1 **Simultaneous infection with porcine reproductive and respiratory**
2 **syndrome and influenza viruses abrogates clinical protection**
3 **induced by live attenuated porcine reproductive and respiratory**
4 **syndrome vaccination**

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19 **Keywords:** Porcine reproductive and respiratory syndrome virus, swine influenza A
20 virus, live attenuated vaccine, pig, co-infection.

21

22 **Abstract**

23 The porcine respiratory disease complex (PRDC) is responsible for significant
 24 economic losses in the pig industry worldwide. Porcine reproductive and respiratory
 25 syndrome virus (PRRSV) and swine influenza virus are major viral contributors to
 26 PRDC. Vaccines are cost-effective measures for controlling PRRS, however, their
 27 efficacy in the context of co-infections has been poorly investigated. In this study,
 28 we aimed to determine the effect of PRRSV-2 and swine influenza H3N2 virus co-
 29 infection on the efficacy of PRRSV modified live virus (MLV) vaccination, which is
 30 widely used in the field. Following simultaneous challenge with contemporary
 31 PRRSV-2 and H3N2 field isolates, we found that the protective effect of PRRS
 32 MLV vaccination on clinical disease and pathology was abrogated, although viral
 33 load was unaffected and antibody responses were enhanced. In contrast, co-infection
 34 in non-immunized animals reduced PRRSV-2 viremia and H3N2 virus load in the
 35 upper respiratory tract and potentiated T cell responses against both PRRSV-2 and
 36 H3N2 in the lung. Further analysis suggested that an upregulation of inhibitory
 37 cytokines gene expression in the lungs of vaccinated pigs may have influenced
 38 responses to H3N2 and PRRSV-2. These findings provide important insights into
 39 the effect of viral co-infections on PRRS vaccine efficacy that may help identify
 40 more effective vaccination strategies against PRDC in the field.

41

42

43 Introduction

44 Porcine reproductive and respiratory syndrome (PRRS) is a viral disease responsible
 45 for major economic losses in the global pig industry (1). The disease can be
 46 subclinical depending on the strain (2,3), however typical clinical signs are
 47 reproductive failure in sows, respiratory distress and reduction of growth
 48 performance in weaned and growing pigs (4). Mortality can be observed in infected
 49 piglets, with rates ranging from 7.5-18.5 % (5) and in some cases up to 75.5 % (6).
 50 The etiologic agents are PRRS viruses (PRRSV), single stranded positive RNA
 51 viruses from the *Arteriviridae* family (7). The first clinical description of PRRS
 52 dates to the late 1980's, with genetically distinct PRRSV isolates described in
 53 Europe and North America, which are now recognized as two separate species
 54 PRRSV-1 (*Betaarterivirus suid 1*) and -2 (*Betaarterivirus suid 2*), respectively (7).
 55 Both species have since spread globally, but PRRSV-1 remains predominant in
 56 Europe, while PRRSV-2 predominates in the Americas and Asia. The rapid
 57 evolution of PRRSV due to a high mutation rate when replicating its genome, and
 58 recombination between strains have resulted in substantial genetic diversity, which
 59 poses challenges for the control of PRRS by vaccination (8).

60 Vaccination is widely practiced as one of measures used to prevent and
 61 control PRRS. Commercially available vaccines include inactivated and live
 62 attenuated (also known as modified live virus; MLV) vaccines for both PRRSV-1
 63 and -2 (9). Although inactivated vaccines are considered safer, MLVs are
 64 preferentially used for their higher protective efficacy (8,10). Studies have
 65 demonstrated an induction of a high level of PRRSV-specific antibodies which is
 66 associated with clinical protection against challenge infection with related PRRSV
 67 strains (9), but rather weak T cell responses after MLV immunization (11). Passive
 68 transfer of purified IgG from PRRS-convalescent pigs have suggested that vaccine-
 69 induced protection against reproductive failure and vertical transmission *in utero* is
 70 also mediated by antibodies (12,13). Since PRRS MLV vaccine-induced antibodies
 71 often lack strong virus neutralizing properties *in vitro* (14), non-neutralizing
 72 antibody functions may also contribute but these remain poorly defined (15).

73 Multiple infections of pigs tend to occur naturally in the field (reviewed in
74 (16)). Indeed, respiratory diseases in pigs in the field are often multifactorial,
75 involving mixed infections with different viral and/or bacterial pathogens, defined as
76 the porcine respiratory disease complex (PRDC). PRRSV and swine influenza A
77 virus (swIAV), an enveloped single stranded segmented negative RNA virus within
78 the *Orthomyxoviridae* family (7), are important contributors to the PRDC. Primary
79 infection with PRRSV and swIAV leads to pneumonia caused by opportunistic
80 pathogens, such as *Pasteurella multocida*, *Mycoplasma hyopneumoniae*,
81 *Actinobacillus pleuropneumoniae* and *Bordetella bronchiseptica* (16). Previous *in*
82 *vivo* PRRSV/swIAV co-infection or superinfections studies demonstrated a
83 potentiation of disease compared to single infection (17,18). It was recently shown
84 that concomitant swIAV infection modulated the immune response to PRRS MLV
85 vaccination, albeit without impacting efficacy (19), but it remains unknown whether
86 such viral co-infection interferes with protection conferred by PRRS vaccination.

87 In the present study, we examined the efficacy of PRRS MLV vaccination to
88 PRRSV-2/H3N2 co-infection. We evaluated clinical signs, viral load, PRRSV-2-
89 specific antibody and T cell responses in PRRS MLV-vaccinated pigs challenged
90 simultaneously with contemporary field-isolated PRRSV-2 and swIAV H3N2
91 strains. We report here that PRRSV-2/H3N2 co-infection abrogated the protective
92 effect of PRRS MLV vaccine on lung pathology, although it did not alter viral load.
93 Moreover, PRRS MLV reversed the beneficial effect of PRRSV-2/H3N2 co-
94 infection in decreasing H3N2 lung viral loads, highlighting a potential interference
95 of PRRS MLV vaccination on the subsequent host immune response against H3N2.

96

97 **Materials and methods**

98 **Cell lines**

99 Madin–Darby canine kidney (MDCK) cells were cultured in Eagle's minimum
100 essential medium (MEM, Merck, Feltham, UK) supplemented with 10 % heat-
101 inactivated fetal bovine serum (HI FBS, Thermo Fisher Scientific, Loughborough,
102 UK) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Thermo
103 Fisher) at 37 °C in a humidified 5 % CO₂. African green monkey kidney (MARC-
104 145) cells were cultured in Dulbecco's modified MEM (DMEM, Merck)
105 supplemented with 10 % HI FBS and antibiotics at 37 °C in a humidified 5 % CO₂
106 atmosphere.

107 **Vaccine and virus strains**

108 PRRSV-2 16CB02 was isolated from pig serum collected from a diseased farm in
109 Chonburi, Thailand in 2016. The serum was adsorbed onto MARC-145 cells and
110 cultured in OptiMEM (Thermo Fisher Scientific) supplemented with 10 % FBS.
111 After 72 h, cytopathic effect (CPE) was observed, and the supernatant was collected.
112 Cells were then infected with a diluted culture supernatant before being overlayed
113 with MEM supplemented with 0.3 % bovine serum albumin (BSA, Merck), 0.22 %
114 sodium bicarbonate (Merck), 1 % penicillin/streptomycin (Merck) and 1 %
115 methylcellulose (Merck). The PRRSV-2 16CB02 isolate (referred to as PRRSV-2)
116 was derived from a single plaque-derived culture, which resulted in obvious CPE
117 after 96 h incubation. Partial genome (ORFs 2-7) sequencing was performed
118 (GenBank, accession number MZ700336) and the ORF5 sequence was compared
119 with sequences in the NCBI database using BLAST showed that 16CB02 belonged
120 to lineage 8.7 of PRRSV-2 (*Betaarterivirus suid 2*). PRRSV-2 16CB02 was
121 propagated in MARC-145 cells by infection with a multiplicity of infection (MOI)
122 of 0.08 for 1 h at 37 °C in 5 % CO₂. After removing the inoculum cells were
123 incubated for 3 days in DMEM with 10 % FBS and antibiotics at 37 °C in 5 % CO₂.
124 Supernatant was harvested, centrifuged at 500 × g for 10 min and stored at -80 °C
125 until use. The viral stock was titrated and expressed as TCID₅₀/mL.

126 SwIAV H3N2 CM5 strain was isolated from a pig farm in the Province of
127 Lumpoon, Thailand in 2018. Nasal swab samples were first screened for IAV by

one-step RT-PCR. Viral RNA was extracted from samples with the GenUP™ Virus RNA kit (biotech rabbit, Henningsdorf, Germany). One-step RT-PCR was conducted using matrix (M) segment specific primers (20) and performed on a T100™ Thermal Cycler (Bio-Rad) utilizing the PrimeScript™ One-Step RT-PCR kit (Takara Bio, Shiga, Japan). PCR products were then separated and visualized by agarose gel electrophoresis. The RT-PCR-positive nasal swab samples were then subjected to IAV isolation using MDCK cells. CPE was observed daily, and CPE positive supernatants collected and confirmed by HA test and RT-PCR for M gene detection as previously described (21). Sequencing of H3N2 RNA segments were performed and alignment of HA (GenBank, accession number MZ665044) and NA (GenBank, accession number MZ665046) sequences against sequences in the NCBI database revealed that CM5 strain was a H3N2 subtype. H3N2 CM5 (referred to as H3N2) was propagated by infecting MDCK cells at MOI 0.001 in serum-free MEM and incubated at 37 °C in 5 % CO₂ for 1 h. After washing the cell monolayer, cells were incubated in serum-free MEM supplemented with 2 µg/mL TPCK-treated trypsin (Merck) for 2 days at 37 °C in 5 % CO₂. Supernatant was harvested, centrifuged at 880 × g for 10 min and stored at -80 °C until use. The viral stock was titrated by plaque assay and expressed as pfu/mL.

PRRS MLV (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany) was used following reconstitution according to manufacturer's instructions.

Immunization and challenge study

The animal experiment was approved by the Animal Welfare and Ethical Review Body at The Pirbright Institute, UK. The treatment, housing, husbandry, and procedures were performed in accordance with the UK Animal (Scientific Procedures) Act 1986 (Project Licence P6F09D691). Thirty-six, 5-7 weeks-old, Large White-Landrace-Hampshire crossbred female pigs were sourced from a high health status commercial herd and were housed in a high biocontainment facility at The Pirbright Institute. Animals were tested for the absence of exposure to IAV and PRRSV prior to their arrival by serological tests via hemagglutination inhibition test against four standard IAV antigens (pdmH1N1, H1N2, H3N2 and avian-like H1N1

159 strains), and antibody (Ab) ELISA and RT-PCR tests against PRRSV (Animal and
160 Plant Health Agency, Weybridge, UK). Pigs were randomly assigned to 6 groups of
161 6 pigs each, which were untreated (naïve), immunized with 2 mL containing $10^{4.5}$
162 TCID₅₀ of Ingelvac® PRRS MLV (Vac) or with 2 mL of PBS (Ctrl) by
163 intramuscular (i.m.) injection (**Figure 1A**). On day 33 post-vaccination (dpv), Ctrl
164 and Vac pigs were inoculated intranasally with 4 mL (2 mL/nostril) containing $5 \times$
165 10^6 pfu of swIAV H3N2 CM5 (Ctrl + H3N2), 10^5 TCID₅₀ PRRSV-2 16CB02 (Ctrl +
166 PRRSV-2 or Vac + PRRSV-2), or concurrently with 5×10^6 pfu swIAV and 10^5
167 TCID₅₀ PRRSV-2 (Ctrl + PRRSV-2/H3N2 or Vac + PRRSV-2/swIAV) diluted in
168 DMEM using a mucosal atomization device (MAD 300, Wolfe Tory Medical, Salt
169 Lake City, USA). To analyze the immune response, pigs from Ctrl + PRRSV-2, Vac
170 + PRRSV-2 and Vac + PRRSV-2/H3N2 groups were bled at 0 dpv, then pigs from
171 all groups at 20 and 38 dpv for peripheral blood mononuclear cell (PBMC) and
172 serum isolation. After the challenge, pigs were observed twice per day until the end
173 of the study for monitoring and scoring of clinical signs (**Supplementary Table**
174 **S1**). Rectal temperatures were taken on -1, 0, 1, 2, 3 and 4 days post-challenge
175 (dpc). Nasal swabs were collected daily for viral detection. At 5 dpc, the animals
176 were humanely euthanized with an overdose of pentobarbital sodium anesthetic.

177 **Pathological and histopathological examination of lungs**

178 Lungs were removed post-mortem and tissue samples were taken from cranial,
179 cardiac, and diaphragmatic lobes of the left lung, and immersed in 10 % neutral
180 buffered formalin for fixation and histological processing. Formalin fixed tissues
181 were paraffin wax-embedded and 4µm sections cut and stained with hematoxylin
182 and eosin (H&E). Immunohistochemical staining of IAV nucleoprotein (NP) and
183 PRRSV nucleoprotein (N) protein was performed in 4 µm tissue sections as
184 previously described (22,23). Histopathological changes in stained lung tissue
185 sections were scored by a board-certified veterinary pathologist blinded to the
186 treatment group. Lung histopathology was scored (“Morgan score”) using five
187 parameters (necrosis of the bronchiolar epithelium, airway inflammation,
188 perivascular/bronchiolar cuffing, alveolar exudates, and septal inflammation) scored
189 on a 5-point scale of 0 to 4 and then summed to give a total slide score ranging from
190 0 to 20 and a total lung score from 0 to 60 (24). Two additional scoring systems

191 were used to analyze the contribution of IAV (“Iowa”) and PRRSV-2 (“Salguero”)
 192 in the lung lesions. Abundance of viral antigen was assessed using the influenza NP
 193 staining (HB-65 mAb, Bio X Cell, Lebanon, USA) and was scored as described
 194 previously (25). Similarly, PRRSV N protein staining was performed (SDOW17-A
 195 mAb, Rural Technologies, Brookings, USA) and the scoring method was adapted
 196 from the Iowa IAV scoring method as described above.

197 **Sample collection and cell isolation**

198 Blood samples were collected at 0, 20 and 38 dpv using BD Vacutainer™ SST™ II
 199 Advance Tubes and BD Vacutainer Heparin Blood Collection Tubes (both Fisher
 200 Scientific). Serum tubes were centrifuged at $880 \times g$ for 10 min and the resulting
 201 serum was aliquoted and stored at -80°C . Heparinized blood diluted 1:1 in PBS
 202 overlaid onto Lymphopure density gradient medium 1.077 g/mL (BioLegend, San
 203 Diego, USA) in Leucosep tubes (Greiner Bio-One, Gloucestershire, UK) and
 204 centrifuged at $800 \times g$ for 15 min. PBMC were harvested from the interface and
 205 washed with PBS. Erythrocytes were removed with RBC Lysis Buffer (BioLegend).
 206 The cell suspension was washed again, filtered through a $100 \mu\text{m}$ cell strainer and
 207 cryopreserved in freezing medium (HI FBS with 10 % DMSO). Bronchoalveolar
 208 lavage (BAL) was performed on the isolated right lung with 300 mL of PBS. Cells
 209 were isolated by centrifugation of the BAL fluid (BALF) at $490 \times g$ for 5 min,
 210 filtered through $100 \mu\text{m}$ cell strainer, and cryopreserved in freezing medium. The
 211 BALF supernatant was aliquoted, and frozen at -80°C for virus titration. For gene
 212 expression analysis, pieces of cranial lung lobe tissues were submerged into
 213 RNeasy™ Stabilization Solution (Thermo Fisher Scientific), placed at room
 214 temperature for 6 h and then stored at -80°C until use. Sampling of nasal secretions
 215 was performed at 0, 1, 2, 3, 4 and 5 dpc using cotton swabs (one in each nostril,
 216 Scientific Laboratory Supplies, Nottingham, UK). Swabs were then placed into virus
 217 transport medium and processed as described previously (26).

218 **RNA extraction from fluids and tissues**

219 Total RNA was extracted from nasal swabs, BALF and serum using QIAamp Viral
 220 RNA kit (QIAGEN, Manchester, UK) according to the manufacturer’s instructions.

221 Piece of lungs (200-300 mg) were weighed and homogenized using gentleMACS
222 Octo dissociator (Miltenyi Biotec) in RPMI 1640 medium in M tubes (Miltenyi
223 Biotec, Woking, UK). After centrifugation at $880 \times g$ for 5 min, supernatants were
224 collected. Total RNA was extracted with RNAeasy kit (QIAGEN) according to the
225 manufacturer's instructions. Extracted RNA were stored at -80°C until use.

226 **Virus titration by plaque assay**

227 SwIAV titers in nasal swabs and BALF were determined by plaque assay as
228 previously described (22). MDCK cells were inoculated with 10-fold serially diluted
229 samples in MEM. After 1 h incubation at 37°C , cells were washed and incubated
230 for further 72 h at 37°C in 5 % CO_2 under an overlay medium consisting of MEM
231 with 0.21 % BSA, 2 mM L-glutamine, 0.15 % sodium bicarbonate, 10 mM HEPES
232 (Merck), 1 % penicillin/streptomycin, 0.01 % Dextran DEAE (Merck), 0.6 % agar
233 (Merck) and 2 $\mu\text{g/mL}$ TPCK trypsin (Merck). The pfu were counted following
234 staining with 1 % (w/v) crystal violet (Merck).

235 **Viral RNA quantification by qRT-PCR**

236 PRRSV-2 RNA quantification in nasal swabs, BALF and serum was performed by
237 quantitative reverse-transcriptase PCR (qRT-PCR) using the one-step Quantinova™
238 Probe RT-PCR kit (QIAGEN). Primers and a TaqMan probe were designed to
239 hybridize to a sequence in ORF7 (encoding N), and sequences are shown in **Table 1**.
240 To determine the viral RNA copies/mL in a sample, an RNA standard was
241 generated. PRRSV-2 RNA was used to amplify cDNA encoding the full-length of N
242 gene by RT-PCR (One-step RT-PCR, QIAGEN) using specific primers with the
243 forward containing a T7 promoter sequence at the 5' end. The PCR product was gel
244 purified (Illustra™ GFX PCR DNA and Gel Band Purification Kit, Merck) and used
245 as template for *in vitro* transcription using the MEGAscript T7 Transcription Kit
246 (Thermo Fisher Scientific), according to the manufacturer's protocol. After DNase
247 treatment, the concentration of RNA was measured using a Nanodrop
248 spectrophotometer (Thermo Fisher Scientific), and the number of RNA
249 molecules/ μL was calculated using Avogadro's number (6.023×10^{23}). The qRT-
250 PCR was performed with 5 μL of the eluted samples and 15 μL of the master mix.

251 Samples and standards were run in duplicate under the following conditions on a
252 Stratagene Mx3500P cycler (Agilent): reverse transcription at 45 °C for 20 min,
253 denaturation at 95 °C for 5 min, 35 amplification cycles of denaturation at 95 °C for
254 5 s and combined annealing/extension at 60 °C for 30 s. The viral genome copy
255 numbers were determined by interpolation of the standard curve and limit of
256 quantification was estimated at 60 copies/mL.

257 **Gene expression by RT-PCR**

258 Total RNA samples were treated with DNA-free Kit (Thermo Fisher Scientific).
259 Absence of residual DNA was verified by using RNA samples as a template for
260 SYBR Green PCR. Relative mRNA expression was evaluated by RT-PCR using the
261 QuantiNova SYBR Green RT-PCR Kit (QIAGEN). Primers of selected cytokines
262 and chemokines used in previous studies (27,28) are listed in **Table 1**. RT-PCR
263 assays were validated and displayed an efficiency between 80 % and 110 %.
264 Reactions utilized 5 µL containing 100 ng of RNA sample with 15 µL of the master
265 mix. Samples were run in duplicate under the following conditions on a Stratagene
266 Mx3500P cycler (Agilent): reverse transcription at 50 °C for 30 min, denaturation at
267 95 °C for 5 min, 40 amplification cycles of denaturation at 95 °C for 10 s and
268 appropriate combined annealing/extension temperature shown in Table 1 for 60 s.
269 Melting curves were generated after each run to confirm the RT-PCR assay
270 specificity. Fold changes in gene expression were calculated using the delta-Cq
271 method with multiple housekeeping genes (29). The housekeeping genes *RPS24*
272 (ribosomal protein S24) and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase)
273 were found to be stably expressed in pig lung cells (27,30) and were used as
274 reference genes to normalize the data. Samples from naïve group were used as
275 calibrators.

276 **Intracellular cytokine staining**

277 Cryopreserved PBMC and BAL cells were thawed, washed, and resuspended in
278 RPMI 1640 medium with 10 % HI FBS and antibiotics. To assess the intracellular
279 cytokine production, 2×10^6 cells/well were seeded in a 96-well-round bottom tissue
280 culture plate with complete RPMI 1640 medium. After 5 h of resting at 37 °C, cells

281 were re-stimulated with H3N2 CM5 (MOI 0.1) and PRRSV-2 16CB02 (MOI 0.1)
 282 for 18 h at 37 °C. Cells cultured in complete RPMI 1640 medium only served as
 283 negative controls. BD GolgiPlug at 1:1,000 (BD Biosciences, Wokingham, UK) was
 284 added into the well for a further 4 h before staining. Cells stimulated with a cocktail
 285 of PMA (phorbol 12-myristate 13-acetate) (20 ng; Merck), ionomycin (500 ng;
 286 Merck) and BD GolgiPlug at 1:1,000 for 4 h was used as positive a control. Cells
 287 were washed and stained with 50 µL of surface marker antibodies listed
 288 in **Supplementary Table S2**, and Near-IR Fixable LIVE/DEAD stain (Thermo
 289 Fisher Scientific) diluted in PBS with 2 % FBS for 20 min at 4 °C. After two
 290 washing steps, the cells were fixed and permeabilized (BD Cytofix/Cytoperm kit;
 291 BD Biosciences) for 30 min at 4 °C. Intracellular cytokine staining (ICS) was
 292 performed according to the manufacturer's directions using 50 µL of cytokine mAbs
 293 listed in **Supplementary Table S2** diluted in Perm/Wash Buffer (BD Biosciences)
 294 for 30 min at 4 °C. Subsequently, cells were washed in Perm/Wash Buffer and fixed
 295 with 2 % PFA in PBS (Santa Cruz Biotechnology, Heidelberg, Germany). Cells
 296 were analyzed with a BD LSRFortessa Flow Cytometer (BD Biosciences). Analysis
 297 was performed with FlowJo version 10.6.2 (FlowJo, LLC). Compensation was set
 298 according to single color staining controls. Isotype controls and fluorescence minus
 299 one (FMO) controls were used to validate the staining and to set the gates.

