DOCTORAT ECOLOGIE BRETAGNE GEOSCIENCES LOIRE AGRONOMIE ALIMENTATION

Oniris
ÉCOLE NATIONALE VÉTÉRINAIRE, AGROALIMENTAIRE ET DE L'ALIMENTATION
SANTÉ ET ALIMENTATION AU CŒUR DE LA VIE

THÈSE DE DOCTORAT DE

ONIRIS

ÉCOLE DOCTORALE N° 600
École doctorale Écologie, Géosciences, Agronomie et Alimentation
Spécialité : Microbiologie, virologie et parasitologie

Par

Georges SAADE

Les co-infections respiratoires du porc

Co-infections des cellules et des tissus respiratoires porcins par le virus de l'influenza A et le virus du syndrome dysgénésique et respiratoire porcin

Thèse présentée et soutenue à Nantes, le 29 Janvier 2021

Unité de recherche : INRAE, Oniris, BIOEPAR, 44300 Nantes, France

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« *Le problème n'est pas de savoir comment faire entrer des pensées nouvelles, innovantes, dans les esprits, mais comment en faire sortir les anciennes* »

Dee HOCK

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Liste des abréviations

PRRSV : *Porcine reproductive and respiratory syndrome virus*
swIAV : *swine influenza A virus*
CRP : Complexe respiratoire porcin
SPF : *Specific pathogen free*
ADV : *Aujeszky disease virus*
HA : Hémagglutinine
NA : Neuraminidase
ARN : Acide ribonucléique
MDCK : *Madin-Darby Canine Kidney*
NLS : *Nuclear localization signal*
ARNv : Acide ribonucléique virale
RNPv : Ribonucléoprotéines virales
CRM1 : *Chromosomal maintenance 1*
NEP : *Nuclear export protein*
IFN : Interféron
IL : Interleukine
CCl : *C-C motif chemokine ligand*
DC : *Dendritic cell*
CCR2 : *C-C chemokine receptor type 2*
AM : A*lveolar macrophage*
NOS2 : *Nitric oxyde synthase 2*
TNFα : *Tumor necrosis factor α*
NK : *Natural killer*
Th : *T helper*
CTL : *Cytotoxic T lymphocyte*
Ig : Immunoglobuline
PRR : *Pattern recognition receptor*
TLR : *Toll like receptor*
RIG-I : *Retinoic acid inducible gene-I*
MDA5 : *Melanoma differentiation-associated gene 5*
NOD : *Nucleotide-binding oligomerization domain*
NLR : *NOD-like receptor*
IRF : *Interferon regulatory factor*
NF-κB : *Nuclear factor-kappa B*
ATF : *Activating transcription factor*
AP-1 : *Activator protein 1*
PRD : *Positive regulatory domain*
IFNAR : *Interferon-alpha/beta receptor*

JAK : *Janus kinase*
TYK2 : *Tyrosine Kinase 2*
STAT : *Signal transducer and activator of transcription*
ISGF3 : *Interferon Stimulated Gene Factor 3*
ISRE : *Interferon-stimulated response element*
ISG : *Interferon Stimulated Gene*
PKR : *Protein kinase R*
OAS : *Oligoadenylate synthetase*
eIF2α : *eukaryotic initiation factor 2 alpha*
Nsp : *Non-structural protein*
PI3K : *Phosphatidylinositol-3-kinase*
IAV : *Influenza A virus*
MAPK : *Mitogen-activated protein kinase*
ERK : *Extracellular signal regulated protein kinase*
JNK : *Jun-N-terminal Kinase*
LDEV : *Lactate dehydrogenase-elevating virus*
LV : *Lelystad Virus*
ORF : *Open reading frame*
GP : Glycoprotéine
Mo-DCs : *Monocyte-derived dendritic cells*
DC-SIGN : *Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin*
CBP : *CREB-binding protein*
KPNA1 : *Karyopherin alpha 1*
SOCS : S*uppressor of cytokine signaling protein*
PIP : *Phosphophatidylinositol phosphate*
MYD88 : *Myeloid differentiation primary response 88*
AMPK : *Adenosine monophosphate-activated protein kinase*
ACC1 : *Acetyl-CoA carboxylase 1*
PIM *: Pulmonary intravascular macrophages*
EOPS : Exempt d'organisme pathogène spécifique

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Préambule

Cette thèse a été effectuée dans l'équipe « Immunocare » au sein de l'UMR-1300 « Biologie, Épidémiologie et Analyse de Risque en Santé Animale » (BIOEPAR) de l'institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE). L'unité est située sur le site de l'école nationale vétérinaire, agroalimentaire et de l'alimentation de Nantes (Oniris).

Le virus responsable du syndrome dysgénésique et respiratoire du porc (ou *Porcine Reproductive and Respiratory Syndrome Virus*, PRRSV), est un virus qui circule beaucoup dans les élevages et qui a des répercussions très importantes sur la filière porcine. Ce virus est connu entre autres pour ses effets perturbateurs de la réponse immune de l'hôte porcin.

Les virus responsables de la grippe porcine sont nombreux et affectent les élevages de porcs dans le monde entier. Le virus de l'influenza A porcin (*swine Influenza A Virus*, swIAV) induit une réponse inflammatoire importante au niveau des voies respiratoires de l'hôte. Une grande partie de la thèse est dédiée à la compréhension de la réponse immune innée du porc face à une co-infection par ces deux virus. Nous nous sommes intéressés également aux interactions potentielles entre les 2 particules virales.

La première partie de la thèse présente une étude bibliographique comportant une revue de littérature publiée en troisième année de thèse et qui introduit la notion du complexe respiratoire porcin (CRP) ainsi que les principaux agents pathogènes bactériens et viraux contribuant à ce complexe. Cette revue décrit aussi les différents types d'interactions entre les agents pathogènes du CRP, leurs conséquences moléculaires ainsi que les mécanismes d'interférence survenant entre ces agents pathogènes en cas de « co-infections ». Un deuxième sous-chapitre de cette première partie est consacré aux deux virus utilisés dans nos travaux. Il détaille l'histoire, la composition et la transmission de ces deux virus ainsi que les mécanismes d'infection des cellules hôtes par chaque virus. Un point est également fait sur la réponse immune du porc suite aux infections simples et aux co-infections.

Le reste des chapitres sera présenté sous forme d'articles scientifiques montrant les résultats de nos différentes expérimentations menées *in vitro* et *ex vivo*. Chaque article sera précédé d'une petite introduction aux travaux menés et suivi d'une conclusion en français. Certaines données non publiées seront également présentées sous forme de données supplémentaires.

Plusieurs techniques conventionnelles ou plus innovantes ont été utilisées au cours des expérimentations dans le cadre de nos collaborations avec différents collègues et partenaires. L'impédencemétrie a été utilisée avec les collègues du LABÉO Franck Duncombe à Caen dans le but d'évaluer en continue la mortalité cellulaire suite à une infection virale. Des techniques d'immunofluorescence ont été utilisées avec les collègues de l'unité de Physiopathologie Animale et bioThérapie du muscle et du système nerveux (PAnTher) de l'INRAE pour suivre la distribution des virus dans le tissu respiratoire. La technique de *Western Blot* a été mise en œuvre par les collègues de l'unité de Physiologie de la Reproduction et des Comportements (PRC) de l'INRAE de Nouzilly pour évaluer les voies de signalisation stimulées par les virus. Des prélèvements réguliers de poumons et de liquide bronchoalvéolaire ont été faits en collaboration avec les collègues de l'Unité Expérimentale de Physiologie Animale de l'Orfrasière de l'INRAE de Nouzilly. Et finalement, une collaboration avec des collègues de l'ANSES de Ploufragan nous a permis d'obtenir les souches virales locales, maîtriser leur amplification et avoir accès à des animaux exempts d'organisme pathogène spécifique (*Specific-pathogen-free,* SPF).

Introduction

Ces dernières années, un surcroît d'études consacrées à l'espèce porcine a été observé. Les pertes économiques dans les élevages de porcs peuvent avoir de graves conséquences économiques et sanitaires, sachant que la viande de cet animal occupe une place très importante dans l'alimentation des populations humaines. Par ailleurs, le porc peut constituer un modèle animal pertinent se rapprochant de l'homme par son régime alimentaire omnivore, sa durée de vie plus longue que celles des rongeurs, son anatomie, sa physiologie et son système immunitaire relativement comparables à ceux de l'homme, (Dawson, 2011; Meurens et al., 2012a; Swindle et al., 2012). Le porc possède des caractéristiques de reproduction très intéressantes tels que la multiparité et la courte durée de gestation par rapport au reste des grands mammifères. Il s'agit d'une espèce dont la manipulation est plus facile que celle des primates et éthiquement plus acceptable. Au niveau viral, les virus influenza du porc, présentent les mêmes sous-types que les virus influenza humains : H1N1, H1N2, H3N2 (Meurens et al., 2012a).

Les atteintes respiratoires d'origine infectieuse affectent le bien-être des animaux et augmentent le taux de mortalité dans les élevages, ce qui pénalise les revenus des éleveurs et engendre d'importantes pertes économiques. Par ailleurs, certaines de ces infections posent un problème de santé publique à cause du potentiel zoonotique de certains agents d'un côté et du recours aux antibiotiques de l'autre. Les pathologies respiratoires porcines d'origine infectieuse constituent une des causes majeures d'usage des antibiotiques en médecine vétérinaire (van Rennings et al. 2015) et les mesures thérapeutiques ou prophylactiques disponibles demeurent insuffisantes. Les antibiothérapies, parfois empiriques, peuvent être à l'origine de phénomènes d'antibiorésistance entrainants une persistance des maladies respiratoires dans les élevages de porc.

Les agents infectieux à l'origine des pathologies respiratoires porcines sont le plus souvent de nature bactérienne ou virale, les virus préparant généralement le terrain pour les bactéries sur-infectantes. La présence de plusieurs agents infectieux aboutit à la formation du « CRP ». Le plus souvent, sur le terrain, les agents pathogènes à tropisme respiratoire se retrouvent associés (Choi et al., 2003; Fablet et al., 2012a, 2012b) rendant le diagnostic, la prévention et le traitement des infections respiratoires plus difficiles. Dans une analyse rétrospective portant sur les agents infectieux responsables de troubles respiratoires chez 2872 porcs du Minnesota (États-Unis) deux ou plus de deux agents pathogènes ont été identifiés simultanément dans 88,2 % des cas (Choi et

al., 2003). Une autre étude française menée sur 125 troupeaux de l'Ouest du pays montre une association entre le pourcentage de porcs positifs au circovirus porcin de type 2 (*Porcine circovirus 2*, PCV2) et le pourcentage de porcs infectés par *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) (Fablet et al., 2012b). Cette même étude décrit une corrélation positive entre le pourcentage d'animaux séropositifs au PRRSV et le pourcentage d'animaux ayant développés des anticorps contre d'autres agents pathogènes tels que swIAV, *M. hyopneumoniae* et *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) (Fablet et al., 2012b).

Au cours des dernières années, les recherches dans l'espèce porcine se sont focalisées le plus souvent sur les infections respiratoires par un seul agent pathogène et ont accordé moins d'importance aux infections impliquant plusieurs agents pathogènes plus représentatives de la situation sur le terrain. D'autre part, les projets de recherches menés autour du CRP se sont le plus souvent intéressés à l'analyse des lésions et des signes cliniques (Van Reeth et al., 2001; Narita and Ishi, 2006; Wei et al., 2010; Niederwerder et al., 2016; Rivera-Benitez et al., 2016). En conséquence, les interactions survenant entre les agents pathogènes et leurs conséquences moléculaires sur l'hôte porcin restent encore mal connues. Ainsi, à ce jour, la compréhension des infections respiratoires impliquant, par exemple, le swIAV sur des élevages où le PRRSV circule reste insuffisante. Une meilleure connaissance de la réponse immune induite par ces co-infections respiratoires et de leurs conséquences sur la santé, le bien-être et la performance des animaux, pourrait aboutir à une réduction des pertes économiques dans les élevages.

Parmi les virus responsables d'infections respiratoires dans l'espèce porcine et jouant un rôle dans le CRP, peuvent être cités le PRRSV, les swIAV, le PCV2, le virus responsable de la maladie d'Aujeszky (*Aujeszky Disease Virus*, ADV) et les alphacoronavirus porcins.

Objectifs de la thèse

Les co-infections impliquant le PRRSV et le swIAV apparaissent très fréquentes dans les élevages de porc (Pol et al., 1997; Choi et al., 2003; Fablet et al., 2012b). L'issue des co-infections est variable selon qu'il y ait antagonisme, synergie ou absence d'interactions mesurables entre agents pathogènes. Du côté de l'hôte porcin, les co-infections peuvent rendre la réponse immune plus ou moins efficace. Les études relatives au PRRSV et au swIAV révèlent des résultats contradictoires concernant l'effet d'une première infection sur une surinfection (Pol et al., 1997; Van Reeth et al., 2001, 2003). Par ailleurs, la grande majorité de ces études a été réalisée *in vivo* sans évaluation des interactions virus-virus et virus-hôte au niveau moléculaire. Fort des deux premières expériences *in vitro* sur la co-infection par ces deux virus dans mon équipe d'accueil (Dobrescu et al. 2014; Provost et al. 2017), il a été proposé dans ma thèse de **poursuivre les études relatives à la compréhension des interactions entre ces agents pathogènes et de leurs conséquences pour l'hôte lors de co-infections simultanées ou différées (surinfections**).

Dans un premier temps, nous avons commencé par un **recensement des études sur les co-infections** impliquant les agents pathogènes viraux et bactériens majeurs contribuant au CRP chez le porc. Ces travaux bibliographiques représentés dans le chapitre 1 ont été menés dans le but de **mettre à jour les connaissances sur les co-infections respiratoires virales et/ou bactériennes dans l'espèce porcine** et de **détailler leurs conséquences moléculaires**.

Le but principal de ce projet de thèse est de **décrypter au niveau de la cellule et du tissu respiratoire les conséquences moléculaires de co-infections impliquant le PRRSV et/ou le swIAV**. Dans le deuxième chapitre, la réponse immune innée des cellules épithéliales et du tissu pulmonaire a été analysée à l'échelle moléculaire en situation de co-infections et de surinfections. Cela nous a amenés à répondre aux questions suivantes :

Comment le PRRSV et le swIAV interagissent ensemble ? Comment interfèrent-ils ? Quel est l'impact de ces interactions sur la réponse immune de l'hôte porcin (sur les réponses IFN plus spécifiquement) ? Est-ce que les virus développent des actions synergiques ? Ou au contraire, ont-ils tendance à se contrecarrer l'un l'autre ?

Quelles sont les causes à l'origine des contradictions dans les résultats des différentes études de co-infections évaluant les atteintes cliniques des porcs co-infectés ?

Notre hypothèse de départ est qu'il existe des interactions entre le swIAV et le PRRSV, dépendant du délai, de la séquence des événements d'infection et de la nature du virus. En effet,

des travaux antérieurs ont montré qu'un délai entre infections par le PRRSV et le swIAV avait des conséquences sur les atteintes cliniques observées sans cependant préciser la nature des interactions entre virus et en restant assez évasif sur le délai (Van Reeth et al., 2001). Il a été envisagé dans ma thèse de faire varier le paramètre délai dans des systèmes de cultures cellulaires ou de cultures d'explants où il est plus aisé de contrôler l'ensemble des paramètres qu'*in vivo*. Nous avons également pour hypothèses que d'autres paramètres comme la nature des virus concernés, les souches virales et le type de cellules cibles peuvent aussi avoir un impact sur les interactions entre les virus.

Dans le but de **définir le niveau de cette interaction entre le swIAV et le PRRSV et d'évaluer l'impact du délai et de l'ordre des infections sur cette interaction,** nos expérimentations ont été effectuées sur des cellules épithéliales connues pour être permissives au swIAV uniquement en modifiant le délais, l'ordre et les souches des virus utilisés dans les co-infections et les surinfections. Ces travaux constituaient la suite logique des deux dernières études menées sur des cellules permissives aux deux virus en même temps (PCLS, AM et la lignée de cellules épithéliales présentant le récepteur CD163) (Dobrescu et al., 2014; Provost et al., 2017).

À partir de ce stade, nous nous sommes posé une nouvelle question :

Le PRRSV n'infecte pas les cellules épithéliales et ne se réplique pas sur ces cellules, mais peut-il les stimuler ou affecter le devenir de leurs infections par le swIAV ?

Nos travaux avaient également pour but d'**étudier les souches locales qui circulent dans le nord-ouest de la France** et **contribuer à la compréhension et à la gestion des co-infections dans les élevages** de porcs de la région des pays de la Loire et de la Bretagne. Pour cela, nous avons utilisé une souche isolée en Finistère pour le PRRSV-1 et une souche isolée en Ile et Vilaine pour le swIAV. Les travaux de la thèse constituent les premiers travaux *in vitro* sur la co-infection comprenant le PRRSV de sous type 1 avec le swIAV.

Enfin une dernière hypothèse nous laisse penser que des phénomènes d'immunité entraînée (Netea et al., 2011; Kleinnijenhuis et al., 2014; Cerwenka and Lanier, 2016; Holmes and Bryceson, 2016; Yao et al., 2018) pourraient être induits après infections par les virus et pourraient aussi être à l'origine de l'hétérogénéité des résultats entre les différentes études.

Dans le chapitre 3, est présentée une étude de terrain qui a été effectuée dans le but d'**identifier les agents pathogènes circulant chez des porcs provenant d'un abattoir local** dans

un premier temps et **d'évaluer l'effet des différentes infections bactériennes et virales sur leur capacité à répliquer le PRRSV** dans un deuxième temps. La deuxième partie de ce chapitre présente une étude **évaluant l'impact d'une infection résolue par le swIAV sur l'immunité entraînée des AMs, leurs activités phagocytaires et sur la réplication virale suite à un contact avec un deuxième virus tel que le PRRSV ou le virus de la maladie d'Aujeszky** (*Aujeszky disease virus*, ADV).

Tous ces travaux favorisent la **compréhension des mécanismes d'interférence entre agents pathogènes et de la réponse immunitaire suites aux co-infections respiratoires chez le porc**. Cela permettra **d'en limiter l'impact en termes de santé dans les élevages porcins** et d'**adapter les recommandations** émises à l'égard des acteurs de la filière **sur la vaccination et l'usage des antibiotiques**.

Chapitre 1

Synthèse bibliographique

Chapitre 1 : Synthèse bibliographique

A- Les co-infections et leurs conséquences moléculaires au niveau du tractus respiratoire porcin

a) Introduction des travaux :

Les co-infections respiratoires sont à l'origine de la majorité des atteintes respiratoires chez le porc, et l'association de deux agents pathogènes semble très fréquente dans les élevages du monde entier (Choi et al., 2003; Fablet et al., 2012b). Ces associations altèrent la représentation classique d'une infection par un seul agent et affectent ses conséquences sur les signes cliniques et la réponse immunitaire de l'animal (Deblanc et al., 2012). Les travaux bibliographiques de ce chapitre consistent à définir le CRP, identifier les agents viraux et bactériens qui contribuent le plus à ce complexe et comprendre les mécanismes sous-tendants leurs interactions avec les cellules cibles. Un recensement des études *in vivo*, *in vitro* et *ex vivo* évaluant les co-infections respiratoires d'origine virale et bactérienne a ensuite été effectué. Une partie a été consacrée pour détailler les différentes combinaisons d'agents pathogènes, l'effet d'un agent sur la réplication de l'autre ainsi que leur effet sur les lésions, les signes cliniques et la réponse immunitaire de l'hôte porcin.

Les différents mécanismes d'interférence ont également été listés en développant les multiples scénarios des interactions directes et indirectes entre les agents infectieux bactériens et/ou viraux. L'analyse fine d'interactions permet une meilleure compréhension des conséquences des co-infections, une anticipation sur les répercussions dans les élevages, et une adaptation des recommandations et des protocoles de gestion.

Finalement, nous avons discuté les limitations et les difficultés rencontrées en proposant des recommandations aux chercheurs pour mieux adapter leurs protocoles et maîtriser les paramètres qui pourraient impacter la fiabilité des résultats.

b) Papier n° 1 : Revue de littérature

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VETERINARY RESEARCH

REVIEW Open Access

Coinfections and their molecular consequences in the porcine respiratory tract

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Abstract

Understudied, coinfections are more frequent in pig farms than single infections. In pigs, the term "Porcine Respiratory Disease Complex" (PRDC) is often used to describe coinfections involving viruses such as swine Influenza A Virus (swIAV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), and Porcine CircoVirus type 2 (PCV2) as well as bacteria like *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica*. The clinical outcome of the various coinfection or superinfection situations is usually assessed in the studies while in most of cases there is no clear elucidation of the fine mechanisms shaping the complex interactions occurring between microorganisms. In this comprehensive review, we aimed at identifying the studies dealing with coinfections or superinfections in the pig respiratory tract and at presenting the interactions between pathogens and, when possible, the mechanisms controlling them. Coinfections and superinfections involving viruses and bacteria were considered while research articles including protozoan and fungi were excluded. We discuss the main limitations complicating the interpretation of coinfection/superinfection studies, and the high potential perspectives in this fascinating research field, which is expecting to gain more and more interest in the next years for the obvious benefit of animal health.

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1 Introduction

Bacterial and viral respiratory diseases are a major health issue in species reared under confined conditions in large groups. Most often multiple infectious agents are involved in the development of these clinical conditions making unsuited the common reductionist approach of host–pathogen interactions by the study of single infection [1]. Infection by more than one type of pathogen (viruses, bacteria and parasites amongst others) is described as a mixed infection. However, the term coinfection is frequently used to describe concomitant infection of a cell or a host by separate pathogens [2]. Since in the literature the definitions of coinfection and mixed infection have been both used to describe the same events, we will use the term "coinfection" in the current review. Additionally, in virology, the term superinfection is used if one virus infects the cell or the host before infection by the second superinfecting virus. We will also use the term "superinfection" in the review. Finally, an opportunistic pathogen is usually considered as a pathogen that would not have infected animals in absence of the primary infection, or alternatively, "pathogen" that would have been asymptomatic in the absence of the primary infection. In some studies, however, the use of the terms "coinfection" is not suitable and "superinfection" should be used instead, as we will see later. This semantic point is responsible for a lot of confusion and makes comparisons between studies sometimes tricky.

The outcome of any coinfection or superinfection can be affected by the interactions taking place between the infectious agents, the nature of the cell/host, adverse environmental and management conditions, intestinal and respiratory microbiomes, and the triggered immune response—innate and adaptive—developed afterwards [2, 3]. When occurring at the same time or with a delay, infections can impact the virulence of causative pathogens with subsequent consequences on the host immune response and its ability to clear the infections [2]. The first contact with a pathogen can change the cell/host response against any other second pathogen, possibly causing a more virulent infection, reducing its severity or suppressing it completely [4]. Thus, different scenarios concerning the pathogen interactions can be observed, the first infectious agent can promote the second one, attenuate its effects or simply prevent its establishment. Conversely, the second pathogen may also influence the first one directly or indirectly.

Coinfections have been described in both humans and animals [1, 2]. Moreover, bacterial and viral infections might be followed by secondary bacterial or viral infections, which in some cases are responsible for the pathology development and the observed clinical signs. In this review, the current knowledge regarding frequent coinfections that occur in the porcine respiratory tract and particularly in the lungs are reviewed. When possible, we focused on the interactions between the mentioned pathogens and the various mechanisms justifying these interactions and their consequences on the host's response. We especially discussed coinfections involving main bacteria and viruses associated with the so-called porcine respiratory diseases, excluding coinfections involving parasites and fungi (including their metabolites, such as mycotoxins). Moreover, we do not discuss the impact of adverse environmental and management conditions which have been shown to be of major importance in the modulation of respiratory infections' severity [3].

