ORF5-7. Strain AUT22-97 is a recombinant virus with AUT15-33 from ORF1–4 as the major and the MLV Ingelvac PRRSFLEX® EU from ORF5–7 as the minor parent. RNA structure predictions of the sequences around the recombination breakpoints revealed a conserved stem loop upstream of ORF5a. We hypothesize that this stem loop might be the driving force of the RdRp template switch, resulting in the crossover events. It is unclear if the recombinants obtain an evolutional advantage compared to their parental wild-type strains from acquiring the MLV sequences. Further research on the pathogenicity and infectivity of the isolates and the molecular function of the putative stem loop has to be done.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15010258/s1, Supplementary File S1: Anamnesis and physical findings; Supplementary File S2: PRRSV accession numbers used for phylogenetic trees in Figure S3.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Primers used for Sanger sequencing of PRRSV isolates.

Primer	Sequence	Forward/Reverse	Binding at nt
PRS328	GYGAWCTYCAAGTTTAYGAGC	forward	527–547
PRS329	GAGCTCCAARCAGGCCATG	forward	2040–2059
PRS330	CCRATACTCGGATGCCTTCC	forward	3506–3527
PRS331	CCACGTGGTGCRGCTGCTG	forward	4993–5011

PRS332	AYGTRATYGTTCTGCTTGGGC	forward	6533–6553
PRS333	TGCCTGGGGTCCTACGCCT	forward	7984–8002
PRS334	GAYGATGATGTYATYTACACACC	forward	9636–9658
PRS335	CTCTCACCGATGTGTACCTY	forward	11,002–11,021
PRS336	CGTCCGGGTACGAYAAYCTY	forward	12,502–12,521
PRS337	GTGTCWCGCGGCCGACTCYTG	forward	14,004–14,024
PRS338	TGGTCRGACACGTGCATGGAG	reverse	606–626
PRS339	RGGCCTTKGAGGAKGGRAG	reverse	2081–2098
PRS340	GCCATCCAAGAACCAAAAACAC	reverse	3572–3593
PRS341	CTGAARGCACCTTCRAGRAGGG	reverse	5105–5126
PRS342	CATTRATRTCGAGGATGGATCC	reverse	6565–6586
PRS343	TGGCCATTRAYCCCTGCCA	reverse	8083–8102
PRS344	GGAATACCTRCAAACTTTRAGAGC	reverse	9690–9713
PRS345	TTCCAGCATTTTGAYGCCGTC	reverse	11,051–11,071
PRS346	MGGATGGAAYTGGGCCGCT	reverse	12,569–12,588
PRS347	AAATGCACATATGTCATGTAYCC	reverse	14,070–14,092

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Supplementary file 1

Anamnesis and clinical findings associated with GER18-258 occurrence

GER18-258 was derived from a farrow to finish farm in Southern Germany harboring 80 sows. The farm produces in a three-week farrowing interval. Piglets weaned at the age of 28 days are nursed on site and transferred to the fattening unit with a body weight of 28kg. The fattening unit with 1500 places is located at a distance of 2km from the remaining buildings and managed by different personnel. In addition to the on-farm produced fattening pigs 150 fattening pigs are purchased every 3 weeks from different sources. The sows and gilts are vaccinated against Porcine Parvovirus and Erysipelas according to the manufacturer's recommendation. Vaccination protocol of piglets includes *Mycoplasma hyopneumoniae* and porcine Circovirus type 2 (PCV-2) in the third week of life. The sow and the nursery units are known to be free of PRRSV since more than ten years, based on routine serological testing of sows and nursery piglets performed every six months. In accordance with the detection of GER18-258 reproductive disorders characterized by stillborn piglets (5%, figure 1a) and weak born piglets (20 %) occurred. Approximately 30% of the sows in the farrowing unit were off feed and showed fever. In the affected batch, 70% of the suckling piglets died prior to weaning and in the two following farrowing batches the pre-weaning mortality accounted for 50% and 30%. Two weeks after the first occurrence of clinical signs in the farrowing unit respiratory distress was observed in the nursery and fattening unit. Based on clinical examination approximately 30% of the nursery pigs and 40% of the fattening pigs showed coughing, sneezing, increased respiratory rates, dyspnoea, and conjunctivitis. In addition, swollen joints (figure 1b) were noticed in individual nursery and fattening pigs. All-cause mortality in the nursery increased from 3% to 5.5% and from 2.5% to 5% in the fattening unit.

a) b)

Figure 1. PRRS affected pigs on the farm of isolate GER18-258. a) stillborn piglet, b) pig displaying swollen joints

For diagnostic workup blood samples from 10 sows, 10 pigs end of nursery and 10 fattening pigs were collected. Blood samples from sows were negative for antibodies against Influenza A and Leptospira. Antibodies against PRRSV were present in 8/10 sows, 8/10 nursery pigs and in all fattening pigs. Six weak born piglets were submitted for necropsy. PRRSV was detected in lung and lymph nodes samples of all six weak born piglets using a

commercial RT-PCR kit. Post mortem examination of nursery and fattening pigs revealed poor retraction of the lungs in all animals as well as purulent arthritis in the nursery pigs. Histologically, interstitial pneumonia including hyperplasia of type II pneumocytes were found in the lungs of all investigated pigs. Bacterial isolation from lung tissue revealed growth of *Streptococcus suis* and *Staphylococcus aureus*. *Streptococcus suis* was also found in the joints of the nursery pigs. PRRSV was detected in lung and tonsil samples, whereas all investigated samples were negative for PCV-2 and IAV.

