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In vitro and *ex vivo* analyses of co-infections with swine influenza and porcine reproductive and respiratory syndrome viruses

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ABSTRACT

Viral respiratory diseases remain problematic in swine. Among viruses, porcine reproductive and respiratory syndrome virus (PRRSV) and swine influenza virus (SIV), alone or in combination, are the two main known contributors to lung infectious diseases. Previous studies demonstrated that experimental dual infections of pigs with PRRSV followed by SIV can cause more severe disease than the single viral infections. However, our understanding of the impact of one virus on the other at the molecular level is still extremely limited. Thus, the aim of the current study was to determine the influence of dual infections, compared to single infections, in porcine alveolar macrophages (PAMs) and precision cut lung slices (PCLS). PAMs were isolated and PCLS were acquired from the lungs of healthy 8-week-old pigs. Then, PRRSV (ATCC VR-2385) and a local SIV strain of H1N1 subtype (A/Sw/Saskatchewan/18789/02) were applied simultaneously or with 3 h apart on PAMs and PCLS for a total of 18 h. Immuno-staining for both viruses and beta-tubulin, real-time quantitative PCR and ELISA assays targeting various genes (pathogen recognition receptors, interferons (IFN) type I, cytokines, and IFN-inducible genes) and proteins were performed to analyze the cell and the tissue responses. Interference caused by the first virus on replication of the second virus was observed, though limited. On the host side, a synergistic effect between PRRSV and SIV co-infections was observed for some transcripts such as TLR3, RIG-I, and IFNβ in PCLS. The PRRSV infection 3 h prior to SIV infection reduced the response to SIV while the SIV infection prior to PRRSV infection had limited impact on the second infection. This study is the first to show an impact of PRRSV/SIV co-infection and superinfections in the cellular and tissue immune response at the molecular level. It opens the door to further research in this exciting and intriguing field.

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1. Introduction

Bacterial and viral respiratory diseases are still a major health issue in pigs reared under confined conditions on intensive breeding farms. Billions of dollars are spent every year to control these diseases. Most often, multiple infectious agents are involved (Bosch et al., 2013; Choi

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et al., 2003; Fablet et al., 2012a,b, 2011; Opriessnig et al., 2011). In a retrospective analysis of diagnostic data from 2872 cases of respiratory disease in pigs received at the Minnesota Veterinary Diagnostic Laboratory over an 18 month period, authors showed that two or more pathogens were detected in 88.2% of the cases (Choi et al., 2003).

Respiratory infectious agents can be divided into primary and secondary pathogens. Primary pathogens include bacteria such as *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, and *Mycoplasma hyopneumoniae*, and viruses including porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies virus, and porcine circovirus type 2 (Opriessnig et al., 2011). Other primary pathogens are described but they are rarely encountered or have less impact on porcine health. Among the secondary pathogens, common bacteria such as *Actinobacillus suis*, *Haemophilus parasuis*, *Pasteurella multocida*, *Salmonella choleraesuis*, and *Streptococcus suis* are frequently reported (Choi et al., 2003; Fablet et al., 2012a,b, 2011; Opriessnig et al., 2011). Together, primary and secondary pathogens are involved in the well-described porcine respiratory disease complex (PRDC) (Hallbur, 1998). PRRSV and SIV, together and individually, are frequently encountered in the fields (Choi et al., 2003; Fablet et al., 2012a, 2011). In a study by Choi and collaborators, 109 samples (17%) of 636 SIV-positive cases were also positive for co-infections with PRRSV, behind *P. multocida* (148 samples, 23.2%) and *M. hyopneumoniae* (122 samples, 19.2%) (Choi et al., 2003). Previous studies dealing with PRRSV/SIV dual infections (Pol et al., 1997; Van Reeth et al., 1996, 2001) showed various outcomes with respect to dual infection. In a study where feeder pigs were infected first with PRRSV, then with porcine respiratory coronavirus or SIV, more severe disease and growth retardation were observed with dual infection than with PRRSV infection alone (Van Reeth et al., 1996). In another study where 3-week-old specific-pathogen-free piglets were intra-nasally infected with PRRSV, followed one week later with a H3N2 SIV strain, observations indicated that the previous PRRSV infection did not influence clinical signs during influenza infection (Pol et al., 1997). Then, in a study with PRRSV and a European H1N1 SIV strain, authors observed variable clinical outcomes of dual PRRSV-SIV infection, depending on both the time interval between infections and the health status of pigs used in the study (Van Reeth et al., 2001). Aside from these various following co-infections, our understanding of the impact of one virus on the other at the molecular level is still extremely limited. Most of the studies on PRRSV/SIV co-infections were performed more than ten years ago at a time where the porcine toolbox was much less developed. Thus, the aim of the current study was to determine at the molecular level how dual infections, compared to single infections, influence the response of porcine alveolar macrophages (PAMs) and precision cut lung slices (PCLS) to PRRSV and SIV. PAMs and PCLS were used because of their relevance in the context of infections with PRRSV and SIV. PAMs, pulmonary intravascular macrophages (PIMs), and interstitial macrophages (ISMs) are the main targets of PRRSV

(Meulenberg, 2000; Sang et al., 2011). PAMs can also be infected by SIV (Crisci et al., 2013; Taubenberger and Morens, 2008). PCLS have previously been used for infection studies in birds (Abd El Rahman et al., 2010), cattle (Goris et al., 2009), and pigs (Punyadarsaniya et al., 2011). The PCLS culture system has several advantages over other systems: (1) slices can be obtained in large numbers; (2) the general architecture of the tissue is preserved so differentiated epithelial cells, which are the main target cells of SIV, and various other cellular types are maintained *in situ*; and (3) the slice viability extends past 7 days (Punyadarsaniya et al., 2011). Moreover, PAMs and PCLS systems together allow us to study host/pathogen interactions in a single cell type population *versus* a multi-cellular tissue, granting more accurate analysis of the contribution of PAMs to the global disease response.

2. Materials and methods

2.1. Ethics statement

A total of 10 eight-week-old York-type crossbred commercial pigs were purchased from the Prairie Swine Centre, University of Saskatchewan. Pigs were healthy and showed no clinical symptoms or serological evidence of respiratory (*e.g.* SIV, *M. hyopneumoniae*, PRRSV) or systemic diseases. All experiments were conducted in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council on Animal Care. Pigs were euthanized with 360 mg/kg sodium pentobarbital (Ethanyl, Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada) administered intravenously. All efforts were made to minimize suffering.

2.2. Precision-cut lung slices

PCLS were prepared from lungs of 4 eight-week-old pigs. Immediately after euthanasia, lungs were carefully removed and the left cranial, middle, and caudal lobes were filled with 37 °C warm low-gelling temperature agarose (Sigma–Aldrich, Oakville, ON, Canada) followed by polymerization on ice. Tissue was excised in cylindrical portions (8-mm tissue coring tool) and around 200 slices/pig approximately 250 μm thick were prepared by using a Krumdieck tissue slicer (model MD6000, TSE systems, Chesterfield, MO, USA) with a cycle speed of 60 slices/min. PCLS were incubated in 1 ml of RPMI 1640 medium (GIBCO®-BRL, Burlington, ON, Canada), supplemented with 1% antibiotic/antimycotic (Anti-Anti 100×, GIBCO®-BRL), clotrimazole 1 μg/ml (Sigma–Aldrich), enrofloxacin 10 μg/ml (Bayer Inc., Toronto, ON), and kanamycin 80 μg/ml (GIBCO®-BRL) in a 24-well plate at 37 °C and 5% CO_2. The medium was changed every hour during the first 4 h and once after 24 h, prior to infection. Viability was analyzed by observing ciliary activity under a light microscope (Olympus CKX31, Tokyo, Japan). In selected samples, slices were analyzed for bronchoconstriction by addition of 10^{-4} M methacholine (acetyl-β-methylcholine chloride, Sigma–Aldrich), as previously described (Vietmeier et al., 2007).

2.3. Cells and viruses

Porcine alveolar macrophages (PAMs) were obtained by lung lavage of 6 eight-week-old pigs and maintained in RPMI 1640 (GIBCO®-BRL) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Anti-Anti 100×, GIBCO®-BRL). In healthy animals, PAMs represent >90% of cells in broncho-alveolar lavage fluid as previously reported (White et al., 2007). Madin–Darby canine kidney (MDCK, ATCC CCL-34) cells were cultured in minimal essential medium (MEM) supplemented with 10% FBS. MARC-145 monkey cells (ATCC CRL-12231) were grown in MEM (GIBCO®-BRL) supplemented with 10% FBS and 1% antibiotic/antimycotic (Anti-Anti 100×, GIBCO®-BRL).