300 **Evaluation of anti-PRRSV antibody responses by ELISA and virus** 301 **neutralization assay**

302 To detect anti-PRRSV IgG, HI sera were tested using PrioCHECK PRRSV
 303 Antibody ELISA Kit (Thermo Fisher Scientific) for detection of PRRSV N protein-
 304 specific antibodies, according to the manufacturer's instructions. Optical density
 305 (OD) was measured at 450 nm using a microplate reader (Synergy™ HT Multi-
 306 Detection Microplate Reader, BioTek Instruments, USA). Percentage positivity (PP)
 307 was calculated using following formula: $PP = (OD \text{ of sample} - OD \text{ of negative control}) / (OD \text{ of positive control} - OD \text{ of negative control}) \times 100$. The cut-off was
 308 determined according to the supplier's protocol i.e., samples above 30 PP were
 309 considered as positive. To measure anti-PRRSV IgG titers in serum, an ELISA using
 311 PRRSV-2 infected cell lysate as antigen was used. Antigen was generated as
 312 described elsewhere (31). Briefly, lysate was obtained by sonicating PRRSV-2

16CB02 infected MARC-145 cell pellets in lysis buffer (1 % Triton X-100, 50 mM borate, 150 mM NaCl, pH 9). The supernatant was clarified and stored at -80 °C. High-binding 96-well plates (Nunc Maxisorp™, Thermo Fisher Scientific) were coated overnight at 4 °C with 100 µL of lysate diluted at 1:40 in carbonate buffer (Merck). After a blocking step with PBS with 4 % milk for 1 h, serial 2-fold dilutions of HI serum were added in duplicates, starting from 1:40 and incubated for 1 h at room temperature (RT). Bound PRRSV-2-specific IgG was detected using goat anti-pig IgG conjugated with horseradish peroxidase (HRP; Bio-Rad) diluted at 1:10,000 in blocking buffer for 1 h at RT. HRP enzymatic activity was revealed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Thermo Fisher Scientific) for 5 min and was stopped by adding 1.2 M sulfuric acid. Antibody endpoint titers were determined as the highest dilution giving twice the OD of the negative control wells (lysate coated well only). PRRSV neutralizing Ab titers were assessed using an adapted protocol previously described (32). Briefly, serial 2-fold dilutions of HI serum incubated with 400 TCID₅₀ of PRRSV-2 for 1 h at 37 °C were added to MARC-145 cell monolayers. After 3 days incubation at 37 °C, cells were fixed and permeabilized (2 % PFA 0.1 % Triton X-100 in PBS) for 10 min at RT and blocked with 10 % goat serum in PBS. Cells were stained using an anti-PRRSV N mAb (SDOW17-A, Rural Technologies) diluted 1:1,600, followed by a secondary goat anti-mouse IgG conjugated to HRP (Bio-Rad) diluted 1:1,000. PRRSV-2 positive cells were revealed using 3, 3'-diaminobenzidine substrate (DAB, Vector Laboratories, Burlingame, USA) for 10 min. Neutralizing Ab titers were calculated as the reciprocal serum dilution that neutralized viral infection in 100 % of the wells.

Statistical analysis

Data were analyzed with GraphPad Prism 8.0.1 software. As distribution was not normal (Anderson-Darling test), the non-parametric unpaired Kruskal-Wallis test followed by Dunn's correction was applied for multiple comparison (lung lesions scores, viral load, proportion of immune cell subsets and genes expression). The unpaired non-parametric Mann-Whitney test was used to compare data between 2 groups. The matched paired non-parametric Wilcoxon test was used to compare the T cell and antibody responses at day 0 and different timepoints within the same group.

345 Results

346 PRRSV-2/H3N2 co-infection abrogates PRRS MLV-induced clinical protection

347 To investigate whether concurrent infection with H3N2 and PRRSV-2 can interfere
 348 with the efficacy of PRRS MLV vaccination, groups of 6 pigs were immunized with
 349 a commercial PRRS MLV (Vac) or mock vaccinated with PBS (Ctrl) by
 350 intramuscular injection (**Figure 1A**). Animals were challenged at 33 day post-
 351 vaccination (dpv) with 5×10^6 pfu of a swIAV H3N2 field isolate (Ctrl + H3N2),
 352 10^5 TCID₅₀ of a PRRSV-2 field isolate (Ctrl + PRRSV-2 and Vac + PRRSV-2) or
 353 both viruses (Ctrl + PRRSV-2/H3N2 and Vac + PRRSV-2/H3N2) by intranasal
 354 inoculation. Pigs were culled 5 days post-challenge (38 dpv). A group of 6 pigs
 355 remained untreated throughout the study and served as naïve controls. Clinical signs
 356 and rectal temperature were monitored daily post-challenge (**Supplementary**
 357 **Tables S1 and S3**). In the unvaccinated and H3N2 infected group (Ctrl + H3N2),
 358 1/6 pigs had an elevated temperature (≥ 40 °C) after the challenge, 2/6 pigs in the
 359 unvaccinated and PRRSV-2 infected group (Ctrl + PRRSV-2) and 1/6 pigs in the
 360 unvaccinated and co-infected group (Ctrl + PRRSV-2/H3N2) (**Figure 1B**). No other
 361 apparent clinical signs were observed after challenge (data not shown), which
 362 indicated that both field strain viruses induced a mild disease during this stage of
 363 infection, and that co-infection did not enhance clinical disease. None of the
 364 vaccinated pigs infected with PRRSV-2 (Vac + PRRSV-2) showed hyperthermia but
 365 4/6 of the pigs vaccinated and co-infected with PRRSV-2/H3N2 (Vac + PRRSV-
 366 2/H3N2) developed fever (≥ 40 °C) which lasted for 3 consecutive days in one pig
 367 (**Supplementary Table S3**).

368

369 Following the experimental infection, microscopic examination of the lungs
 370 collected at 5 dpc revealed mild to moderate bronchointerstitial pneumonia in H3N2
 371 (Ctrl + H3N2 or Ctrl + PRRSV-2/H3N2) or PRRSV-2 infected groups (Ctrl +
 372 PRRSV-2 or Vac + PRRSV-2/H3N2) in comparison to the naïve controls or
 373 vaccinated group and infected with PRRSV-2 (Vac + PRRSV-2) (**Supplementary**
 374 **Figures S1**). Lesions were characterized by bronchial epithelial necrosis, an
 375 expansion and thickening of alveolar septa, and lymphocyte cuffing and histiocytic
 376 cellular infiltration in the peribronchial and perivascular space as previously

described after H3N2 (33,34) and PRRSV (23,35) infection. Immunohistochemistry analysis indicated that IAV NP was observed in the relevant group, i.e., mainly observed in cells within the epithelium of bronchi and bronchioles in Ctrl + H3N2, Ctrl + PRRSV-2/H3N2 and Vac + PRRSV-2/H3N2 groups, and not in naïve and Ctrl + PRRSV-2 and Vac + PRRSV-2 groups (**Supplementary Figures S2A**). Similarly, PRRSV N was localized, as expected, in alveolar and interstitial macrophages in the Ctrl + PRRSV-2, Ctrl + PRRSV-2/H3N2, Vac + PRRSV-2 and Vac + PRRSV-2/H3N2 groups (**Supplementary Figures S2B**).

The severity of lung lesions was compared between unvaccinated groups after a single and co-infection using three different scoring systems, with similar results (**Figures 1C-E**). Single infection of unvaccinated pigs with H3N2 or PRRSV-2 induced lung lesions (mean scores of 5.6 and 9.6, respectively) in comparison to naïve pigs (mean scores of 1), although significant differences in lung lesions were found only after PRRSV-2 infection ($p < 0.05$ vs naïve). Simultaneous PRRSV-2/H3N2 infection did not significantly increase the lung lesions in the unvaccinated group (mean score of 7) compared to single virus infection groups. To assess whether PRRS vaccine conferred protection, lung lesion scores were compared between Ctrl + PRRSV-2 and Vac + PRRSV-2 groups. A significant reduction of the score was observed in Vac + PRRSV-2 (mean score of 3.5) in comparison to Ctrl + PRRSV-2 group (mean score 9.6; $p < 0.05$ all scoring systems). However, PRRSV-2/H3N2 co-infection abrogated the protective effect of the PRRS MLV since the Vac + PRRSV-2/H3N2 animals exhibited high lung lesion scores in comparison to Vac + PRRSV-2 animals (mean score 13.2 versus 3.5, respectively) ($p < 0.05$, all scoring systems).

Collectively, these results indicate that co-infection with H3N2 abrogates the PRRS MLV mediated protective effect, by enhancing lung lesions and clinical disease.

Assessment of PRRSV-2 and H3N2 viral loads

To assess whether PRRSV-2/H3N2 co-infection affects the ability of PRRS MLV vaccine to reduce virus loads, PRRSV-2 RNA was quantified in nasal swabs, BALF

409 and serum (**Figure 2**). Shedding of PRRSV-2 was observed in nasal swabs from 3
410 to 5 dpc in all PRRSV-2 challenged groups and not in the samples from naïve or Ctrl
411 + H3N2 group (**Figure 2A**). At these early timepoints post-infection, we found great
412 variability within group. PRRSV-2 genome was detected in nasal swabs in 4/6 pigs
413 from Ctrl + PRRSV, in 1/6 pigs from Ctrl + PRRSV-2/H3N2, in 5/6 pigs from Vac
414 + PRRSV and in 1/6 pigs from Vac + PRRSV-2/H3N2. These data indicate that
415 PRRS MLV did not reduce viral shedding. Of note, PRRSV-2 RNA was detected in
416 only 1/6 in co-infected groups (Ctrl or Vac), suggesting that co-infection may reduce
417 PRRSV-2 viral shedding.

418

419 In the lower respiratory tract, high levels of PRRSV-2 RNA were measured
420 in BALF after single PRRSV-2 infection in both Ctrl + PRRSV-2 or Vac + PRRSV-
421 2 groups and not in naïve and Ctrl + H3N2 groups (**Figure 2B**). A reduction
422 (average 4-fold) of the PRRSV-2 RNA load was observed in the Vac + PRRSV-2
423 group compared to Ctrl + PRRSV-2 group although the difference was not
424 significant, indicating that intramuscularly administered PRRS MLV may help to
425 limit the viral replication in the lung at 5 dpc. Interestingly, in both vaccinated and
426 unvaccinated groups, there was a trend towards lower PRRSV-2 RNA loads in
427 BALF following the co-infection compared to the single infection. These results
428 indicate that co-infection seems to be efficient to reduce viral load (**Figure 2B**). In
429 the serum, PRRSV-2 RNA was also detected at 5 dpc in Ctrl or Vac + PRRSV-2
430 groups and not in naïve and Ctrl + H3N2 groups (**Figure 2C**). However, levels of
431 PRRSV-2 RNA were significantly reduced in Vac + PRRSV-2 compared to Ctrl +
432 PRRSV-2 (mean 4-fold, $p < 0.05$) indicating that the vaccine conferred a degree of
433 protection. A significant reduction of PRRSV-2 viral RNA was also measured in
434 Ctrl + PRRSV-2/H3N2 group compared to Ctrl + PRRSV-2 (mean 19-fold; $p < 0.05$).
435 The co-infection also slightly reduced PRRSV-2 RNA in Vac + PRRSV-2/swIAV
436 group as compared to the Vac + PRRSV-2 group, although the difference was not
437 statistically significant (mean 5-fold; $p = 0.06$).

438 H3N2 load was also assessed in nasal swabs and BALF. Virus titers were
439 detectable in nasal swabs of groups challenged with H3N2 (alone or with PRRSV-2)
440 and not in naïve and PRRSV-2 only infected animals (**Figure 2D**). After a single

infection with H3N2 (Ctrl + H3N2), a peak of the virus shedding was reached at 2 dpc, followed by a plateau until 5 dpc. After PRRSV-2/H3N2 co-infection, the peak of nasal shedding was also reached at 2 dpc in the Ctrl + PRRSV-2/H3N2 group, whereas this was seen earlier at 1 dpc in Vac + PRRSV-2/H3N2 group. A significant reduction of H3N2 was measured in the Ctrl + PRRSV-2/H3N2 at 5 dpc (mean 50-fold; $p < 0.01$), however, this reduction disappeared in animals vaccinated with PRRS MLV (Vac + PRRSV-2/H3N2 group) which exhibited similar pattern of H3N2 shedding to Ctrl + H3N2.

In BALF, H3N2 was detected at low levels after the single (Ctrl + H3N2 – mean of 47 pfu/mL) or after co-infection (Ctrl + PRRSV-2/H3N2 – mean of 2 pfu/mL) although these differences were not significant (**Figure 2E**). After co-infection, PRRS MLV vaccination markedly increased the H3N2 titers in the Vac + PRRSV-2/H3N2 group compared to the non-immunized co-infected group (mean 69 and 2 pfu/mL respectively, $p = 0.05$).

Together, these data indicated that PRRS MLV immunization reduced PRRSV-2 viraemia but did not reduce PRRSV-2 replication in the respiratory tract after challenge. PRRSV-2/H3N2 co-infection did not alter the effect of PRRS MLV vaccination on PRRSV-2 load. However, in unimmunized animals, co-infection had a beneficial effect by reducing PRRSV-2 viraemia and H3N2 shedding, with a trend for lower PRRSV-2 and H3N2 loads in the BALF. However, the reduction of H3N2 viral load was reversed in PRRS MLV immunized animals.

PRRSV-2-specific antibody responses

We next determined whether PRRSV-2/H3N2 co-infection modulates the PRRS MLV induced Ab responses. Ab in serum were evaluated using a commercial PRRSV N-specific ELISA at 0 dpv, 20 dpv and 5 dpc (38 dpv) (**Figure 3A**). Sera from immunized pigs at 20 dpv were above the cut-off and displayed significant levels of PRRSV N-specific Abs ($p < 0.05$; 0 vs 20 dpv) indicating a seroconversion following PRRS MLV immunization. There was no significant difference in the level of PRRSV N-specific Abs between the Vac groups at 20 dpv. However, after

the challenge, an increase of PRRSV N-specific Abs was detected in the Vac + PRRSV-2/H3N2 group ($p < 0.05$; 20 vs 38 dpv) but not in the Vac + PRRSV-2 group. The levels of PRRSV N-specific antibodies were comparable between Vac + PRRSV-2 and Vac + PRRSV-2/H3N2 groups at 5 dpc ($p = 0.13$).

Serum Ab titers were also measured by an in-house ELISA using lysate from PRRSV-2 16CB02 infected cells as antigens (**Figure 3B**). Consistent with the results found with the N-based ELISA test, significant anti-PRRSV-2 IgG titers were measured after immunization ($p < 0.05$; 0 vs 20 dpv) which were similar in both Vac + PRRSV-2 and Vac + PRRSV-2/H3N2 groups ($p = 0.13$; 20 dpv). However, after challenge, the Vac + PRRSV-2/H3N2 group had significantly higher Ab titers compared to the Vac + PRRSV-2 group (mean Ab titer: 40,647 vs 17,126, respectively; $p < 0.01$), suggesting that the co-infection with H3N2 enhanced the recall of B cell responses primed by PRRS MLV immunization. Remarkably, none of the sera collected at 20 and 38 dpv were able to neutralize the PRRSV-2 16CB02 challenge strain (**Figure 3C**). The lack of neutralization might be explained by differences between the vaccine and challenge strains (**Supplementary Table S4**).

These data indicated that PRRS MLV vaccination induced a significant PRRSV-2-specific Ab response, which was enhanced after PRRSV-2/H3N2 co-infection. In contrast, the PRRSV-2 only challenge did not significantly alter the magnitude of the specific antibody response (**Figure 3C**).

PRRSV-2-specific T cell responses in PBMC

In swine, conventional $CD4^+$ T cells are defined as $CD3^+CD4^+CD8\alpha^{+/-}CD8\beta^-$ and $CD8^+$ T cells as $CD3^+CD4^-CD8\beta^+$ (36,37). PRRSV-2 specific $CD4^+$ and $CD8^+$ T cell responses were assessed by intracellular staining of IFN- γ , TNF, IL-2, IL-4, and IL-17 after *in vitro* restimulation of PBMC isolated at 0, 20 and 38 dpv (5 dpc) with PRRSV-2 (**Supplementary Figures S3 and S4**). Overall, the frequencies of cytokine-producing cells were low in T cell populations after PRRS MLV immunization, as demonstrated previously (11) (**Figure 4**). Similar results were obtained after the challenge with either PRRSV-2 alone or together with H3N2,

although a substantial increase in IFN- γ expressing CD8⁺ T cells was detected after in the Vac + PRRSV-2/H3N2 group but not the Vac + PRRSV-2 group (mean 0.11 % and 0.06 %, respectively) (**Figure 4B**). Similarly, an increased proportion of PRRSV-2-specific TNF expressing CD8⁺ T cell population was detected in these two groups (mean 0.24 % and 0.35 %), but this was not boosted after challenge. In addition, no IL-2, IL-4 and IL-17 expressing CD4⁺ and IL-2 expressing CD8⁺ T cells were detected after challenge (**Figure 4** and **supplementary Figure S5**).

Overall, PRRS MLV immunization induced TNF and IFN- γ expressing CD8⁺ T cells in PBMC, which were not boosted by the subsequent PRRSV-2 or PRRSV-2/H3N2 challenge. PRRSV-2-specific CD4⁺ T cell responses were weaker compared to CD8⁺ T cells and there was no significance between the groups for any of the measured cytokines.

PRRSV-2- and H3N2- specific T cell responses in BALF

Local T cell responses in the lung likely to play a role in controlling both IAV (38–40) and PRRSV-2 infections and pathology (11,41). We therefore assessed the $\alpha\beta$ and $\gamma\delta$ T cell responses against both PRRSV-2 and H3N2 in BALF at 5 dpc. Cells were restimulated with PRRSV-2 or H3N2, and cytokine production was assessed by ICS. Staining of IFN- γ , TNF, IL-2 were performed for CD4⁺ and CD8⁺ $\alpha\beta$ T cells, and staining of IFN- γ , TNF, IL-17 for $\gamma\delta$ T cells (**Supplementary Figure S6**), which were divided into CD2⁺ and CD2⁻ subsets (42). CD4⁺ and CD8⁺ T cell responses to PRRSV-2 overall were very low after single infection with PRRSV-2 in either Ctrl or Vac groups (**Figure 5A**). However, T cell responses to PRRSV-2 stimulation were higher after PRRSV-2/H3N2 co-infection in both Ctrl and Vac pigs. The highest proportion of IL-2⁺ CD4⁺ (mean 0.14 %) and IFN- γ ⁺ CD8⁺ T cells (mean 0.11 %) was observed in Ctrl + PRRSV-2/H3N2 group. In the Vac group, PRRSV-2/H3N2 co-infection induced a proportion of IFN- γ ⁺ CD8⁺ T cells (mean 0.23 %) and a significantly greater proportion of TNF⁺ CD8⁺ T cells compared to Vac + PRRSV-2 (mean 0.35 %; $p < 0.5$). Similarly, co-infection induced the highest proportion of IFN- γ ⁺ CD2⁺ $\gamma\delta$ T cells in the Vac group (mean 0.22 %), and IL-17⁺

531 CD2⁺ γδ T cells in the Ctrl group (mean 0.30 %) (**Figure 5B**). Higher frequencies of
532 TNF⁺ and IL-17⁺ CD2⁻ γδ T cells were detected in the Ctrl + PRRSV-2/H3N2
533 compared to Ctrl + PRRSV-2 group (mean 0.73 % versus 0.31 % and mean 0.40 %
534 versus 0.23 %, respectively) though this was not statistically significant. In
535 vaccinated pigs, co-infection increased the frequency of TNF⁺ (mean 0.47 %) and
536 IL-17⁺ (mean 0.51 %) CD2⁻ γδ T cells, although these differences did not reach
537 statistical significance.

538 Stimulation of BALC with H3N2 also showed the greatest frequencies of
539 cytokine producing T cells in co-infected animals (**Figure 6A**). The proportion of
540 IL-2 expressing CD4⁺ T cells in the Ctrl + PRRSV-2/H3N2 group was higher
541 compared to Ctrl + H3N2 (mean 0.23 % versus 0.05 %). Similarly, the frequency of
542 IFN-γ⁺ CD8⁺ T cells in the Ctrl + PRRSV-2/H3N2 group was higher compared to
543 the Ctrl + H3N2 (mean 0.35 % versus 0.07 %), but none of these differences reached
544 statistical significance. A greater frequency of TNF⁺ CD2⁺ γδ T cells was again
545 measured in the Ctrl + PRRSV-2/H3N2 group compared to Ctrl + H3N2 group
546 (mean 0.29 % versus 0.01 %) (**Figure 6B**). Similar results were obtained for TNF⁺
547 and IL-17⁺ CD2⁻ γδ T cells in the Ctrl + PRRSV-2/H3N2 group compared to the Ctrl
548 + H3N2 group (mean 1.18 % versus 0.23 % and 1.02 % versus 0.28 %, respectively). Intriguingly, lower H3N2-specific cytokine producing T cell
549 responses were induced by the co-infection in PRRS MLV vaccinated group in
550 comparison to the unvaccinated group although no statistically significant
551 differences were observed (**Figures 6A and 6B**).

553 Overall, these data indicate that PRRSV-2/H3N2 co-infection induces the
554 highest frequency of cytokine producing T cells in response to both PRRSV-2 and
555 H3N2 in BAL, although these did not reach statistical significance, most likely due
556 to the sample size. The data also highlight the effect of PRRS MLV vaccination on
557 the immune responses after the co-infection. The increased PRRSV-specific, but
558 lowered H3N2-specific T cell responses in the Vac + PRRSV-2/H3N2 compared to
559 the Ctrl + PRRSV-2/H3N2 suggest that PRRS MLV may potentially drive the host
560 immune response toward PRRSV-specific, and rather away from the H3N2-specific
561 responses.

562 Cytokine expression in the lung

563 To further characterize the immune responses in the lungs of singly and co-
 564 infected pigs, the gene expression of a panel of cytokines and chemokines was
 565 assessed in lung tissues and data were normalized to the naïve group (**Figure 7**).
 566 After single infection with H3N2, an elevated mRNA expression of pro-
 567 inflammatory cytokines TNF and IFN- γ (mean fold change of 2.22 and 1.86,
 568 respectively), and anti-inflammatory cytokines IL-10 and TGF- β (mean fold change
 569 of 1.92 and 2.40 respectively), was observed (**Figure 7**). In PRRSV-2-infected
 570 group (Ctrl + PRRSV-2), a modest increase of TNF, IL-12p40 and IL-10 transcripts
 571 (mean fold change of 1.67, 1.69 and 1.80, respectively) was measured. After co-
 572 infection (Ctrl + PRRSV-2/H3N2), TNF, IFN- γ , IL-12p40, IL-4 and CXCL-13
 573 transcripts were all up-regulated (mean fold change of 2.43, 1.86, 3.47, 2.49 and
 574 2.83, respectively). In both vaccinated single and co-infected groups, an up-
 575 regulation of TNF (mean fold change 2.0 in both Vac groups) and IFN- γ transcripts
 576 were quantified (mean fold change 1.8 in both Vac groups). Notably both IL-10
 577 (mean fold change of 2.5 and 2.4, respectively) and TGF- β (mean fold change of 1.6
 578 and 2.0, respectively) mRNA expression was also upregulated in these vaccinated
 579 groups. A significant down-regulation of IL-12p40 mRNA was observed in Vac +
 580 PRRSV-2/H3N2 pigs compared to the Ctrl + PRRSV-2/H3N2 group ($p < 0.05$).

581 Despite a lack of statistical significance, these data suggest that upregulation
 582 of inhibitory cytokines gene expression in the lungs of previously immunized pigs
 583 might have influenced responses to H3N2 and PRRSV-2.

584

585

586 Discussion

587 The PRDC is responsible for major economic losses in the pig industry worldwide.
 588 The PRDC commonly results from mixed infections, in combination with
 589 environmental stressors (16). PRRSV and swIAV, alone or in combination, are two
 590 major viral pathogens involved in the PRDC, often leading to secondary infections
 591 with opportunistic bacteria. Previous experimental *in vivo* PRRSV/swIAV co-
 592 infection studies reported variable outcomes in terms of clinical or virological
 593 parameters, which may reflect differences in the timing of infections (18,43,44).
 594 Superinfection, i.e., infection of pigs with H1N1 3 days later a primary infection
 595 with PRRSV-1 led to more severe disease and a delayed shedding of H1N1
 596 compared to pigs that were infected 14 days later (45), whereas simultaneous
 597 PRRSV-1/H1N1 co-infection did not alter the clinical and virological course of
 598 infection of either virus (44). Several studies have also assessed the effect of
 599 concurrent infections and superinfections on vaccines efficacy and demonstrated
 600 that PRRSV infection decreases the efficacy of swIAV (46), *Mycoplasma*
 601 *hyopneumoniae* (47), and classical swine fever (48) vaccines although underlying
 602 mechanisms involved have not been demonstrated. Whilst vaccination with PRRS
 603 MLV is widely practiced in efforts to control PRRS, swIAV vaccination is less often
 604 used. Reflecting the field situation, a recent study experimentally assessed the
 605 impact of swIAV superinfection on vaccination with PRRS MLV efficacy and found
 606 a delay in MLV replication and Ab responses, but this did not affect vaccine
 607 efficacy (19). In our study here, we investigated the effect of PRRSV-2/H3N2 co-
 608 infection on the protection provided by a commercial PRRS MLV vaccine. The
 609 PRRS MLV protected against clinical disease, reduced lung pathology and viremia,
 610 but not virus shedding, as previously reported (14,49–51). However, PRRSV-
 611 2/H3N2 co-infection abrogated the protective effect of PRRS MLV vaccination on
 612 clinical disease and pathology. Co-infection did not affect the vaccine-induced
 613 reduction in PRRSV-2 load and enhanced CD8⁺ T cell responses in the lung and Ab
 614 responses. In contrast, co-infection in non-immunized animals had a beneficial
 615 effect by reducing PRRSV-2 viremia and H3N2 virus loads in BALF and nasal
 616 swabs at day 5 post-infection and did not affect clinical signs or pathology.

617

618 We sought to dissect the mechanisms underlying these opposing effects of co-
619 infection in immunized and non-immunized animals at day 5 post-infection. The
620 reduced PRRSV load in unimmunized co-infected animals has been suggested to be
621 due to the early type I IFN response triggered by H3N2, and interference and/or
622 competition for substrates required for replication (19,52). In this study, analysis of
623 gene expression in the lungs, did not reveal significant differences in the expression
624 of IFN- α , which may be due to the timing of sampling (5 days post-infection).
625 However, there was significantly more IL-12p40 gene expression, in the lung, and a
626 trend for higher number of H3N2-specific T cell responses (IL-2⁺ CD4⁺, IFN- γ ⁺
627 CD8⁺, IL-17⁺ CD2⁻ $\gamma\delta$ and TNF⁺ CD2⁻ $\gamma\delta$ T cells) and PRRSV-2-specific T cell
628 responses (IL-17⁺ CD2⁻ $\gamma\delta$ T cells TNF⁺ CD2⁻ $\gamma\delta$ T cells) in the BALF of the co-
629 infected unimmunized animals. This trend for enhanced cell-mediated response,
630 may have contributed to the control of virus replication in the lung in the co-infected
631 groups compared to the singly infected groups. In vaccinated pigs, co-infection
632 induced a greater PRRSV-specific TNF⁺ CD8⁺ T cell response in the BALF and an
633 increased Ab response. However, this was not the case for the specific response to
634 H3N2, and the cytokine gene expression profile in the lung did not differ
635 significantly from the singly infected animals. The precise mechanism for
636 abrogation of the PRRS MLV protective effect against lung injury by co-infection
637 remains unclear but might be due to exuberant cytokine production by other cell
638 types producing more pro-inflammatory cytokines such as TNF and IL-6 (53),
639 which were not detected at the timepoint sampled here. Although effectors CD8⁺ T
640 cells in the BAL aids to eliminate PRRSV-infected cells, the local response might
641 contribute to pulmonary inflammation and injury. Alternatively, co-infection may
642 have enhanced the production of low affinity non-neutralizing antibodies that may
643 augment infection and exacerbate disease.

644 Whilst PRRS MLV vaccines can provide a significant clinical benefit, the
645 protection against virus shedding is limited which may drive the evolution of
646 PRRSV (54,55). This has been attributed to these vaccines being weakly
647 immunogenic, especially for cellular responses (8). In line with previous reports, we
648 confirmed that PRRS MLV vaccinated pigs recorded lower lung lesions, viraemia
649 and viral load in lungs after a single infection with PRRSV-2, which was associated

650 with a robust, although non-neutralizing, antibody response and a weak peripheral
651 PRRSV-specific T cell response (11,14,50,51).

652 PRRSV has been shown to suppress host immune response through the
653 induction of regulatory T cells (56), alteration of peripheral NK cell cytotoxic
654 activity (57), and inhibition of cytokine responses (58,59). Moreover, PRRS MLV
655 vaccine can induce systemic secretion of IL-10 (60). It is therefore plausible that the
656 vaccine used in our study may exhibit similar immunomodulatory features. We
657 observed that, in comparison to unvaccinated pigs, lungs from vaccinated pigs co-
658 infected with PRRSV-2/H3N2 displayed a lower level of pro-inflammatory IL-
659 12p40 gene expression, along with a trend for higher expression of anti-
660 inflammatory IL-10 and TGF- β as measured by RT-qPCR. In a study on
661 *Litomosoides sigmodontis* helminth infection (60), helminth-infected mice exhibit
662 lower HA-specific antibody responses post-IAV vaccination, linked with a higher
663 viral load in the lung compared to non-infected mice. The level of HA-specific
664 induced by the vaccine was restored after the blockade of IL-10 using an anti-IL-10
665 receptor mAb. PRRS MLV vaccination may have potentially impaired the host
666 immune response to H3N2 and perhaps facilitated its replication. In addition to the
667 highest lung pathology seen in the PRRS MLV vaccinated co-infected pigs, the
668 beneficial effect of the co-infection in reducing H3N2 viral loads was also abrogated
669 in vaccinated pigs. These data suggests that PRRS MLV initiates a strong PRRS
670 specific response and may suppress H3N2 specific responses leading to increased
671 pathology and poor disease outcome.

672

673 Whilst confirmatory studies are required, the demonstration that co-infection
674 with H3N2 can abrogate the clinical protective effect of PRRSV MLV suggests that
675 better control measures against swIAV might be warranted to further increase
676 protection against PRRSV. Large number of herds are endemically infected with
677 swIAV and suffer intermittent bouts of disease. SwIAV contributes to suboptimal
678 weight gain and reproductive performance and is occasionally associated with fever-
679 induced abortion in sows (61). Immunization can be a cost-effective control measure
680 to combat swIAV, but the rapid evolution of the virus is a major obstacle (62). Not

all swIAV-endemic countries use vaccines to control the disease, for example, current UK policy does not involve immunization against swIAV, although it is used in some European countries and widely in the USA (63). We have used recent field PRRS and swIAV strains from Thailand. As in the UK, immunization of pigs against swIAV is not mandatory in Thailand, although some farms use the GRIPORK[®] vaccine (Hipra, Spain) containing inactivated H1N1 and H3N2 strains. Inactivated vaccines induce neutralizing Ab against the immunizing strain but do not induce sufficient heterologous protection due to the rapid viral escape that occurs through antigenic drift of the surface glycoproteins (64), so that coincident infection with PRRS and swIAV remains possible. There is a need to develop more broadly protective influenza vaccines that could provide a better control of swIAV, reducing the zoonotic risk (65), the contribution to the PRDC and its potentially harmful effect on PRRS vaccine efficacy.

Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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711

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717

718 **Author Contributions**

719 ET, SG, NW and SK acquired funding for the project. ET, SG and TC contributed to
720 the conception, design, and coordination of the study. TC and EM performed
721 experiments. TC acquired, analyzed, and interpreted the data. ET, SG, EM, EV,
722 VM, BP, AM, TM, and ME contributed to sampling during post-mortem. FS carried
723 out pathological analysis. TC wrote the first draft of the manuscript. ET, SG, NW,
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991 **Table 1:** Primers used for RT-qPCR and RT-PCR

Primers	Sequences (5'-3')	μM	Tm (°C)	Size (bp)	Eff (%)	R ²	Slope	PMID
N PRRSV-2	F: GCAAAATCAGTCCAGAGGCAAG	400	60	183	98.6	0.99	-3.33	-
	R: TGACAGGGTGCAAGTTCCAG	400						-
	P: FAM-CCCATTTCCCTCTTGCGACCGAAGATGACG-BHQ	250						-
IFN-α	F: TCTGCAAGGTTCCCAATGG R: GGCATTGCAGCTGAGTAGCA	350 350	60	69	100	0.99	-3.32	30333837
IFN-γ	F: TGGTAGCTCTGGGAAACTGAATG R: TGGCTTTGCGCTGGATCT	350 350	60	79	89.3	0.89	-3.60	30333837
TNF	F: TGGTGGTGCCGACAGATG R: CAGCCTTGGCCCCTGAA	350 350	60	64	108	0.96	-3.12	30333837
IL-4	F: GCCGGGCCTCGACTGT R: TCCGCTCAGGAGGCTCTTC	350 350	63	68	94.2	0.99	-3.46	30333837
IL-6	F: CTGCTTCTGGTGATGGCTACTG R: GGCATCACCTTTGGCATCTT	350 350	60	69	101.1	0.99	-3.29	30333837
IL-12p40	F: GGAGCACCCCACATTCCTACT R: TTCTCTTTTGTCTTGCCCTGAA	350 350	60	68	92.7	0.98	-3.51	30333837
IL-21	F: AAATAGTCATCTGCCTGATGGTCAT R: AGGCGATCTTGTCCTTGGA	350 350	60	76	103	0.97	-3.25	30333837
CXCL-13	F: ATCTCTGCTTCTCGTGCTG R: ACTTCTCTGGTTGGACATCC	350 350	60	180	92	0.99	-3.52	30819249
IL-10	F: GAGCCAACTGCAGCTTCCA R: TCAGGACAAATAGCCCACTAGCTT	350 350	60	65	109.8	0.98	-3.10	30333837
TGF-β	F: GAAGCGCATCGAGGCCATTC R: GGCTCCGGTTCGACACTTTC	350 350	60	162	91.1	1	-3.55	30333837
RPS24	F: AAGGAACGCAAGAACAAGATGAA R: TTTGCCAGCACCAACGTTG	350 350	60	62	94.2	0.99	-3.46	30333837
GAPDH	F: CACCATCTTCCAGGAGCGA R: CCAGCATCACCCCACTTGAT	350 350	60	51	101.5	0.98	-3.28	30333837

992 F: Forward; R: Reverse; P: Probe

993

994 Figures

995

996 **Figure 1: Clinical signs and lung lesions.** (A) Pigs were vaccinated
997 intramuscularly with Ingelvac PRRS[®] MLV (Vac) or with PBS (Ctrl) or were
998 untreated (naïve group). Thirty-three days after the vaccination, pigs were
999 challenged by intranasal inoculation with H3N2, PRRSV-2 or simultaneously with
1000 PRRSV-2/H3N2. Nasal swabs were daily taken after the challenge and pigs were
1001 culled 5 days later (38 days post-vaccination (dpv)). Sera and PBMC were collected
1002 at 0, 20 and 38 dpv. Clinical signs and rectal temperature were monitored daily after
1003 the challenge (dpc). (B) Table indicating the number of pigs that developed fever
1004 after the challenge. (C-E) Lungs sections were scored for histopathological lesions
1005 (C; Morgan score), lesions with presence of influenza NP-positive cells (D; Iowa
1006 score) or lesions with presence of PRRSV N-positive cells (E; Salguero score). Each
1007 symbol represents an individual animal within the indicated group (n=6 per group).
1008 The horizontal lines represent mean $\pm$ SD. Comparisons between 2 group were
1009 analyzed using Mann-Whitney test and asterisks indicate significant differences (*p
1010 < 0.05; **p < 0.01).

Figure 2: PRRSV-2 and H3N2 loads. Quantification of PRRSV-2 viral RNA in the nasal swabs (A), BALF (B) and serum (C) were determined by qRT-PCR. H3N2 titers in nasal swabs (D) and BALF (E) were determined by plaque assay. The mean values (A and D) or individual values (B, C, D and E) for each group $\pm$ SD are indicated (n=6 per group). P values were determined using Mann-Whitney test and asterisks indicate significant differences (*p< 0.05).

Figure 3: Antibody responses against PRRSV-2. (A) Detection of PRRSV N-specific Abs in the serum at 0, 20 and 38 dpv was performed with a commercial ELISA test. The positive threshold is indicated with dashed lines. (B) PRRSV-specific Ab titers were measured in the sera of PRRS-immunized pigs at 0, 20 and 38 dpv with an in-house ELISA test. (C) Levels of virus neutralizing Ab titer in the serum of PRRS-vaccinated pig at 0, 20 and 38 are shown. Sera from PRRSV-2 infected pigs from an unrelated study were used as positive controls (red symbols). Each pig serum is shown as a symbol within the indicated group (n=6 per group) and the mean $\pm$ SD is represented. The comparison between the percentage positivity values or Ab titers measured at 20 and 38 dpv versus at 0 dpv for each vaccinated group were performed using the Wilcoxon test. Comparisons between groups were made using the Mann-Whitney test. Asterisks indicate significant differences (*p < 0.05; **p < 0.01). nd: not determined.

Figure 4: PRRSV-2- specific T cell responses in PBMC. PBMC isolated at 0, 20 and 38 dpv were restimulated *in vitro* for 18h with PRRSV-2 (MOI 0.1) or cultured with medium. Intracellular staining of IFN- γ , TNF, IL-2, was performed and frequencies of IFN- γ , TNF and IL-2 producing CD4⁺ (A) and CD8 β ⁺ (B) T cells were analyzed. The corrected frequencies values are shown (percentage of cytokine-producing cells subtracted with medium only). Data for individual pigs and the group mean $\pm$ SD are displayed (n=5-6 per group). The Wilcoxon test was used to compare the T cell responses at day 0 and 20 dpv within the same group. Comparisons between 2 groups were performed using Mann-Whitney test. nd: not determined.

A α T cells – PRRSV-2

B γ δ T cells – PRRSV-2

○ Naïve ● Ctrl + H3N2 ▲ Ctrl + PRRSV-2 ■ Ctrl + PRRSV-2/H3N2 ▲ Vac + PRRSV-2 ■ Vac + PRRSV-2/H3N2

Figure 5: PRRSV-2- specific T cell responses in bronchoalveolar lavage. Cells isolated from BAL at 5 dpc were restimulated with PRRSV-2 (MOI 0.1) or cultured with medium. (A) Frequency of IFN- γ -, TNF- and IL-2-producing CD4⁺ and CD8 β ⁺ T cells are shown. (B) Frequency of IFN- γ -, TNF- and IL-17-producing CD2⁺ and CD2⁻ γ δ T cells are represented. The corrected frequencies (percentage of cytokine-producing cells subtracted with medium only) of each individual pig and the group mean $\pm$ SD are displayed (n=5-6 per group). Comparisons between groups were made using Mann-Whitney test. Asterisk indicates significant difference (*p < 0.05).

A $\alpha\beta$ T cells – H3N2

B $\gamma\delta$ T cells – H3N2

○ Naïve ● Ctrl + H3N2 ▲ Ctrl + PRRSV-2 ■ Ctrl + PRRSV-2/H3N2 ▲ Vac + PRRSV-2 ■ Vac + PRRSV-2/H3N2

Figure 6: H3N2- specific T cell responses in bronchoalveolar lavage. Cells isolated from BALF at 5 dpc were restimulated with swIAV H3N2 (MOI 0.1) or cultured with medium. Cytokine secretion measured in CD4⁺ and CD8⁺ (A), and CD2⁺ and CD2⁻ $\gamma\delta$ (B) T cells are represented. Corrected frequencies of individual value and the mean $\pm$ SD are displayed (n=5-6 per group). Comparisons between 2 groups were made using the Mann-Whitney test.

Figure 7: Gene expression in lung tissues. Total RNA was extracted from lung tissue collected at 5 dpc and the relative mRNA expression of IFN- α , IFN- γ , TNF, IL-12p40, IL-4, IL-6, IL-21, IL-10, TGF- β and CXCL-13 was assessed by qRT-PCR. Fold changes are shown over naïve group (dash line) after normalization with *GAPDH* and *RPS24* genes. Individual pig values and the group mean $\pm$ SD are displayed (n=5-6 per group). Comparisons were made using Kruskal-Wallis test and asterisks indicate significant differences (* p < 0.05).