Initial phylogenetic analysis of two qRT-PCR PRRSV positive samples was performed at IVD GmbH, Seelze, Germany using ORF 5 sequencing. ORF 5 sequencing revealed 97% nucleotide identity to Ingelvac PRRSFLEX® EU (Boehringer Ingelheim Vetmedica GmbH, Germany) for sample one and 99% nucleotide identity to Ingelvac PRRSFLEX® EU for the second sample. The nucleotide identity between these two samples and PRRSV-1 prototype strain Lelystad virus (LV) was only 91% and 95% respectively.

Anamnesis and clinical findings associated with AUT20-1664 occurrence

Isolate AUT20-1664 was obtained from a nursery unit, which was newly built in 2019; piglets from two different sow farms of the same owner are housed in this unit from seven weeks of age until 30 – 35 kg body weight ,when they are sold to different finishing sites. The two sow farms both produce in a four-week batch farrowing interval with a two-week period in between farrowings of the two farms. Sow farm A operates with 350 sows and is classified as PRRS stable according to Holtkamp *et al.* [1]; sows are vaccinated against PRRSV (ReproCyc® PRRS EU, Boehringer Ingelheim Vetmedica GmbH, Germany) every three months after the introduction of a new field virus strain led to a severe PRRS outbreak in 2015. Piglets from this herd are vaccinated against PRRSV (Ingelvac PRRSFLEX® EU, Boehringer Ingelheim Vetmedica GmbH, Germany) at weaning (three weeks of age), when they are transferred to a weaning unit located within the sow farm. After three and a half weeks, piglets are moved to the newly built nursery unit which is located about 300 meters from the sow farm. Sow farm B produces piglets with 600 sows and is free of PRRS based on routine serological testing; piglets from this herd are housed in a nursery unit within the sow farm for 3.5 weeks after weaning before they are transferred to the newly built nursery unit located around 100 km from the sow farm. Piglets from farm B are vaccinated against PRRSV (Ingelvac PRRSFLEX® EU, Boehringer Ingelheim Vetmedica GmbH, Germany), when entering the nursery unit at the age of approximately 6.5 weeks. The set-up led to the placement of piglets with different PRRS status, i.e. vaccinated 3.5 weeks prior to entry or vaccinated directly at entry, into the nursery unit every other week. The nursery unit consisted of four different rooms with 6 pens in each room to house a total of 2600 nursery piglets. No strict all-in/all-out was performed and piglets from the two sources shared the same air space. According to the farmer, production parameters in the nursery were satisfying with 1-2 % average piglet all-cause mortality. Nevertheless, about 1 % of the piglets showed respiratory symptoms and retarded growth. Diagnostic investigations of such runt pigs revealed a mixed infection with PRRSV, PCV2 (no further genotyping done) and Influenza A virus (swine H1N1 of avian origin). Histologic lesions of the lung were described as purulent bronchopneumonia, peribronchial interstitial pneumonia and partially severe damage of alveoli with type 2 pneumocyte proliferation. Pathologists summarized their report as lung lesions caused by viral infection with secondary bacterial colonization. Following

bacteriological examination *Streptococcus suis*, *Pasteurella multocida* and *Mycoplasma hyorhinis* could be isolated.

Anamnesis and clinical findings associated with AUT22-97 occurrence

In January 2022, an Austrian piglet-producing farm harboring 70 sows faced respiratory distress and increased mortality in the nursery unit. The farm is located in Styria, the southern part of Austria, in an area with a high density of pig farms. The farm has a three-week batch farrowing rhythm and a suckling period of 28 days. Approximately 26 piglets are weaned per sow and year. Gilts are bought from a conventional gilt-producing farm with unknown PRRS-status. After arrival on the farm, all gilts are kept in isolation units for six weeks. During isolation, gilts are not routinely tested for the presence of certain pathogens or antibodies against common pathogens, like PRRSV. Semen is acquired from a conventional boar stud located in Styria; one teaser boar is kept in the service center. Cleaning and disinfection are performed between batches in the farrowing rooms as well as in the nursery, whereas a strict "all-in/all-out" is not possible in the nursery unit, since two age groups have to be kept together for structural reasons.

The sows and gilts are vaccinated against porcine parvovirus and Erysipelas (Parvoruvac®, Ceva Santé Animale, France) according to the manufacturer's recommendation. Vaccination protocol of piglets includes *Mycoplasma hyopneumoniae* (Hyogen®, Ceva Santé Animale, France) and PCV-2 (Ingelvac CircoFLEX®, Boehringer Ingelheim Vetmedica GmbH, Germany) in the third week of life. Additionally, an inactivated vaccination against *Escherichia coli* and *Clostridium perfringens* (Enteroporc Coli AC, Ceva Santé Animale, France) is used in sows for passive immunization of piglets. Prior to the PRRSV detection, the PRRSV-status of the farm was unknown. Investigated thymus pool samples from aborted fetuses were PRRSV negative by PCR in 2018. The farm has recurring problems with post-weaning diarrhea. In January 2022 hemolytic *Escherichia coli* (virulence genes for fimH-fimbriae, F4-fimbriae, heat-labile enterotoxin LT, and hlyA-hemolysin) were detected. Colistin was used for treatment.

The clinical signs started in the rearing period. Respiratory distress, wasting and increased mortality rates up to 10% were observed. The herd-attending veterinarian suspected an outbreak of *Actinobacillus pleuropneumoniae*, which could be confirmed in necropsy and sampling of lung tissue. Additionally, 10 serum samples from 10-week-old nursery pigs were taken and investigated for PRRSV as predisposing pathogen. Antibodies were investigated by IDEXX PRRS X3® ELISA (IDEXX PRRS X3® Ab Test, IDEXX Europe B.V., Netherlands), with positive results in 10/10 samples (S/P ratios ranged from 1.56 to 2.25; cut-off: 0.4). PRRSV1 ORF1 RT-qPCR was performed in pools of five with positive results (2.84×10^7 genome equivalents [GE]/mL and 4.85×10^7 GE/mL). In addition, four sows aborted at the same time clinical signs occurred in the nursery unit. Increased return-to-heat and increased numbers of stillborn or weak born piglets could not be observed. Fattening pigs didn't show respiratory signs, but tail biting could be observed.

Immediately after occurrence of clinical signs, all gilts and sows were vaccinated against *Actinobacillus pleuropneumoniae* (Coglapix®, Ceva Santé Animale, France) and against PRRSV (UNISTRAIN® PRRS, Laboratorios Hipra, S.A., Spain). Additionally, all piglets were routinely vaccinated against PRRSV (UNISTRAIN® PRRS, Laboratorios Hipra, S.A., Spain) within the third week of life. After mass vaccination, gilts are vaccinated twice against PRRSV in the isolation unit. The sows are re-vaccinated every four months. Clinical signs in the nursery vanished after three batches and production parameters reached levels prior to the outbreak. The next batch of gilts was sampled within quarantine (April 2022), with negative PRRSV antibody and PCR results.

References

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4. Discussion

PRRSV is one of the most relevant porcine pathogens worldwide. It is prevalent in most countries with commercial pig farming and causes a significant amount of production and financial losses (Neumann et al. 2005; Zhang et al. 2022). Due to the big genetic diversity of circulating PRRSV strains, the prevention of PRRS outbreaks by developing safe and efficient vaccines has become a major challenge. MLV vaccines are regularly used, and it is indisputable that their use outweighs their risks - nevertheless they are still far from perfect. First of all, MLV vaccines have the potential to revert to virulence, by mutations (Nielsen et al. 2001), adaption (Wang et al. 2022b), and recombination (Eclercy et al. 2019). Recombination of MLV strains with other field strains can potentially result in fitter chimeric viruses that outcompete their parental strains (Kristensen et al. 2020; Kvisgaard et al. 2020; Eclercy et al. 2019; Sun et al. 2022). At last, several studies show that MLV vaccines do not elicit a cross-protective antibody response against heterologous strains (Park et al. 2015; Mengeling et al. 2003; Park et al. 2014). Subsequently, cellular immune responses are considered important factors in PRRSV immunology. With our research, we aimed to elucidate two of these issues: the search for cross-protective PRRSV-1 epitopes with the ability to restimulate $CD8^+$ T cells, and the investigation of PRRSV-1 recombination mechanisms with MLV vaccines. Both topics aimed to improve the current knowledge and possible refinement of the development of novel PRRSV vaccines.

4.1. PRRSV $CD8^+$ T cell epitopes

Our approach for the isolation of MHC-I bound PRRSV-1 peptides from *in vitro* infected cells was designed to identify epitopes that are naturally synthesized by the cell. The immunoproteasome has a specific pattern of hydrolyzing proteins, and not all peptides are bound by the MHC-I α-chains' peptide binding cleft, due to specific anchor residues and spatial limitations (Murphy et al. 2017). Many immunopeptidome studies focus on peptides that are predicted by specific algorithms, or randomly designed peptide libraries (Pan et al. 2019; Liang et al. 2021; Wang et al. 2011). Many of these hypothetical peptides are too large or small, or do not possess the preferred amino acid interaction partners of the specific MHC-I anchor residues.

Our data relies on *in vitro* generated immunoproteasomal peptides, that are isolated from PAMs 18 hours p.i. with PRRSV-1. We chose this time point, since data from our preliminary experiments suggests that MHC-I starts to get downregulated at 24 hours p.i. With this strategy we wanted to ensure an optimal sample yield. Our isolated MHC-I bound peptides essentially follow the pattern of hydrophobic and basic amino acids as anchor residues. Furthermore, most isolated peptides are 9-mers, which agrees with the literature (Murphy et al. 2017). These results strongly support our MHC-I/peptide isolation protocol. Hence, our approach ensures that isolated epitopes are truly presented by MHC-I molecules upon infection. This is important, since the reinfection of an animal after previous infection or vaccination would be dependent on pre-existing PRRSV-specific T cell receptors. Such receptors are tailored to recognize epitopes they have been encountered previously. If these epitopes are not produced by the proteasome and displayed by an antigen-presenting cell, the infected cell would not be recognized, and subsequently not neutralized by $CD8^+$ T cells.

To identify the sequences of our peptide isolates, we analyzed the samples with liquid chromatography tandem mass spectrometry (LC-MS/MS). This data revealed that the most confident PRRSV-1-specific peptides from infected PAMs all derive from the nsps of ORF1. To elaborate, whether these identified peptides also have the potential to trigger $CD8^+$ T cell responses, we developed an *in vitro* restimulation assay. This assay aided PBMCs of previously vaccinated and/or infected pigs, that were restimulated with a pool of our identified peptides, and the single peptides only. These results were compared to unstimulated cells, cells stimulated with endogenous (or porcine) peptides, and a positive control. After a staining of cell surface markers and intracellular cytokines, the PBMCs were gated and analyzed with a flow cytometer. Our results show that especially the pooled peptides elicit a PRRSV-1-specific IFNγ production of $CD8^+$ T cells. Furthermore, several single peptides show an elevated IFNγ production, compared to the controls. No TNFα, or IFNγ/ TNFα double producers could be detected upon stimulation with the PRRSV-1 peptides. At last, we could show that there was a higher frequency of $CD27^+CD8^+$ T cells in the PRRSV-1 peptide-restimulated PBMCs, compared to the positive controls. CD27 is a marker for naïve T cells, that is depleted upon differentiation into an effector phenotype (Cossarizza et al., 2019). Subsequently, there are less effector (memory) cells present upon stimulation with our PRRSV-1-derived peptides. This could be a result of the short stimulation time of 17 hours only. A longer stimulation should be

considered for future experiments to investigate, whether our peptides have the potential to cause differentiation into memory T cells. This is an important feature that peptides used for the development of effective subunit vaccines should have.

To address the issue, that our analyses show the most confident LC-MS/MS results of peptides derived from the PRRSV-1 nsps, we hypothesize that these proteins are plausible targets of ubiquitin-mediated immunoproteasomal degradation, since they are produced and reside in the cytosol. The PRRSV structural proteins of ORF2-6 are translocated into the ER to receive post-translational modifications. Nevertheless, there must be a more thorough investigation of the hypothesis, that nsps are the main targets of MHC-I presentation. For this purpose, the LC-MS/MS analyses have to be expanded by producing more replicates, investigating different PRRSV strains, and cells with different SLA-I haplotypes. Therefore, we designed a Classical Swine Fever Virus (CSFV) based vector, where a gene of insert can be inserted. This insert is fused to polyubiquitin, to mark it for ubiquitin-mediated proteasomal degradation. This recombinant vector can be used to infect porcine cells and to take a closer look at T cell epitopes from defined viral proteins. Additionally, this vector is not restricted to primary cells, but is able to infect permanent cell lines that are susceptible to CSFV, for example SK6 or MAX cells. We aspire to use this vector system in the future to gain a more detailed insight into the MHC-I presentation of a defined proteins from defined ORFs. Special proteins of interest would be the PRRSV nsps, but also the nucleocapsid protein of ORF7. This structural protein also remains in the cytoplasm (Spilman et al. 2009) and could be a target of E3 ligases and immunoproteasomal degradation.

In order to define the PRRSV immunopeptidome in more detail, different viral strains would have to be considered for MHC-I/peptide isolation and identification. Our data suggests that several PRRSV-1 epitopes, recognized by $CD8^+$ T cells, are conserved among other strains. Nevertheless, the generation of a pool of epitopes, protecting against a broad quantity of PRRSV-1 and PRRSV-2 strains, would be important in regard of vaccine developments. Even though the two PRRSV species show different prevalence at certain geographical areas, the transmission between different counties is not unlikely due to the commercialized animal trade.

Another issue that has to be considered during the search for cross-protective viral epitopes are the diverse SLA-I haplotypes. Our data provides isolated MHC-I peptides from the haplotype

SLA-I Lr-Hp 35.0/24mod and the PBMCs used for restimulation all possessed one similar allele. This similarity of the four different PBMC batches showed an $CD8^+$ T cell-specific IFNγ response upon restimulation with our identified PRRSV-1 peptides. Nevertheless, the SLA-I haplotypes in the overall pig population are very diverse and a subunit vaccine, with the aim to stimulate $CD8^+$ T cells, would have to protect numerous of these haplotypes. Therefore, optimally, a pool of PAMs with the most common SLA-I haplotypes should be analyzed for PRRSV-specific MHC-I epitopes. A limitation of this proposal is the availability of PAMs. These primary cells are extracted from lungs of euthanized pigs and are not available in an unlimited amount. Therefore, the establishment of a permanent PAM cell line, susceptible to PRRSV infection, is an urgent matter. This cell line could be genetically modified to express different SLA-I haplotypes, by removing the customary SLA-I locus with CRISPR/Cas9 and replace it with a different allele. Subsequently, this would allow a more detailed look into haplotype-specific MHC-I bound PRRSV peptides and would reduce the need of live animals for PAM extraction. At last, a permanent cell line has the additional advantages of providing an unlimited amount of cells, a smaller risk of contamination, robustness, and a better reproducibility of experiments.

Comparing our results with other studies strengthens our suspicions, that the PRRSV immunopeptidome is much more diverse. An investigation of overlapping peptides from nsp9 and nsp1 by restimulation of PBMCs revealed several immunogenic peptides (Parida et al. 2012). The authors' identified reactive peptide sequences do not show an overlap. A similar study identified one immunogenic peptide from nsp9, after MHC-I binding predictions with NetMHCpan 4.0 (Pan et al. 2019). This peptide sequence does not match any of our immunogenic PRRSV-1 peptides. Furthermore, the creation of a library of overlapping 20-mer PRRSV peptides and restimulation of PBMCs (Chung et al. 2016) identified several epitopes triggering $CD8^+$ T cell responses. One sequence shows an overlap of peptide 7 (nsp4) with one of Chung et al., and two more peptides partially overlap with our peptides 1 and 2 (nsp1α). However, nsps are not the only targets of immunopeptidome studies. A study investigating T cell epitopes of the PRRSV-2 M protein identified three immunogenic peptides (Liang et al. 2021). Furthermore, GP3 epitopes have been shown to trigger IFNγ responses of $CD8^+$ T cells (Cao et al. 2016). These findings underline the importance of a more thorough investigation of the PRRSV immunopeptidome to identify more immunogenic MHC-I epitopes.

With our MHC-I/peptide isolation protocol, LC-MS/MS based identification, and validation of the immunogenicity of these epitopes with an intracellular cytokine staining, we established a solid method for PRRSV immunopeptidome studies in the future. Furthermore, we provide first immunogenic peptide sequences from the PRRSV-1 nsps. This could be helpful for the development of a rational novel vaccine that includes the stimulation of $CD8^+$ T cells.

4.2. PRRSV recombination mechanisms

Recombination is a common process in positive stranded ssRNA viruses (Pérez-Losada et al. 2015), induced by discontinuous RNA replication. During this process, the RdRp dissociates from its RNA template and re-associates with another complementary strand. This can result in the generation of chimeric genomes with two or more parental strands. Even though this mechanism can potentially result in evolutionary advantageous strains, as it has been shown in HIV-1 (Yusa et al. 1997; Moutouh et al. 1996; Nora et al. 2007), it can also generate less fitter strains, that are possibly eliminated from the gene pool. Nevertheless, together with high mutation rates, the recombination of RNA viruses is a main contributor to viral evolution and the quasispecies concept.

To date, many recombinant PRRSV strains have been reported, and due to advanced sequencing techniques and recombination detection tools, these reports and the submission of full-genome sequences to data banks are increasing. These isolates can be chimeric viruses between two or more wild-type strains (Liu et al. 2018; Wang et al. 2020), wild-type and vaccine strains (Li et al. 2009; Marton et al. 2019), or different vaccine strains (Kvisgaard et al. 2020; Eclercy et al. 2019; Sun et al. 2022). Some of these recombinants have been isolated from PRRS-affected farms (Kvisgaard et al. 2020), whereas others have been found by chance during whole-genome screenings of existing isolates or sequences (Vandenbussche et al. 2021). This supports the evidence, that recombination does not necessarily result in more pathogenic or infectious strains but is a random process.

Our work presents three recombinant PRRSV-1 strains, originating from different farms in Germany and Austria, between 2018 and 2022. Strain GER18-258 originates from Southern Germany and was isolated in 2018. This farrow-to-finish farm reported reproductive disorders, characterized by still and weak born piglets and a high pre-weaning mortality. The nursery and

fattening pigs displayed typical PRRS symptoms, and overall mortality increased by 2.5%. Clinical samples revealed a co-infection of PRRSV-1 with *Streptococcus suis* and *Staphylococcus aureus*. Strain AUT20-1664 was isolated in Austria in 2020. This farm harbored a nursery unit with sows from two different farms. 1% of piglets showed retarded growth and respiratory symptoms. These piglets were diagnosed with a co-infection of PRRSV-1, PCV2, Influenza A, Streptococcus suis, *Pasteurella multocida*, and *Mycoplasma hyorhinis*. Strain AUT22-97 was isolated in Austria in 2022. This piglet-producing farm reported an increased mortality, abortions, and respiratory symptoms in the nursery. PRRSV-1 and *Actinobacillus pleuropneumoniae* were detected in clinical samples from this farm.

We were able to isolate PRRSV-1 from clinical samples of all farms and sequenced their genomes. Initial BLAST analysis revealed all three isolates to be recombinants between different wild-type strains and the Ingelvac PRRSFLEX EU® vaccine strain. Further investigations with a recombination detection program disclosed, that GER18-258 is most likely a recombinant strain with GER09-613 as a major and PRRSFLEX as a minor parent, AUT20-1664 a recombinant with an unknown major and PRRSFLEX as a minor parent, and AUT22-97 most likely a recombinant of AUT15-33 as a major and PRRSFLEX as a minor parent. The similarities of the major parents are below 93%, which cannot rule out a different, more similar, not yet isolated PRRSV strain to be the actual major parental strain. The recombination breakpoint of all recombinant strains was assigned to the beginning of ORF5. This made us suspect, that the RdRp template-switch did not occur randomly. RNA structure predictions of the ORF5 recombination hotspots of the parental strains revealed the presence a stem-loop. This structure is a plausible cause for the RdRp to switch templates, since such structures have been shown to cause of polymerase dissociation from RNA strands (Cheng et al, 1991). Having observed this structure, we analyzed other common PRRSV strains for the presence of the stem-loop. Interestingly, this structure revealed to be conserved among other PRRSV-1 wild-type and vaccine strains, but not among PRRSV-2 strains. BLAST analysis of ORF5-7 resulted in the clustering of several PRRSV isolates from Belgium (Vandenbussche et al., 2021) with our recombinant strains. The authors conducted a broad whole-genome sequencing study of 124 PRRSV isolates and detected several recombinants of wild-type and vaccine strains. Interestingly, four of these strains are chimeric viruses of different wild-type strains and the Ingelvac PRRSFLEX EU® vaccine strain, with a recombination breakpoint at

the beginning of ORF5. These results support our hypothesis, that PRRSV-1 strains and the PRRSFLEX vaccine strain possess a recombination hotspot at the beginning of ORF5. This lets us conclude, that a closer investigation of PRRSV strains in the field is necessary to monitor the emergence and spread of such chimeric viruses. Interestingly, one of the farm where our recombinant strains was isolated never applied a PRRSV vaccine. This means that GER18-258 was spread from another farm, where the virus originates. This underlines that biosafety measures are to be taken seriously to avoid contamination of other pig populations with a potentially harmful virus.

With this evidence, we suspect there will be more similar recombinant PRRSV strains emerging in the future. PRRSV demonstrates a considerably fast evolution with nucleotide exchange numbers between $1*10^{-6}$ and $1*10^{-4}$ mutations per nucleotide site per year (Peck und Lauring 2018). Recombination further contributes to this fast evolution, which helps PRRSV to rapidly adapt to host to changes and generate strains with immune escape properties. As there is a big selective pressure on PRRSV field strains due to vaccination, the virus will evade immunity by all means. In this context it is difficult to comprehend which advantage recombined genes from MLV strains might provide. Even though veterinarians agree that the benefits of PRRSV vaccination outweighs their risks, a monitoring of isolates would help to further distinguish recombinant strains and could help prevent their spread. Additionally, a closer characterization of the ORF5 stem-loop has to be done to help understand recombination mechanisms of PRRSV, and other RNA viruses subsequently.

4.3. Conclusion

Taken together, both our manuscripts are part of a prevailing topic in PRRSV research: the search for a safe and potent vaccine. As already discussed in chapter 1.1.5. inactivated and subunit vaccines have so far not been providing a sufficient protection. Nevertheless, the available MLV vaccines used in the field are often not cross-protective against heterologous strains within the PRRSV-1 and PRRSV-2 species and bear the potential to revert to virulence or recombine with other strains. With our immunopeptidomics study, we provide a method to isolate and identify PRRSV-specific MHC-I bound epitopes with the ability to stimulate IFNγ production of $CD8^+$ T cells and first immunogenic peptide sequences. It is especially important

to find conserved PRRSV MHC-I epitopes to avoid immune evasion by viral evolution. Some of our identified epitopes are conserved among other PRRSV strains, but it remains to be investigated, whether they elicit an IFNγ response of PBMCs from pigs with different SLA-I haplotypes. Furthermore, a follow up study to identify more epitopes from different viral strains has to be conducted, to provide a broad PRRSV immunopeptidome library. This library could be used in the future to design a reasonable subunit vaccine, causing a solid and cross-protective PRRSV-specific IFNγ response. Furthermore, the vaccine should optimally generate a long-term immunologic memory to protect animals from (re)infections.

Our finding of a conserved stem-loop within the PRRSV-1 ORF5 RNA sequence that is a recombination hotspot in PRRSV-1 strains, raises concerns about the emergence of more recombinant strains in the field. This strengthens the claim, that ideally PRRSV MLVs are to be replaced by safer alternatives. At last, we hypothesize that the function of the stem-loop within ORF5 is likely not a recombination tool. These RNA structure often have other functions, like translational initiation. It remains to be instigated, whether the original function of the stem-loop is to start the translation of a yet unknown PRRSV alternative ORF.

A recent SARS-CoV-2 immunopeptidomics study discovered immunogenic out-of-frame T cell epitopes (Weingarten-Gabbay et al., 2021). This would be the missing link of our two studies – to look for out-of-frame epitopes within the PRRSV immunopeptidome, to not only identify immunogenic peptides, but potentially new ORFs. This would not only contribute to finding candidates for novel vaccines stimulating $CD8^+$ T cell responses but would help to better understand PRRSV immunology and the virus as an entity.

5. Summary

PRRSV is an enveloped single-stranded RNA virus of positive polarity, and one of the most devastating porcine pathogens worldwide. Clinical signs of infected animals include respiratory disease and reproductive disorders, causing huge production and financial losses of affected farms. MLV vaccines are available and widely used, but they are often not cross-protective against heterologous virus strains and able to recombine with wild-type or other vaccine strains. These two issues are the focus of this thesis: The search for cross-protective PRRSV-1 epitopes with the potential to elicit a $CD8^+$ T cell response, and the investigation of recombination mechanism of PRRSV-1 wild type and MLVs strains.

For the identification of PRRSV-1 epitopes with the potential to restimulate $CD8^+$ T cells, we isolated MHC-I/peptide complexes of PRRSV-1 infected PAMs by immunoprecipitation. Furthermore, we analyzed the bound peptides with LC-MS/MS and compared them to the pig proteome. For the confirmation of the immunogenicity of these epitopes, we conducted *in vitro* restimulation assays of PBMCs followed by an ICS and flow cytometry. We were able to successfully establish a convenient MHC-I/peptide complex isolation protocol for the LC-MS/MS identification of PRRSV-1 epitopes. Furthermore, we conducted a workflow for the restimulation of PBMCs with these identified peptides to measure $CD8^+$ T cell cytokine responses. Our analyses reveal the most confident MS matches of MHC-I-bound peptides derive from the PRRSV-1 nsps of ORF1. Additionally, we confirmed the elicitation of an IFNγ response by $CD8^+$ T cells after restimulation with several of these peptides. At last, we compared these peptide sequences with proteomes of other PRRSV strains and detected the conservation of several epitopes in other PRRSV-1, PRRSV-2, and PRRSV vaccine strains. We enforce a more detailed research of the PRRSV immunopeptidome by investigating other strains and other SLA-I haplotypes to gain a deeper understanding of MHC-I presentation toward $CD8^+$ T cells after infection.

The second part of the thesis focuses on PRRSV recombination mechanisms. After the isolation and sequencing of three recombinant PRRSV-1 strains from farms with mild to severe PRRS cases, our attention was drawn towards a specific MLV vaccine strain. Recombination analyses of these isolates confirmed the recombination of three different wild-type strain with the same

MLV strain at the beginning of ORF5. RNA structure predictions revealed a conserved stem-loop within ORF5, which might be a plausible cause of the RdRp to switch temples during replication upon the co-infection of the same animal with different strains. We suggest a more detailed exploration of this stem-loops structure and function and underline the surveillance of PRRSV recombination in the swine population.

Taken together, we suggest a more thorough investigation of the PRRSV immunopeptidome for the development of PRRSV vaccines with the ability to elicit a cross-protective $CD8^+$ T cell response, in order to eliminate the risk of introducing more recombinant strains in the field.

Zusammenfassung

PRRSV ist ein umhülltes, einzelsträngiges RNA-Virus mit positiver Polarität und einer der verheerendsten Krankheitserreger bei Schweinen weltweit. Zu den klinischen Symptomen infizierter Tiere gehören Atemwegserkrankungen und Fortpflanzungsstörungen, die in den betroffenen Betrieben enorme finanzielle Verluste verursachen. Modifizierte Lebendimpfstoffe sind verfügbar und weit verbreitet, aber sie sind oft nicht kreuzprotektiv gegen heterologe Virusstämme und können mit Wildtyp- oder anderen Impfstämmen rekombinieren. Diese beiden Probleme stehen im Mittelpunkt dieser Arbeit: Die Suche nach PRRSV-spezifischen MHC-I Epitopen, die das Potenzial haben, eine $CD8^+$ T-Zell-Antwort hervorzurufen, und die Suche nach dem Rekombinationsmechanismus von PRRSV-1 Impf- und Wildtyp-Stämmen.

Zur Identifizierung von PRRSV-1 Epitopen die $CD8^+$ T-Zellen stimulieren, isolierten wir MHC-I/Peptid-Komplexe von PRRSV-1-infizierten Alveolarmakrophagen durch Immunpräzipitation. Dann analysierten wir die isolierten Peptide mit LC-MS/MS und verglichen sie mit dem Proteom des Schweins. Zur Bestätigung der Immunogenität dieser Epitope führten wir *in vitro* Restimulationsversuche mit PBMCs durch, gefolgt von einer intrazellulärem Zytokinfärbung und Durchflusszytometrie. Es gelang uns, ein erfolgreiches MHC-I/Peptidkomplex-Isolierungsprotokoll für die LC-MS/MS-Identifizierung von PRRSV-1 Epitopen zu entwickeln. Außerdem führten wir eine PBMC-Restimulation mit diesen identifizierten Peptiden durch, um die Zytokinreaktionen von $CD8^+$ T-Zellen zu messen. Unsere Analysen zeigen, dass die zuverlässigsten MS-Übereinstimmungen der MHC-I-gebundenen Peptide von den PRRSV-1 Nichtstrukturproteinen vom Leseraster 1 stammen. Darüber hinaus bestätigten wir die Generierung einer IFNγ-Antwort durch $CD8^+$ T-Zellen nach Restimulation mit mehreren dieser Peptide. Schließlich verglichen wir diese Peptidsequenzen mit den Proteom anderer PRRSV-Stämme und stellten fest, dass mehrere unserer Epitope in anderen PRRSV-1-, PRRSV-2- und PRRSV-Impfstoff-Stämmen konserviert sind. In der Zukunft beabsichtigen wir eine detailliertere Analyse des PRRSV-Immunopeptidoms, indem wir andere Virusstämme und andere SLA-I-Haplotypen untersuchen, um ein tieferes Verständnis der MHC-I-Präsentation gegenüber $CD8^+$ T-Zellen nach der Infektion zu gewinnen.

Der zweite Teil der Arbeit befasst sich mit Rekombinationsmechanismen von PRRSV. Nach der Isolierung und Sequenzierung von drei rekombinanten PRRSV-1-Stämmen aus Betrieben mit leichten bis schweren PRRS-Fällen wurde unsere Aufmerksamkeit auf einen spezifischen modifizierten Lebendimpfstoffstamm gelenkt. Rekombinationsanalysen dieser Isolate bestätigten die Rekombination von drei verschiedenen Wildtyp-Stämmen mit demselben Impfstamm am Anfang vom Leseraster 5. RNA-Strukturvorhersagen ergaben eine konservierte Haarnadelstruktur innerhalb des Leserasters 5, die ein plausibler Grund dafür sein könnte, dass das RdRp während der Replikation, bei der Koinfektion desselben Tieres mit verschiedenen Stämmen, die RNA-Vorlage wechselt. Wir schlagen eine genauere Untersuchung der Struktur und Funktion dieser Stammschleife vor und unterstreichen die genauere Überwachung von rekombinanten PRRSV Stämmen in der Schweinepopulation.

Zusammenfassend schlagen wir eine gründlichere Untersuchung des PRRSV Immunopeptidoms vor, um Impfstoffe zu entwickeln, die in der Lage sind, eine kreuzprotektive $CD8^+$ T-Zellen-Antwort auszulösen, um das Risiko der Einführung weiterer rekombinanter Stämme in der Praxis zu vermeiden.

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7. Supplement

Supplementary file from part 3.2: S2 Accession numbers of PRRSV strains used for phylogenetic trees of figure 3

A26843.1	JX215551.1	KC862576.1
AF046869.1	JX215552.1	KC862577.1
AF494042.1	JX215553.1	KC862578.1
AY032626.1	JX215554.1	KC862579.1
AY588319.1	JX235365.1	KC862580.1
EU200962.1	JX235366.1	KC862581.1
EU624117.1	JX235367.1	KC862582.1
FJ797690.1	JX235370.1	KC862583.1
GU047344.1	JX258843.1	KC862584.1
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GU067771.1	JX857698.1	KF001144.1
GU737264.2	KC492504.1	KF183946.1
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JF802085.1	KC492506.1	KF815525.1
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JX187609.1	KC862567.1	KJ523894.1
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JX192639.1	KC862575.1	KP889243.1

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