The influenza strain A/Sw/Saskatchewan/18789/02 (SIV/Sk02) of H1N1 subtype was isolated from pigs on a 1200-sow, farrow-to-finish farm in Saskatchewan in May 2002 (Karasin et al., 2004). It was isolated and grown in MDCK cells in the presence of 0.5 μg/ml human neutrophil elastase (Serva Electrophoresis GmbH, Heidelberg, Germany). Titer was determined on MDCK cells by a plaque assay, as described previously (Shin et al., 2007). Stock of the virus reached titer of 9.5×10^7 plaque forming units (pfu)/ml after purification.

The virulent PRRSV strain ISU-12-SAH was obtained from ATCC (ATCC VR-2385, Hanassas, VA, USA). Quantitation of PRRSV stock was performed in MARC-145 cells and the titer (1.5×10^6) was calculated and expressed as $TCID_{50}$/ml (Reed and Muench, 1938).

2.4. Virus infection

Six wells of PAMs (10^5 cells/well, one well corresponding to one pig) in a 24-well plate were single-infected or co-infected with SIV and PRRSV at a MOI of 10. Additionally 6 non-infected wells were used as controls. The same six pigs were used for each condition. Virus attachment was allowed for 1 h at 4 °C. Cells were then incubated at 37 °C. One hour after the temperature shift, the cells were washed once with phosphate buffered saline (PBS) and maintained at 37 °C in 1 ml of RPMI 1640 (GIBCO®-BRL) supplemented with 10% FBS and 1% antibiotic/antimycotic (Anti-Anti 100×, GIBCO®-BRL). Eighteen hours after the temperature shift the culture medium was removed, clarified twice by centrifugation ($1000 \times g$), divided into aliquots, and stored at −80 °C. For PCLS single-infections and co-infection, the procedure was identical except that 10^6 pfu of SIV and 10^6 $TCID_{50}$ of PRRSV were used as it is not possible to determine the number of target cells in a slice. Six slices, prepared from the same left lung lobe, were used for each condition. The experiment was repeated four times using 4 different animals.

For superinfections, six wells of PAMs (10^5 cells/well, one well corresponding to one pig) were first infected with SIV (MOI of 10), then superinfected with PRRSV (MOI of 10) 3 h later. In parallel, six wells of PAMs were infected with PRRSV (MOI of 10) and superinfected with SIV (MOI of 10) 3 h after infection with PRRSV. The 3 h delay between infections was selected based on previous studies where interference between related viruses of another family was intensively assessed *in vitro* and *in vivo* (Banfield et al., 2003;

Glazenburg et al., 1994; Meurens et al., 2004a,b; Schynts et al., 2003). Many interference mechanisms take place early in the viral cycle (Meurens et al., 2003). After the first infection, virus attachment was allowed for 1 h at 4 °C. Cells were then further incubated at 37 °C and superinfections were performed 3 h after the temperature shift. One hour after the temperature shift and 1 h after each superinfection cells were washed once with PBS and further incubated at 37 °C in 1 ml of RPMI 1640 (GIBCO®-BRL) supplemented with 10% FBS and 1% antibiotic/antimycotic (Anti-Anti 100×, GIBCO®-BRL). Additionally 6 non-infected wells were used as controls. Fifteen hours post-superinfection, the culture medium was removed, clarified twice by centrifugation ($1000 \times g$), divided into aliquots, and stored at −80 °C. For PCLS the procedure was identical except that 10^6 pfu of SIV and 10^6 $TCID_{50}$ of PRRSV were administered. Six slices, prepared from the same left lung lobe, were used for each condition. The experiment was repeated four times using 4 different animals.

2.5. Immunofluorescence analysis of precision-cut lung slices

Staining was performed after fixation of the slices with 3% paraformaldehyde (Sigma–Aldrich). For permeabilization, cells were treated with 0.2% Triton X-100 (Sigma–Aldrich), and then immunostained with sequential incubations of appropriate antibodies. To identify cells infected by SIV, rabbit polyclonal antibodies (Predicala and Zhou, 2013) recognizing viral nucleoprotein (dilution 1/500) were used followed by an appropriate goat anti-rabbit secondary antibody coupled to Alexafluor594 (dilution 1/400) (Invitrogen, Carlsbad, CA, USA) or Cy2 (dilution 1/400) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). To identify cells infected by PRRSV, a monoclonal antibody-fluorescein conjugate targeting the virus nucleo-capsid protein was used (dilution 1/100) (Rural Technologies Inc., Brookings, SD, USA). Cy3-labeled monoclonal antibody recognizing beta-tubulin (dilution 1/600) (Sigma–Aldrich) was used as ciliated cell marker.

Cell nuclei of prepared slides were stained by incubation with 4′,6′-diamidino-2-phenylindole (DAPI) (Life Technologies Inc., Burlington, ON, Canada). For this purpose, DAPI was added to the slices and removed after incubation for 15 min (37 °C). Then, the cells were washed three times with PBS, and finally embedded in Mowiol 4-88 resin (Sigma–Aldrich) covered by no. 1½ circular micro-cover glass (12 mm) (Electron Microscopy Sciences, Hatfield, PA, USA). Image data were collected using a Leica SP5 laser-scanning microscope (Leica Microsystems Inc., Concord, ON, Canada).

2.6. Validation of reference genes and transcript expression analysis using quantitative real-time polymerizing chain reaction

All the selected transcript sequences were available in genome databases (http://www.ensembl.org/index.html and http://www.ncbi.nlm.nih.gov/nucleotide). Real-time PCR Primers were designed and optimized using Clone Manager 9 (Scientific & Educational Software, Cary, NC, USA) and were purchased from Invitrogen (Carlsbad, CA, USA) (Table 1).

Table 1
Primer abbreviations, full names, sequences, amplicon size (bp), annealing temperature, and accession number or reference.

Primer abbreviation and full name	Primer sequences: sense (S) and anti-sense (AS)	Amplicon size (bp)	Annealing temperature (°C)	Accession number or reference
(1) Viral transcripts				
SIV (M protein)	(S) AGATGAGTCTTCTAACCGAGGTCG (AS) TGCAAAAACATCTTCAAGTCTCTG	100	60	Richt et al. (2004)
PRRSV (Open reading frame 7)	(S) GGCCAGCCAGTCAATC (AS) TCAGTCGCTAGAGGAAAATGG	136	60	Yang et al. (2006)
PRRSV (Nucleoprotein)	(S) TGGTGAATGGCACTGATTGAC (AS) CACACGGTCGCCCTAATTG	63	60	Calzada-Nova et al. (2011)
(2) Reference genes				
ActB (Beta actin)	(S) CACGCCATCCTGCGTCTGGA (AS) AGCACCGTGTTGGCGTAGAG	100	63	Nygard et al. (2007)
B2MI (Beta-2-microgobulin)	(S) CAAGATAGTTAAGTGGGATCGAGAC (AS) TGGTAACATCAATACGATTTCTGA	161	58	Nygard et al. (2007)
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	(S) CTTCACGACCATGGAGAAGG (AS) CCAAGCAGTTGGTGGTACAG	170	63	AF017079
HMBS2 (Hydroxymethylbilane synthase 2)	(S) AGGATGGGCAACTCTACCTG (AS) GATGGTGGCCTGCATAGTCT	83	58	Nygard et al. (2007)
HPRT1 (Hypoxanthine phosphoribosyltr ansferase 1)	(S) GGACTTGAATCATGTTTGTG (AS) CAGATGTTTCCAAACTCAAC	91	60	Nygard et al. (2007)
RPL19 (Ribosomal protein L19)	(S) AACTCCCGTCAGCAGATCC (AS) AGTACCCTTCCGCTTACCG	147	60	Meurens et al. (2009)
TBP1 (TATA box binding protein 1)	(S) AACAGTTCAGTAGTTATGAGCCAGA (AS) AGATGTTCTCAAACGCTTCG	153	60	Nygard et al. (2007)
YWHAZ (Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide)	(S) TGATGATAAGAAAGGGATTGTGG (AS) GTTCAGCAATGGCTTCATCA	203	60	Nygard et al. (2007)
(3) Viral recognition				
DAI/ZBP1 (DNA-dependent activator of interferon-regulatory factors)	(S) CCATGGCTGCCTTCTACCTC (AS) CCGGGAAGCTGTGAAGTCTC	162	62	NM_001123216
LGP2/DHX58 (Laboratory of genetics and physiology 2)	(S) AGAGGGACCAGCAAGAAGTG (AS) ATTGGTCAGGAGCCCATAGC	134	61	NM_001199132
MDA5 (Melanoma differentiation-associated protein 5)	(S) AGCCCACCATCTGATTGGAG (AS) TTCTTCTGCCACCGTGGTAG	133	62	NM_001100194
RIG-I (Retinoic acid-inducible gene 1)	(S) CGACATTGCTCAGTGCAATC (AS) TCAGCGTTAGCAGTCAGAAG	126	60	NM_213804
TLR3 (Toll like receptor 3)	(S) GACCTCCCGGCAAATATAAC (AS) GGGAGACTTTGGCACAATTC	155	60	NM_001097444
TLR7 (Toll like receptor 7)	(S) CGGTGTTTGTGATGACAGAC (AS) AACTCCCACAGAGCCTCTTC	174	61	NM_001097434
TLR8 (Toll like receptor 8)	(S) CACATTTGCCCGGTATCAAG (AS) TGTGTCACTCCTGCTATTCG	145	60	NM_214187
TLR9 (Toll like receptor 9)	(S) GGCCTTCAGCTTCACCTTGG (AS) GGTCAGCGGCACAAACTGAG	151	64	NM_213958
(4) Interferons				
IFNα (Interferon alpha (Type I))	(S) GGCTCTGGTGCATGAGATGC (AS) CAGCCAGGATGGAGTCCTCC	197	62	Sang et al. (2010)
IFNβ (Interferon beta (Type I))	(S) AGTTGCCTGGGACTCCTCAA (AS) CCTCAGGGACCTCAAAGTTCAT	70	60	Razzuoli et al. (2011)
IFNγ (Interferon gamma (Type II))	(S) GCTCTGGGAAACTGAATGAC (AS) TCTCTGGCCTTGGAACATAG	167	60	Meurens et al. (2009)
(5) Interferon-induced genes				
Mx1 (Myxovirus resistance 1)	(S) AGTGTCGGCTGTTTACCAAG (AS) TTCACAAACCCTGGCAACTC	151	60	NM_214061
Mx2 (Myxovirus resistance 2)	(S) CCGACTTCAGTTCAGGATGG (AS) ACAGGAGACGGTCCGTTTAC	156	62	AB258432
OAS1 (2′-5′-Oligoadenylate synthetase 1)	(S) CCCTGTTCGCGTCTCCAAAG (AS) GCGGGCAGGACATCAAACTC	303	64	NM_214303
RNAseL (Ribonuclease L (latent))	(S) AACGTGGTGACGTTCTATGG (AS) ATGTTTCGGGCAGACTCATC	146	60	NM_001097512
PKR (Protein kinase RNA-dependent)	(S) CACATCGGCTTCAGAGTCAG (AS) GGGCGAGGTAAATGTAGGTG	166	61	NM_214319

Table 1 (Continued)

Primer abbreviation and full name	Primer sequences: sense (S) and anti-sense (AS)	Amplicon size (bp)	Annealing temperature (°C)	Accession number or reference
(6) Suppressor of cytokine signaling				
SOCS1 (Suppressor of cytokine signaling 1)	(S) CGCCCTCAGTGTGAAGATGG (AS) GCTCGAAGAGGCAGTCGAAG	110	62	Delgado-Ortega et al. (2011)
(7) Inflammation				
IL1β (Interleukine 1 beta)	(S) AGAAGAGCCCATCGTCCTTG (AS) GAGAGCCTTCAGCTCATGTG	139	62	Meurens et al. (2009)
IL6 (Interleukine 6)	(S) ATCAGGAGACCTGCTTGATG (AS) TGGTGGCTTTGTCTGGATTC	177	60	Meurens et al. (2009)
NLRP3 (NLR family, pyrin domain containing 3)	(S) GCAACCTGGCTGTAACATTC (AS) GATCCAGTTCCACCAACTTC	116	60	JQ219660
(8) Other cytokines				
IL10 (Interleukine 10)	(S) GGTTGCCAAGCCTTGTCAG (AS) AGGCACTCTTCACCTCCTC	202	60	Zanello et al. (2011)
TNFα (Tumor necrosis factor alpha)	(S) CCAATGGCAGAGTGGGTATG (AS) TGAAGAGGACCTGGGAGTAG	116	60	Meurens et al. (2009)
TRAIL (Tumor-necrosis-factor related apoptosis inducing ligand)	(S) AGAGTGGCTGCTCACATAAC (AS) GATAACCAGCTCTCCATTCC	168	60	NM_001024696

Slices and cells were suspended in Trizol reagent (Invitrogen) with ceramic beads (BioSpec Products, OK, USA) and total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Mississauga, ON, Canada). The absence of genomic DNA contamination was verified using prepared RNA as a template for reverse transcription-quantitative real-time PCR (RT-qPCR). RNA concentration was determined by measuring optical density at 260 nm (OD260) and the RNA quality was assessed by calculating OD260/OD280 ratio and by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies Inc., Santa-Clara, USA). cDNA was generated from 100 to 200 ng of RNA per reaction and RT-PCR was performed using the SuperScript™ III Platinum® Two-Step RT-qPCR Kit as per the manufacturer's recommendations (Invitrogen). The generated cDNA was stored at −80 °C. Diluted cDNA (3×) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. The qPCR conditions were: 95 °C for 3 min, 45 cycles each of 15 s at 95 °C (denaturation), 30 s at the appropriate annealing temperature (Table 1) and 30 s at 72 °C (elongation). Real-time assays were run on a Bio-Rad Cycler iQ (Bio-Rad). The specificity of the qPCR reactions was assessed by analysing the melting curves of the products and size verification of the amplicons. Samples were normalized internally using an average Cycle quantification (Cq) of the three most suitable reference genes out of seven in each sample to avoid any variation or artifacts in the target gene. These suitable reference genes were selected amongst beta-actin (ActB), beta-2-microglobulin (B2MI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hydroxymethylbilane synthase 2 (HMBS), hypoxanthine phosphoribosyltransferase-1 (HPRT-1), ribosomal protein L-19 (RPL-19), and TATA box binding protein 1 (TPB-1). The stability of these reference genes was determined using the geNorm application software (Vandesompele et al., 2002). The correlation coefficients of the standard curves were >0.995 and the concentration of the test samples were calculated from the standard curves, according to the formula $y = -M \times Cq + B$, where M is the slope of the curve, Cq the first positive second derivative maximum of amplification curve calculated using PCR Miner (http://www.ewindup.info/miner/version2/data_submit.htm) (Zhao and Fernald, 2005), and B the y-axis intercept. All qPCRs assays, displaying efficiency between 90% and 110%, were performed following MIQE guidelines (Bustin et al., 2009; Taylor et al., 2010). qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002) using the Cq from the samples for the different transcripts.

2.7. Interferon alpha and beta enzyme-linked immunosorbent assays

Pig IFN enzyme-linked immunosorbent assays (ELISAs) were performed with a homemade ELISA using an R&D Systems antibody (Minneapolis, MN, USA) for IFNα and a MyBioSource kit (San Diego, CA, USA) commercial ELISA for IFNβ. For IFNα detection, polystyrene microtiter plates (Immulon 2, Dynex Technology Inc., Chantilly, VA, USA) were coated with the capture antibody mouse anti-recombinant porcine IFNα clone K9 (R&D no. 27100-1) at a concentration of 1 μg/ml in coating buffer. Recombinant porcine IFNα (Endogen, Rockford, IL, USA; rPo IFNα; 2000 pg/ml) was used as standard. Standards and culture supernatants were diluted in tris-buffered saline and Tween 20 (TBST, Sigma–Aldrich)–0.1% skim milk and added to the coated plates. After overnight incubation at 4 °C, biotinylated mouse anti-recombinant porcine IFN-α clone F17 (R&D no. 27105-1; 1/1000) detection antibody was added to the appropriate wells. Finally, the plates were developed, and the responses were measured as previously described (Masic et al., 2009). Sample concentrations were calculated using Softmax Pro 5.2 version software (Molecular Devices, Sunnyvale, CA, USA). IFNβ ELISA was performed according to the supplier's protocol.

2.8. Statistical analysis

Data for the comparison of differences in relative mRNA expression between cells and tissues were expressed as relative values. All statistical analyses were done using computer software Prism 6 for Windows (version 6.02; GraphPad Software, San Diego, CA, USA). One-Way ANOVA was used to detect differences amongst the groups. To account for the non-normal distribution of the data, all data were sorted by rank status prior to ANOVA statistical analysis. Tukey's test was used to compare the means of the ranks among the groups. P values less than 0.05 were considered significant.

3. Results

3.1. Viability of porcine precision-cut lung slices

Around 200 PCLS per lung were generated for each pig, sufficient to perform all of the experiments. In the PCLS, the beating of the ciliated bronchial epithelium was observable 24 h and 96 h after their preparation (Fig. 1A). Additionally, bronchoconstriction could be triggered by the use of methacholine 10^{-4} M in the four days following slice preparation (Fig. 1B) and subsequently reversed by removal of the drug (Fig. 1C). These

observations provide evidence that porcine PCLS remained viable for up to 96 h under the incubation conditions described.

3.2. Different cellular targets for PRRSV and SIV in lung explants

Confocal microscopy was utilized to visualize cells *in situ* upon infection with PRRSV and/or SIV. In all the infected slices, we observed that SIV was restricted to the bronchial epithelial cells (Fig. 2A and C), and no viral nucleoproteins were detected in other parts of the slices – for instance, alveolar epithelial cells and macrophages–. Even if the possible infection of the cells cannot be excluded, it appears to be an infrequent event in the conditions examined here. On the contrary, PRRSV nucleocapsid was only detected in deeper layers of the tissue, below the epithelium and in alveoli (Figs. 2B and C and 3). In the alveolus, the PRRSV staining was associated with cells stretched across a large surface of the alveolus, likely type 1 pneumocytes (Fig. 3). Additionally, the staining was also associated with cells presenting a macrophage-like appearance (Fig. 3). No detection was observed in cells co-infected by PRRSV and SIV in slices (see for instance Figs. 2C and 3), while many single-infected cells were observed throughout the tissue.
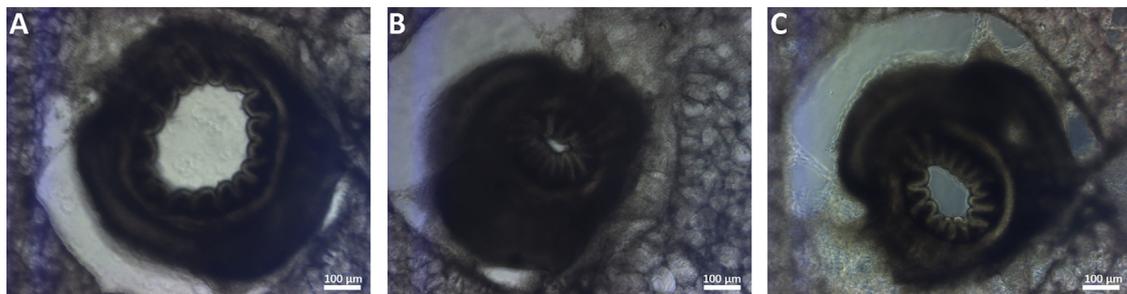

Fig. 1. Viability of PCLS evaluated by bronchoconstriction 96 h after the PCLS preparation. Untreated slice (a) was incubated with 10^{-4} M methacholine (b) to induce bronchoconstriction. Removal of the drug resulted in a reverse effect (c). Viability was tested at 24 h and 96 h after the PCLS preparation. Representative of two independent experiments.
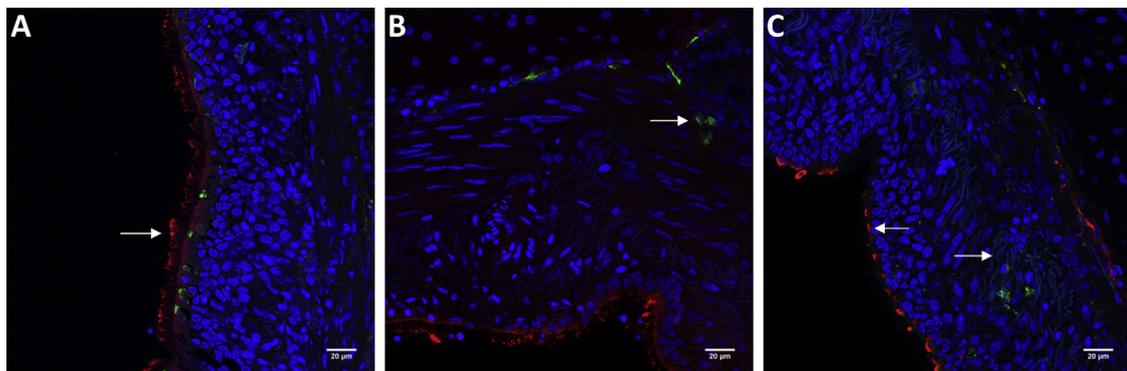

Fig. 2. Infection of PCLS by SIV and PRRSV characterized by immunostaining. PCLS were infected by SIV (A), PRRSV (B) or both viruses (C). Cryosections were prepared after 18 h of infection and image data was collected using a laser-scanning confocal microscope. Infected cells were stained with an anti-nucleoprotein polyclonal antibody (green in A and red in C) for the detection of SIV (green in A and red in C) and with an anti-nucleocapsid monoclonal antibody to detect PRRSV (green in B and C). Ciliated cells were stained using an anti-beta-tubulin monoclonal antibody (red in A and B). White arrows indicated infected cells in each panel, scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
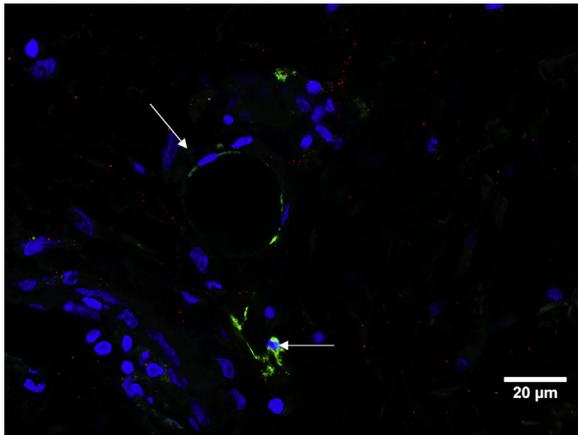
Fig. 3. PRRSV cellular targets in alveolar tissue. PCLS was infected by PRRSV and SIV. Cryosections were prepared after 18 h of infection and used for detection of infected cells in the alveoli. Infected cells were stained with an anti-nucleocapsid monoclonal antibody for the detection of PRRSV (green). Ciliated cells were stained using an anti-beta-tubulin monoclonal antibody (red). Arrows indicate infected cells (presumably type 1 cell and macrophage, horizontal arrow), scale bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Viral transcript expression in the precision-cut lung slices and the alveolar macrophages

Viral transcription was assessed in PCLS and PAMs 18 h post-infection using RT-qPCR assays (Tables 1 and 2 and Fig. 4). The 18 h post-infection was selected based on preliminary experiments showing a good balance between the cell and/or tissue response and the lysis post-viral infection. Furthermore, the amount and quality of the RNA at time intervals past 18 h post-infection were not optimal for subsequent analyses (data not shown).

Regarding PRRSV VR-2385 nucleoprotein transcript expression in the PCLS, no expression was observed in the control slices or the slices only infected with SIV H1N1 (Fig. 4). In most of the conditions where the PRRSV viruses were used, PRRSV transcript expression was detected at similar levels (detection around Cq 22) with no statistically significant differences between conditions (Fig. 4). PRRSV transcript expression was only significantly lower

($P < 0.05$) than in the "PRRSV" condition when slices were superinfected with PRRSV 3 h after SIV infection (SIV-3h-PRRSV) (Fig. 4). Similar observations were made for SIV (M protein transcripts) in PCLS (detection around Cq 20), except for "SIV-3h-PRRSV", where the transcript expression was not significantly lower than in the other conditions where SIV had been used (Fig. 4).

In PAMs, PRRSV transcript expression was also not identified in the control slices or the slices infected only with SIV (Fig. 4). When PRRSV was used to superinfect the cells 3 h after SIV (SIV-3h-PRRSV), the transcript expression was significantly lower ($P < 0.05$) than in the condition where PRRSV was used alone (detection around Cq 13) and the condition "PRRSV-3h-SIV" (Fig. 4). Regarding SIV, transcript expression was only observed in the conditions where the virus was used, as anticipated (Fig. 4) (detection around Cq 18). Conditions "PRRSV + SIV" or "PRRSV-3h-SIV", showed lower expression of the SIV transcripts. The expression of SIV transcripts was significantly lower ($P < 0.05$) in the co-infection conditions than in the condition "SIV-3h-PRRSV" (Fig. 4).

3.4. Host transcript expression in the precision-cut lung slices and the alveolar macrophages

Next, various host genes involved in the response to the viral infections were analyzed (Tables 1 and 2) for alterations in mRNA levels. To study the response of PCLS and PAMs to both viruses using RT-qPCR assays, a selection of seven reference genes was chosen based on previous studies (Delgado-Ortega et al., 2013; Erkens et al., 2006; Nygard et al., 2007). Amongst these genes, we identified HMBS2, HPRT-1, and TBP-1 as the three most suitable genes for transcript normalization for the PCLS. For these three reference genes, the M values (0.211, 0.211, and 0.305, respectively) were below the threshold (M value = 1) defined for stably expressed reference genes in heterogeneous samples (Hellemans et al., 2007). Regarding PAMs, the three most stable reference genes were HPRT-1, RPL-19, and HMBS2 (M values: 0.441, 0.366, and 0.366, respectively) (supplementary Fig. 1). The M values for these genes were below the threshold (M value = 0.5) defined for stably expressed genes in homogeneous sample panels (Hellemans et al., 2007).

Table 2
Viral and host transcript expression summary.

|	PCLS	PAMs
Conditions:		
PRRSV	-*Viral*: Moderate expression (Cq: 22) -*Host*: Low response	-*Viral*: High expression (Cq: 13) -*Host*: Low to moderate response
SIV	-*Viral*: Moderate expression (Cq: 20) -*Host*: Moderate response	-*Viral*: Moderate expression (Cq: 18) -*Host*: Moderate response
PRRSV + SIV	-*Viral*: Similar to single infections -*Host*: Additive effects and synergy	-*Viral*: SIV replication reduced -*Host*: Similar to single infection
PRRSV-3h-SIV	-*Viral*: Similar to single infections -*Host*: Decreased response	-*Viral*: Similar to single infections *Host*: Similar to single infections or slightly increased (IFNα)
SIV-3h-PRRSV	-*Viral*: PRRSV replication reduced -*Host*: Similar to single infections	-*Viral*: PRRSV replication reduced -*Host*: Similar to single infections
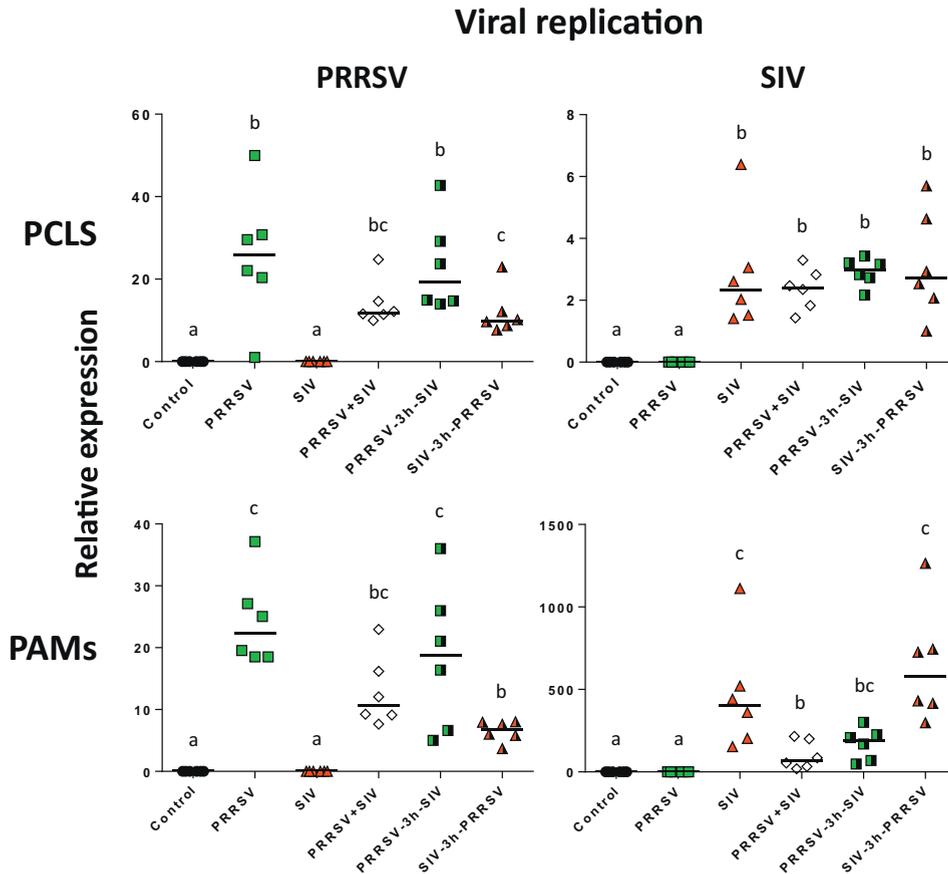
Fig. 4. Viral replication – relative expression of viral transcripts (nucleoprotein-PRRSV and M protein-SIV) after 18 h of infection of PCLS and PAMs. For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common superscripts are significantly different ($P < 0.05$).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.11.037.

3.4.1. Transcripts involved in viral recognition

Regarding transcripts involved in viral recognition (Tables 1 and 2), several observations were made in experiments where PCLS (Fig. 5A) and PAMs (Fig. 5B) were used. None of the pathogen recognition receptor (PRR) transcripts was significantly over-expressed in response to PRRSV in the PCLS although one of them (TLR9) had a small, but not statistically significant, increase (Fig. 5A). On the contrary, SIV significantly ($P < 0.05$) induced the expression of DAI, LGP2, MDA5, RIG-I, and TLR3 genes (Fig. 5A). These transcripts were generally expressed at a similar level to when PRRSV was superinfecting the PCLS 3 h after SIV (SIV-3h-PRRSV) (Fig. 5A). However, when PRRSV was administered to the slices 3 h prior to SIV (PRRSV-3h-SIV), a lower expression than in the co-infection condition "PRRSV + SIV" was observed for most of the transcripts, with the exception of TLR8 and TLR9. This lower induction was statistically significant for MDA5, RIG-I, and TLR3 transcripts in some conditions (see Fig. 5A). Except for TLR3 and RIG-I, the induction of transcript expression was not statistically higher in the

situation of co-infection compared to single infections (Fig. 5A). In PAMs, PRRSV did not significantly induce the expression of viral recognition transcripts, similarly to PCLS (Fig. 5B). However, for DAI, LGP2, MDA5, RIG-I, and TLR7 there was a marked increase in the expression of the transcripts in response to PRRSV when compared to controls (Fig. 5B). Similarly to what was observed in PCLS, the expression of transcripts for DAI, LGP2, MDA5, and RIG-I was significantly higher in the SIV infected cells ($P < 0.05$) than in the controls (Fig. 5B). Regarding TLR3, TLR8, and TLR9 transcripts, no significant difference was observed between control and SIV conditions (Fig. 5B), although expression of TLR3 transcripts seemed higher with SIV than PRRSV. However, this observation was not confirmed by statistical analysis. Furthermore, no statistically significant differences were identified between the various conditions for TLR transcripts, with the exception of TLR7 transcripts (Fig. 5B). Moreover, "SIV" condition was not significantly different amongst the co-infection and superinfection conditions for all the transcripts (Fig. 5B).

3.4.2. Interferon transcripts

Next, the expressions of interferon transcripts were analyzed using the same viral infection combinations
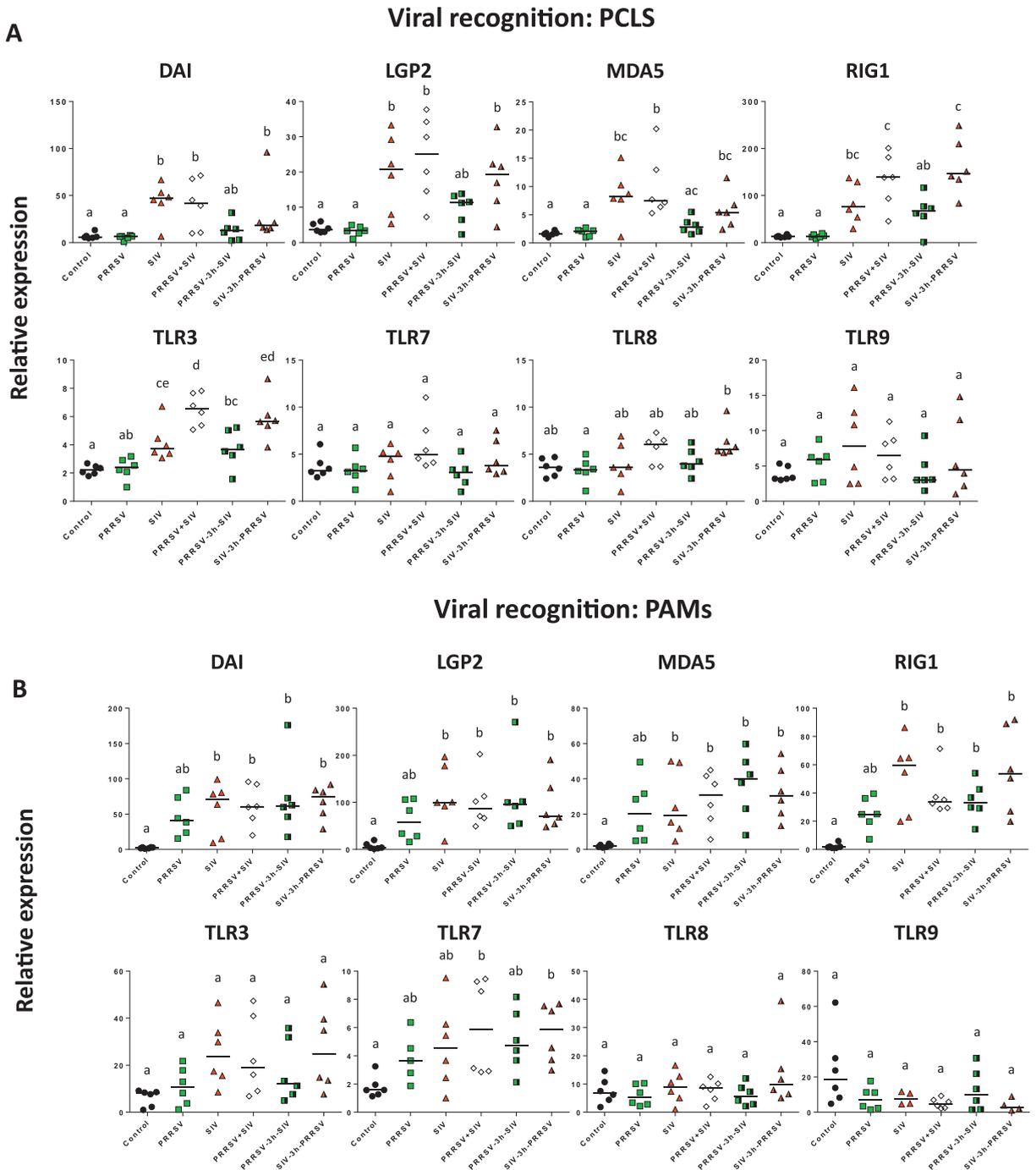
Viral recognition: PCLS

Viral recognition: PAMs

Fig. 5. Viral recognition – relative expression of viral recognition transcripts after 18 h of infection of PCLS (A) and PAMs (B). For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common superscripts are significantly different ($P < 0.05$).

discussed above in PCLS and PAMs (Fig. 6 and Table 2). In PCLS, no statistically significant differences between conditions were reported for IFNα mRNA (Fig. 6). IFNβ transcripts were expressed at higher levels in response to SIV alone or in association with PRRSV (Fig. 6) ($P < 0.05$). When SIV was co-administered with PRRSV, the expres-

sion of the transcript was significantly higher than when SIV and PRRSV were administered alone (Fig. 6) ($P < 0.05$). The median of relative expression in the "PRRSV + SIV" condition is higher than the sum of the median of relative expression of "PRRSV" and "SIV" conditions. For IFNγ, only the conditions "PRRSV + SIV" and "SIV-3h-PRRSV" showed
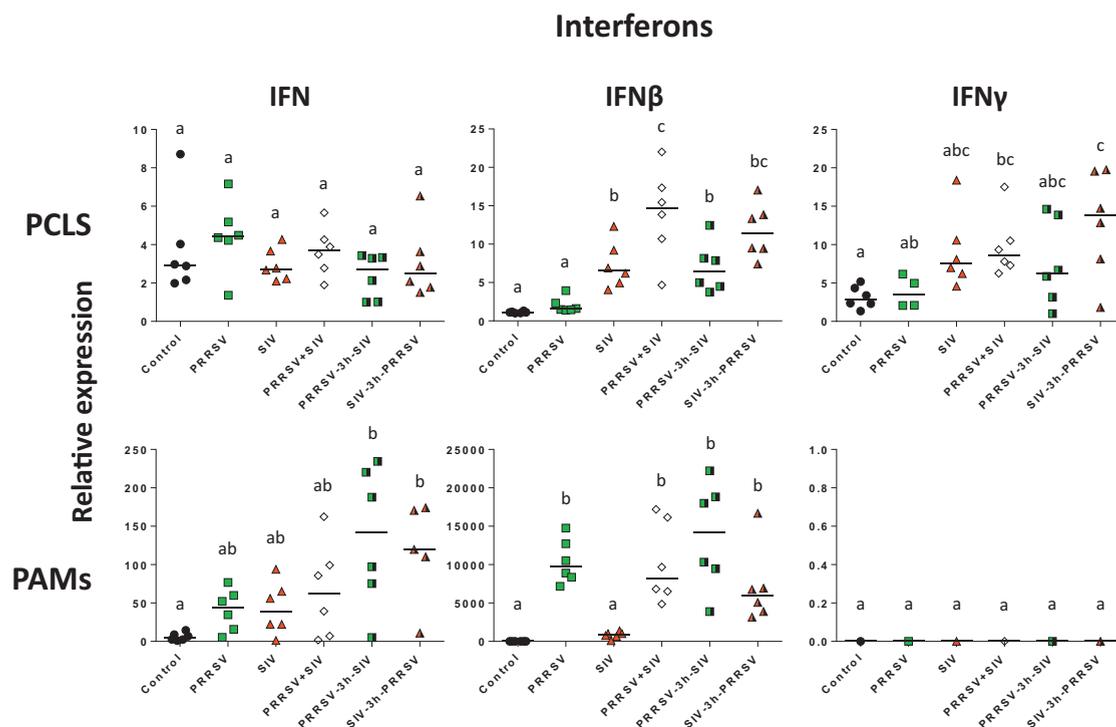
Interferons

Fig. 6. Interferons – relative expression of interferon transcripts after 18 h of infection of PCLS and PAMs. For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common superscripts are significantly different ($P < 0.05$).

significantly higher expression of the transcripts than in the "control" and "PRRSV" conditions (Fig. 6). In PAMs, a significant increase of IFNα transcript expression compared to the control was only observed in superinfection conditions (Fig. 6). For IFNβ the transcript expression was higher than in the control in all the conditions where the PRRSV was administered (Fig. 6). No expression of IFNγ transcripts was observed in any conditions (Fig. 6).

3.4.3. Antiviral transcripts in response to interferons

In response to the IFNs, various antiviral transcripts may be expressed (Tables 1 and 2). In PCLS, PRRSV did not induce any significant increase in the expression of the transcripts under study, while SIV was able to significantly induce ($P < 0.05$) their expression (Fig. 7). Similarly, when co-infected (PRRSV + SIV), the expressions of antiviral transcripts were significantly higher ($P < 0.05$) than in the "control" conditions (Fig. 7). In the superinfection condition, "PRRSV-3h-SIV", the expression of the transcripts was slightly higher for some (Mx2 and PKR) but not all (Mx1 and OAS1), relative to the control (Fig. 7). When PRRSV was administered 3 h after SIV (SIV-3h-PRRSV), the expressions of transcripts were similar to when both viruses were administered together or when the SIV was administered alone (Fig. 7). In the PAMs, PRRSV was unable to induce a statistically significant stimulation in the expression of the various transcripts, despite the subtle increase observed (Fig. 7). In nearly all the conditions where SIV was administered, a higher expression of the transcripts ($P < 0.05$) was observed (Fig. 7). However, as

previously observed (Figs. 5A and 6), the increase was less important and sometimes not statistically significant comparatively to the control when the PRRSV was administered 3 h before SIV (Fig. 7).

3.4.4. Cytokines and related transcripts

Regarding major inflammatory cytokines involved in innate immune response such as IL1β and TNFα, regulatory cytokine IL10, and the inflammasome-associated PRR, NLRP3, no significant differences were observed between the different infection combinations in PCLS (supplementary Fig. 2 and supplementary Fig. 3). Again, PRRSV administration did not alter the expression of any transcripts in the PCLS comparative to control, despite a minimal trend toward an increase for IL10 and TNFα (supplementary Fig. 3). IL6, SOCS1 and TRAIL transcripts were significantly ($P < 0.05$) induced in response to SIV administration in the PCLS but were not remarkable when PRRSV was administered 3 h before SIV (supplementary Fig. 2 and supplementary Fig. 3). IL1β transcripts were not detected in PAMs in any conditions under study (supplementary Fig. 2). In PAMs, most of the transcripts (IL6, NLRP3, SOCS1 and TRAIL) had little impact upon PRRSV infection (supplementary Fig. 2 and supplementary Fig. 3), while for IL10 and TNFα there was a significant increase ($P < 0.05$) in response to the virus (supplementary Fig. 3). SIV alone induced marked expression of SOCS1 and TRAIL transcripts only in PAMs ($P < 0.05$) (supplementary Figs. 2 and 3). In the co-infection and superinfection conditions, IL6, SOCS1, TNFα, and TRAIL transcripts were substantially
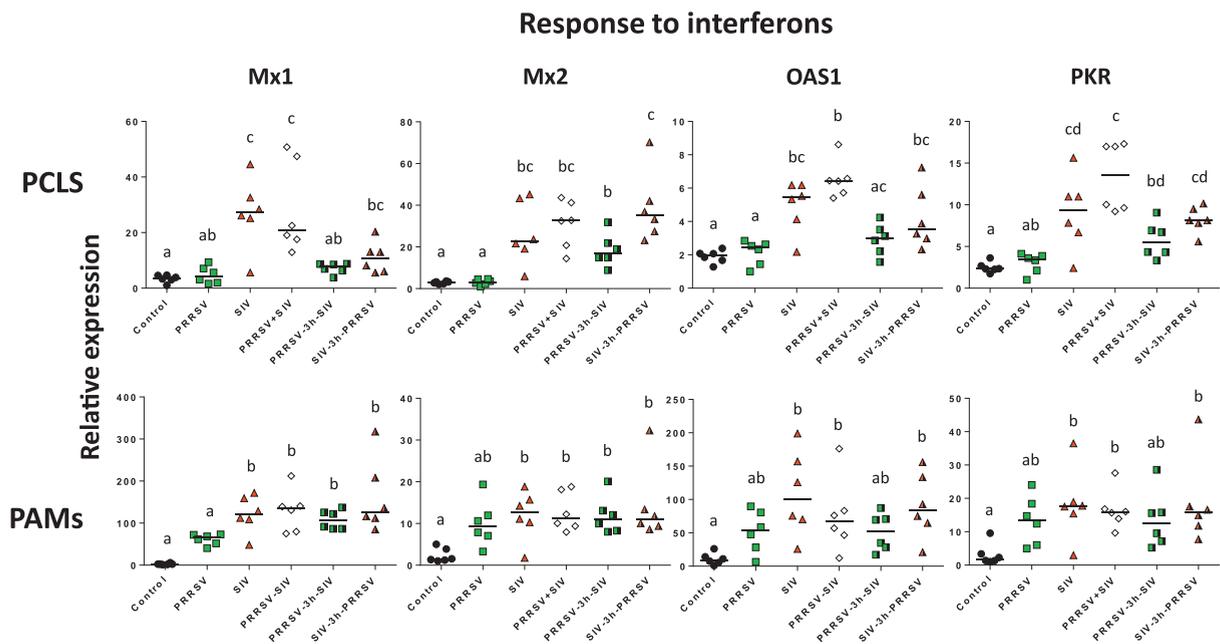
Response to interferons

Fig. 7. Response to interferons – relative expression of interferon induced gene transcripts after 18 h of infection of PCLS and PAMs. For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common superscripts are significantly different ($P < 0.05$).

increased ($P < 0.05$) *versus* control in PAMs (supplementary Figs. 2 and 3).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.11.037.

3.5. IFNα and IFNβ quantification

Protein expression of IFNα and IFNβ was assessed by ELISA using the supernatants collected from the same experimental conditions as those for RT-qPCR analysis. No protein was detected 18 h post-infection in all conditions examined (control, single infected, co-infected, and superinfected) (data not shown).

4. Discussion

Among viruses contributing to porcine lung infectious diseases, PRRSV and SIV, alone or in combination, are the two main known contributors (Choi et al., 2003; Fablet et al., 2012a, 2011; Opriessnig et al., 2011). Studies assessing the impact of co-infections with these two viruses have not been frequent since the 1990s (Pol et al., 1997; Van Reeth et al., 1996, 2001). Moreover, none of these studies specifically addressed the question of the impact of polymicrobial infections at the molecular level. In the current report, the impact of PRRSV/SIV co-infection *versus* single infections on the immune response of PAMs and PCLS with respect to alterations in viral replication and associated host gene transcripts was assessed.

In a study by Van Reeth and collaborators, results suggested that the SIV replication was only slightly affected by the prior infection with the Lelystad virus

strain of PRRSV (Van Reeth et al., 1996). In that experiment, the pigs were inoculated first by aerosol with PRRSV and three days later with SIV (Van Reeth et al., 1996). Viral excretion in the PRRSV-SIV group was delayed by two days, not only with regard to the presence of virus, but also with respect to the peak amount (Van Reeth et al., 1996). In our PCLS experiment, SIV transcript expression was not altered in the condition where SIV was administered to slices 3 h after PRRSV. The timing, the experimental settings (*in vivo versus ex vivo*), and the different genotypes of the viral strains used could account for this small difference in the results obtained. Similarly, superinfection with PRRSV after SIV infection did not impact the SIV replication. On the contrary, PRRSV superinfection 3 h after SIV infection significantly reduced the replication of the PRRSV. Because no other studies have looked at the impact of PRRSV superinfection on SIV replication, it is difficult to make comparisons. The reduction in the PRRSV replication, however, has to be interpreted carefully since a lower number of PRSSV transcripts detected could also be a consequence of the shorter replication time allowed for the superinfecting virus (15 h *versus* 18 h). Interestingly, when the two viruses were co-administered, there was a noticeable decrease in PRRSV replication. However, it was not statistically significant. The results obtained in PAMs were noticeably different than in the PCLS. SIV replication was reduced when the PRRSV was co-administered with SIV ($P < 0.05$), or prior to SIV (however, $P > 0.05$). These results corroborate those reported by Van Reeth et al. (1996). Regarding PRRSV replication, again the pre-infection of the PAMs with SIV was accompanied by a significant decrease in PRRSV replication ($P < 0.05$). Similarly, the replication of PRRSV seemed also decreased

when PAMs were co-infected with PRRSV and SIV ($P > 0.05$). Taken together, our data suggest a slightly negative impact of the first virus on the replication of the second virus regardless the order of viral infection especially in PAMs. As both viruses are RNA viruses using the same cellular machinery, especially in the PAMs where infections were performed at high MOI, an interference of one virus cycle on the cycle of the second is not surprising. Penetration kinetics of both viruses could also account for the observed differences as previously described in another family of respiratory viruses (Meurens et al., 2004a).

To analyze the transcript expression in PCLS and PAMs (Table 2) three stable reference genes were used for each system. As previously observed in lung tissue (Delgado-Ortega et al., 2011), HPRT-1, along with RPL-19, were two of the three most stable reference genes in both PCLS and PAMs. The third gene, HMBS2, was not tested in the study mentioned above, but was chosen based on rank from the reference gene stability analysis. In the PCLS, PRRSV had very little impact on the transcriptional expression of viral recognition, interferon, interferon-induced, and cytokine genes, while in the PAMs, we observed a statistically significant increase in transcript expression of IFNβ, IL10, and TNFα transcripts in response to the viral infection.

Regarding the PRR transcripts (DAI, LGP2, MDA5, RIG-I, and TLR7) in PAMs, PRRSV showed a positive trend toward an increase in their expression; however it was not statistically significant, possibly due to the small number of samples examined. One hypothesis to explain the complete absence of transcript over-expression in response to the PRRSV in the PCLS is the relatively small percentage of macrophages in the slices and their cellular heterogeneity in comparison to a pure population such as PAMs. In the literature, PRRSV was associated with an increase of most, if not all, viral-sensing TLRs in the lungs, including TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 (Liu et al., 2009; Xiao et al., 2010a,b). In our study, after 18 h of infection, an increase in expression was demonstrated for TLR7. Notably, the relative expression of TLR3 transcripts was particularly high in "PRRSV/SIV" condition in PCLS. For TLR8 and TLR9, there were no statistically significant alterations in transcriptional expression in all infection conditions. In agreement with previous reports (Xiao et al., 2010a,b), our data suggest a significant stimulation of RIG-I and MDA5 after infection of lungs with another type 2 PRRSV. Additionally, after PRRSV infection, we observed an increase in the mRNA expression of two known detectors of virally derived RNA and DNA, DAI and LGP2 transcripts (Pichlmair and Reis e Sousa, 2007; Sang et al., 2011). Contrary to PRRSV, SIV significantly induced the up-regulation of the expression of all the selected transcripts (i.e. DAI, LGP2, MDA5, and RIG-I) and TLR3 transcripts in the PCLS ($P < 0.05$). In the PAMs, increases in the expression of viral recognition transcripts were statistically significant (DAI, LGP2, MDA5, and RIG-I) or showed a slight but not significant increase (TLR3 and TLR7). These results are in agreement with previous studies in pigs (Husser et al., 2011; Li et al., 2011) and other species (Wu et al., 2011). Conversely, NLRP3, a detector of SIV replication (Wu et al., 2011), was not induced but rather

slightly repressed in our conditions. This is possibly due to the chosen time point after viral infection. In co-infection and superinfection conditions, relative expression of the viral recognition transcripts was not generally statistically different from relative expression of the same transcripts in response to SIV infection alone. However, in the case of TLR3 and RIG-I transcripts in the PCLS, they were clearly additive and even displayed synergistic effects of the two viruses with possible contribution of TLR7 transcripts to the additive effects (Fig. 5A). When a 3 h delay was introduced between the administration of the two viruses, observations were similar, with the exception of "PRRSV-3h-SIV" in PCLS, where expression of the transcripts was generally lower. Again, the 15 h incubation time versus 18 h could account for the observed difference even if we cannot exclude a PRRSV-specific effect.

IFNα transcript expression was very similar in all PCLS, while some significant expression differences were observed in the PAMs especially in superinfection conditions. The higher expression of IFNα transcripts in "PRRSV-3h-SIV and SIV-3h-PRRSV" conditions could be a consequence of both additive effects and viral kinetics. Indeed, based on other findings in our laboratory (unpublished data) and another study in pig PAMs (Genini et al., 2008), it was shown that IFNα mRNA is usually detected earlier than IFNβ mRNA after SIV infection. In the PCLS, IFNβ transcripts were mostly produced in response to SIV virus with a synergy in their expression when SIV was co-administered with PRRSV. The same trend was observed in response to PRRSV infection in PAMs. This observation is a bit surprising because it has been shown that PRRSV actively suppresses IFNβ in macrophages, at least in MARC-145 cells and some human cells (Miller et al., 2004; Sang et al., 2011). However, in a study using porcine PAMs (Genini et al., 2008), the authors observed a strong up-regulation of IFNβ transcripts 12 h post-infection, in support of our data. Regarding IFNγ, transcript expression was only observed in the PCLS with a significant increase in co-infection and superinfection conditions, suggesting again synergistic effects of the two infections. At the protein level, no interferons (α and β) was detected at 18 h post-infection, suggesting either very low expression under the limits of detection of the kit or some post-transcriptional regulatory mechanisms preventing protein expression as previously described (Lee et al., 2004; Wang and Christopher-Hennings, 2012). However it seems, to some level, sufficient amounts of interferons were produced; at least in response to SIV, as interferon response genes (IRGs) were induced in PCLS and PAMs. Generally, interferons need to be produced in order to trigger the induction of IRGs, although a direct induction of IRGs such as OAS1 and protein kinase R by viral nucleic acids has been reported (Player and Torrence, 1998; Williams, 2001).

Amongst the cytokines tested in response to single, co- and superinfections, IL6, IL10, TNFα and TRAIL were frequently induced. IL6 mRNA was up-regulated in response to SIV but not to PRRSV in PCLS. In PAMs, the up-regulation was more significant in co-infection and superinfection conditions, TNFα mRNA in particular, in agreement with previous studies (Choi et al., 2002; Gao

et al., 2012; Van Reeth et al., 2002). TRAIL, which is produced by alveolar macrophages and contributes to epithelial cell apoptosis (Herold et al., 2008; Wu et al., 2011), showed the most significant induction by SIV in both PCLS and PAMs. IL10 transcript expression was significantly up-regulated in PAMs in the conditions examined here, similar to previous observations (Suradhat et al., 2003; Thanawongnuwech and Suradhat, 2010). SOCS1, a regulator of immune response (Delgado-Ortega et al., 2013), was clearly up-regulated in response to SIV in both PCLS and PAMs while there was only a slight increase in response to PRRSV, contrary to what was observed in other studies (Wysocki et al., 2012; Zhou et al., 2011). This discrepancy could be explained by some differences in experimental conditions (infection time, heterogeneous mixed versus single cell population). Moreover, while the role of SOCS1 in the host response to influenza virus has been clearly demonstrated in other species (Pothlichet et al., 2008), its role in PRRSV pathogenesis is still unclear.

Confocal microscopy was utilized to visualize infected cells in situ upon infection with PRRSV and/or SIV. Upon co-infection of PCLS, we did not detect any co-infected cells, confirming the importance of bronchial epithelial cells and alveolar macrophages as the main target of SIV and PRRSV in lung, respectively (Crisci et al., 2013; Meulenberg, 2000; Sang et al., 2011; Taubenberger and Morens, 2008). However, our study also confirms that some alveolar epithelial cells such as pneumocyte type 1 can be infected by PRRSV virus as previously reported (Sur et al., 1996). Many different cell types are present in the PCLS, e.g. macrophages, epithelial cells, pneumocytes type 1 and 2, endothelial cells, fibroblast, and dendritic cells. This cellular diversity could account for some of the differences observed in our study between PAMs and PCLS. Moreover, many PAMs are probably removed from the PCLS because of the multiples washes performed during their preparation while PIMs and especially ISMs are more intimately associated to the tissue.

Altogether, the results of our study (Table 2) show that co-infection with PRRSV VR-2385 and SIV (A/Sw/Saskatchewan/18789/02) demonstrate additive effects on the expression of several types of virally induced transcripts. Moreover, a synergy was observed for some specific targets such as TLR3, RIG-I, and IFNβ transcripts in the PCLS when the two viruses were administered concomitantly. The impact of such a synergy on the clinical outcome is difficult to establish as it can either increase symptoms and be detrimental for the host or, on the contrary, assist in the rapid clearance of the infections. The lower host response to superinfecting virus after an initial infection with PRRSV, if confirmed, could contribute to the development of more severe forms of SIV infection and needs further study to accurately determine how PRRSV modulates the immune response to superinfecting SIV. On the virus side, the absence of synergy between the two viruses for their replication is already beneficial for the host. The delay introduced between the two infections had most generally limited impact and we only observed a decrease in the tissue response when the PRRSV was administered first. However, the cause of that decrease is not clear and remains to be further investigated. The limited impact of superinfection delay may be due to the differences in cellular targeting between the two viruses. In conclusion, the current study opened the door to further research in the exciting and intriguing field of viral co-infection and superinfection research.

Acknowledgements

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