2 Porcine respiratory disease complex and the associated pathogens

Respiratory diseases have been formally described in pigs as early as the 1960′s [5] and several studies have been carried out to identify associated agents. The role of the infectious pathogens has been assessed by using two main approaches: direct research of the pathogens (by culture or Polymerase Chain Reaction—PCR for instance) from tissue samples of diseased (acute or chronic stage) and non-diseased pigs or indirect detection by serological tests to look for antibodies produced after exposure to specific pathogens. These studies indicated that frequently under field conditions, several infectious pathogens are simultaneously detected from lung lesions (see [6–8] amongst others). Combinations of several infectious pathogens in particular bacteria and viruses frequently occur and are responsible for respiratory diseases in pigs reared under confined conditions in large communities [1]. However, the type of combinations and associated infectious agents change over time with the emergence of new viral pathogens generally complicating disease severity [e.g. new strains of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), new types of Porcine CircoVirus (PCV), new strains of Porcine Respiratory alphaCoronaVirus (PRCoV) and new reassortants of swine Influenza A Virus (swIAV)].

Causative respiratory infectious agents can be divided into primary and secondary or opportunistic pathogens. Primary pathogen being defined here as pathogen that can infect the animal as first unique pathogen and then facilitate secondary or opportunistic coinfection. These primary pathogens include common bacteria such as highly virulent *Actinobacillus* (*A.*) *pleuropneumoniae*,

M. hyopneumoniae, *Bordetella* (*B.*) *bronchiseptica* in young piglets and common viruses such as swIAV [1]. PRRSV and PCV2 are not strictly respiratory pathogens as swIAV, however, since they also frequently affect the respiratory system and since they can act as facilitators of secondary respiratory infections, they must be considered too. Other primary pathogens such as Aujeszky's Disease Virus (ADV) and PRCoV are reported but they are far less frequently encountered today or they have less impact on porcine health [1]. Then, some viruses like the porcine cytomegalovirus can also inhibit host immune functions—particularly the action of T lymphocytes—and promote respiratory diseases such as the porcine reproductive and respiratory syndrome [9]. Among the secondary pathogens common bacteria such as lower virulence strains of *A. pleuropneumoniae*, *A. suis*, *Glaesserella parasuis*, *Pasteurella multocida*, and *Streptococcus* (*S.*) *suis* are reported. Together primary and secondary pathogens are involved in the "Porcine Respiratory Disease Complex" (PRDC) [10].

Several studies have assessed the nature of the infectious agents directly or indirectly associated with respiratory diseases in pigs [7, 8, 11, 12]. In one of these studies involving breeding sows in five French farrow-to-finish herds [12], results indicated that *S. suis*, a secondary pathogen, was quite widespread among sows—67.1% of the animals being positive using a PCR assay—and PCV2 and swIAV infections were highly prevalent (75% of the sows with antibodies against PCV2 and between 91.7% and 100% of the sows with antibodies against swIAV). Other infectious agents such as *A. pleuropneumoniae*, *G. parasuis* and *P. multocida* were detected in 31%, 25%, and 23% of the sows, respectively [12]. In another study evaluating infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds in France, it has been shown that *M. hyopneumoniae*, PRRSV, and swIAV subtype H1N1 were the major pathogens involved in pneumonia-like gross lesions [8]. For extensive pleuritis, PRRSV was frequently associated with *A. pleuropneumoniae* [8, 12]. Regarding bacteria associated with lung lesions in 3731 French slaughter pigs [8], a report mentioned lesions of pneumonia and pleuritis as the most frequent lesions. In these lesions, bacteria such as *M. hyopneumoniae*, *P. multocida*, *A. pleuropneumoniae*, *S. suis*, and *G. parasuis* were detected in 69.3%, 36.9%, 20.7%, 6.4%, and 0.99% of the lungs, respectively [13]. In a retrospective analysis of the etiologic agents associated with respiratory diseases in pigs in USA, two or more infectious agents were identified in 88.2% of the analyzed cases [7]. PRRSV (35.4% of the samples), *P. multocida* (31.6%), *M. hyopneumoniae* (27%), swIAV (22.2%), *G. parasuis* (22.0%) and PCV2 (18.6%) were the infectious agents most frequently encountered [7]. In Korean pigs, PRRSV and PCV2 were frequently identified associated or not to various bacteria such as *S. suis* (25.2%), *M. hyopneumoniae* (20.1%), *P. multocida* (12.9%), and *A. pleuropneumoniae* (5%) [11].

Below we review the main primary pathogens as defined above, common viruses such as PRRSV, PCV2, swIAV, PRCoV and ADV as well as bacteria like *A. pleuropneumoniae*, *M. hyopneumoniae* and *B. bronchiseptica*. Conversely, other pathogens involved in the PRDC are not presented in the following sections while considered in Additional file 1 presenting the different coinfections' situations.

2.1 Porcine reproductive and respiratory syndrome virus

PRRSV is an enveloped single stranded positive RNA virus belonging to the *Arteriviridae* family. Two different species, PRRSV-1 (also known as Betaarterivirus suid 1), from European origin, and PRRSV-2 from American origin, are now distinguished [14]. This enveloped virus replicates mainly or exclusively in macrophages such as Alveolar Macrophages (AMs), but also macrophages from the nasal mucosa and Pulmonary Intravascular Macrophages (PIMs) [15, 16]. In vitro, PRRSV can also replicate in cultured monocytes and monocyte-derived cells including macrophages [17] and in vitro-derived Dendritic Cells (DCs) generated either from Bone Marrow hematopoietic cells (BMDCs) or blood Monocytes (MoDCs), depending on the in vitro culture conditions [18, 19]. However, such in vitro generated DCs are not representative of in vivo primary DCs which do not seem to be permissive to viral replication [20]. In fact, MoDC and BMDC (at least when generated using Granulocyte Macrophage Colony-Stimulating Factor, GM-CSF) although possessing functional overlaps with the DC family, do not represent *bona fide* DCs, which represent an own lineage of hematopoietic cells distinct from the monocytic lineage [21]. Different cell surface molecules are involved in PRRSV entry and infection of cells: heparan sulfate, porcine sialoadhesin—also known as sialic acid-binding immunoglobulin-type lectin 1 (Siglec-1), Siglec-10, CD151 and CD163 [22, 23]. Heparan sulfate is a GlycosAminoGlycan (GAG) that seems to play a modest or secondary role in PRRSV infection since the blocking of this receptor on AMs induced only a mild decrease in PRRSV infectivity. Moreover, this effect was not observed with all the PRRSV isolates tested, suggesting that the involvement of heparan sulfate depends on the antigenic diversity of PRRSV [22]. Siglec-1/CD169 is a member of the sialic acid-binding lectins (Siglecs) family and is expressed on macrophages [22] and Siglec-10 has been identified as an alternative receptor to Siglec-1 [23]. Binding of PRRSV to Siglecs induces its internalisation by clathrin-mediated endocytosis. Expression of

recombinant porcine sialoadhesin is sufficient to induce the internalisation of PRRSV by non-permissive cells, but not replication [24]. CD163 is a scavenger receptor involved in PRRSV infection [22]. Its expression on non-permissive cells makes them susceptible to infection with PRRSV and allows productive replication of the virus [22]. Moreover, CD169-KO animals are still susceptible to PRRSV-2 infection [22], whereas CD163-KO animals are resistant to PRRSV-1 and PRRSV-2 [25, 26]. Finally, MYH9 has been recently identified as an indispensable partner of CD163 for PRRSV cell entry for both PRRSV-1 and PRRSV-2 [27].

PRRS clinical signs can be nearly absent to severe depending on the considered PRRSV species and strains. When observed, there are, amongst the most frequent, lethargy, dyspnea, tachypnea, as well as a reproductive disease [16]. PRRSV can persist in infected pigs for several months after the initial infection particularly in lymphoid tissues and has the ability to alter the host's immune system to escape it (for review see [16]). PRRSV interferes with the porcine innate immune response through downregulation of type I InterFeroNs (IFNs—IFNα and IFNβ mostly), which are cytokines known for their antiviral properties [28]. PRRSV-infected macrophages also had a reduced capacity to produce the pro-inflammatory cytokines TNFα and IL1β [28] and the production of the anti-inflammatory cytokine IL10 was found enhanced during infection [29]. Nevertheless, the role of cytokine modulation during PRRS is unclear considering that the effects appeared to depend on the PRRSV species, as well as on the PRRSV isolates, since opposite results can be found with different PRRSV strains [30, 31]. In fact, some PRRSV-2 isolates were shown to enhance IFNα production while other PRRSV-1 isolates suppressed it. Results seemed also very variable for the immunoregulatory IL10 along different isolates of PRRSV-1 [30, 31], making general conclusions about how PRRSV alters innate immune responses difficult. PRRSV impact on adaptive cellular immunity seems also to be highly variable according to the species and the strain [20]. Conversely, whereas non-protective antibody response against the viral nucleocapsid is found within a week post-infection, neutralizing antibodies appearance is highly delayed for all PRRSV species and strains, appearing only after 3 or 4 weeks of infection and peaking even later [16].

2.2 Porcine circovirus type 2

PCV2 is a naked circular single stranded DNA virus belonging to the *Circoviridae* family and responsible for Porcine CircoVirus Disease (PCVD). The attachment of PCV2 to target cells occurs through chondroitin sulfate B and probably other receptors [32]. Internalisation is not fully known but it does not seem to involve a specific receptor and the GAGs could mediate internalisation and binding to the target cells [33]. Most of the time the infection is subclinical but in some circumstances such as coinfections with other respiratory pathogens it can cause the Post-weaning Multisystemic Wasting Syndrome (PMWS), clinically characterized by wasting respiratory disease, and enteritis [34]. Infection with PCV2 can occur in utero, resulting in stillborn piglets and mummified fetuses, or death at different ages after birth [34]. In young and older animals, PCV2 was found in cells expressing monocytes ($CD14^+$), and T and B cells ($CD4^+$, $CD8^+$, IgM^+) markers [35]. Further results showed that active replication of the virus was supported by T and B cells, with enhanced replication in proliferative cells [36]. In vitro, PCV2 can also infect many other cell types including endothelial cells, gut epithelial cells, fibrocytes, and DCs [37]. In DCs the virus seems to persist and remain infective for a prolonged period without replication indicating that these cells might serve as a vehicle for virus spread in the host [38]. PMWS is characterized by the depletion of lymphoid cells affecting T cells, B cells, and NK cells [39]. This lymphopenia was also associated with impaired responses of Peripheral Blood Mononuclear Cells (PBMCs) to mitogen stimulation with lower levels of IL2, IFNγ, and IL4 production compared to PBMCs from non-infected pigs [40]. Another feature of PMWS is an elevated level of IL10 found in lymphoid organs, especially in the T cells rich areas [41]. IL10-mediated immunosuppression could play an important role in the PCV2 infection and the development of PMWS. PCV2 has also the ability to alter the innate immune response [42]. Even though the virus does not productively infect DCs, evidence shows that it can interfere with the normal plasmacytoid DCs (pDCs) response. Upon stimulation with CpG-ODN, pDCs' ability to produce IFNα and TNFα was impaired in cells previously infected with PCV2 [43]. PCV2 DNA isolated from infected cells induced the suppression of pDC IFNα production [43].

2.3 Swine influenza A virus

Influenza A viruses are enveloped single stranded negative RNA viruses belonging to the *Orthomyxoviridae* family. These enveloped viruses can infect a broad range of hosts, with pigs being one of their natural hosts (for a review see [44]). The three main IAV subtypes encountered in pigs are H1N1, H1N2, and H3N2 [44], but many genetic lineages and antigenic variants within these subtypes are co-circulating in the pig population worldwide. Subclinical infections with swIAVs are common in pigs, but they can also induce a disease similar to what is observed in humans, with upper respiratory tract

distress associated with fever, cough, rhinitis, high morbidity, and low mortality [44]. The main targets of swIAVs are epithelial cells of the respiratory tract but IAVs can also non-productively infect alveolar macrophages [45]. Two major glycoproteins are present at the surface of the virus: HemAgglutinin (HA) and NeurAminidase (NA). Binding of HA with the sialic acid molecules at the surface of the host cells will induce the endocytosis of the viral particle [44]. The NA molecule plays the main role in the budding of the virus by removing the sialic acid, allowing the release of neoformed virus particles from the infected cell [44]. The innate response against the virus includes production of high levels of pro-inflammatory cytokines such as IFNα, TNFα, and IL6. DCs, in particular pDCs play an important role in this response [46]. An important observation was that the production of these cytokines correlated to the viral loads and the severity of the disease. Infection with swIAV induces cellular and humoral specific immune responses in pigs recovering from the disease and the serum IgG and the mucosal IgA can protect the animal from re-infection [44]. NS1 and PA-X are the main viral proteins that alter the innate immune response, mainly by blocking the type I IFN response [47] as well as the NLRP3 inflammasome activation [48] in infected-epithelial cells and alveolar macrophages. Finally, the main mechanisms through which the swIAV escapes the adaptive host immune system are the antigenic drift and the antigenic shift concerning mainly HA and NA which are also the two major antigenic proteins expressed on the surface of the virus and against which the neutralizing humoral response is directed [44].

2.4 Porcine respiratory alphacoronavirus

PRCoV is an enveloped single stranded positive RNA virus belonging to the *Coronaviridae* family. In pigs, four *Alphacoronavirus*, one *Betacoronavirus* and one *Deltacoronavirus* have been described [49, 50]. Thus, most of the porcine coronaviruses are from the genus *Alphacoronavirus*. The only respiratory porcine coronavirus, PRCoV, is a variant of Transmissible Gastroenteritis Virus (TGEV) where a large 5′ region deletion (nucleotides 621–681) in the Spike gene of the virus altered the tropism and the virulence. Even if pigs have been shown to be susceptible to the first SARS-CoV (serological evidence and isolation of the virus in a pig farm in the Xiqing County of Tianjin, China) [51] they have not been successfully experimentally infected, at this stage, by SARS-CoV-2 [52]. PRCoV uses aminopeptidase-N (CD13) domain IV to enter cells [53] and replicates to high titers in the lungs (1×10^7–10^8 Tissue Culture Infectious Dose 50—$TCID_{50}$) specifically in type 1 and 2 pneumocytes. Moreover, it can infect epithelial cells of the nares, trachea, bronchi, bronchioles, alveoli, and, occasionally, alveolar macrophages [49]. Infections with the PRCoV are usually subclinical, but there is variation between strains and some can induce a more severe disease. PrCoV can infect pigs of all ages by direct contact transmission or aerosol [49]. The clinical signs are associated to the respiratory system and are mild to severe—bronchointerstitial pneumonia—depending the strain and the context (environmental and management factors as well as the presence of other pathogens).

2.5 Aujeszky's disease virus or PseudoRabies Virus (PRV)

Suid herpesvirus 1, usually known as PRV or ADV is the responsible agent of Aujeszky's disease in pigs. It is a double stranded enveloped DNA virus from the *Herpesviridae* family and *Alphaherpesvirinae* subfamily targeting respiratory and/or genital mucosae for its replication [54]. ADV has a very broad host range varying from domestic animals like pigs, cattle, goats, sheep, cats and dogs to wild animals such as ferrets, foxes, hares, raccoons, and wild deer, and where it induces different diseases [54]. Infected animals usually show fever, sneezing, coughing and vomiting accompanied occasionally with typical nervous manifestations like convulsions, aggressiveness and lack of coordination. Mortality rate can reach 100% in suckling piglets while in mature pigs the infection is inapparent or mild [54].

ADV possesses eleven types of envelope glycoproteins playing major roles in the interaction with host cells and the induction of immune response [54]. Viral binding and fusion with the plasma membrane of the target cell—epithelial cells, neurons and alveolar macrophages—are controlled by a cascade of events orchestred by glycoproteins C (gC), gB, gD, gH and gL. The binding process starts with an interaction of gC with heparin sulfate proteoglycans [54, 55]. Stabilization of this interaction is then assured by the binding of gD to specific cellular receptors known as herpesvirus entry mediators such as HveA (TNFRSF14), HveB (PRR2, nectin 2), HveC (PRR1, nectin 1), HveD (PVR, CD55), and 3-*O*-sulfated heparin sulfate [54, 56]. At this stage, Tyrosine-based or dileucine-based endocytosis in parallel with clathrin-mediated endocytosis occur by the mediation of gB, gH and gL, leading to the penetration of the capsid and the tegument into the cellular cytoplasm. Finally, the interaction of the capsid with dynein leads to the release of viral DNA into the cellular nucleus after a transport along microtubules from the periphery to the nuclear pores [55].

Porcine humoral immune response is induced by ADV and neutralizing antibodies are mainly directed against gC [57]. Specific cell mediated immune responses are also triggered and MHC class I restricted, gC-specific,

cytotoxic cells are induced. ADV also alters the IFN signaling pathway by suppressing STAT1 tyrosine phosphorylation leading to an inhibition of IFN-Stimulated Genes (ISGs) expression [54, 57].

ADV may be involved in the PRDC and can be isolated alone or with other pathogens. Accordingly, a study conducted in Taiwan reported the association of ADV with PCV2 in 10.3% of the evaluated pigs using a multiplex PCR [58].

2.6 *Actinobacillus pleuropneumoniae*

Animals affected with this Gram negative bacterium develop a pleuropneumonia characterized by fibrinohemorrhagic necrotizing bronchopneumonia and fibrinous pleuritis which can reach a high mortality rate [59, 60]. Although the disease is best known in its acute/peracute forms, subacute and/or chronic presentations with low or no mortality are highly prevalent, especially in the presence of antibiotic treatments. Many herds are subclinically infected without previous or present episodes of clinical disease and in the absence of suggestive lesions at the slaughter house. Animals are, nevertheless, carriers of the pathogen. This happens in several conventional herds which may be simultaneously infected not only with several low/intermediate virulent strains, but also, in some cases, with strains highly likely to cause disease. In the latter case, outbreaks may suddenly appear in the presence of concomitant diseases or as a consequence of changes in management and/or environment [59, 60]. Eighteen serotypes of the bacterium have been described, which can all induce disease, although clear differences in virulence have been described [59, 60]. These bacteria can be found mainly in tonsils of carrier animals; virulent strains have a tropism for the lower respiratory tract where they preferentially bind to ciliated cells of the terminal bronchioli and pneumocytes [59, 60]. Different virulence factors expressed by *A. pleuropneumoniae* are involved in the colonization and the development of the disease. Adhesion to cells could be mediated by type IV fimbriae that are expressed upon contact with respiratory epithelial cells in vitro and during lung infection [59, 60]. Adhesion of *A. pleuropneumoniae* to respiratory epithelial cells also involves the binding of bacterial lipopolysaccharides to glycosphingolipids on the surface of the cells [59, 60]. The formation of biofilm by the bacteria is likely to play an important role in the colonization of the host [61]. After attachment to the target cells, the bacteria can produce four different pore-forming exotoxins (Apx I, II, III and IV) inducing the lysis of alveolar epithelial cells, thus allowing the acquisition of nutrients by the bacteria, but also participating in the development of the lesions [60, 62]. Some of the virulence factors expressed by *A. pleuropneumoniae* interfere with the host's immune response. The toxins Apx I, II and III induce the lysis of not only respiratory epithelial cells, but also of cells involved in the innate immune response such as macrophages and neutrophils [60, 63]. At lower concentrations, these toxins lose their lytic properties but can still impair macrophages chemotactic activity and their phagocytic abilities [64]. The capsular polysaccharides of *A. pleuropneumoniae* interfere with macrophage phagocytosis and enable resistance to complement-mediated killing [60]. *A. pleuropneumoniae* may also interfere with the antibody response by producing proteases that can degrade porcine IgA and IgG [59, 60].

2.7 *Mycoplasma hyopneumoniae*

This cell wall-free bacterium is considered to play a primary role in PRDC and is the causative agent of porcine Enzootic Pneumonia (EP), a disease with high morbidity but low mortality rates [65]. The main pathological mechanisms involved in *M. hyopneumoniae* infections are: (i) adhesion to the ciliated cells of the tracheal epithelium inducing ciliostasis, loss of cilia and exfoliation, dysregulation of cellular homeostasis (with increased intracellular calcium concentration) and secretion of cytotoxic factors, (ii) alteration of the mucociliary tract, (iii) inflammatory reactions sometimes exacerbated and prolonged, and (iv) manipulation of the innate and adaptive immune responses [65, 66]. Among the adhesins described in *M. hyopneumoniae*, P97 is reported to be a major determinant of cell adhesion [65–67]. Several other adhesins were reported: P102 linked to P97, LppS, LppT, MgPa, P65, P76, P110, P146, P159, and P216 [65, 66]. Most adhesins are transcribed and translated during *M. hyopneumoniae* infection and then undergo post-translational cleavage to result in diverse products on the membrane surface [65, 67, 68]. The diversity of surface proteins can also derive from the variation in the number of repeats in genes encoding adhesins [69]. These mechanisms of antigenic variation enable the bacterium to escape from immune system recognition and to invade the host [66]. Adhesins can also recruit extracellular matrix components (plasminogen, fibronectin and actin amongst others), and therefore can promote invasion and inflammatory response [65, 70].

The immune response induced against *M. hyopneumoniae*, may have a double action: over-activation of the local immune response resulting in a pathologic inflammatory reaction or local immunosuppression explaining the chronic nature of the associated pathologies [65, 66]. Acute *M. hyopneumoniae* infection leads to the recruitment and activation of various innate immune cells, essentially through the involvement of a large range of cytokines: IL1, IL6 and TNFα in lungs; CXCL8, IL1, IL2, IL4, IL6, TNFα and IL10 in Bronchus-Associated

Lymphoid Tissue (BALT) or TracheoBronchial Lavage Fluid (TBLF) [65, 71, 72]. Some of these inflammatory cytokines (TNFα, CXCL8, IL1β, IL6) are produced chronically in the lungs and play powerful roles in apoptosis (TNFα), differentiation and chemotaxis of neutrophils (respectively, IL6 and IL8), and macrophage activation (TNFα, IL1β). Chronic infections are typically associated with intense lymphoid hyperplasia [71] and are characterized by an accumulation of IgG- and IgA-expressing plasma cells, $CD4^+$ T cells, macrophages and DCs in the BALT of inflamed lung tissue [73]. Involvement of T cell activation in chronic inflammation is also supported by the presence of T-cell cytokines such as IL-2 and IL-4 in bronchoalveolar exudates [72].

In vitro studies conducted with macrophages co-cultured with *M. hyopneumoniae* highlighted a strong activation of inflammatory pathways inducing the production of cytokines and chemokines, and expression of receptors or pathways inducing cell apoptosis [65, 66, 74, 75]. Moreover, *M. hyopneumoniae* is described as an inhibitor of macrophages phagocytic activity, which may explain the chronicity of *M. hyopneumoniae* infections and the greater host susceptibility to other pathogens [65, 66, 74].

Mycoplasma hyopneumoniae was found to activate co-stimulatory molecule expression on *bona fide* DCs with poor TNFα production, contrasting with monocytes. Interestingly, a strong mitogenic activity for B cells was observed [76]. Altogether, these data indicate that *M. hyopneumoniae* is well sensed by the innate immune system, but the presence of immune evasion mechanisms targeting antigen presenting cells remains a possibility that needs further investigations.

Antibody responses after infection develop slowly and do not appear to correlate with protection [65, 66]. The literature on *M. hyopneumoniae* infections coupled with information from mouse models indicates that adaptive immune responses represent a fragile balance between pathogenic and protective Th- cell responses, probably belonging to the Th1 or Th17 types [65, 66].

2.8 *Bordetella bronchiseptica*

This aerobic Gram-negative bacterium can be found in the respiratory tract of several animal species and it presents a worldwide distribution in the porcine rearing [77]. *B. bronchiseptica* has a strong tropism for ciliated cells from the respiratory tissue and is mostly detected in the apical portion of the ciliated cells of turbinates, trachea and lungs [77, 78]. It can also be found in the cytoplasm of neutrophils and macrophages and rarely in the alveolar lumen associated with small tufts of cilia [77, 78]. Hence, infected pigs show cilia loss in the bronchial and bronchiolar epithelium associated with multifocal erosion, fibrosis, and hyperplasia. Neutrophil infiltrates are noted in the peri-conchal meatus and the submucosa of the bronchioles and alveoli, while lymphocyte and plasma cell infiltrations occur at the level of the *lamina propria* [77, 78].

Cell adhesion of *B. bronchiseptica* is a multifactorial process involving two main virulence factors; Filamentous HemAgglutinin (FHA) and PeRtactiN (PRN) [77, 79]. The expression of both adhesins is controlled by the *Bordetella* virulence genes (Bvg)AS signal transduction system. FHA is an adhesin with several binding domains including a carbohydrate- recognition domain responsible of the adhesion to macrophages and ciliated epithelial cells, a heparin-binding domain that mediates the binding to sulfated polysaccharides, and an Arg-Gly-Asp domain (RGD) regulating the InterCellular Adhesion Molecule 1 (ICAM1) by epithelial cells after interaction with the NF-κB signalling pathways [79]. This RGD domain is also present in the structure of PRN and contributes to the binding process [77, 79]. On the other hand, non-opsonic adhesion mechanisms play a role in binding to the host cells such as carbohydrate-specific mechanisms and those involving sialic acid-containing compounds [77].

Virulence of the bacteria depends on the strains; therefore, clinical signs can be different going from sneezing and transient nasal discharge for moderate and non-toxic strains to bronchopneumonia and atrophy of the nasal turbinate bones for virulent strains, especially if they are associated to other bacteria such as *P. multocida* [77]. Thus, *B. bronchiseptica* is usually described as primary lung pathogen in young pigs where it causes necrohemorrhagic bronchopneumonia whereas in older pigs this bacterium is mostly known as an opportunistic pathogen contributing to the PRDC [77]. The immune response against *B. bronchiseptica* is mainly triggered by the different toxins expressed such as adenylate cyclase, tracheal cytotoxin and DermoNecrotic Toxin (DNT).

3 Coinfections and superinfections and the resultant consequences for the porcine host

3.1 Selection and exclusion criteria for considered studies

In the following section and Additional file 1, we have used the published studies evaluating multiple infections including viral-viral, bacterial-viral, viral-bacterial and bacterial-bacterial respiratory coinfections and superinfections in swine. Both in vivo and in vitro studies comparing single to multiple infections were included. Studies evaluating vaccinations and the development of diagnostic techniques such as ELISA or qPCR were excluded as well as trials testing antiviral or antibacterial molecules when there was no clear comparison between

single and multiple infections. An attempt to present a synthetic view of coinfections is depicted in the heat maps (see Figures 1, 2, 3). However, we recommend readers to refer to Additional file 1 for each coinfection couple to get a more detailed view.

In these heat maps, we were interested in the effect of the first pathogen on the multiplication of the second one (named "assessed pathogen" in the figures) and on the host immune response and/or clinical signs. These effects were evaluated and a grade from −5 to +5 was given to every pathogen depending on the intensity of its impact on the multiplication of the second agent and on the immune response or on the clinical signs. Negative grades were given to pathogens decreasing the multiplication of other pathogens, while positive grades were given to those inducing an increase. Similarly, negative grades were attributed to pathogens with a tendency to decrease clinical signs or immune response related to the other pathogen. Positive grades were given in case of an increase. The sum of the grades was calculated if the same pathogen combination was evaluated in several studies except in the case of discordant results. This grading was represented in the following heat maps and the number of the identified studies for the same pathogen combination is shown on the maps. In the heat maps, other pathogens, that are less associated to PRDC such as *G. parasuis*, *M. hyorhinis*, *M. floculare*, *P. multocida*, and *S. suis* or even not considered as respiratory pathogens like *Staphylococcus aureus*, Classical Swine Fever Virus, Hepatitis E Virus, Porcine RubulaVirus, PPV, and Torque Teno sus Virus 1 have also been included. Indeed, these pathogens can also impact the outcome of respiratory infections and deserve, at least, to be mentioned.

3.2 The different types of coinfections

3.2.1 Virus–virus

Viral/viral respiratory coinfections have always had an important role in the porcine respiratory disease complex [1]. Several studies assessed the presence of two or more viral pathogens in pigs showing respiratory clinical signs in farms located in endemic regions [7, 8, 13]. The main viruses contributing to the porcine respiratory disease are swIAV, PRRSV, PCV2, and to a lower extent the PRCoV and the ADV. Due to their fast-spreading and

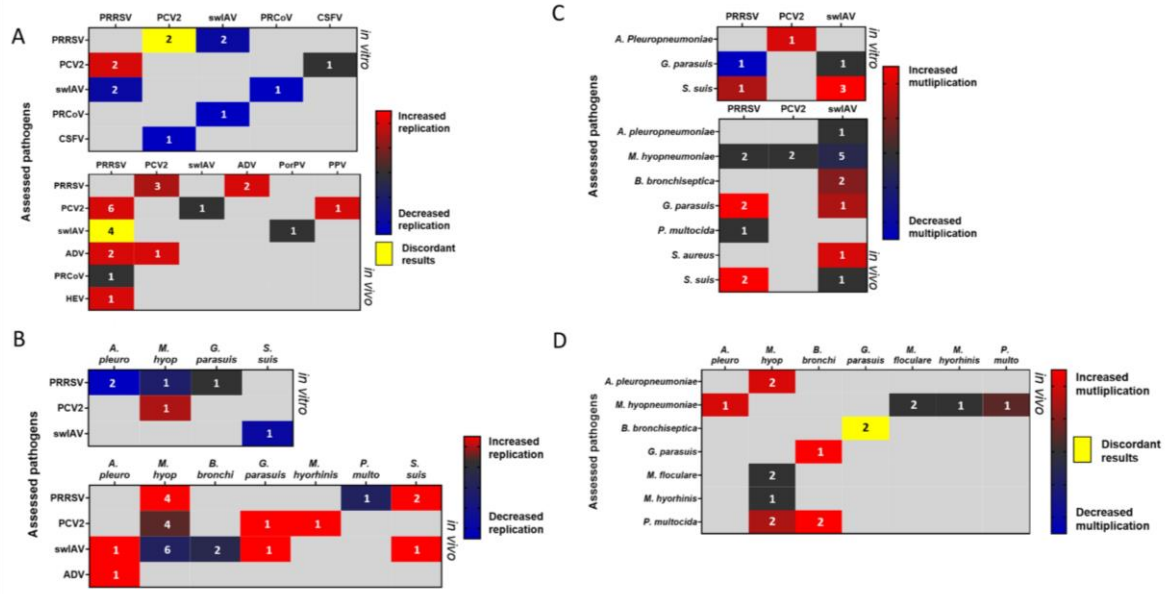

Figure 1 Impact of coinfection on multiplication/replication of the microorganisms. Heat maps depicting: **A** the impact of a secondary viral infection (top) on the replication of the virus (side) responsible of the primary infection in virus-virus dual infections (coinfections and superinfections) in vitro (up) and in vivo (down). **B** the impact of a bacterial infection on the replication of the infecting virus in virus-bacterium dual infections (coinfections and superinfections) in vitro (up) and in vivo (down). **C** the impact of a viral infection on the multiplication on the infecting bacteria in virus-bacterium dual infections (coinfections and superinfections) in vitro (up) and in vivo (down). **D** the impact of a secondary bacterial infection (top) on the multiplication of the bacteria (side) responsible of the primary infection in bacterium-bacterium dual infections (coinfections and superinfections) in vivo. The numbers shown on the maps correspond to the number of the identified studies for the same pathogen combination (see Additional file 1A and B). PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, swIAV: swine Influenza A Virus, PCV2: Porcine Circovirus type 2, ADV: Aujeszky's Disease Virus, PRCoV: Porcine Respiratory alphaCoronaVirus, CSFV: Classical Swine Fever Virus, HEV: Hepatitis E Virus, PorPV: Porcine RubulaVirus, PPV: Porcine ParvoVirus.

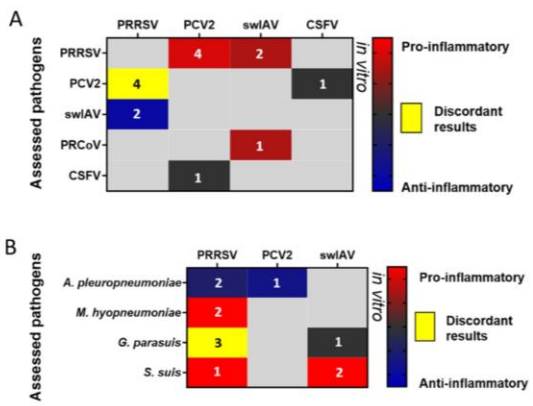

Figure 2 Impact of coinfection on inflammation. Heat maps depicting: **A** the impact of dual virus-virus infections (coinfections and superinfections) and **B** the impact of virus-bacterium infections (coinfections and superinfections) on the immune response of hosting cells (in vitro). The numbers shown on the maps correspond to the number of the identified studies for the same pathogen combination (see Additional file 1). PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, swIAV: swine Influenza A Virus, PCV2: Porcine Circovirus type 2, PRCoV: Porcine Respiratory alphaCoronaVirus, CSFV: Classical Swine Fever Virus.

their economic consequences, some viruses were more studied than others in the last 20 years, especially PCV2, PRRSV, and swIAV as shown in Additional file 1A and B. We will thus put more emphasis on these three viruses as causes of primary infections.

Many in vivo studies were carried out to assess the severity of the clinical signs and the development of the microscopic/macroscopic lesions. This approach enabled a comparison between coinfection/superinfection and single-infection conditions. Then, viral interference was progressively more frequently measured as a way to better understand the consequences of coinfections. In the last decades, the strong development of molecular biology and various tools enabled the evaluation of the immune response developed following polyviral infections.

In Additional file 1, the selected studies that were carried out on viral coinfections are presented from the oldest in vivo experiments to the latest in vitro and ex vivo experiments (Additional file 1A and B). This data synthesis highlights the major impact of PRRSV primary infection, which can both increase the titre of the following virus (PCV2, Hepatitis E Virus—HEV) in vitro [80] and in vivo [81–83] (Figure 1A), but can also worsen the clinical score associated to the disease (Figure 3A). Interestingly, even when the PRRSV does not increase the viral production of the other virus, as observed in coinfections

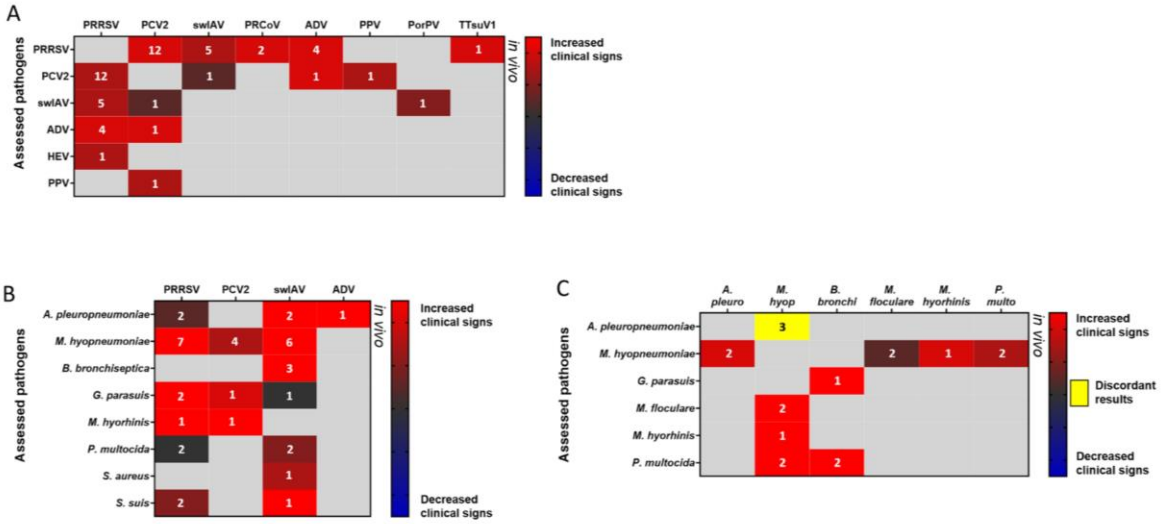

Figure 3 Impact of coinfection on clinical signs. Heat map depicting: **A** the clinical impact of dual virus-virus infections (coinfections and superinfections), **B** the clinical impact of virus-bacterium infections (coinfections and superinfections) and **C** the clinical impact of bacterium-bacterium infections (coinfections and superinfections) on the developed clinical signs (in vivo). The numbers shown on the maps correspond to the number of the identified studies for the same pathogen combination (see Additional file 1). *M. hyop*: *Mycoplasma hyopneumoniae*, *A. pleuro*: *Actinobacillus pleuropneumoniae*, *B. bronchi*: *Bordetella bronchiseptica*, *P. multo*: *Pasteurella multocida*, PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, swIAV: swine Influenza A Virus, PCV2: Porcine Circovirus type 2, ADV: Aujeszky's Disease Virus, PRCoV: Porcine Respiratory alphaCoronaVirus, HEV: Hepatitis E Virus, PorPV: Porcine RubulaVirus, PPV: Porcine ParvoVirus, TTsuV1: Torque Teno sus Virus 1.

involving swIAV [84] or PRCoV [85] (Figure 1A), it can also worsen the associated clinical signs. SwIAV and PCV2 as primary infectious agents have been less studied. However, it can be observed that swIAV can interfere with other virus productions (PRRSV and PRCoV) [85, 86] whereas PCV2 has some detrimental impact on the clinical outcome of secondary viral infections (PRRSV, swIAV, and PPV) [87–89] (see Additional file 1 and Figure 1A). Then, regarding the inflammation induced in coinfection conditions, various outcomes were observed depending which viruses were considered (Figure 2A).

3.2.2 Bacterium–virus and virus–bacterium

Many in vitro and in vivo experiments, with different bacterium-virus and virus-bacterium combinations, have been performed to identify the underlying mechanisms of the PRDC (see Figures 1B, C, 2B, C, and 3B). The main studies are presented in Additional file 1C and D.

Bacterium-viral coinfections can also involve various primary respiratory pathogens. Among them, the most frequently studied bacterium is *M. hyopneumoniae*, a pathogen that induces a chronic respiratory disease and can influence the outcome of a subsequent viral infection. However, mycoplasma infection needs to be already well established in the respiratory tract at the time of the viral infection to potentiate it. Indeed, *M. hyopneumoniae* inoculated to pigs simultaneously or shortly before the virus did not strongly impact the severity of the viral infections (PCV2, swIAV, PRRSV) [90–92], while its impact was clearly evidenced when inoculated 3 weeks before viral infections [93].

It is well-known that viral infections can induce an ideal environment for a bacterial superinfection through different mechanisms such as the destruction of the epithelial barrier, the over-expression of the receptors involved in the bacterial adhesion to the cells, and the alteration of the host immune response [1, 2, 94, 95]. The swIAV infection has been shown, for instance, as a way to facilitate the colonization of epithelial cells by *S. suis*, but only for the serotypes containing sialic-acid in their capsule [96]. The swIAV infection induces a loss of ciliated cells leading to the impairment of the mucociliary clearance function, but induces also the presence of the viral HA on the surface of infected cells that interacts with the sialic acid of the bacterial capsule, leading to increased adherence of *S. suis* [96, 97]. Although these swIAV effects on *S. suis* have been clearly shown in vitro, no clear in vivo impact of swIAV infection on *S. suis* pulmonary load has been described [98]. It was clearly shown that the presence of both pathogens significantly induces more inflammation than single infections [98, 99].

Overall, studies carried out in pigs showed that a bacterium-virus or a virus-bacterium coinfection frequently induces an aggravation of pulmonary lesions (Figure 3B) and a higher inflammation (Figure 2B) and immune response, with increased production of pro-inflammatory cytokines. In many bacterium-virus and virus-bacterium associations, this worsened outcome seems to be the result of additive effects from both pathogens rather than a real synergy [100, 101]. However, a potentiation of the viral infection by bacteria can also be observed in other cases, such as in the *M. hyopneumoniae*-PRRSV coinfection [102]. In that case, higher amounts of PRRSV genomes were detected in lymphoid tissue and blood [102] and a slower viral clearance was observed [75] (Figure 1B), suggesting that the recruitment of immune cells in the lung parenchyma upon established *M. hyopneumoniae* infection may provide a steady supply of susceptible cells for PRRSV [1]. Then, in porcine AMs and in the "African green monkey" (originally described as porcine origin) St-Jude Porcine Lung (SJPL) cell line, PRRSV infection has been shown to be blocked by a pre-infection with *A. pleuropneupmoniae*, this antiviral activity being due to the *A. pleuropneumoniae* metabolites [103] (Figure 1B). Given the fact that in vivo studies involving PRRSV and *A. pleuropneumoniae* did not always investigate the impact of an *A. pleuropneumoniae* pre-infection on the subsequent PRRSV infection [104, 105], as done in experiments performed in vitro [103], it cannot be easily concluded if this interference would be observed in the target species. However, in vivo, PRRSV-*A. pleuropneumoniae* interactions were reported as absent or mild [104, 105] (Figure 3B).

3.2.3 Bacterium–bacterium

In virus-bacterium coinfections, the dogma usually encountered is that viruses play an immunomodulatory role, which favors bacterial superinfections. Nevertheless, a pre-disposing effect is also described for *M. hyopneumoniae*, which promotes viral but also bacterial superinfections [65] (Figure 1D). Few studies of experimental coinfections or superinfections with *M. hyopneumoniae* and/or other bacteria involved in PRDC were performed compared to coinfections involving viruses. These studies are reported in Additional file 1E. Overall, these coinfections or superinfections induce more clinical signs and lung lesions and poorer technical performances when compared to single infections with the same infectious pathogens (Figure 3C). The bacterial-bacterial coinfections are also responsible for immune response alterations (for reviews see [106, 107]). For example, macrophages from pigs infected by *M. hyopneumoniae* decrease their phagocytosis capacity against *A. pleuropneumoniae* [60, 65]. *M. hyorhinis* and *M. flocculare*, two mycoplasmas commonly co-isolated with *M. hyopneumoniae* in gross pneumonia-like lesions, may

also impact the immune response by inducing the cytotoxicity of immune cells and/or the secretion of cytokines affecting its outcome [108]. Co-stimulation of porcine BMDCs with *M. hyopneumoniae* and *M. hyorhinis* induces a strong IL12 production. In this last in vitro model, *M. hyopneumoniae* associated with *M. flocculare* reduces TNFα production compared to BMDCs stimulation by *M. flocculare* alone producing a TNFα concentration greater than that observed after stimulation with *M. hyopneumoniae* alone and *M. hyorhinis* alone [108]. Therefore, *M. flocculare* might play an initial role in pulmonary inflammation by inducing the production of TNFα by resident myeloid cells. Supplementary investigations will be needed to elucidate the role of this cytokine in the pathogenesis of the disease [108]. Other examples of bacterium-bacterium in vivo coinfections are presented in Additional file 1E.

3.3 Mechanisms of coinfections interferences

Regarding coinfections and superinfections, most studies assessed the clinical outcome of the process but less is known about the mechanisms of interactions between pathogens and the consequences for the pathogens themselves, the infected cells and more generally for the host.

The outcome of dual infection is variable depending on the antagonism, neutrality or synergy between the infectious agents. On the host side, coinfection can make the host response ineffective, and vice versa. If we look now at the possible interactions that can occur between pathogens we have to consider the nature of the infectious agents (summary provided in Figure 4). Different situations can be observed and coinfections can involve virus with virus, bacterium with virus and vice versa, and bacterium with bacterium.

3.3.1 Virus–virus interactions

Regarding virus–virus interactions, consequences are diversified and many studies looking at virus replication in coinfection situations have been carried out [2]. The first consequence of coinfection could be the so-called viral interference, a situation whereby one virus interferes with the replication of the other one making the cells resistant to the superinfecting virus [109].

The most common way for viral interference is indirect and based on the production of type 1 and 3 IFNs which induce the expression of ISGs after interacting with their cognate receptors [110, 111]. These proteins then activate numerous mediators of the cellular antiviral system that may non-specifically block the replication of viruses.

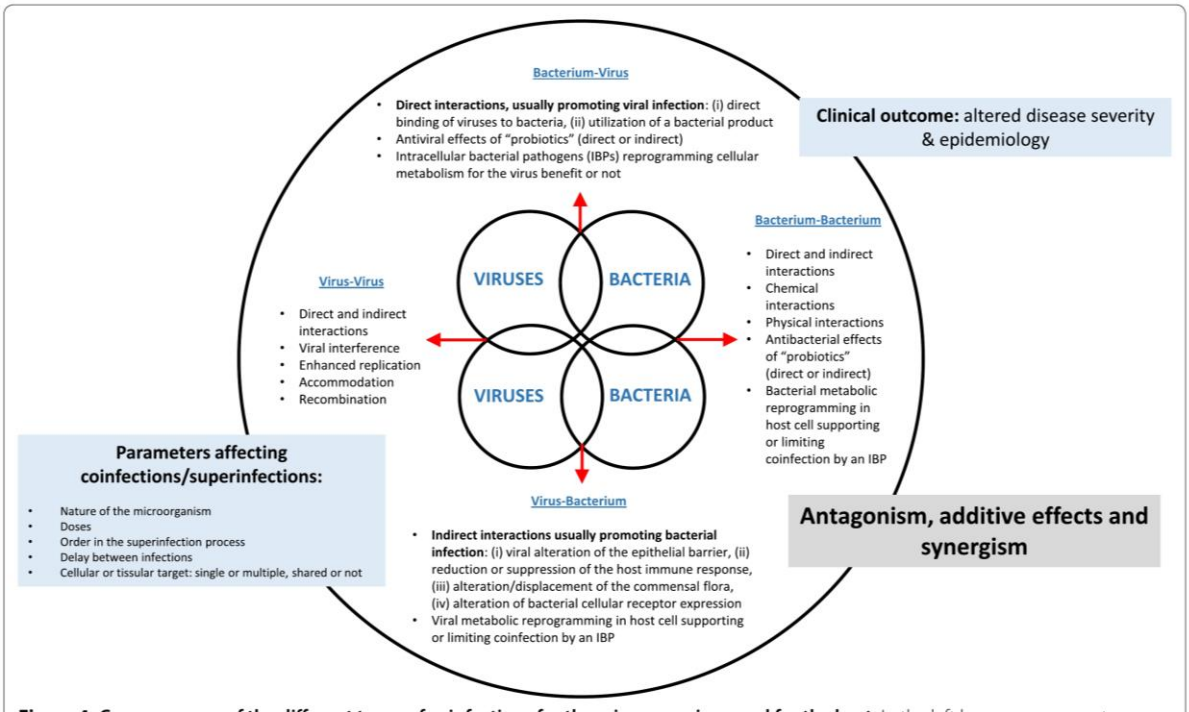

Figure 4 Consequences of the different types of coinfections for the microorganisms and for the host. In the left box some parameters enable to affect coinfections and superinfections are listed. IFN: Interferon, IBP: Intracellular Bacterial Pathogen.

They may also interfere, to a certain extent, with bacterial multiplication since IFN can also be induced by Intracellular Bacterial Pathogens (IBPs) or some extracellular bacteria [107, 112]. Nevertheless, in some situations type 1 IFNs can also increase the host susceptibility to subsequent bacterial infection [113] through impaired macrophage recruitment with a reduced CXCL1 and CXCL2 transcription [114] and a reduced IL17 [115] production. Then, there is also the non-interferon-mediated viral interference (or intrinsic interference) which is a cellular state of resistance induced by the virus to new viral infection by closely related or unrelated viruses [116]. Various mechanisms are described to explain this cellular state of direct or indirect resistance (for examples see [2]). In this type of interference, which can occur between viruses but also between viruses and bacteria [107, 117], there is a competition between pathogens for the metabolites and all the host factors that allow their multiplication. Besides the mechanisms involving a competition for common cellular factors, there are also several other mechanisms of interference described. These relies on viral Defective Interfering (DI) particles [118], RNA interference (RNAi) [119], non-specific double stranded RNA (dsRNA) [120] as well as trans-acting proteins [121]. Interference can occur at specific steps or multiple steps of the viral replication cycle (attachment, penetration, genome replication and/or budding) and can be direct or indirect. Inhibition of superinfection (superinfection exclusion and superinfection suppression) is one of several consequences that can be observed in the interference between related and unrelated microorganisms. In superinfection exclusion, an established infection interferes with a subsequent, closely related infection [2, 122]. An example of this phenomenon in pigs is the exclusion of highly virulent classical swine fever virus strain Margarita in wild boars persistently infected with this virus upon a challenge infection with the same Margarita strain [123]. Superinfection suppression is a quite close concept where this time persistently infected cells resist to a challenge with a heterologous virus [2]. Furthermore, when the host immune response—innate or adaptive—is considered in the study of the complex interactions taking place in viral coinfections, additional mechanisms of indirect interference linked to cellular and humoral cross-protection—resulting from a first viral contact with a wild-type or a vaccine strain—can be described.

Conversely, in some situations, viral coinfections can directly or indirectly result in enhanced replication and virulence for one or both pathogens as observed in several studies involving porcine viruses [80, 83, 124–126]. In other cases, coinfection/superinfection has no effects on virus replications and the viruses can coexist in a relation called accommodation [2]. Besides consequences in terms of viral replication, there are also consequences for the genetic of the viruses and their evolution through events of recombination between closely related viral genomes. Recombination, the parameters influencing it and its consequences were reviewed in RNA and DNA viruses [127, 128]. Then, as a result of all these possible interactions between viruses, the severity of the resulting disease and its epidemiology can be altered as exemplified in Additional file 1. In the pig studies, most often, however, the exact mechanisms controlling interactions between viruses were not elucidated.

3.3.2 Bacterium–virus and virus–bacterium interactions

Several mechanisms explaining bacterium–virus and virus–bacterium interactions have been identified (for reviews see [1, 94, 117]). The interactions can have either a positive or a negative impact on both pathogens depending on the bacterial and viral species involved. Usually, when the interactions are direct they promote viral infection without affecting the bacterial species [1, 94, 117]. Examples of these direct interactions are (i) direct binding of the virus to a bacterium or (ii) the utilization of a bacterial product by the virus. An example of direct interactions in the respiratory tract is the cleavage of the IAV HA into HA1 and HA2 by a *Staphylococcus aureus* protease helping the viral particle to become infectious [129]. On the contrary, when interactions are indirect they often provide an advantage to bacterial infections. Four mechanisms dealing with indirect interactions have been described: (i) viral alteration of the epithelial barrier, (ii) reduction or suppression of the host immune response, (iii) viral alteration/displacement of the microbiota, and (iv) virus-induced alteration of bacterial cellular receptor expression [94]. All these mechanisms can operate together for the benefit of the superinfecting bacteria. A typical example of these indirect interactions is provided by PCV2 and swIAV and porcine pathogenic bacteria such as *A. pleuropneumoniae* [130] and *S. suis* [96, 97, 131] where the bacteria benefit from the prior viral infections. However, bacteria can also directly benefit from a previous viral infection as observed in a study demonstrating that *Staphylococcus aureus* was able to bind viral HA [132]. The consequence of that binding was an enhanced bacterial internalisation by two mechanisms: (i) binding to HA exposed at the surface of infected cells, and (ii) binding to free extracellular virions.

In some other situations, non-pathogenic bacteria can also directly or indirectly protect the host from viral infection as typically observed with probiotic bacteria which can show antiviral activity through the binding/capture of the viruses and/or the competition for cell adhesion (for a review see [117]). This type of interaction

has been frequently observed with enteric bacteria [117] and an example in pigs is the reduced infection of IPEC-J2 cells by vesicular stomatitis virus after pre-incubation of the cells with multiple probiotic bacteria [133]. An intriguing relationship is occurring between IBP and viruses where metabolic reprogramming in host cells triggered by viruses might support or conversely limit coinfection by an intracellular bacterial partner (for a review see [107]). Different possibilities can be identified in that type of interaction [107]: (i) the first pathogen can reprogram cellular metabolism related to cellular immunity and decrease the defense against the other pathogen, (ii) the metabolic changes triggered by the first pathogen can facilitate the adhesion, the penetration, and the replication of the other, and (iii) the coinfection transform the active replicative state of the first pathogen into a stable persistent state. The first possibility associated to a decrease of the cellular defense is a commonly accepted mechanism [134] while the second and the third possibilities are less experimentally demonstrated [107].

3.3.3 Bacterium–bacterium interactions

Looking at bacterium–bacterium interactions, they are extremely complex to assess because of the large diversity of the bacterial world and because little is known about the mechanisms underpinning these interactions during infections. Moreover, it is now also clear that intestinal and respiratory microbiomes affect the interactions between pathogenic bacteria and the porcine host [135]. Some examples of the complex interactions occurring in bacteria–bacteria coinfections are presented in Additional file 1E, but little is known about the mechanisms controlling these interactions. However, some mechanisms were provided above and interesting reviews dealing with that subject were published recently [106, 107] discussing the possible direct interactions between bacteria—mainly chemical and physical. Indirect interactions between bacteria were not reviewed in these articles but were discussed to some extent in other review papers focusing on polymicrobial infections [1, 136].

3.4 Limitations of coinfection studies

The first observation coming from this review must be, even if several studies have been carried out on the subject, a lack of data about some specific coinfections and many discrepancies between studies. For instance, there are only a few in vivo studies about PCV2 in virus/virus coinfections and about PCV2 and PRRSV in virus/bacterium coinfections (Additional file 1 and heat maps in Figures 1, 2, 3). Discrepancies are not surprising because of the definition of coinfection is not always the same between studies in addition to huge variations in the coinfection parameters amongst studies.

In this review we focused on experimental (in vivo and in vitro) coinfections, it is worth to underline that these studies are inspired by field veterinarians and epidemiologists observations. However, the definition of epidemiologist coinfection can also vary between studies. Indeed, in some cases there is concomitant direct identification of two microorganisms in the same animals, sometimes in the same farms, while in other cases it is just an indirect identification of the microorganisms' presence at some points through indirect serological assays. Moreover, as stated before, the term coinfection is sometimes used to describe some situations of superinfections where the delay can be significant.

Regarding the experimental parameters, the Multiplicities Of Infection (MOI), the strains, the potential delays between successive infections, the routes of inoculations, the types of cellular hosts considered (more or less susceptible to one of the microorganism), the genetic background (breeds) and the sanitary status (specific pathogen free or conventional breeding) of the pigs, and the readout to assess coinfection outcome varied a lot between studies. To fully compare studies, a standardization of the assays would be needed. Interestingly also, whereas in vitro studies' usefulness is not in question, it is important to underline here that in vitro observed interplay between pathogens cannot be automatically applied in vivo. For instance, whereas *M. hyopneumoniae* decreases the PRRSV titre in vitro [75], it increases PRRSV shedding in vivo and indeed worsens the clinical signs upon coinfection [102]. Consequently, the use of intermediary settings, such as co-culture of different cell types (see [2] for examples), Precision Cut Lung Slices (PCLS) [137] or organoids [138], could help to understand the complexity of coinfections in the respiratory tissue. In vitro approaches usually consider one or a few cell types with some limitations during the evaluation process of the coinfection consequences. Some viruses can contribute to the elimination of other viruses just because of their ability to replicate faster on a particular cell type [139]. Thus, results obtained in vitro cannot mimic the field situation when both agents coinfect the same pig, providing inaccurate conclusions about the coinfection dynamics. Under such circumstances, pathogens may simply have different host cells and no longer be under direct competition for resources [140]. Besides the different interactions that infecting agents can have between them through a competition to resources, studies showed clearly that the immune system and the immunological responses can highly affect these interactions by inducing the competitive power of a pathogen or abolishing it and making it less competitive on the resource [141, 142]. The effects of the immune system (especially humoral parameters) are often not taken

into consideration in selected in vitro models [140]. On the other hand, in vivo coinfection experiments have to deal with numerous constraints (health status of the animals used, cost, husbandry, and ethics amongst others) and therefore are not always easy to perform. Hence, although in vivo experiments are required in this very complex field, they surely need to be combined to in silico/in vitro/ex vivo analyses of potential interactions between pathogens. Moreover, multiple parameters of the coinfection protocols appeared difficult to set without any a priori such as the choice of the pathogen that will be inoculated first and the delay between infections. One possibility to deal with multiple parameters is to use intra-host infection mathematical modelling [143] allowing to play, at limited cost, with the different parameters of the coinfections. However, these models need to be fed with data coming from conventional in vitro experiments as well as more complex in vivo studies. The other possibility is to rely on field prevalence studies monitoring the very presence of the pathogens (isolation, PCR) instead of the sero-conversion, in order to have a clear epidemiological picture of when and where coinfections occur.

Consequently, ex vivo models such as PCLS generated from freshly sacrificed pigs [137] or organoids [138] are developing. These models are closer to mimic the in vivo situation than usual in vitro approaches, combining different types of cells and providing the pathogens with a wider range of cell hosts. However, the contribution of the immune response to the interaction between different pathogens is rarely considered [97]. Furthermore, the MOI cannot be controlled because the number of infected cells in the slice or the organoid cannot be monitored easily either.

Another limiting factor in coinfection studies is the cell regeneration, which can vary between in vivo and in vitro models. Cell regeneration can highly affect the dynamics of a coinfection, giving some pathogens extra target cells guarantying their longer existence while contributing to the clearance of others [140]. Finally, other potential technical limitations could always be discussed such as the lack of precision or sensitivity in the different diagnostic techniques especially in the presence of multiple agents. Hence, the detection of coinfecting pathogens could be compromised or reduced as compared to their detection level in the context of single infections.

4 Conclusion and perspectives

As shown in this review many works have been dedicated to the study of coinfections and superinfections in pigs. Usually, when the experiments were carried out in vivo, the researchers were more interested in the clinical outcome than in the interactions occurring between pathogens. Indeed, in most of the cases the fine interactions between pathogens and especially the mechanisms behind these interactions and its potential consequences, at the molecular level, on the immune response were not studied for several reasons including technical limitations. Also, in the studies assessing the occurrence of coinfections/superinfections in the fields, coinfection identification based on molecular tools such as PCR would be more accurate than sero-prevalence approaches which are less prone to identify currently present pathogens and thus coinfective pathogens. Then, a better knowledge of each pathogen involved is crucial. We thus would like to make recommendations for future studies dealing with respiratory coinfections in pigs: (i) Authors should clearly summarize their coinfection or superinfection experimental setup—doses of pathogens, delays between infections—in their Materials and methods section; (ii) in this summary they would need to clearly present the pathogens they use and they should, as often as possible, select well-characterized strains; (iii) environmental and management conditions would need a strict control and monitoring; (iv) animal genetic and sanitary status would need to be carefully described and monitored during the study; and (v) the multiplications of all the pathogens shall be followed during the experiment using highly sensitive and specific assays. A clear description of all these parameters would help the scientific community to compare studies and progress in the understanding of the complex interactions between microorganisms.

In the last years, the concept of innate immune memory or trained immunity has gained a lot of interest. This concept is coming from old observation, in 1946 [144], recognizing that the bacterial vaccine strain "*Bacille de Calmette et Guérin*" (BCG) was protecting not only against *Mycobacterium tuberculosis* but also against antigenically different microorganism causing childhood mortality, suggesting an "adaptation" of the cellular innate immune system. Since then, many interesting studies about innate immune memory or trained immunity have been published (for a review see [145]) and it is recognized that cells such as myeloid cells, NK cells, and even epithelial cells [146] can have a higher and quicker response upon re-exposure to a pathogen. Trained immunity is accompanied by epigenetic changes and most often associated with modifications in cellular metabolism. A close look at potential epigenetic changes and cellular metabolism modifications would be of high interest in respiratory coinfection studies in the porcine species. Recently an alternative to the mechanism of trained immunity in resident lung innate immune cells named "epigenetic legacy" has been described [147]. In that study, the authors demonstrated that following IAV clearance and clinical recovery (1-month post-infection),

mice were better protected from *Streptococcus pneumoniae* infection by adult bone-marrow-derived AMs displaying transient transcriptional and epigenetic distinct profiles. This newly described consequence of a first viral infection also needs additional studies about PRDC with an identification of the mechanisms shaping the complex interactions between pathogens.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13567-020-00807-8.

Additional file 1. Studies about coinfections in the pig respiratory tract and their consequences.

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All the authors were involved in the writing of the review. GS, NB, and FM generated the figures. GS, CD, JB, CF, CM-C, GSi, NB and FM prepared the Additional file 1. All the authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

a) Ce qu'il faut retenir :

Ce chapitre montre une grande variabilité entre les nombreux agents pathogènes respiratoires et leurs interactions en cas de co-infections. Les *heat maps* résumant les résultats des différentes études sur les co-infections respiratoires ont montré :

- Certaines contradictions au niveau des conséquences de la même co-infection (B. *bronchiseptica*/G. *parasuis*, PRRSV/PCV2 et swIAV/PRRSV) sur la réplication/multiplication du même micro-organisme.
- Une aggravation des signes cliniques dans la majorité des co-infections à l'exception de la co-infection *M. hyopneumoniae*/*A. pleuropneumoniae* et quelques co-infections qui ne montrent pas de variation au niveau des signes cliniques par rapport aux infections simples (swIAV/*G. parasuis* et *P. multocida*/PRRSV).
- Une contradiction au niveau de la réplication du swIAV, mais qui est toujours traduite par l'augmentation des signes cliniques en présence du PRRSV.

Toutes ces contradictions peuvent être expliquées par les variations qui existent entre les différentes souches utilisées et par les différences de l'ordre et du délai entre les 2 infections. La maîtrise de tous ces paramètres d'expérimentation reste une tâche très complexe et un obstacle dans la réalisation des expérimentations sur les co-infections respiratoires du porc. Viser une plus grande maîtrise est cependant essentielle pour limiter l'expérimentation animale, réussir à mimer les infections naturelles et bien comprendre les différentes interactions entre les agents pathogènes.

c) Données supplémentaires

Tableau 1. *Description of experimental assays carried out to study viral/viral coinfections and superinfections involved in PRDC (*in vitro + ex vivo*)*

Coinfections	Protocols	Selected cells or tissues	Identified target cells	Viral interference	Immune responses	Reference PMIDs
swIAV **PRRSV**	swIAV: H1N1 Sw/Saskatchewan/18 789/02 PRRSV: ISU-12-SAH Interv: 3h/Inf: 15h +18h	NPTr-CD163	NPTr-CD163	PRRSV reduced the réplication of swIAV in coinfection and when inoculated 3h after. swIAV decreased réplication of PRRSV when inoculated 3h before.	Both RNA viruses interfere with each other. PRRSV primary infection has less effect than swIAV primary infection. No clear synergistic nor additive effects observed IFNλ1 are more expressed than other IFN.	28757015
	swIAV: H1N1 Sw/Saskatchewan/18 789/02 PRRSV: ISU-12-SAH Interv: 3h/Inf: 18h	Lung slices AM	swIAV: Epithelial cells PRRSV: Pneumocytes type 1/MΦ No coinfected cells	PRRSV réplication suppressed by swIAV PRRSV did not alter swIAV réplication in PCLS but reduced its réplication in AMs.	PRRSV reduced the response to swIAV. swIAV showed low impact on PRRSV infection. swIAV alters the ISG expression but not PRRSV. PCLS, synergy for TLR3, RIGl, and IFNβ expressions	24418046
swIAV **PRCoV**	swIAV: H1N1 A/sw/Bad Griesbach/IDT5604/2006 and H3N2 A/sw/Bissendorf/IDT 1864/2003 PRCoV: Bel85 Interv: 0h/Inf: 72h	NPTr Lung slices	NPTr more permissive to swIAV than to PRCoV	Viral titers in coinfections were lower than in single-infections.	swIAV single- and coinfections with PRCoV showed stronger effects on ciliary activity than PRCoV single-infections.	28779714
PCV2 **PRRSV**	PCV2: Local field strain	AM	AM permissive to both viruses	PRRSV and PCV2 réplication enhanced	The coinfection induced IκBα degradation and phosphorylation → induced NF-κB activation.	27080155

	PRRSV: JS-1 Interv: 2h/Inf: 6h to 96h					
	PRRSV: VR2385 PCV2b: NC-16845 Interv: 0d/Inf: 24h	MoDC	MoDC	ND	DCs develop a regulatory phenotype upon PCV2 + PRRSV coinfection. IL10 production increased in coinfection. CD86 decreased in coinfection. PDL1 increased in co- and PCV2 single-infection.	26446939
	PRRSV: VR2385 PCV2a: ISU-40895 Interv: 0h/Inf: 36h	DC	DC	ND	DC permissive to PCV2 + PRRSV PRRSV coinfection increased Treg lymphocytes. TGFβ is upregulated in coinfection condition.	22633482
	PRRSV: tw91 PCV2: Local strain Interv: 0h/Inf: 18, 36, 54, 72, 90, 108h	AM	AM permissive to both viruses	PRRSV infection rate was reduced in the presence of PCV2. However, the infectivity of PCV2 was unaffected.	Cell death and apoptosis were reduced in coinfection conditions compared to PRRSV single infected AMs. The reduction of PRRSV infection by PCV2 is related to the increased production of IFNα.	15936905
PCV2 **CSFV**	PCV2: HZ0201 CSFV: HCLV Interv: 0/Inf: 72h	PK15 ST AM	PK15 and ST permissive to both viruses AM permissive to PCV2 and not to CSFV	PCV2 not influenced by CSFV PCV2 suppressed CSFV.	No effect of coinfection on apoptosis	26431319

swIAV: swine Influenza A Virus, PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, PCV2: Porcine Circovirus type 2 (subspecies a and b when indicated), CSFV: Classical Swine Fever Virus, PRCoV: Porcine Respiratory Coronavirus, AM: Alveolar Macrophage, NPTr: Newborn Pig Trachea cell line, DC: Dendritic Cells, MoDC: Monocyte Derived Dendritic Cells, PK15: Pig Kidney cells 15, ST: Swine Testicle cells, Interv: Interval, Inf: Duration of the infection assay

Tableau 2. *Description of experimental assays carried to study viral/viral coinfections and superinfections involved in PRDC (*in vivo*)*

Coinfections	Protocols	Clinical signs	Macroscopic lesions	Microscopic lesions	Other observations	Reference PMIDs
PRRSV swIAV	PRRSV: 10PL01 swIAV: H1N1 A/swine/Thailand/CU-PL65/2010 PRRSV then swIAV Interv: 6 days	No major differences in clinical signs were registered except a prolonged fever in coinfected pigs compared to single-infected ones.	Coinfected group showed higher lung lesion scores than PRRSV single-infected group.	Interstitial pneumonia was more severe following infection with PRRSV. Additionally, coinfected animals showed epithelial necrosis and mononuclear cells infiltration in the alveolar septum.	-H1N1 viral load was more important in coinfected group compared to single-infected.	(no PMID) The Thai Journal of Veterinary Medicine, 49(1), 71-79
	PRRSV: VR2385 swIAV: H1N1 A/Swine/IA/40776/92 PRRSV: at 4 and 7 wks of age swIAV: at 7 wks of age	-Fever and reduced weight gain in coinfected animals -Coinfected animals had the worst clinical disease.	Coinfected pigs showed a high percentage of swIAV-induced pneumonia.	Coinfection induced important interstitial pneumonia.	-No increase in swIAV shedding -Increased lymphocyte proliferation after PRRSV infection -PRRSV infection reduced swIAV vaccine efficacy.	19595522
	PRRSV: Lelystad swIAV: H1N1 A/Sw/Belgium/1/83 PRRSV then swIAV Interv: 3, 7, and 14 days	-Differences in the clinical signs between coinfections and single infections were negligible. However, weight gain tends to be less important in coinfected animals.	ND	ND	-The time interval between both infections can affect the clinical outcome. Multiple infections with an interval of 3 or 7 days resulted in an acute disease while an interval of 14 days between the viruses resulted in a subclinical infection. -Differences between coinfected and single infected pigs were negligible.	15129583
	PRRSV: Lelystad swIAV: H3N2 PRRSV then swIAV	ND	Coinfected pigs showed larger bronchiolar and	Coinfection induced more pronounced inflammation of the bronchiolar wall.	PRRSV infection had no effect on swIAV infection.	9220621

	Interv: 1 wk		lung lymph nodes than single infected.			
	PRRSV: Lelystad swIAV: H1N1 A/Sw/Belg/ l/83 PRRSV then swIAV Interv: 3 days	More severe clinical signs in coinfected animals than others	ND	ND	swIAV excretion delayed by PRRSV infection (2 days)	9054128
	PRRSV: PL15-33 swIAV: H1N1 A/Poland/Swine/14131/2014 Simultaneous infections	Most severe clinical signs in the coinfected pigs	ND	ND	-The mean PRRSV load in BALF was higher in PRRSV-infected pigs than in coinfected pigs. -IL6 and IL10 transcripts were upregulated in their expression in coinfected pigs but not in PRRSV-infected animals.	31934657
PRRSV ADV	PRRSV: EDRD-1 strain ADV: YS-81 strain PRRSV then ADV interv: 14 days	More severe clinical signs in coinfected pigs	Macroscopic lesions like brain congestion, pneumonia and thymus atrophy were more important in coinfected pigs.	Coinfected pigs showed more pronounced microscopic lesions (encephalitis, pneumonitis, tonsillitis and lymphadenitis).	ADV réplication is enhanced by PRRSV.	16423576
	PRRSV: EDRD-1 strain ADV: YS-81 strain PRRSV then ADV interv: 14 days	Clinical signs were more severe in the coinfected group.	Conifected pigs showed higher macroscopic lesions such as thymic atrohy, brain congestion and diffused pneumonia.	Neuronal microscopic lesions were more important incoinfected pigs.	Shedding of both viruses was enhanced in coinfected group.	15511536
	PRRSV: E4 strain ADV: Yamagata-S81 strain PRRSV then ADV Interv: 7 days	Prolonged febrile response in coinfected pigs	Coinfected pigs showed mottled or diffusedly tan and red lungs with failure to collapse.	More severe pneumonic lesions in coinfected group	PRRSV excretion was higher with coinfection conditions.	12710495
	PRRSV: Lelystad virus ter huurne ADV vaccine: strain M141 ADV: wild-type NIA-3	PRRSV pre-infected pigs only showed fever	ND	Lymphoproliferative response to ADV was delayed and	Inhibition of ADV viral shedding by vaccination not affected by PRRSV	10973691

	PRRSV then ADV vaccine then ADV challenge interv: 2 weeks then 8 weeks simultaneously	following ADV vaccination.		reduced but not inhibited in co-infected pigs.		
PRRSV PRCoV	PRRSV: Lelystad PRCoV: 91V44 PRRSV then PRCoV Interv: 3 days	More severe clinical signs in coinfected animals than others	ND	ND	PRCoV excretion not affected by PRRSV	9054128
	PRRSV: SD23983 PRCoV: ISU-1 PRRSV then PRCoV Interv: 10 days	Clinical signs were more severe in coinfected animals.	ND	ND	Coinfected pigs showed: -Synergistic decrease in NK-cytotoxicity. -Higher production of IL6, IL10 and TGF-β than other pigs. -Increased myeloid cells and CD4+CD8+ lymphocytes.	20883160
PRRSV TTsuV1	TTSuV1 natural infection + PRRSV vaccination + PRRSV challenge Interv: 4 wks	Prior infection with TTSuV1 increases PRRSV clinical signs.	Macroscopic lung lesions more severe in case of TTSuV1 natural infection	ND	TTSuV1 suppresses immunization by PRRSV vaccines and exacerbates PRRSV clinical signs.	22327391
PCV2 **swIAV**	PCV2: ADDLPP 10069 swIAV: H1N1 PCV2 then swIAV Interv: 7 days	Higher clinical scores in coinfected animals for a longer time	ND	No difference between coinfected and single-infected groups	-swIAV did not affect the réplication of PCV2. -No difference in the antibody titer to PCV2 in the presence of swIAV	20158948
PRRSV PCV2	PRRSV: HF6-7 PCV2: THF601-7 Simultaneous infections	More severe clinical signs in coinfected pigs	More severe lesions in coinfected pigs	More severe lesions in coinfected pigs	-Positive synergistic effect on the CD14 mRNA expression in coinfection condition	26022073
	PRRSV: KS62 PCV2b: isolate 06-06274	Coinfected pigs were divided into worst and best clinical outcome	The worst clinical outcome coinfected pigs showed interstitial	In the worst clinical outcome groups, coinfection induced an	-PRRSV/PCV2 coinfection increased the rate of *Bacillus cereus* in the serum.	27139023

Simultaneous infections	groups. The best outcome group consisted of pigs without any clinical disease.	pneumonia, granulomatous nephritis, multifocal fibrous adhesions in the abdomen and between the lungs.	interstitial pneumonia and a lymphoid depletion.	-PRRSV and PCV2 réplication was higher in the worst outcome group. -The microbial diversity was lower in the worst clinical group.	
PRRSV: MLV vaccine PRRSV: KS62 PCV2b: isolate 06-06274 Vaccination then simultaneous infections	Coinfection clinical signs were first reduced but increased later.	ND	After 22 days, PCV2 infection induced depletion of lymphocytes.	PRR vaccination decreased PRRSV viremia but increased PCV2 viremia.	26446422
4 PCV2 vaccines PCV2b: SNUVR000463 PRRSV: SNUVR090851 Simultaneous infections post-vaccination	Coinfected group showed tachypnea, abdominal breathing, and severe dyspnea. No clinical signs in all vaccinated groups	Vaccinated groups showed less pulmonary lesions than coinfected non vaccinated group.	ND	-Coinfection prolonged severe clinical respiratory signs similarly to field PRDC. -The vaccination against PCV2 was efficient in PCV2/PRRSV coinfected pigs.	24403524
PRRSV: HBR PCV2b: YJ PRRSV then PCV2b and PCV2b then PRRSV Simultaneously or 7 days of delay	PRRSV/PCV2 group showed higher clinical scores than PCV2/PRRSV group.	PRRSV/PCV2 group showed the most severe haemorrhages. Dead pigs showed swollen, brown kidneys, and pulmonary congestion.	PRRSV/PCV2 group showed the most severe histological lesions.	-PRRSV/PCV2 group showed the highest viral loads and to the lowest antibody titers.	23971711
PRRSV: VR2385 and NC16845b PCV2: 40895 and NC16845 Simultaneous infections	Coinfection induced more fever.	More severe macroscopic lung lesions for coinfected groups than others.	Increased levels of lymphocytes in coinfected pigs	-More severe lung lesions in coinfected pigs than others -Stronger anti-PRRSV IgG response in response to coinfection	22406346

PRRSV: VR2385 PCV2: 4089 Simultaneous infections	No differences between groups	Coinfection induced higher severe lung lesions than PRRSV single infection.	Microscopic lesions were more severe in PCV2/PRRSV than single infection group.	/	20637549
PRRSV: VR2385 PCV2a: 40895 PCV2b: NC16845 Simultaneous infections	Coinfected pigs showed mild respiratory signs	Some coinfected pigs showed lymphohistiocytic interstitial nephritis.	Mild lung lesions in coinfected pigs	-PRRSV prolonged the infection with PCV2 and increased its réplication. -No differences between the two PCV2 subtypes	21641124
PCV vaccines PCV2: 40895 PRRSV: ISU12 Simultaneous challenges	No difference in clinical signs between coinfected group and PRRSV single infected	Coinfected group showed the highest lung lesions. Vaccination reduced theses lesions in coinfected pigs.	The highest scores of microscopic lesions in the coinfected group animals.	-PCV2 single-infected pigs showed higher levels of Anti-PCV2-IgM than coinfected group.	18430525
PRRSV: HB-2 (sh)/2002 PCV2: BJ-HB Simultaneous infections	Clinical signs in the coinfection group were more pronounced.	Gross lesions were more sever in coinfection group.	ND	-Coinfection prolonged the disease, increased the réplication of both viruses, and decreased the antibodies production and the number of WBCs.	18164875
PRRSV: Olot/91 PCV2: / PRRSV then PCV2 7 days of delay	Coinfected pigs suffered from growth retardation and fever.	ND	Coinfection has aggravated the lymphocyte depletion and the histiocytic infiltration.	-PRRSV increased the réplication and the propagation of PCV2 in the lymphoid tissue and in the blood.	18164875
PRRSV: NADC-20 PCV2: 35358 Simultaneous infections	Coinfection mortality rate of 100% on day 20 compared to 26% for PCV2 and 0% for PRRSV alone	Coinfected pigs showed more pronounced hepatic lesions and severe proliferative interstitial pneumonia.	Microscopic lesions in coinfected pigs were also present in PCV2 single-infected pigs.	-ND	11572560

PCV2 **ADV**	ADV: YN1 strain PCV2: ND Simultaneous infections	Coinfection increased clinical signs severity and mortality rate.	Higher neurologic and respiratory macroscopic lesions in the coinfected group	Microscopic lesions were more severe in coinfected pigs.	PCV2 increased ADV shedding in lungs and brain.	31585642
PCV2 **PPV**	PCV2: ISU-40895 PPV: NADL-8 2 PPV vaccinations before coinfection Simultaneous infections	Coinfected animals had more pronounced fever.	Some coinfected pigs showed icterus, bleeding gastric ulcers, and pneumonia.	Lymphoid depletion was more important in coinfection conditions.	-Vaccination for PPV enhanced the réplication of PCV2 in coinfected pigs. -Vaccination against PPV did not affect the severity of PCV2 clinical signs.	15036529
HEV **PRRSV**	HEV: Genotype 3 PRRSV: Finistère Half of the animals inoculated / half infected by contacts Simultaneous infections	ND	Coinfection caused dramatic increase in HEV lesions at slaughter time	ND	-PRRSV increased HEV shedding. -Immune response delayed -PRRSV increased HEV transmission and infection susceptibility. -Coinfection leads to chronic HEV infection.	26048774
PorPV **swIAV**	H1N1: H1N1 A/Swine/New Jersey/11/76 PorPV: PAC-3 PorPV then H1N1 Interv: 44 days	Coinfected pigs showed increased clinical signs.	No macroscopic lesions were registered.	All groups presented interstitial pneumonia hyperplasia of the bronchiolar lymphoid tissue.	-Both viruses infect the bronchiolar epithelium. -PorPV enhances the spread of H1N1 in the respiratory tissues without affecting the shedding.	26854342

swIAV: swine Influenza A Virus, PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, PCV2: Porcine Circovirus type 2 (subspecies a and b when indicated), CSFV: Classical Swine Fever Virus, PRCoV: Porcine Respiratory Coronavirus, TTsuV1: Torque Teno sus Virus 1, HEV: Hepatitis E Virus, PPV: Porcine Parvovirus, PorPV: Porcine Rubulavirus, WBCs: White Blood Cells, PRDC: Porcine Respiratory Disease Complex

Tableau 3. *Description of experimental assays carried to study bacterial/viral coinfections and superinfections involved in PRDC (*in vitro + ex vivo*)*

Bacterium	Virus	Conditions	Cell types	Target cells	Réplication/multiplication	Immune responses	Reference PMIDs
Mycoplasma hyopneumoniae	PRRSV	HP-PRRSV: NJGC Mhp: XLW-1 PRRSV then Mhp Interv: 1h, Duration: 6h, 15h	AM	AM	ND	-Several pathways are induced in coinfected cells.	25445346
		PRRSV: VR2385 Mhp: 91-3 Duration: 24 and 48h	AM	ND	Mhp decreased the réplication of PRRSV in AM at 24h.	-Increased production of IL1β, IL8 and IL10 in coinfection conditions	11356254
	PCV2	PCV2: 2010AHCY Mhp: WX, AH, NJ, TH, XLW-1, and 168 PCV2 then Mhp, reverse, and simultaneous infections Interval: 0h to 24h, Duration: 72h	PK15	Both pathogens detected in the same cell	PCV2 réplication was enhanced by subsequent inoculation with Mhp but not by prior or simultaneous co-inoculations.	-ND	27033909
Actinobacillus pleuropneumoniae	PRRSV	PRRSV: IAF-Klop App: S4074 serotype 1 PRRSV then App Interv: 4h, Duration: 48h	MARC-145 SJPL AM	3 cell types permissive to PRRSV	PRRSV infection of SJPL and AM blocked by a pre-infection with App	-Enhanced cytotoxicity with coinfection -Anti-PRRSV activity due to App metabolites -IFNγ contributed to this antiviral activity.	24878741
		PRRSV: IAF-Klop App: MBHPP147 from S4074 PRRSV then App culture supernatant App culture supernatant for 2 hours then PRRSV then App culture supernatant Interv: 4h, Duration: 48h	MARC-145 SJPL AM	3 cell types permissive to PRRSV	Pre-treatment of AM with App culture supernatant reduced PRRSV réplication in AM and SJPL but not in MARC-145 cells.	-The treatment of AM with App culture supernatant before the PRRSV infection decreased mRNA expression of IFNα and IFNβ.	29293082
	PCV2	PCV2: CC1 App: L20 PCV2 then App or App then PCV2 or simultaneous Interv: 0h or 2h, Duration: 2h and 4h	AM	AM are infected by PCV2 and App.	App invasion and adhesion to AM was enhanced by PCV2.	-Clearance of App was reduced in coinfection conditions. -Reduction of TNFα, IL4, and IFNγ production in coinfection conditions	31176418

Glaesserella parasuis	swIAV	swIAV: H3N2 A/swine/Spain/SF32071/2007 Hp: SW114 (serovar 3) or Nagasaki (serovar 5) strains swIAV then Hp or Hp alone Interv: 1h, Duration: 1 or 8h	BMDC	-BMDC internalises Hp and swIAV. -Hp and swIAV in the same phagolysosome	ND	-No clear impact of swIAV pre-infection	23157617
	PRRSV	PRRSV: Lelystad- CAPM V-490 Hp: HP 132 - CAPM 6475 PRRSV then Hp Interv: 24h, Duration: 28h and 48h	AM	AM	ND	-Increased IL1β, IL8, and TNFα production in coinfection situation -Coinfection decreased ROS production.	26358898
		PRRSV: Lelystad - CAPM V-490 Hp: HP 132 - CAPM 6475 PRRSV then Hp Interv: 24h, Duration: 28h and 48h	AM MDM	MDMs are more sensitive to PRRSV than AM.	Réplication of PRRSV not affected by Hp but coinfection reduced the growth of Hp.	-Coinfected macrophages produced less ROS than PRRSV single-infected cells.	28472979
		PRRSV: Lelystad Hp: HP 132 - CAPM 6475 PRRSV then Hp Interv: 24h, Duration: 28h and 48h	AM MDM	MDMs and AM are permissive to PRRSV.	MDMs mortality is not affected by Hp in coinfection conditions.	-Coinfection of MDMs: Reduced gene expression and production of IL1β and IL8 compared to single infections Reduced mRNA expression of CD86, CD14, and CXCL10 compared to Hp single infection Increased mRNA levels of TNFα -Unlike AMs, MDMs increased expression of IFNα following PRRSV infection.	30322536
Streptococcus suis	swIAV	swIAV: H1N1 A/swine/St-Hyacinthe/148/1990 Ss: 31533 swIAV then Ss Interv: 12h, Duration: 12h	NPTr	ND	ND	-CCL5, IL8, VCAM1, and COX2 significantly more upregulated in the presence of both pathogens compared to single-infection	24708855

	swIAV: H1N1 A/sw/Bad Griesbach/IDT5604/2006 and H3N2 A/sw/Herford/IDT5932/2007 Ss: 10 swIAV + Ss or Ss alone Interv: 0h, Duration: 72h	Lung slice	Ss selectively attached to swIAV-infected cells.	-swIAV promoted bacterial adherence and colonization. -Bacterial adherence facilitated by swIAV -swIAV-impairment of the mucociliary barrier plays a major role in promotion of bacterial infection.	ND	25916988
	swIAV: H3N2 A/swine/St-Hyacinthe/148/1990 Ss: 31533 swIAV then Ss Interv: 12h, Duration: 24h	NPTr	Colocalization of Ss and swIAV	Bacterial adhesion and invasion significantly increased by swIAV	-Higher levels of CCL2, CCL4, IL6, IL8, and TNFα in coinfected cells than in mon-infected	24082069
	swIAV: H1N1 A/sw/Potsdam/15/1981, H1N1 A/sw/Bad Griesbach/IDT5604/2006, H3N2 A/sw/Herford/IDT5932/2007, H3N2 A/sw/Damme/IDT5673/2006, H1N1 A/Swine/Shanghai/1/2005 Ss: 10 and 10 cpsΔEF swIAV then Ss Interval: 2h or assay: up to 72h	NPTr	Co-localization of Ss and swIAV	-swIAV enhanced bacterial adhesion. -Ss adhesion increased via the capsular α-2,6-linked sialic acid recognized by HA expressed by swIAV-infected cells -Bacterial coinfection had a negative effect on the réplication of swIAV.	ND	26297001
PRRSV	PRRSV: IAF-Klop Ss: P1/7 PRRSV then Ss Interv: 2h, Duration: 5h, 12h	BMDC Monocytes	-BMDC more permissive to PRRSV than monocytes -Ss intracellular	-PRRSV impaired the phagocytosis of Ss.	- Increased pro-inflammatory response of BMDC to Ss after a pre-infection with PRRSV -Additive effects for CCL4, CCL14, CCL20, and IL15 -Synergistic effects for IL6 , CCL5, and TNFα -Little effect of PRRSV pre-infection on monocyte response to Ss	27213692

BMDC: Bone Marrow Dendritic Cell, NPTr: Newborn Pig Trachea, PK15: Porcine Kidney 15, MDMs: Monocyte Derived Macrophages, ROS: Reactive Oxygen Species, SJPL: St-Jude Porcine Lung cell, swIAV: swine Influenza A Virus, PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, PCV2: Porcine Circovirus type 2 (subspecies a and b when indicated)

Tableau 4. *Description of experimental assays carried to study bacterial/viral coinfections and superinfections involved in PRDC (*in vivo*)*

Bacterium	Virus	Protocols	Clinical signs	Macroscopic lesions	Microscopic lesions	Pathogen detection	Results	Reference PMIDs
Mycoplasma hyopneumoniae	**swIAV**	swIAV: H1N1 A/Swine/IA/40776/92 Mhp: 232 Mhp then swIAV Interv: 21 days	Coinfected pigs coughed significantly more than the others.	More severe pneumonia in coinfected pigs than in others	No difference	Similar distribution	No impact on antibody levels in serum	11427564
		swIAV: H1N1 A/Sw/Hok/2/81 Mhp: E-1 Mhp then swIAV Interv: 7/21 days	Clinical signs (coughing and fever) were more observed in coinfected pigs.	H1N1 infection, 7 or 21 days after Mhp infection, resulted in a greater percentage of dark red-lung lesions.	Bronchial epithelial lesions and interlobular oedema only observed in the dual infection groups	-No impact of swIAV on the Mhp titers -No impact of Mhp on virus duration shedding	No impact of Mhp on the antibody levels against H1N1	15036530
		swIAV: H1N1 A/Sw/Cotes d'Armor/0231/06 and H1N2 A/Sw/Cotes d'Armor/0113/06 Mhp: 116 Mhp then swIAV Interv: 21 days	Mhp pre-infection increased influenza clinical signs.	Mhp increases the mean lesion score for the Mhp+H1N1 group not for the Mhp+H1N2 group.	Exacerbation of bronchial pneumonia and superinfection with cellular exudates in the alveoli, more marked in the lungs of Mhp+H1N1 group than in Mhp+H1N2 group	-No impact of Mhp on both swIAV shedding nor on H1N2 multiplication in lungs -At 7dpi H1N1 detected in co- but not in single inoculated pigs -Less Mhp in the Mhp+H1N2 group than in the Mhp or Mhp+H1N1 groups	Higher humoral immune response to H1N2 infection in the Mhp+H1N2 group than in the H1N2 group at 7dpi swIAV	22261237
		swIAV: H1N1 A/Sw/Cotes d'Armor/0231/06 Mhp: 116 Mhp then swIAV Interv: 21 days No single-infected group in this study	Some clinical signs were observed in the coinfected group (fever, cough and decreased mean weight gain).	Extensive pneumonia lesions	bronchiolitis, broncho-interstitial, and interstitial pneumonia	Detection of both pathogen in all lung lobes	Mhp infection induced an oxidative stress before H1N1 infection.	23266108
		swIAV: H1N1 A/Sw/Cotes d'Armor/0231/06	Feed-restricted pigs presented shorter	All coinfected pigs had macroscopic	All coinfected animals developed bronchiolitis,	-Mhp genome detected in all lung lobes -swIAV genome detected	Both infection and feed restriction reduced postprandial glucose	25101681

	Mhp: 116 Mhp then swIAV Interv: 21 days No single-infected group in this study	hyperthermia and a positive mean weight gain over post-H1N1 infection whereas animals fed *ad libitum* lost weight.	lesions of pneumonia.	broncho-interstitial and interstitial pneumonia.	in nasal swabs and lung tissues	concentrations, indicating changes in glucose metabolism.	
	swIAV: H1N1 A/Sw/Cotes d'Armor/0231/06 Mhp: 116 Mhp then swIAV Interv: 21 days No Mhp single-infected group in this study	No impact of the Mhp pre-infection on clinical signs usually induced by swIAV	No impact of the Mhp pre-infection on pneumonia lesions	Earlier and more extended inflammatory lesions in coinfected compared to single-infected pigs	No impact of the Mhp pre-infection on viral excretion and multiplication	Coinfected pigs presented an earlier influx of CD163+ cells in cardiac lobes and a greater influx of neutrophils and of pro-inflammatory cytokines in BALF than single-infected pigs.	27498789
PRRSV	PRRSV: MN-30100 and MN-184 Mhp: 232 Mhp then PRRSV Interv: 21 days	Coinfection exacerbated the disease and increases the duration and the severity of the clinical signs.	ND	ND	Mhp increased the réplication of PRRSV in lymphoid tissue and blood.	ND	16506914
	PRRSV: VR2385 Mhp: 11 Interval: few hours	Coinfection PRRSV/Mhp increased clinical signs of respiratory disease.	Dual infection PRRSV/Mhp increased pneumonia lesions.	Microscopic lesions (Mhp) were most severe in coinfected pigs.	No observation regarding coinfections	No observation regarding coinfections	10649626
	PRRSV: VR2385 Mhp: 232 Interv: 0	Clinical disease was more severe in coinfected pigs.	Pneumonia was more frequent and pronounced in coinfected pigs.	No difference registered between single and dual infections	-PRRSV detection was prolonged in the presence of Mhp. -Mhp infection was not altered by PRRSV.	-IL12 and IL10 transcript expressions were higher in coinfected pigs than in single-infected. -Higher production of IFNγ and IL10 in BAL fluids in coinfected animals than others	14583150

	PRRSV: VR2385 Mhp: 11 Mhp and PRRSV simultaneously or with an interval: -Mhp 21 days before PRRSV -Mhp 10 days after PRRSV	Pigs infected with both Mhp and PRRSV had more severe clinical. respiratory disease.	Coinfection induced an acute interstitial pneumonia.	PRRSV infection increased the microscopic lesions typical of Mhp.	-No evidence of increased numbers of cells containing PRRSV antigen in tissues with Mhp lesions -No significant differences in Mhp titer between groups	-PRRSV infection increased the severity of the Mhp-induced pneumonia. -Mhp-infected pigs showed increased PRRSV-induced pneumonia lesions.	9986823
	PRRSV: VR2385 Mhp: 232 Simultaneous infections	Increased clinical respiratory disease and slower viral clearance	Coinfection increases the percentage of lung showing visible lesions.	ND	ND	-The pigs coinfected with PRRSV and Mhp had increased levels of IL1β, IL8, IL10, IL12, and TNFα.	15358650
	PRRSV: MN-30100 and MN-184 Mhp: 232 Simultaneous infections	Coinfection with PRRSV and Mhp increased the severity of the clinical disease.	ND	ND	-Large quantities of virus were detected in the aerosols	-Coinfection did not influence the concentration of PRRSV in aerosols.	17042383
	PRRSV: IND-5 Mhp: P5722-3 Interv: 7 days	No difference between single and coinfected pigs	ND	No difference between single and coinfected pigs	ND	-PRRSV infection did not increase the severity of Mhp infection in piglets.	8734647
PCV2	PCV2: ISU-40895 Mhp: 232 Mhp then PCV2 Interval: 14 days	Dual-infected pigs had moderate dyspnea, lethargy, and reduced weight gain.	Increased severity of pneumonia lesions in coinfected pigs	Higher PCV2-associated microscopic lesions in lung and lymphoid tissues of coinfected pigs than others	-Mhp pre-infection increased the amount of PCV2.	-Higher serum antibody response to Mhp and higher antibody response to PCV2 in dual-infected pigs	15557072
	PCV2: ISU-40895 Mhp: 232 Mhp then PCV2 Interv: 14 days	Dual-infected pigs showed mild-to-severe respiratory disease. No difference with Mhp single-infected pigs	Pigs infected with Mhp (alone or in association) had higher lung lesion scores than pigs infected with PCV2.	Lymphoid depletion and histiocytic infiltration in tracheobronchial lymph nodes in groups infected with PCV2	-No impact of Mhp on PCV2 detection in sera	-No effect of coinfections	21176971

		PCV2: I-12/11 Mhp: 98 Simultaneous infections	No impact of infections	No differences in the mean lung score detected between Mhp-inoculated groups	Mild microscopical lesions in one or two pigs infected with PCV2 (single and coinfections)	-No differences in PCV2 load or duration of viremia between groups -No differences in Mhp load in nasal swabs between groups	-No impact of Mhp on PCV2-antibody titer -No impact of PCV2 on Mhp seropositive pigs	22522076
		PCV2: SNUVR000463 Mhp: SNU98703 Interv: 14 days	ND	Higher gross lung lesions in the dually infected groups	Dually infected pigs had more severe PCV2-induced pulmonary and lymphoid lesions than others. Coinfection did not significantly potentiate the Mhp-induced pulmonary lesions.	-Dually challenged pigs had increased PCV2-viremia but no difference in Mhp nasal shedding.	-No impact of the coinfection on the immunological responses against Mhp -Higher immunological responses against PCV2 in the PCV2 single-infected group compared to the coinfected group	24631087
Actinobacillus pleuropneumoniae	**swIAV**	swIAV: H1N1 A/Poland/Swine/ 14131/20141 App: 4226 serotype 2 Simultaneous infections	Clinical signs were more severe in the coinfected group.	Macroscopic lesions were more severe in the coinfected group.	ND	ND	Increased concentrations of IL1β, IL8, and IFNα in the lungs of coinfected animals	29978082
		swIAV: H1N1 A/Poland/Swine/ 14131/2014 App: PIWetHps192/2015 Simultaneous infections	Clinical signs were more severe in the coinfected group.	swIAV-like lesions were more severe in coinfected pigs.	ND	-Coinfection enhanced the nasal swIAV shedding and virus réplication. -No impact of swIAV on App load and shedding	-Earlier detection of anti-HI antibodies in the coinfected group -Strongest SAA and Pig-MAP responses in coinfected pigs	29202835
	PRRSV	PRRSV: LV-Ter Huurne App: 17415 Interv: 8 days PRRSV then App	ND	ND	ND	ND	-The infection affected the IgM, not the IgG isotype.	29126442
		PRRSV: Lelystad	PRRSV/H3N2: PRRSV infection does	Coinfection PRRSV/H3N2: Inflammation of the	ND	-PRRSV-positive macrophages in the lungs and rare influenza-positive	-No impact of a previous PRRSV infection on the	9220621

		swIAV: H3N2 App: 1421 -PRRSV/swIAV: Interv: 7 days PRRSV then swIAV -PRRSV/App: Interv: 2 days App then PRRSV	not aggravate the acute stage of H3N2 infection but could make influenza infection more chronic. -Coinfection PRRSV/App: App produced more severe disease.	bronchiolar wall more pronounced		cells in the bronchiolar epithelium -App lesions were surrounded by PRRSV-positive macrophages.	acute of chronic stage of influenza infection -Lung alveolar macrophages and bronchiolar epithelial cells do not become more susceptible for infection after App infection.	
		PRRSV: LV-Ter Huurne App: 17415 PRRSV then App Interv: 8 days	ND	Coinfection significantly increased the patho-histological score.	Coinfection increased lympho-monocytic cell infiltration in the lung.	ND	ND	27606818
	ADV	ADV: strain YS-81 App: ZF-867 serotype 1 ADV then App Interv: 7 days	Coinfected pigs showed more severe clinical signs, especially fever.	Pneumonic lesions were more important in coinfected pigs.	ND	ADV shedding was increased in presence of App.	App increased the severity of ADV infection in pigs.	8399736
Bordetella bronchiseptica	**swIAV**	swIAV: H1N1 A/Swine/Minnesota/37 866/1999 (MN99) Bb: KM22 Simultaneous infections	ND	Pneumonia lesions were more severe in the coinfected group.	Lesions were more severe in the coinfected group (peribronchiolar lymphocyte infiltration, accumulation of neutrophils, and alveolar epithelial cell necrosis)	-No impact of coinfection on swIAV shedding but enhancement of Bb burden in the coinfected group.	-Higher type I IFN response and enhanced IL1β and IL8 mRNA expression in coinfected group than others	20558274
		swIAV: H1N1 A/Swine/Minnesota/37 866/1999 Bb: KM22 Simultaneous infections	ND	ND	ND	ND	Coinfection induced an enhanced expression of TLR3.	21561668

		swIAV: H1N1 A/sw/Poland/KPR9/2004 Bb: field strain Simultaneous infections	Hyperthermia for a longer period in the coinfected group than others	More severe and extensive lesions in coinfected piglets than others	ND	-No impact of swIAV on Bb in lung -Bb infection delays the swIAV clearance in the lung.	-Higher mRNA levels for IFNα and IL8 in BALF of coinfected pigs than others	24629899
		swIAV: H1N2 A/swine/Minnesota/03012/2010 Bb: KM22 Interv: 28 days	ND	Higher percentage of the lung tissue affected in the coinfected group than others	More severe in coinfected pigs than others	-No impact of swIAV on the Bb respiratory tract colonization -No impact of Bb on virus multiplication in the lung	-Higher level of MCP1 in the coinfected group compared to single-infected groups	30337924
Pasteurella multocida	**swIAV**	swIAV: H1N1 A/sw/Poland/KPR9/2004 Pm: field strain Simultaneous infections	Coinfected pigs had clinical signs (no single-infected).	-Lung lesions observed at 3, 5, and 10 days post-infection -Atrophy of turbinates observed at 10 days post-infection only	ND	-Viral shedding from 2 to 7 dpi -No virus detected at 10 dpi -Pm detected in nasal swabs from 3 to 10 dpi and lungs	-Increased concentrations of CRP, SAA, haptoglobin, and MAP in serum	23332090
		swIAV: H3N2 Sw/Ghent/172/2008 Pm: field strain Simultaneous infections	-All coinfected pigs had clinical signs. -Less severe disease than after H1N1+ Pm coinfection	Various lesions in the coinfected pigs	ND	-Virus detected in nasal swabs and lungs in the first days after inoculation -Pm detected in nasal swabs and lungs	-Lower levels of CRP, SAA, and MAP than after H1N1+Pm coinfection	26161700
	PRRSV	PRRSV: NADC-21 Pm: P-3480 Bd: KM22 PRRSV then Pm Interv: 7 days	Coinfected animals showed no difference in clinical signs from single-infected ones.	No difference between coinfected and single-infected regarding gross lesions	ND	No effect of coinfection on pathogens' réplication	ND	11327458
		PRRSV: VR2332 ADV: 4892 Pm: A52 and A24	Central nervous clinical signs detected only in animals	ADV/Pm: More extensive pneumonic lesions than in other groups	-ADV/Pm and PRRSV/Pm: interstitial pneumonia, catarrhal	ND	-PRRSV/Pm, pneumonic lesions very slight compared to ADV/Pm	9220619

		Interv: 5 days PRRSV then Pm ADV then Pm	challenged with ADV		purulent bronchopneumonia and polymorphonuclear neutrophils in alveoli -ADV/Pm: Meningo-encephalitis and purulent rhinitis		-The role of PRRSV on the development of pulmonary lesions is unclear.	
Glaesserella parasuis	**swIAV**	swIAV: H1N1 A/Poland/Swine/ 14131/2014 Hp: PIWetHps192/20 15 Simultaneous infections	No differences between swIAV and swIAV/Hp groups	No differences between swIAV and swIAV/Hp groups	ND	-No effect of swIAV on the Hp shedding but Hp infection increased swIAV shedding. -Enhanced Hps and swIAV lung réplication in coinfected animals	-More important increase in cytokine production (IFNγ, TNFα, IL1β, IL6 and IL10) and APP (haptoglobin, C-reactive protein, SAA and MAP) after coinfection	29202835
	PRRSV	PRRSV: VR2332, SDSU73, rJXwn06, rSRV07 Cocktail of Hp, *Streptococcus suis*, and *Actinobacillus suis* Bacteria then PRRSV, 1 week later	-More severe clinical signs in coinfected groups -Severity was also dependent of the PRRSV strain.	ND	Bacteria/PRRSV: bronchopneumonia with alveoli and larger airways containing neutrophils and/alveolar necrosis	ND	-PRRSV infection causes interstitial pneumonia and predisposes to secondary bacterial bronchopneumonia in a strain dependent manner. -The severity of disease could depend of the virulence of the PRRSV strain and the development of secondary bacterial infection.	28619168
		PRRSV: HuN4 HUN4 Hp: Nagasaki Only alveolar macrophages were coinfected	/	/	/	-Bacterial sequencing in broncho-alveolar lavage: 11 bacterial species were increased after PRRSV infection, including Hp.	Hp proliferates faster in PRRSV-infected piglets.	28532803
		PRRSV: SX-1 Hp: LZ Interv: 5 days PRRSV then Hp	-Single- and coinfections increase rectal temperatures.	More lesions of the ears and dorsum in coinfected pigs	Coinfection PRRSV/Hp leads to severe interstitial pneumonia.	-Higher Hp loads were observed in coinfected pigs than in single-infected pigs.	-Coinfection increases the amount of Hp in blood at 3 days post-infection. -PRRSV could accelerate HP infection.	22460022

			-Higher temperatures in coinfected pigs than others -High mortality in coinfected pigs					
	PCV2	PCV2: WH (2b) Hp: MD0322 Simultaneous infections	Coinfected piglets developed dyspnea, anorexia, prostration, and weight lost (more than single-infected pigs)	ND	Coinfection resulted in severe widened alveolar septa, lymphocyte infiltrations in lungs, and lymphocyte depletion.	-Increased amount of virus in serum and tissues in coinfected pigs	-Coinfection resulted in slower generation and lower levels of anti-PCV2 antibodies. -Lower numbers of blood leukocytes in coinfected pigs than others	29157279
Streptococcus suis	**swIAV**	swIAV: H1N1 A/swine/Hubei/101/2009 Ss: 05ZY Interv: 3 days swIAV then Ss	More severe clinical signs in the coinfected group than in other groups	More serious lung lesions in the coinfected groups than in other groups	More important lung damages (extensive cellular infiltrates, bleeding, and cellular debris) in the coinfected pigs than others	-Higher viral titers in nasal swabs and lung 6 days post-infection in the coinfected pigs than others -No impact of swIAV on the bacterial loads	Genes related with the immune response and apoptosis were highly overexpressed in the coinfected group.	25906258
	PRRSV	PRRSV: VR2385 Ss: ISU VDL 40634/94 - Interv: 7 days PRRSV then Ss	Highest mortality rate in the dually infected group	No significant differences between single- and coinfected groups	No significant differences between single- and coinfected groups	Presence of PRRSV and Ss more important in dually infected pigs	ND	10714643
		PRRSV: HuN4 Ss: WC0711 Simultaneous infections	Coinfected pigs died of an acute disease.	Several pigs in the coinfected group had pleuritis.	Severe lesions in died pigs	-Bacteremia and viremia were observed in all the coinfected pigs.	-The coinfection increased the pathogenicity and the mortality.	20696031
Mycoplasm a hyorhinis	**PRRSV**	PRRSV: LMY Mhr: EH5 Simultaneous infections	/	The lung tissue from coinfected animals showed more severe histopathological signs.	The lung tissue from coinfected animals showed more severe histopathological signs.	ND	-More severe histopathological signs in coinfected piglets than in others	27436444

	PCV2	PCV2: YJ Mhr: DL PCV2 then Mhr or Mhr then PCV2 or simultaneous infections Interv: 0 to 7 days	Lower average daily weight gains in coinfected pigs than in others	Dual infection resulted in more severe macroscopic lung lesions.	Dual infection resulted in more severe microscopic lung lesions.	-Larger amounts and wider range of tissue distribution of PCV2 in coinfected pigs than in others	-Higher levels of PCV2 and Mhr antibodies in the coinfected groups -Significant increase of TNFα, IL2, and IL6 in coinfected pigs compared to single-infected	26711038
Staphylococcus aureus	**swIAV**	swIAV: H3N2 A/Swine/Minnesota/1145/2007 Sa: NRS123 swIAV then Sa Interv: 3 to 6 days	Animals infected with Sa 5 days after swIAV infection showed a second increased body temperature.	Dually infected animals showed more red hepatization of the lung than other animals.	No difference between H3N2 and H3N2/Sa groups	-Only dually infected animals had viable Sa in the lung, lymph nodes, and spleen 48h after bacterial infection.	Sa impacted swIAV infection.	23074662

Interv: Interval, dpi: days post-infection, ND: Not Determined, ADV: Aujeszky's Disease Virus, swIAV: swine Influenza A Virus, PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, PCV2: Porcine Circovirus type 2 (subspecies a and b when indicated)

Tableau 5. *Description of in vivo experimental assays carried to study the bacterial/bacterial infections and superinfections involved in lung diseases of PRDC*

Bacterial species	Protocol / interval between infections (CI or SI*)	Clinical signs	Macroscopic lesions	Main laboratory results			Reference PMID
				Microscopic lung lesions	Bacterial detection	Immune responses	
M. hyopneumoniae (Mhp) and *A. pleuropneumoniae* (App)	-Mhp and App serotype 9 / 0 day (CI) -App serotype 9 then Mhp / 28 days (SI) -Mhp and App serotype 9 / 28 days (SI) -Duration of the experiment: 30-65 days	In the three groups: mortality, hyperthermia, severe coughing, and decreased average daily weight gain. *App* infection is potentiated by *Mhp*.	In the three groups: pneumonia, pleurisy, fibrinous and haemorrhagic pleuro-pneumonia, pulmonary necrosis, and hypertrophy of the tracheo-bronchial lymph nodes	In the three groups: lesions of early phase of App infection, haemorrhage, vascular thrombosis, oedema, necrosis and the presence of fibrinous exudate / lesions of chronic phase of App infection, marked fibrosis around areas of necrosis and fibrinous pleuritis / lesions of Mhp infection, infiltrating lymphocytes in the peribronchiolar area, interstitial pneumonia, lymphoid nodules, and collapse of the alveoli	*Mhp* and *App* were detected by PCR and culture without further information on multiplication.	-In the three groups: IgG anti-Mhp and anti App serotype 9 -2–3 weeks after infection, similar ELISA titers	18977616
	-Mhp then App serotype 5/ 14 days (SI) -Mhp then App serotype 5/ 28 days (SI) -Mhp then App serotype 5/ 35 days (SI) -Duration of the experiment: 6 weeks - + 3 days	ND	ND	ND	ND	Phagocytosis suppression is more important in dually infected pigs.	2301832

	-Mhp then App type I / 7 days (SI) -Duration of the experiment: 14 days	Higher mortality and clinical signs in App single infected animals	Fibrinous and haemorraghic pleuro-pneumonia are less severe in pigs pre-infected with Mhp compared to App single infected pigs.	ND	ND	-IgG anti App: seropositivity 1 week after infection -Titers of these IgG were lower in double infected pigs compared to App single infections.	2531628
	-Mhp then App /16 days (SI) -Duration of the experiment: 35 days	Mortality and severe clinical signs in coinfection conditions	Coinfections showed more extensive and widespread pneumonic lesions than single-infections.	Haemorrhagic fibrinous pleuropneumonia, chronic form with necrotic nodules	-Mhp and App titers in the upper respiratory tract were higher in dually infected animals	No correlation between antibody titers to App and intensity of the lung lesions	6513243
M. hyopneumoniae and *P. multocida* (Pm)	-Mhp then Pm / 23 days (SI)	-Aggravated fever, cough and dyspnea in dually infected animals -Similar ADWG but dually infected pigs consumed 60% more food.	Extensive exudative pneumonia	Enlarged septa, large increase in the number of PMN in alveoli and bronchi, alveolar and interstitial haemorrhages, marked alveolar macrophage proliferation, and perivascular and alveolar lymphocytic infiltration with fibrin deposition	-Pm isolated exclusively from dually infected pigs -Mhp positive pigs were higher in dually infected animals.	ND	3196973
	-Mhp then Pm / 13 weeks (SI) – group 1 -Mhp vaccination (3 and 8 week-old) then Mhp inoculation then Pm / 4	-Coughing in all groups with the highest frequency of coughing in Mhp and Pm	Pneumonia in all groups with the most severe lesions in dually infected group 3	-Peribronchiolar lymphocytic inflammation in all groups (lowest score in group-4 pigs)	Mhp induced the multiplication of Pm since Pm was detected in	Seroconversion to Mhp in all groups with varying frequencies of	8125807

	weeks respectively (SI) – group 2 -Mhp inoculation then Pm / 4 weeks (SI) – group 3 -Pm alone – group 4 -Duration of the experiment: 15 weeks	inoculated group (group 3) -Dyspnea in group 3 (Mhp+Pm)		-Alveolitis in all groups (highest score in group-3 pigs)	dually infected pigs of group 3 only but not in single infected or dually infected vaccinated pigs against Mhp of group 2. -Percentage of lung surface area with pneumonia correlated with the number of Pm colonies isolated in group 3.	seropositive pigs	
	-Mhp then Pm / 20 days (SI)	Coughing	Pneumonia	Bronchial and perivascular lymphoid accumulations in some pigs	Mhp Pm App in nasal swabs	Seroconversion to App was higher in pigs pre-infected with Mhp.	22632286
	-Mhp then Pm / 13 days (SI)	Coughing without significant differences between groups	Pneumonia in all the groups	ND	Mhp multiplication is not affected by App.	ND	28499212
M. hyopneumoniae and *P. multocida* and *A. pleuropneumoniae*	-Mhp then Pm then App serotype 2 / 7 days and 7 days, respectively (SI)	Sneezing, depression, forced breathing, coughing, and elevated rectal temperature at some points	Catarrhal pneumonia and purulent foci in the diaphragmatic lobes	-Lymphohistiocytic bronchitis and peribronchitis and interstitial pneumonia peribronchitis	Mhp and App detection	Seroconversion to Mhp	11768127

		after Pm or App infections		-Hyperplasia of the peribronchial lymph nodes, lymphohistiocytic, and infiltration of the nasal mucosa			
M. hyopneumoniae and *M. hyorhinis* (Mhr)	-Mhp at 6 weeks of age then Mhr at 7 weeks of age / 7 days (SI) -Duration of the experiment: 33-36 days	Coughing, Hyperthermia, and lower ADWG during the third week	Pneumonia, pleuritic, and pericarditis	ND	Mhp and Mhr (PCR)	-IgG anti-Mhp: seropositivity from 13 dpi -Higher haptoglobin concentration (additive effect)	31030844
M. hyopneumoniae and *M. flocculare* (Mfloc)	-Mhp at 6 weeks of age then Mfloc at 7 weeks of age / 7 days (SI) -Duration of the experiment: 33-36 days	Coughing and lower ADWG during the third week	Pneumonia	ND	Mhp and Mfloc (PCR)	-IgG anti-Mhp: seropositivity from 13 dpi - Higher haptoglobin concentration (additive effect)	31030844
	-Mhp and Mfloc / 0 days (CI) -Mfloc then Mhp / 6 weeks (SI) -Duration of the experiment: 7 to 24 weeks post-infection	Clinical signs following Mhp infection such as coughing are unchanged in presence of Mflo	No difference registered between dually infected and Mhp single infected pigs	Histopathology revealed characteristic pneumonia induced by Mhp in all pigs without any difference between single and dually infected groups.	-Mhp and Mfloc were detected by IFA and cultutre. -The poor colonization of the respiratory tract by Mfloc was not	Mhp induced a stronger antibody response that was not influenced in the presence of Mfloc in dually infected groups.	1570675

					affected by Mhp.		
B. bronchiseptica (Bb) and *H. parasuis* (Hps)	Bb and Hps / 0 day (CI) Bb then Hps / 7 days (SI) Duration of the experiment: 2 weeks	Coughing was reported in case of Bb and/or Hp infection while Bb caused sneezing in infected pigs.	-Pneumonia: areas of tan-red consolidation with well-demarcated borders and a cranial ventral distribution (no statistical differences between the groups)	ND	Bb predisposes to Hps colonization while Hps reduced the multiplication of Bb in the nasal cavity.	ND	15019114
B. bronchiseptica and *P. multocida*	Bb then Pm / 4 days (SI) Duration of the experiment: 25 days	ND	Atrophic rhinitis scores were higher in dually infected compared to Pm single infected pigs.	ND	Bb enhanced the multiplication of Pm.	ND	17624695
	Bb then Pm / 7 days (SI) Duration of the experiment: 25 days	Clinical signs such as sneezing, coughing, fever and weight loss were higher in dually infected group compared to Pm single infected group.	Atrophic turbinates and mucopurulent nasal discharge were exclusively noted in animals pre-infected with Bb.	ND	Bb enhanced the multiplication of Pm in the upper respiratory tract.	ND	11327458

ADWG: Average Daily Weight Gain, CI: Coinfection, IFA: Immunofluorescence assay, SI: Superinfection, swIAV: swine Influenza A Virus, PRRSV: Porcine Reproductive and Respiratory Syndrome Virus, PCV2: Porcine Circovirus type 2 (subspecies a and b when indicated), PRDC: Porcine Respiratory Disease Complex

B- Les co-infections par le swIAV et le PRRSV

a) Le virus de l'Influenza A porcin

1. La grippe porcine dans l'histoire

Le virus H1N1 classique a émergé en 1918 dans les élevages de porcs aux États-Unis. Cette souche a causé une epizootie et a circulé dans les porcheries européennes jusqu'à l'émergence d'une nouvelle souche d'origine aviaire en 1980 (Pensaert et al., 1981). Les réassortants issus de cette dernière souche appelée « *avian-like* » H1N1 ($H1_{av}N1$) se sont bien adaptés à l'espèce porcine et certains ont réussi à re-traverser la barrière d'espèce pour infecter l'homme et causer la fameuse pandémie de l'influenza A de sous type H1N1 en 2009 (Brown, 2013). L'espèce porcine s'est donc montrée comme un hôte très important pour la transmission des virus de l'influenza d'une espèce à une autre. Le porc est d'ailleurs parfois qualifié de *mixing vessel*, chaudron de réassortiment, en anglais. Cela a été confirmé par les différentes études et séquençages des virus circulant chez le porc révélant des origines virales aviaires ou humaines (Brown, 2013).

2. Épidémiologie

Il existe plusieurs sous-types du swIAV et un grand nombre de souches dont la circulation varie selon les régions étudiées. Il est très commun d'isoler plusieurs souches dans une même zone géographique. En Europe de l'Ouest, des sous-types tels que le H1N1 classique, le $H1_{av}N1$ et le « *human-like* » H3N2 ($H3_{hu}N2$) sont toujours à l'origine d'enzooties (Brown, 2013). Plusieurs souches parmi ces sous-types partagent certaines ressemblances avec les souches qui ont émergé à travers l'Amérique ou l'Asie (Brown, 2013). Avec le temps, de nouveau réassortants et de nouvelles souches ont été identifiés. Ainsi, 169 nouvelles souches ont été isolées entre 2006 et 2008 à travers la Belgique, l'Italie, la France, l'Espagne et le Royaume-Uni. Quatre-vingt-une souches classées comme $H1_{av}N1$, 36 comme $H3_{hu}N2$ et 47 comme H1N2 (Kyriakis et al., 2011). Les études au Royaume-Uni montrent que 50 % des porcs domestiques ont déjà été infectés par une ou plusieurs souches de swIAV dont 40 % ayant rencontrés à la fois des virus d'origine humaine et d'origine porcine (Brown et al., 1995). Cette prévalence s'élève à 76 % dans les élevages espagnols condensés dans une petite zone géographique (Maldonado et al., 2006). En Europe centrale, le $H1_{av}N1$ semble le sous-type dominant avec une faible prévalence en comparaison avec celle

observée dans les pays d'Europe de l'Ouest (Brown, 2013). Cependant, l'émergence des sous-types H1N2 et H3N2 reste très faible et limitée. Cela est peut-être lié aux conditions d'élevage moins extensives en Pologne et en République Tchèque (Van Reeth et al., 2008). Enfin, l'Europe du Sud constitue la région européenne la plus touchée par le $H3_{hu}N2$ avec une forte prévalence dans les élevages (Kyriakis et al., 2011). Les causes sous-jacentes à ces importantes variations épidémiologiques entre les différents sous-types de swIAV restent inconnues. Cependant, cette complexité est liée à la variation génétique entre les souches virales, à la réponse immune du porc, aux pratiques d'élevage variées et aux différents facteurs climatiques locaux et régionaux (Brown, 2013).

3. Description de la particule virale et de son génome

Le virus de l'influenza est un virus enveloppé à ARN simple brin de polarité négative, appartenant à la famille des *Orthomyxoviridae*. Selon le comité international de taxonomie virale (*International Committee on Taxonomy of Viruses*, ICTV), cette famille comprend sept genres, les *Alphainfluenzavirus* (l'influenza A), les *Betainfluenzavirus* (l'influenza B), les *Gammainfluenzavirus* (l'influenza C), les *Deltainfluenzavirus* (l'influenza D), les *Quaranjavirus*, les *Isavirus* et les *Thogotovirus*. L'influenza A comporte plusieurs sous-types déterminés par les deux glycoprotéines virales ; l'hémagglutinine (HA) et la neuraminidase (NA). Dix-huit HA (H1-H18) et onze NA (N1-N11) ont été identifiées (Crisci et al., 2013; Hutchinson, 2018). Le génome de l'influenza A est constitué de 8 molécules d'ARN simple brin à polarité négative codant pour 11 ou 12 protéines virales (Medina and García-Sastre, 2011) (figure 1).

L'enveloppe du virus formée à partir de la membrane cytoplasmique de la cellule hôte pendant le bourgeonnement est constituée d'une bicouche lipidique, de glycoprotéines transmembranaires telles que la HA et la NA et d'autres protéines dont les protéines de matrice, M1 et M2 (Crisci et al., 2013). La HA est la protéine majeure de l'enveloppe qui forme des « *spikes* » à la surface du virion et qui comprend le site d'adhésion des anticorps neutralisants (Webster et al., 1992). La NA est plutôt impliquée dans le bourgeonnement du virus après la réplication dans la cellule (Nayak et al., 2004). La M2 est un canal ionique jouant un rôle principal dans la décapsidation du virus et la dissociation de la ribonucléocapside et de la M1 après l'endocytose (Nayak et al., 2009). Le cœur du virus est constitué d'une ribonucléocapside hélicoïdale enfermant L'ARN viral (ARNv), la

nucléoprotéine (NP), la protéine non-structurale NS2 et le complexe de l'ARN polymérase virale (Arranz et al., 2012; Nayak et al., 2004) (figure 1).

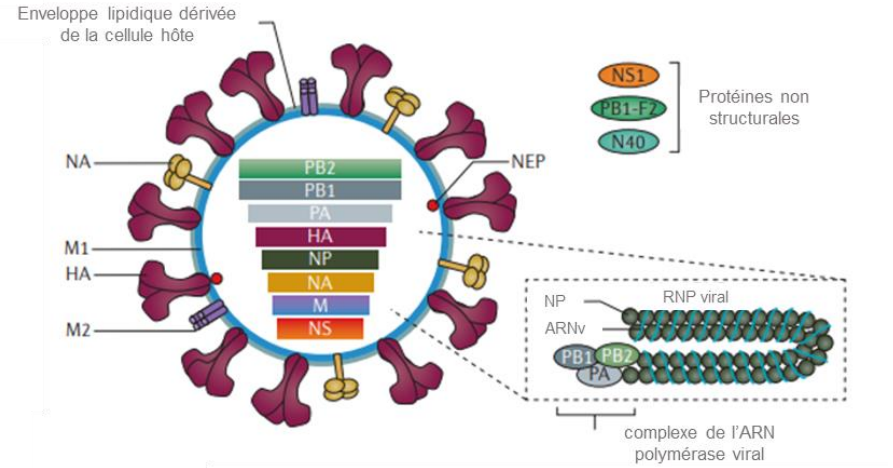

Figure 1. *Le virus de l'influenza A et ses différents éléments (Traduite de Medina et al. 2011)*

4. Les signes cliniques et l'impact économique

Les infections subcliniques par le swIAV sont très communes, et la majorité des porcs infectés par un ou plusieurs sous-types ne présentent aucun signe clinique (Van Reeth, 2007). Les signes cliniques de la grippe chez le porc ressemblent souvent aux symptômes déclenchés chez les humains (Brown, 2000). Ainsi, la réaction inflammatoire induite après le contact avec le virus est à l'origine de signes cliniques généraux et respiratoires tels que la fièvre, l'anorexie, l'abattement et la toux (Goraya et al., 2015).

Aujourd'hui, la grippe porcine est considérée parmi les maladies les plus importantes et surtout les plus contagieuses menaçant les élevages de porc dans le monde entier (Brown, 2013). Les infections par le swIAV entraînent de fortes pertes économiques liées à la perte pondérale chez les porcs qui risquent souvent de développer une infection secondaire par un autre agent pathogène virale ou bactérien (Brown, 2013).

Au Royaume-Uni, les pertes économiques causées par la baisse de production liée aux infections par l'influenza porcin sont estimées à 60 millions de livres par an (Kay et al., 1994). Les études sur le sujet au Mexique ont estimé le déficit dans le commerce de la viande porcine à 27 millions de dollars américains à la fin de l'année 2009 suite à la pandémie H1N1 survenue cette

année-là (Rassy and Smith, 2013). Une perte de 2,8 milliards de dollars en 5 mois a été aussi rapportée dans le secteur du tourisme (Rassy and Smith, 2013).

L'aspect zoonotique de la grippe porcine s'accompagne aussi de répercussions sanitaires et de conséquences économiques sur les systèmes de santé humains. Une étude menée aux États-Unis a estimé de 89 000 à 207 000 le nombre de décès dans la population humaine américaine suite à une épidémie de grippe porcine. Ces décès sont accompagnés de pertes économiques dans le secteur de la santé allant jusqu'à 166,5 milliards de dollars américains sans compter le coût de la vaccination (Meltzer et al., 1999).

5. Hôte cellulaire et tropisme tissulaire

Les cellules épithéliales des voies respiratoires constituent les premières cibles de l'infection par le swIAV (Crisci et al., 2013). L'adhésion à ces cellules s'effectue par liaison aux acides sialiques membranaires des glycoprotéines HA exprimées à la surface du virus (Medina and García-Sastre, 2011). Les différentes études menées *in vitro* montrent la capacité de ce virus à infecter et à se répliquer dans d'autres types de cellules telles que les cellules épithéliales intestinales porcines (*South Dakota - porcine jejunal epithelial cells*, SD-PJEC) (Sun et al., 2012) et les cellules rénales du chien (*Madin-Darby Canine Kidney, MDCK*) ou du singe (*African Green Monkey Kidney cells*) (Genzel et al., 2010; Tobita et al., 1975). Des cellules immunitaires comme les monocytes et les macrophages dérivant de monocytes sont aussi sensibles à l'infection par le virus de la grippe, cependant l'infection de ces cellules n'est pas productive dans la plus grande partie des cas (Bussfeld et al., 1998; Hofmann et al., 1997; Meischel et al., 2020a).

6. L'infection des cellules hôtes et la réplication virale

La première étape de l'infection des voies respiratoires est l'adhésion du virus à l'acide sialique exprimée sur les cellules épithéliales grâce à la glycoprotéine virale HA (Hamilton et al., 2012). Cette adhésion a lieu au niveau des liaisons α-2,6 ou α-2,3 de l'acide sialique avec le galactose (Medina and García-Sastre, 2011) (figure 2). D'autres études sur des récepteur alternatifs tels que les lectines confirment leur rôle dans l'adhésion et l'internalisation du virus (Upham et al., 2010). Suite à l'adhésion, le virus entre dans la cellule par endocytose dépendante de la chlatrine et de la

cavéoline, et les ribonucléoprotéines virales (RNPv) sont ensuite libérées dans le cytoplasme par effet du pH acide de l'endosome (Fontana et al., 2012; Leung et al., 2012) (figure 2). Cette baisse de pH est importante pour la fusion de la membrane virale à la membrane de l'endosome et pour l'ouverture des canaux ioniques M2 qui contribuent à l'acidification et à la dissociation de la protéine M1 et des ribonucléoprotéines (Pinto and Lamb, 2006). La transcription et la réplication du génome du swIAV s'effectue au niveau du noyau (Samji, 2009). Grâce à différents signaux de localisation nucléaires (*Nuclear Localization Signal, NLS*) présents dans les protéines virales, l'importation au noyau se fait après adhésion à des karyophérines comme l'importine α et β (Boulo et al., 2007). À ce stade, une conversion de l'ARN viral (ARNv) à polarité négative en ARNv positif par l'ARN polymérase ARN dépendante du virus (*viral RNA dependent RNA polymerase, RdRp*) est essentielle pour la production de l'ARNv (Samji, 2009). L'exportation des ribonucléoprotéines virales (RNPv) récemment produites s'effectue par une voie d'exportation dépendante de l'exportine 1 (nommée aussi *Chromosomal Maintenance 1, CRM1*) à travers les pores nucléaires. Seules les RNPv à polarité négative seront exportées par liaison à la protéine M1 et à la protéine d'export nucléaire (NEP) (figure 2), qui se lie à la CRM1 et franchit la membrane nucléaire. Finalement, les protéines virales seront rassemblées et transportées vers le pôle apicale de la cellule pour effectuer le bourgeonnement et se servir de la membrane cellulaire pour constituer l'enveloppe virale (Nayak et al., 2009). La NA joue un rôle très important à cette étape en clivant la liaison α-2,3 ou α-2,6 de l'acide sialique au galactose pour faciliter le bourgeonnement des nouvelles particules virales produites (McAuley et al., 2019).

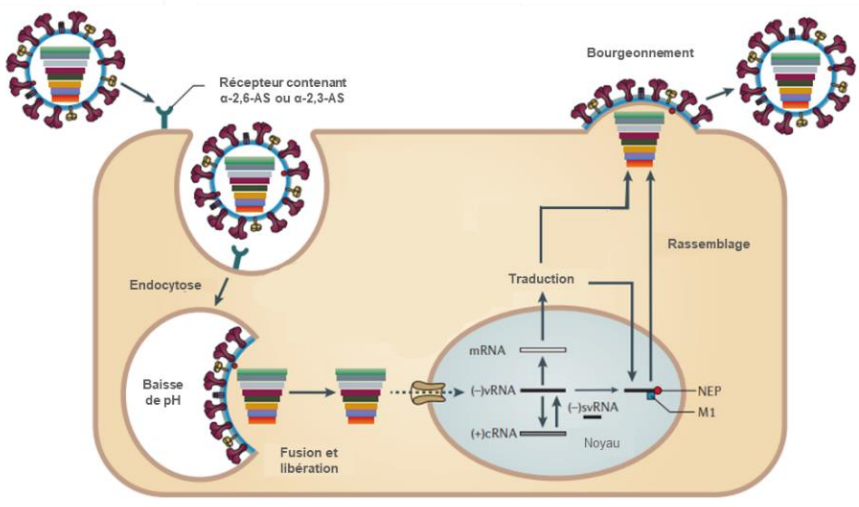

Figure 2. *Les étapes de la réplication du swIAV dans une cellule hôte (traduite et adaptée de Medina et al. 2011)*

7. La transmission et la persistance du virus

Les pratiques d'élevage influencent directement l'évolution et la propagation du swIAV, en effet la transmission du virus est liée aux mouvements des animaux infectés dans des troupeaux susceptibles (Brown, 2000). L'introduction d'un animal infecté entraîne la contamination du troupeau et la persistance du virus chez les porcelets nouveaux nés et récemment introduits à l'élevage. Des épisodes cliniques sont donc observés d'une façon saisonnière et annuelle dans le troupeau contaminé. L'infection prend alors une forme récurrente qui se traduit par une persistance du virus et une apparition des signes cliniques à un âge plus avancé (Easterday, 1980). L'excrétion du virus s'effectue sur une courte durée variant entre 7 et 10 jours post-infection et donc la persistance du virus dans les élevages est assurée grâce à la transmission continue du virus aux jeunes porcelets (Hinshaw et al., 1978).

La détection d'un foyer dans des élevages de la même région n'est pas toujours liée au transport ou aux échanges des animaux infectés entre les troupeaux, mais plutôt à une dissémination du virus par aérosols entre les élevages sans introduction d'animaux infectés (Brown, 2000). La principale voie de transmission du swIAV est la voie nasopharyngeale, le virus est cependant transmis dans les secrétions nasales des porcs contaminés. Cette transmission est souvent favorisée par la mauvaise hygiène des élevages, la densité et l'état de stress des animaux et par les facteurs météorologiques et environnementaux tels que l'humidité et le froid (Brown, 2000).

8. La gestion et la lutte contre le virus

Le contrôle de la transmission du swIAV au sein de la filière porcine est un défi mondial pour les autorités de santé et les éleveurs. Les bonnes pratiques d'élevage telles que la maintenance d'une bonne hygiène des locaux, l'application d'une quarantaine avant toute introduction d'un nouvel animal et la séparation des porcelets récemment sevrés contribuent à la lutte contre la dissémination du swIAV (Kothalawala et al., 2006). Vu la forte persistance de ce virus dans le troupeau récemment contaminé, la dépopulation partielle ou totale reste l'une des techniques les plus efficaces pour se débarrasser du virus (Brown, 2000). L'utilisation des anti-inflammatoires dans les abreuvoirs est souvent pratiquée, cependant ces traitements sont limités à la réduction des signes cliniques notamment la fièvre (Linden, 2013).

La vaccination contre le swIAV a montré une efficacité partielle dans la réduction des signes cliniques, de l'excrétion du virus et de sa transmission d'un porc infecté à un porc sain. Cet échec de protection des vaccins commerciaux est souvent lié à la diversité génétique du swIAV et à l'apparition continue de nouveaux réassortants (Van Reeth and Ma, 2013). Malheureusement, le renouvellement des souches vaccinales n'est pas toujours possible pour des raisons économiques.

9. La réponse immunitaire de l'hôte porcin à l'infection par le swIAV

La réponse immune innée de l'hôte contre le swIAV fait appel à plusieurs barrières physiques telles le mucus et l'épithélium pseudo-stratifié dans les voies respiratoires supérieures et le surfactant et les macrophages dans les alvéoles (Holt et al., 2008). Cette immunité innée qui constitue la première ligne de défense suite à une infection par le swIAV est rapide. Cependant, elle manque de spécificité et de mémoire (Goraya et al., 2015). Elle se traduit par une libération de cytokines, telles que les interférons de type 1 et 3, les interleukines IL1β, IL6, IL8 et IL10 (Pomorska-Mól et al., 2014) et les CCL2, CCL3 et CXCL10 (Skovgaard et al., 2013). Cette première production de cytokines et chimiokines par les cellules épithéliales entraîne le recrutement de macrophages, de cellules dendritiques (DCs) et de neutrophiles (figure 3) (Achdout et al., 2003; Perrone et al., 2008). Le recrutement et l'activation des macrophages et des monocytes s'effectuent grâce à leur récepteurs CCR2 (Herold et al., 2006; Perrone et al., 2008). Suite à leur activation, les macrophages alvéolaires (*alveolar macrophage*, AMs) augmentent la production de cytokines pro-inflammatoires comme le facteur de nécrose tumorale α (TNFα), les IFN et l'IL6 qui sont à l'origine de la pathologie induite par le virus (Jayasekera et al., 2006; Perrone et al., 2008; van Riel et al., 2011a). En phagocytant les cellules apoptotiques et les particules virales opsonisées (figure 3) les AM contribuent à la diminution de la charge virale et de l'inflammation et donc réduisent la pathogénie de l'infection par le swIAV (Hashimoto et al., 2007; Heui Man Kim et al., 2008).

Les cellules *natural killers* (NK) participent également à la réaction immune innée contre le virus influenza en détruisant les cellules infectées. L'action des NK est médiée par la reconnaissance du HA exprimé à la surface des cellules infectées (figure 3) (Mendelson et al., 2010; Mair et al., 2012).

La réponse immune adaptative suite à l'infection par swIAV est une réaction plus spécifique et durable, mais son effet est plus tardif (Kreijtz et al., 2011). Elle consiste en une différenciation et une prolifération des lymphocytes T CD4, T CD8 et B spécifiques après la présentation des antigènes du virus par les DC. Les lymphocytes T CD4 qui se différencient en lymphocytes auxiliaires ou « *helper* » (Th) sont ainsi classés en différents types tels que Th1, Th2, Th17 ou Treg selon les cytokines produites. Quant aux lymphocytes T CD8, ils se différencient en lymphocytes T cytotoxiques (CTL) qui lyseront les cellules infectées (Charley et al., 2006). En parallèle, les lymphocytes B se différencient en plasmocytes et acquièrent la capacité de secréter des anticorps, neutralisants ou pas (figure 3). Les anticorps neutralisants vont bloquer le virus et empêcher son adhésion à ses cellules hôtes, alors que les anticorps non-neutralisants vont contribuer à l'opsonisation des particules virales pour faciliter leur élimination par d'autres cellules effectrices.

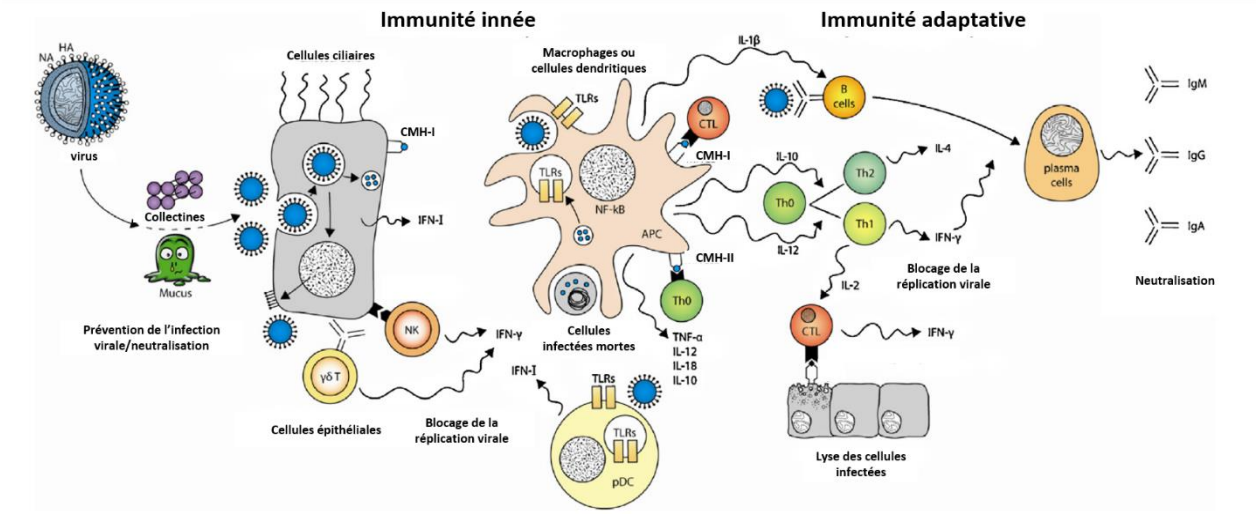

Figure 3. *Les composants de la réponse immune innée et adaptative suite à une infection par le virus de l'influenza (traduite de Crisci et al. 2013)*

10. Les voies de signalisation induites par le swIAV

Les cellules épithéliales infectées sont équipées de récepteurs qu'on appelle *pattern recognition receptors* (PRR) et qui servent à signaler la présence d'un danger, par exemple un virus, et à initier l'activation des différentes voies de signalisation cellulaires pour lutter contre l'infection virale (Bleiblo et al., 2012). Parmi ces PRR, peuvent être cités les *toll like receptors* (TLRs), le *retinoic acid inducible gene-I* (RIG-I), le *melanoma differentiation-associated gene 5* (MDA5) et le *nucleotide-binding oligomerization domain* (NOD) (Pang and Iwasaki, 2011).

Suite à une infection par le swIAV, la stimulation de certains récepteurs PRR induit des événements de phosphorylation et de dimérisation, et entraîne l'activation des facteurs régulateurs des IFN (IRF3/7). Cela conduit à l'exposition des signaux de localisation nucléaire (*nuclear localization signal*, NLS) qui à leurs tours entraînent une translocation des IRF3/7 au noyau (Goraya et al., 2015) (figure 4A). En parallèle, et suite à la signalisation d'autres récepteur PRR (RIG1 et MDA5), la dégradation de l'inhibiteur du facteur nucléaire-kappa B (NF-κB) entraîne la translocation de ce dernier dans le noyau *via* son NLS (Hayden and Ghosh, 2004). Cette translocation du NF-κB est aussi favorisée par la phosphorylation de c-jun et du facteur de transcription (ATF-2) qui constituent les 2 composants hétérodimériques de la protéine activatrice 1 (AP-1). L'assemblage de ces facteurs de transcription dans le noyau forme un complexe nucléoprotéique transcriptionnel appelé « enhanceosome » qui se lie à ses domaines de régulation positive (*positive regulatory domain*, PRDs). La fixation de IRF3, IRF7, NF-κB et AP-1 aux PRDI/III, PRD II, et PRD IV respectivement, induit la transcription des IFN type 1 et 3 et des cytokines pro-inflammatoires TNFα, IL6 et IL1β (Goraya et al., 2015) (figure 4A). Suite à leur production, les IFN se fixent à leur récepteur spécifique IFNAR, ce qui entraîne l'activation des kinases associées aux récepteurs comme la *janus kinase* 1 (JAK1) et la *tyrosine kinase* 2 (TYK2). Cette activation induit le recrutement et la phosphorylation de l'IRF9 à l'hétérodimère STAT1/STAT2 pour former le facteur 3 des gènes stimulés par les interférons (*Interferon-stimulated gene factor,* ISGF3) (Mukaigawa and Nayak, 1991). Après sa translocation dans le noyau, l'ISGF3 se lie aux éléments stimulés par les interférons (*interferon-stimulated response element*, ISREs) et induit la transcription des gènes stimulés par les interférons (*Interferon-stimulated genes,* ISG) (Kessler et al., 1990) déclenchant à proprement parler la réponse immune innée de l'hôte au swIAV (figure 4B).

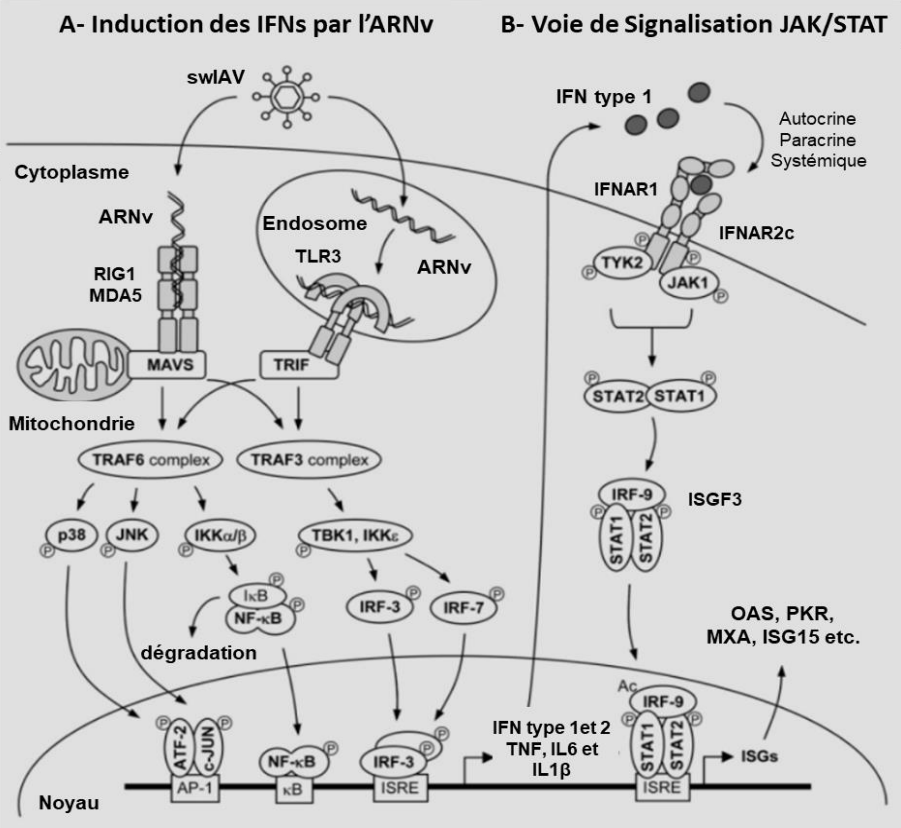

Figure 4. *La voie d'induction des IFN suite à une infection par le swIAV (A) et la voie de signalisation JAK/STAT induite par ces IFN (adaptée et traduite de Fensterl and Sen 2009)*

Parmi ces ISG, peuvent être cités les ISG12 et ISG15, les gènes de résistance aux orthomyxovirus (MX) ainsi que les gènes codant pour la « *protein kinase R, PKR* » et la « *2'- 5' oligoadenylate synthetase, OAS* » (Ehrhardt et al., 2010). PKR inhibe la traduction cellulaire en phosphorylant le facteur d'initiation eukaryotique 2α (eIF2α) et limite toute synthèse de nouvelles protéines cellulaires et virales (García-Sastre and Biron, 2006). Elle est aussi capable de réguler la voie de signalisation de NF-κB et d'augmenter la production des IFN (Ehrhardt et al., 2010). Quant à l'OAS, cette protéine contribue à l'activation des endoribonucléases qui dégradent l'ARN virale et cellulaire (Samuel, 2001). Alors que les MXs, s'associent à la sous-unité PB2 de la polymérase du swIAV et aux protéines de la nucléocapside pour inhiber la transcription cellulaire et bloquer la réplication du swIAV (Sadler and Williams, 2008).

Parallèlement, une autre kinase a été identifiée (Ehrhardt et al., 2006). Celle-ci interagit avec la protéine non-structurale nsp1 du virus. Il s'agit de la « *phosphatidylinositol-3-kinase,*

PI3K » connue pour son rôle antiviral et qui induit une phosphorylation complète et une activation du facteur IRF3 (Ehrhardt et al., 2006). Un effet pro-viral de la PI3K a également été décrit. Il s'agit donc d'une induction biphasique suite à une infection par l'IAV (Ehrhardt et al., 2010). L'activation précoce de PI3K suite à l'adhésion du virus contribue à l'internalisation de la particule virale, alors que son activation tardive, induite par l'adhésion de la NS1 à sa sous-unité P85, est responsable de la prévention de l'apoptose prématurée (Ehrhardt and Ludwig, 2009).

D'autre part, l'induction de quatre cascades de protéines kinases activées par les mitogènes (*mitogen-activated protein kinase, MAPK*) a été décrite suite à une infection par le IAV (Ludwig et al., 2006) (figure 5). La famille de ces MAP kinases comprennent quatre membres prototypes tels que les deux isoformes de la kinase régulée par le signal extracellulaire (*extracellular signal-regulated kinase,* ERK1 et ERK2), la kinase *Jun-N-terminal* (JNK), la p38 et la ERK5 (figure 5). Ces cascades effectuent la conversion des signaux extra et intracellulaires en réponses cellulaires dans le but de réguler la prolifération, la différenciation et l'activation des cellules, ainsi que la réponse immunitaire pour lutter contre l'infection par le IAV et éliminer le virus (Dong et al., 2002). L'activation des MAPK se produit par double phosphorylation induite par les protéines kinases kinases activées par les mitogènes (*mitogen-activated protein kinase kinase,* MKK et MEK) (figures 5). JNK et p38 réagissent principalement aux conditions de stress et aux cytokines pro-inflammatoires, tandis que ERK5 peut être activée à la fois par des mitogènes et des inducteurs de stress (Ludwig et al., 2006). JNK, p38 et ERK5 jouent un rôle antiviral suite à une infection par le IAV, alors que les ERK1/2 sont essentielles à la réplication du virus en facilitant l'export du RNPv. L'activation de p38 suite à l'infection virale induit une production de chimiokines telles que CCL5 (connue sous le nom de RANTES) et L'IL8 impliquée dans le recrutement des neutrophiles (figure 5). La phosphorylation de JNK est aussi induite lors d'une infection par IAV et conduit à l'activation des facteurs de transcription AP-1, ATF2 et c-Jun, qui sont essentiels pour réguler non seulement l'expression des IFNβ et du CCL5, mais également l'apoptose (figure 5). Enfin, le rôle de ERK5 activé par l'infection reste inconnu sachant qu'il n'affecte pas la réplication virale (Ludwig et al., 2008). JNK, p38, and ERK5 sont induites par le terminal 5' triphosphate présent sur l'ARNv, alors que les ERK1/2 sont activées par l'hémagglutinine HA sous l'action de la protéine kinase C α (PKCα) et induisent l'exportation de la RNPv. Ainsi, les études montrent que la voie de signalisation PKCα/Raf/MEK/ERK est activée tardivement au moment de l'assemblage et du bourgeonnement du virus (Pleschka et al., 2001) (figure 5).

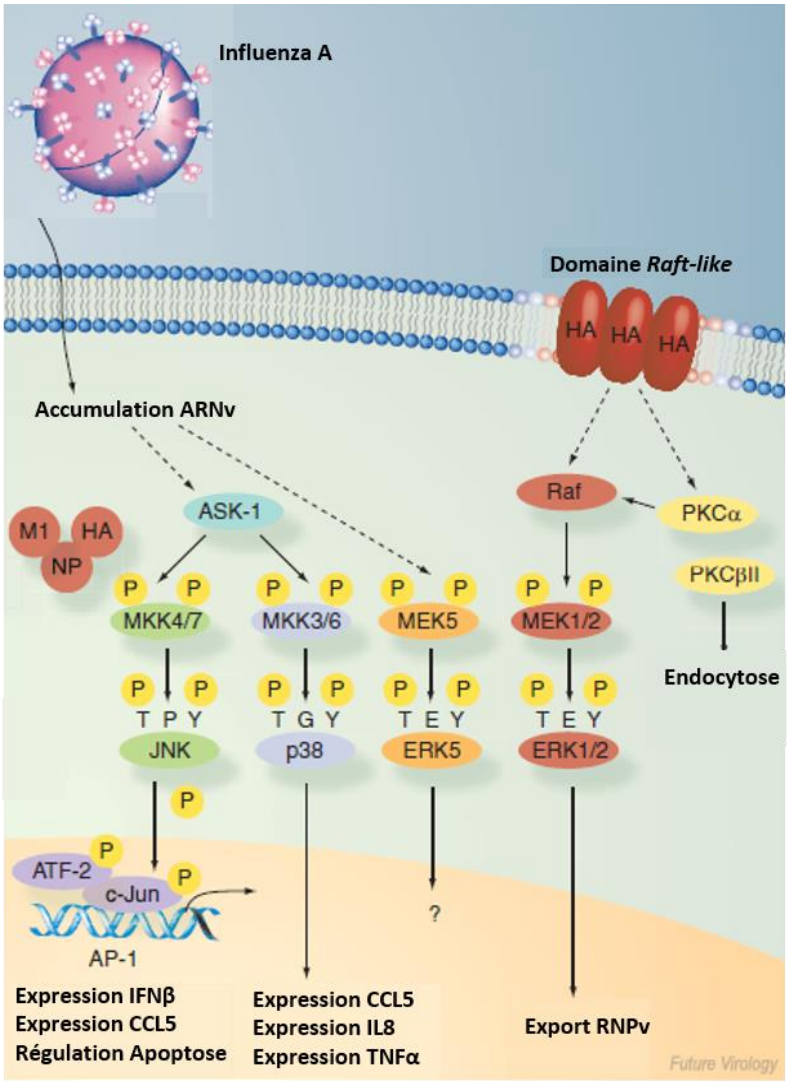

Figure 5. *Les voies de signalisation intracellulaires impliquant les quatre MAP kinases activées suite à une infection par swIAV et leurs fonctions dans la cellule infectée (Ludwig et al. 2006)*

Finalement, une étude sur les AM a montré une interaction croisée entre la voie NF-κB et la voie de signalisation des MAPK (Gao et al., 2012). Par conséquent, les ERK1/2 et les JNK1/2 impliquées dans la voie MAPK peuvent impacter l'activation de la NF-κB en induisant RIG1 et l'expression de l'IFNβ suite à l'infection par le swIAV H1N1 (Gao et al., 2012).

b) Le PRRSV

1. Le PRRSV dans l'histoire

Le virus est apparu pour la première fois chez les porcs domestiques en Europe centrale et à l'ouest des États-Unis à la fin des années 1980 (Lunney et al., 2010). L'origine de ce virus reste inconnue, cependant une hypothèse suggère qu'un artérivirus mutant chez la souris (*lactate dehydrogenase-elevating virus*, LDEV) est à l'origine du PRRSV après une infection de sangliers jouant le rôle d'hôtes intermédiaires. Le virus a bien circulé chez les sangliers en Europe centrale avant d'être transmis en Amérique en 1912 par l'importation de sangliers. Il est ensuite passé aux porcs domestiques vers 1985 (Plagemann, 2003). Le syndrome dysgénésique respiratoire porcin a été nommé « la maladie porcine mystérieuse » jusqu'à ce que le virus soit isolé et clairement identifié pour la première fois en 1991 (Wensvoort et al., 1991). Ce premier isolat européen du virus a été nommé Lelystad Virus (LV) en référence à une ville des Pays-Bas qui constituait avec l'Allemagne les premiers pays touchés en Europe. Un an plus tard, un autre génotype du virus (ATCC VR-2332) causant les même signes cliniques a été isolé dans un élevage aux États-Unis (Collins et al., 1992). Cet isolement du virus a eu lieu sept ans après la première identification d'un porc séropositif dans un élevage en Iowa aux États-Unis en 1985 (Plagemann, 2003). Les deux génotypes décrits pour ce virus présentent une différence de 40 % au niveau de leurs génomes (Nelsen et al., 1999). Aujourd'hui, ils sont considérés comme 2 espèces différentes nommées PRRSV-1 et PRRSV-2 (Kuhn et al., 2016). Cela signifie que le PRRSV possède une vitesse d'évolution élevée par rapport à la plupart des virus à ARN (Hanada et al., 2005).

2. Épidémiologie

Depuis le premier isolement du virus en 1991, ce dernier est devenu endémique dans toutes les porcheries du monde (Wensvoort, 1993). La prévalence peut varier de 0 à 80 % selon la région et le pays (Guo et al., 2019, 2018). Les deux espèces de PRRSV, d'abord nommées génotypes 1 et 2, étaient originellement définies comme génotype Américain (type 2), le plus virulent et souvent rencontré en Amérique du Nord et en Asie et le génotype européen (type 1), relativement moins virulent et qui a toujours circulé en Europe de l'Ouest (Ropp et al., 2004) (figure 6). Cette répartition, liée à l'origine de chaque espèce, n'est cependant plus valable, et les deux espèces se sont trouvées en cohabitation dans plusieurs régions dans le monde entier (Fang et al., 2004; Lee

et al., 2010) (figure 6). Le Danemark est un bon exemple après la détection des deux espèces dans les élevages danois. En 1996, 25% des élevages du pays ont été contaminés par le PRRSV type 1 qui était la seule espèce circulante dans le pays. Un programme de vaccination non adapté utilisant un vaccin vivant de PRRSV type 2 à la place du PRRSV-1 a permis l'introduction de la deuxième espèce dans le pays (Mortensen et al., 2002).

En 2006, une souche virulente associée à des taux de létalité compris entre 20 et 100% émerge en Chine et cause une épidémie touchant des milliers d'élevages et impliquant d'importantes pertes économiques (Tong et al., 2007). En 2007, le centre Nord des États-Unis témoigne de l'émergence d'une nouvelle souche à haute virulence (souche 1-18-2) non liée à la souche virulente détectée en Chine et identifiée comme du PRRSV-1 (Lunney et al., 2010). Selon les données publiées en 2017 (Niederwerder and Rowland, 2017) et selon la base de données de l'organisation mondiale de la santé animale, certains pays comme l'Australie, la Suisse, la Suède, la Norvège, la Finlande et la Nouvelle-Zélande sont toujours indemnes de ce virus (figure 6). Malgré la déclaration de quelques foyers dans des élevages suédois en 2007 et suisses en 2012 ces deux pays ont réussi à contrôler la dissémination du PRRSV (Carlsson et al., 2009; Nathues et al., 2016).

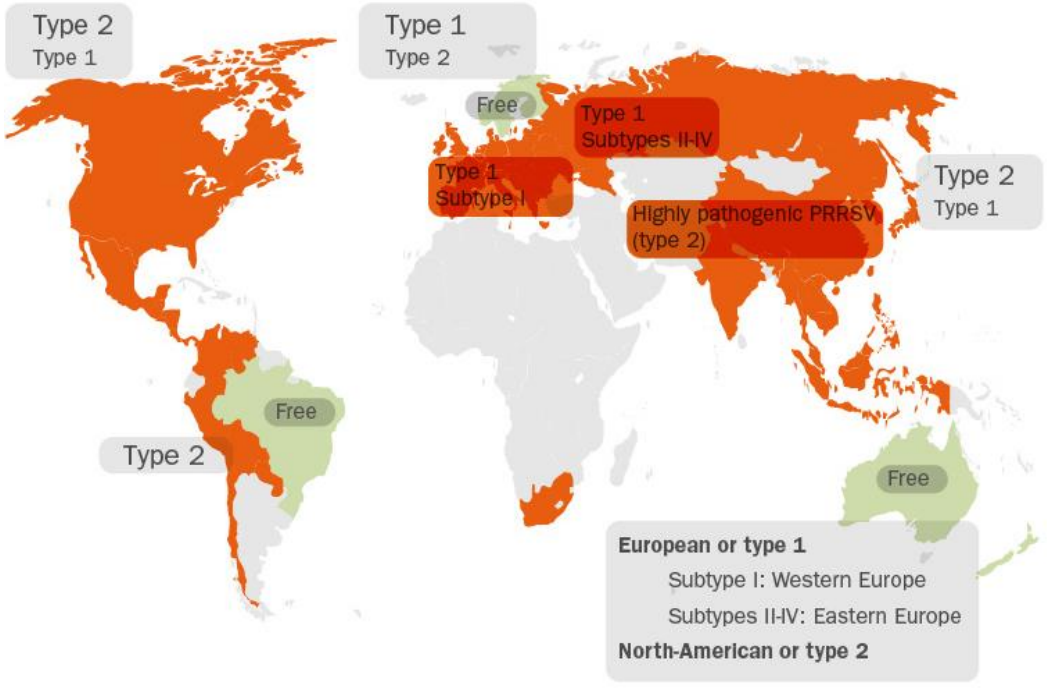

Figure 6. *Distribution globale du PRRSV selon la base de données de l'information sur la santé animale mondiale (*World Animal Health Information Database*, WAHIS) actualisée par l'organisation mondiale de la santé animale, (OIE). https://www.oie.int/wahis*

3. Description de la particule virale et de son génome

Il s'agit d'un virus enveloppé à ARN monocaténaire à polarité positive appartenant à l'ordre des *Nidovirales*, famille des *Arteriviridae* et au genre Porartevirus (Lunney et al., 2016). La famille des *Arteriviridae* comprend le virus de la fièvre hémorragique simien, le virus de l'artérite virale équine, le virus élévateur de la lactate déhydrogénase chez la souris et le PRRSV (Snijder and Meulenberg, 1998). Avec la famille des *Coronaviridae*, les *Arteriviridae* forment l'ordre des *Nidovirales* (Meulenberg, 2000) (figure 7).

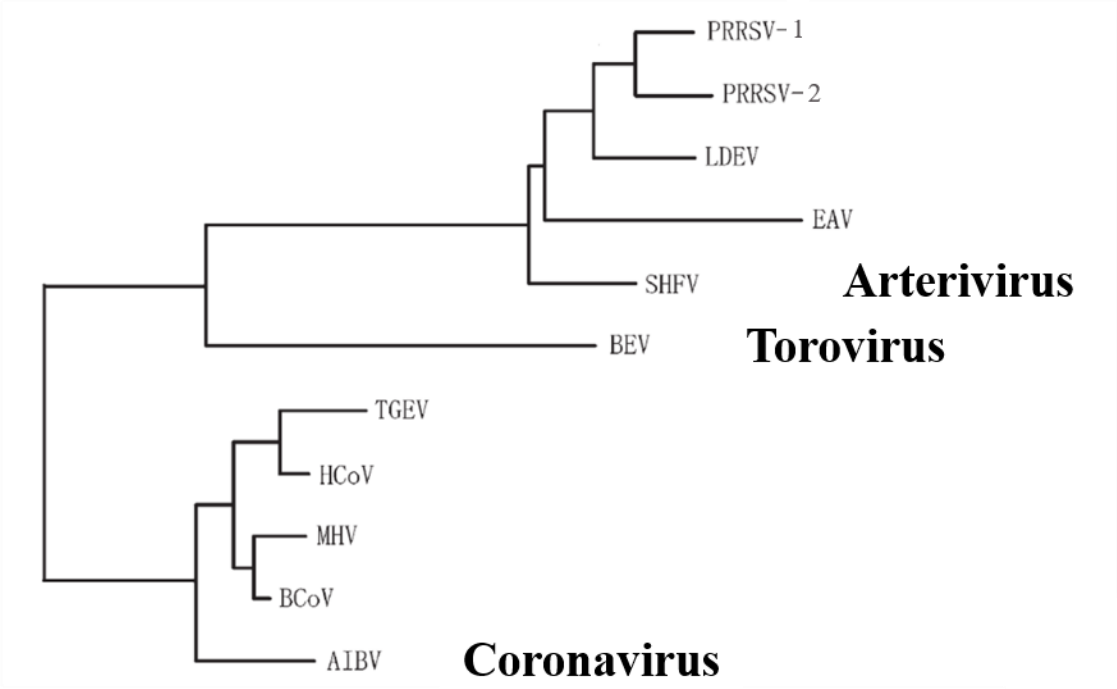

Figure 7. *L'arbre phylogénétique de l'ordre des Nidovirales (adaptée de Hanada et al. 2005)*

Le génome du PRRSV mesure 15kb et comprend neuf cadres ouverts de lecture (*open reading frames*, ORF). Les ORF1a et ORF1b constituent 80 % de ce génome et codent pour des protéines non-structurales (nsp) impliquées dans la réplication du virus telle que la *RNA-dependant RNA polymerase* (nsp9) (Lc et al., 1996; Meulenberg, 2000). Les ORF2 à 7 codent pour des protéines structurales, de l'enveloppe ou de la nucléocapside et donc jouent un rôle dans l'adaptation du virus à l'espèce porcine (Hanada et al., 2005) (figure 8).

Les principales protéines structurales des artérivirus sont la protéine N de la nucléocapside (codée par l'ORF7), la protéine intégrale M (codée par l'ORF6) et la glycoprotéine primaire de l'enveloppe GP5 (codée par l'ORF5). La protéine M et la GP5 forment un hétérodimère via un pont di-sulfure (Meulenberg, 2000). Cet hétérodimère est essentiel pour l'infectiosité et pour la formation de nouvelles particules virales, alors que le reste des protéines structurales telles GP2a, GP3, et GP4 forment un complexe multimérique essentiel pour l'interaction avec la cellule hôte (Wissink et al., 2005). La protéine E semble faire partie de ce complexe (Wu et al., 2001). Quant à la protéine N qui est très immunogène, elle est trouvée dans le cytoplasme et dans le noyau et contribue à l'antagonisme de la fonction des gènes cellulaires (Lunney et al., 2010). Contrairement aux glycoprotéines GP2, GP3, GP4, et GP5, les protéines N et M ne sont pas N-glycosylées (Meulenberg, 2000).

La GP5 est la protéine structurale qui varie le plus entre les 2 espèces du PRRSV, tandis que la protéine M semble la plus conservée avec 78 à 81 % de ressemblance dans la séquence des acides aminées (figure 8). La plus grande différence est notamment observée au niveau de la protéine non-structurale nsp2 avec seulement 32 % de similarité dans la séquence des acides aminées (Allende et al., 1999; Nelsen et al., 1999).

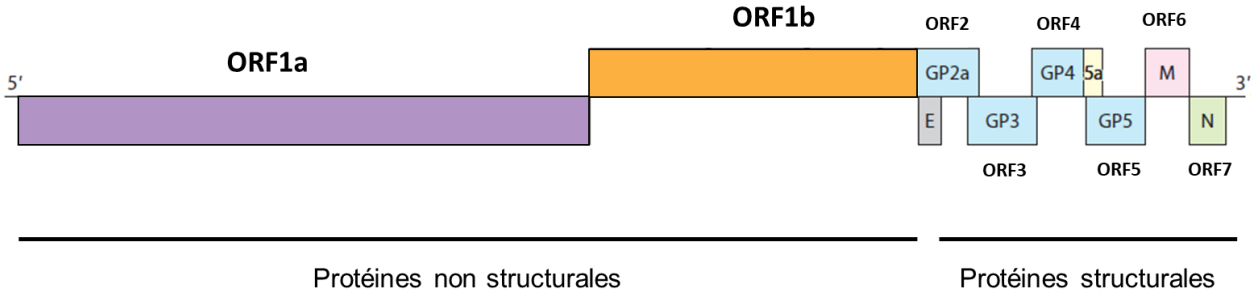

Figure 8. *Présentation du génome du PRRSV (adaptée et traduite de Lunney et al. 2016)*

4. Les signes cliniques et l'impact économique

Le PRRSV est considéré comme le plus important agent infectieux à l'origine d'atteintes reproductives et de pneumonies chez le porc domestique (Hanada et al., 2005). Le nom de la maladie causée par ce virus a connu beaucoup de variations au cours du temps. Après l'identification de ses signes cliniques, « la maladie porcine mystérieuse » fut appelée « la maladie

de l'oreille bleu » ou « le syndrome de l'avortement épidémique et respiratoire porcin » en Europe. Ensuite, et après l'isolement du virus, les Américains ont proposé « le syndrome de l'infertilité et respiratoire porcin » avant que l'appellation actuelle soit choisie durant le premier congrès international dédié à ce virus en 1992 (Lunney et al., 2010). