Dual infections of CD163 expressing NPTr epithelial cells with influenza A virus and PRRSV

Chantale Provost\textsuperscript{1§}, Glenn Hamonic\textsuperscript{2§}, Carl A. Gagnon\textsuperscript{1}, and François Meurens\textsuperscript{3*}

\textsuperscript{1}Swine and Poultry Infectious Diseases Research Center (CRIPA) et \textit{Groupe de Recherche sur les Maladies Infectieuses en Production animale} (GREMIP), Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada

\textsuperscript{2}Vaccine and Infectious Disease Organization (VIDO)-International Vaccine Centre (InterVac) and the Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

\textsuperscript{3}BIOEPAR, INRA, Oniris, La Chantrerie, 44307, Nantes, France

\textsuperscript{§}Contributed equally to this work

\textsuperscript{*}Corresponding author: Email: francois.meurens@oniris-nantes.fr; Phone: +33 240 68 77 02; Fax: +33 240 68 28 02.

\textbf{Short Running Title:} Dual viral infection of CD163 expressing NPTr cells

\textbf{Keywords:} CD163, Co-infection, PRRSV, Influenza A virus, Pig epithelial cells
ABSTRACT

In the pig, respiratory co-infections involving various pathogens are far more frequent than single infections. Amongst respiratory viruses, swine influenza type A virus (swIAV) and porcine reproductive and respiratory syndrome virus (PRRSV) are frequently associated. Previously, we performed co-infections with swIAV and PRRSV in porcine alveolar macrophages (PAM) and precision cut lung slices (PCLS). With these two approaches it was practically impossible to have co-infections of the same cells as the main target cell of swIAV is the epithelial cell while the main target of PRRSV is the PAM. This constraint makes the study of interference between the two viruses difficult at the cellular level. In the current report, an epithelial cell line expressing, CD163, the main receptor of PRRSV was generated. This cell line receptive for both viruses was used to assess the interference between the two viruses. Results showed that swIAV as well as PRRSV, even if they interacted differently with the modified epithelial cells, were clearly interfering with each other regarding their replication when they infected a same cell with consequences within the cellular antiviral response. Our modified cell line, receptive to both viruses, can be used as a tool to assess interference between swIAV and PRRSV in a same cell as it probably happens in the porcine host.

INTRODUCTION

In pigs, as in many species, respiratory co-infections are far more frequent than single infections (Choi et al., 2003; Fablet et al., 2012). Amongst viral co-infections, the association between swine influenza type A virus (swIAV) and porcine reproductive and respiratory syndrome virus (PRRSV) is frequent (Choi et al., 2003; Fablet et al., 2012). However, the assessment of the outcome of co-infections at molecular level is still very limited in the literature. Previously we assessed the impact of swIAV/PRRSV in vitro in alveolar
macrophages (Porcine Alveolar Macrophage, PAM) and *ex vivo* using precision cut lung slices (PCLS) (Dobrescu et al., 2014). Results showed that whereas a first infection of PCLS or PAMs by PRRSV did not affect the local H1N1 swIAV infection 3 h later, primary infection of PCLS or PAMs with swIAV partially inhibited their infection by the PRRSV 3 h later and some modifications in the host response. However, it was not possible to identify clearly co-infected cells in PCLS even if cells such as small populations of dendritic cells and in some circumstances type 1 pneumocytes can be targeted by both viruses. Moreover, there are still questions regarding full replication of swIAV in PAMs. These constraints make difficult the study of interference between the two viruses at the cellular level. To overcome that issue it was decided to generate a cell line susceptible to both viruses. Thus, newborn pig trachea (NPTr) cell line (Ferrari et al., 2003) was modified to express CD163, the main receptor of PRRSV (Calvert et al., 2007), allowing infection with both viruses. Using the cells we selected a protocol similar to the one used previously (Dobrescu et al., 2014), we evaluated the impact of one virus on the replication of the other, and we determined the consequences of co-infection at the host level by an assessment of the expression of various transcripts involved in viral recognition and resistance. Generated data were then compared to previous results obtained in a less constrained system based on PAMs and PCLS.

**MATERIALS AND METHODS**

**NPTr cell line**

The newborn pig trachea epithelial cell line (NPTr) was kindly provided by Dr. M. Ferrari (*Instituto Zooprofilattico Sperimentale*, Brescia, Italy) (Ferrari et al., 2003). The NPTr cell line was cultured in Minimum Essential Medium (MEM) (GIBCO®-BRL, ON, Canada) supplemented with 10% Fetal Bovine Serum (FBS) (Wisent Bioproducts, QC, Canada), 1 mM
sodium pyruvate, 10 I.U./mL of penicillin, 10 μg/mL of streptomycin and 250 g/L amphotericin B solution (Wisent Bioproducts) (Ferrari et al., 2003).

Creation of PRRSV-permissive NPTr cells

In order to modify the NPTr cell line to be permissive to PRRSV, a cDNA copy of the mRNA sequence of CD163, a known receptor of PRRSV (Calvert et al., 2007), was stably integrated to the genome. To do so, primary porcine alveolar macrophages (PAMs) were isolated from pathogen-free pig lungs (Provost et al., 2012). After RNA extraction with Trizol Reagent (Invitrogen™, New-Mexico, USA), a RT-PCR was performed using primers CD163-\textit{Hind}III-F (5' - AAGCTTAAGCTTAGACATACTCAGAATGGTGCTAC- 3') and CD163-\textit{Cla}I-Reverse (5' - ATCGATATCGATTATGTACTTCAGAGTGGTCTCCTGTAGGGATTTAG-3') with SuperScript® One-Step RT-PCR for long template (Invitrogen), the full mRNA sequence of CD163 was expected at approximately 3623 bp (Genbank: DQ067278.1). The resulting product was digested by \textit{Hind}III and \textit{Cla}I restriction enzymes (New England Biolabs, Ipswich, MA, USA) and inserted into the retroviral plasmid pLNCX2 (Clontech Laboratories Inc., California, USA). The integrated sequence was analysed to accurately match the cDNA sequence of CD163 (Genbank DQ067278). To stably integrate the coding sequence of CD163, the protocol from a retroviral transduction kit, the Retro-X System® (Clontech Laboratories Inc.) was followed. Briefly, the CD163 cDNA was introduced in the pLNCX2 plasmid. The pLNCV2-CD163 plasmid was then co-transduced with pVSV-G®, coding for mammalian retrovirus envelop proteins, in provided Eco-Pack® GP2-293 cells (Clontech Laboratories Inc.). This enabled the production of non-replicating mammalian cell specific retrovirus containing the pLNCX2-CD163 plasmid. NPTr cells were then infected with the supernatant of the GP2-293 cells for 8 hours (h), left for 12 h with fresh medium, then re-infected with the same retrovirus for 8 h. Cells were then cultured in G418 (Sigma-
Aldrich, Missouri, USA) for clone selection. CD163 integration was determined by PCR and transcription by RT-PCR, as described below, and expression of the protein was assessed by IFA with mouse monoclonal anti-pig CD163 antibody (AbD Serotec, Oxford, UK), as previously described (Provost et al., 2012).

Detection of CD163 mRNA by RT-PCR

The extraction of total RNA was done by Trizol® reagent (Invitrogen). A reverse-transcription was performed following protocol of QuantiTech Rev. Transcription kit (Qiagen™, Missisauga, ON, Canada). To facilitate detection, a new reverse primer was designed: CD163-detect-R (5’-CCAGAGAAACTGACAGCACTTCCACATTCA- 3’) to be used with the forward primer CD163-HindIII-F, as described above.

Sequence analyses

The nucleotide sequencing of CD163 gene was performed by the Diagnostic Laboratory of the Faculté de médecine vétérinaire of Université de Montréal and the gene analysis was done using the Geneious bioinformatics version 5.4.6 (Biomatters, Ltd., Auckland, New Zealand).

Cells and viruses

For influenza virus propagation, Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) and NPTr cells were cultured while for PRRSV propagation MARC-145 monkey cells (ATCC CRL-12231) were used. These cells were cultured as previously described (Delgado-Ortega et al., 2014b; Dobrescu et al., 2014). PAM cells were obtained from lungs of 2 to 14 week-old pigs as previously explained (Lévesque et al., 2014). To collect the PAM cells, animals were humanely sacrificed following the ethic protocol 12-Rech-1640. This protocol was approved by Université de Montréal ethic committee, which is following the guidelines of the Canadian
Council of Animal Care. The swine influenza strain A/Sw/Saskatchewan/18789/02 (swIAV/Sk02) of H1N1 subtype was isolated from pigs in Saskatchewan in 2002 (Karasin et al., 2004). The titer of influenza virus was determined on MDCK and NPTr cells by a plaque assay, as described previously (Shin et al., 2007). Stock of the virus reached titer of $9.5 \times 10^7$ plaque forming units (pfu)/mL after purification. The virulent PRRSV strain ISU-12-SA was obtained from ATCC (ATCC VR-2385, Hanassas, VA, USA) and the quantitation of PRRSV was performed in MARC-145 cells. The titer ($4.8 \times 10^6$) was calculated and expressed as TCID$_{50}$/mL (Reed and Muench, 1938).

Series of six wells of CD163 expressing NPTr cells (NPTr-CD163) ($2 \times 10^5$ cells/well) in a 24-well plate were single-infected or co-infected with swIAV and PRRSV at a MOI of 5. Additionally 6 non-infected wells were used as controls. 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 twice with phosphate buffered saline (PBS) and maintained at 37°C in 1 mL of MEM (GIBCO®-BRL) supplemented with 10% FBS and 1% antibiotic/antimycotic (Anti-Anti 100x, GIBCO®-BRL). Fifteen and eighteen hours after the temperature shift the culture medium was removed, clarified twice by centrifugation (1,000 x g), divided into aliquots, and stored at -80°C. For superinfections, six wells of NPTr-CD163 cells ($2 \times 10^5$ cells/well) were first infected with swIAV (MOI of 5), then superinfected with PRRSV (MOI of 5) 3 h later. In parallel, six wells of NPTr-CD163 cells were infected with PRRSV (MOI of 5) and superinfected with swIAV (MOI of 5) 3 h after infection with PRRSV. The 3 h delay between infections was selected based on previous studies where interference between the same viruses or others was intensively assessed in vitro and in vivo (Dobrescu et al., 2014; Meurens et al., 2004b; Schynts 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 twice with PBS and further incubated at 37°C in 1 mL of MEM (GIBCO®-BRL) supplemented with 10% FBS and 1% antibiotic/antimycotic (Anti-Anti 100x, GIBCO®-BRL). Additionally 8 non-infected wells were used as controls. Fifteen hours post-superinfection, the culture medium was removed, clarified twice by centrifugation (1,000 x g) and stored at -80°C.

### Gene expression analysis using quantitative real-time polymerizing chain reaction

Real-time PCR Primers (targeting M protein gene of swIAV, Open Reading Frame 7 (ORF7) of PRRSV, DAI, LGP2, MDA5, RIG1, TLR3, TLR8, IFNα, IFNβ, IFNλ1, IL6, CCL20, MX2, OAS, and PKR transcripts) were designed and optimized using Clone Manager 9 (Scientific & Educational Software, Cary, NC, USA) and were purchased from Invitrogen (Carlsbad, CA, USA) as previously described (Table 1) (Dobrescu et al., 2014). NPTr-CD163 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). The absence of genomic DNA contamination was verified using prepared RNA as a template for quantitative real-time PCR (qPCR). RNA concentration was determined by measuring optical density at 260nm (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 100-200 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. qPCR assays were carried out as previously described using the three most stable reference genes (Delgado-Ortega et al., 2014a; Dobrescu et al., 2014). qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002) using the Cycle quantification (Cq) from the samples for the different transcripts.
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 among 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.

RESULTS-DISCUSSION

Creation of a new model to study PRRSV and influenza co-infections in vitro

To study PRRSV and swIAV co-infection, NPTr cells have been genetically modified to express the protein CD163, which is one of PRRSV’s principal cell receptor (Calvert et al., 2007; Das et al., 2010). Many other papers have shown that expression of CD163 protein enabled PRRSV permissivity to cells (Lee et al., 2010; Lee and Lee, 2010; Patton et al., 2009; Van Gorp et al., 2010). NPTr cell line did not show any CD163 mRNA production by RT-PCR (Figure 1A). As such, CD163 cDNA was isolated from PAMs and cloned into the NPTr cell line using a retroviral transduction kit. To verify the stable introduction of the CD163 cDNA into the new genetically modified NPTr cells genome (NPTr-CD163 cells) and the expression of the protein, a RT-PCR and a specific porcine anti-CD163 immunofluorescence assay (IFA) was performed (Figure 1). RT-PCR detection revealed the presence of CD163 mRNA in NPTr-CD163, as compared to non-modified NPTr cells (negative control) and primary PAMs (positive control) (Figure 1A). IFA by porcine anti-CD163 confirms high expression of the protein (Figure 1C). Furthermore, the permissiveness of the modified NPTr-
CD163 cells to PRRSV type II Quebec reference strain (IAF-Klop) was evaluated by IFA with polyclonal pig anti-PRRSV serum (data not shown).

**PRRSV and swIAV single and co-infections of NPTr-CD163 cells**

NPTr-CD163 cells were then single or co-infected with PRRSV and swIAV for a maximum of 18 h (Figure 2). Co-infections were carried out simultaneously or with a 3 h delay between the two viruses.

The replication of both viruses was assessed by RT-qPCR at the end of the experiment. We observed that PRRSV impacted the replication of swIAV especially when the cells were simultaneously co-infected and when PRRSV virus was added to the cells 3 h after swIAV ($P<0.05$) (Figure 2). On the contrary co-infections with PRRSV added to the cells 3 h before swIAV did not show any impact on swIAV replication. Regarding PRRSV replication it could be observed that PRRSV replication was clearly decreased when swIAV infected NPTr-CD163 cells 3 h before PRRSV (Figure 2). Thus, when these data were compared to previous ones where PAMs and PCLS were similarly single and co-infected with the same viruses a difference was identified (Dobrescu et al., 2014). Indeed, in cells permissive to both viruses PRRSV could interfere with swIAV replication which was not the case in co-infections of PAMs or PCLS (Dobrescu et al., 2014). Interference between viruses was clearly observed in NPTr-CD163 cells. Since both viruses are RNA viruses mobilizing similarly cellular machinery and defenses this strong interference was expected and here confirmed. Variations in the impact of interference on the replication of the second virus could be explained, for instance, by differences in penetration kinetics between viruses as previously observed with other enveloped viruses (Meurens et al., 2004a).

Regarding the antiviral response of the NPTr-CD163 cells and the potential impact of co-infection on it, several observations could be made. First, in line with previous data (Dobrescu
et al., 2014), our strain of PRRSV – ISU-12-SAHI – was a very poor inducer of the cellular antiviral response as observed with the low expression of several transcripts related to its response (Figure 3) confirming previous observations with PAMs and PCLS (Dobrescu et al., 2014). On the contrary the strain of swIAV (A/Sw/Saskatchewan/18789/02) was clearly triggering the expression of various transcripts (see LGP2, TLR8, IFN types 1 and 3 in Figure 3), especially IFNλ1 mRNA. However, it did not seem to have consequences on the interferon stimulated genes (ISGs) we assessed. IFN type 3 mRNA were more expressed than IFN type 1 mRNA suggesting a particular role for these IFNs in epithelial cells. This observation confirms previous results showing a preferential expression of IFN type 3 in primary airway epithelial cells (Ioannidis et al., 2013) and clear expression of IFNλ1 mRNA in NPTr cells and PCLS in response to an European strain of H3N2 swIAV (Delgado-Ortega et al., 2014b).

When PRRSV was added to the cells on the same time or after swIAV, transcript levels of expression were similar or decreased (see for instance IFNλ1, Figure 3). PRRSV effect was less obvious when the virus was infecting the cells before swIAV. For ISGs, even if both viruses were poor inducer after 18 h of stimulation, it appears that transcript levels of expression were very low in the experimental conditions involving a PRRSV infection before or after swIAV infection (Figure 3). Globally, the two viruses were also interfering with each other at the cellular level, usually with one decreasing the induction triggered by the other, probably as a consequence of their altered replications. However, neither synergistic nor additive effects were observed at the cellular level when the two viruses were simultaneously infecting NPTr-CD163 cells. Additive and synergistic effects between PRRSV and swIAV were reported previously (Dobrescu et al., 2014) but it was not in a single pure cell population like here, but in tissue slice where various cell types were present and could communicate through cytokines network. Thus, it seems that at the cellular level, early in the infection process, both viruses are mostly interfering. While at tissue level, even if there is still
interference there is also the establishment of synergistic and additive effects when the host
response is considered.

In the current report interactions between two strains of PRRSV and swIAV were assessed
early in co-infections of a same genetically modified target epithelial cell line expressing the
PRRSV receptor, CD163. Results using this new tool showed that both RNA viruses can
clearly interfere with each other when infecting the same cell with consequences on the
antiviral cell response. Further researches are needed to assess the impact of this observed
interference later in the infection process and on the protein production that cannot be
assessed at early times. Moreover, interactions between various cells types exposed to both
viruses would undoubtedly need further assessment too. Finally, additional in vivo and field
experiments taking advantages of the recent developments in porcine immuno-microbiology
and the new tools available should be performed to clarify complex relations between micro-
organisms in the respiratory tract and the lung.

ACKNOWLEDGEMENTS

FM is supported by an establishment grant from the Région Pays de la Loire (RFI Food for
tomorrow-Cap aliment). GH is a recipient of the University of Saskatchewan Department of
Veterinary Microbiology Devolved Scholarship. CAG is financially supported by a Natural
Sciences and Engineering Research Council of Canada (NSERC) discovery grant. We are
very grateful to Ken Lai and Meghanne Rieder for virus stock preparation and technical
assistance. The manuscript was published with permission of the Director of VIDO as
manuscript # 800.

COMPETING INTERESTS STATEMENT

The authors declare that they have no conflict of interests.
REFERENCES


viruses. Vet Microbiol 169, 18–32. doi:10.1016/j.vetmic.2013.11.037


Meurens, F., Keil, G.M.M., Muylkens, B., Gogev, S., Schynts, F., Negro, S., Wiggers, L,


**FIGURE CAPTIONS**

**Figure 1: NPTr-CD163 cells express the CD163 protein**
NPTr cells were infected with a CD163-coding retrovirus, selected, cloned and tested for expression of the protein CD163. RT-PCR detection of mRNA of partial CD163 in NPTr, NPTr-CD163, and PAMs (A). Immunofluorescence against CD163 protein in NPTr (B) and modified NPTr-CD163 (C) cells. White scale bar represent 200 µm.

**Figure 2: Viral replication** - Relative viral expression (ORF7-PRRSV and M protein-swIAV genes) after 15 h or 18 h of infection of NPTr-CD163 cells. For every situations n=6 + median except control where n=8 + median. Dot plots within each graph with no common superscripts are significantly different (P < 0.05).

**Figure 3: Viral recognition (LGP2, MDA5, and TLR8), Interferons (IFNα, IFNβ, and IFNλ1), and Response to Interferons (MX2, OAS, and PKR)** - Relative expression of transcripts after 15 h or 18 h of infection of NPTr-CD163 cells. For every situations n=6 + median except control where n=8 + median. Dot plots within each graph with no common superscripts are significantly different (P < 0.05).
**Figure 1**

A. Gel electrophoresis showing bands of 400 bp.

B. Immunofluorescence for NPTr and NPTr-CD163.

C. Higher magnification of NPTr-CD163 stained with Anti-CD163.
Figure 2
Figure 3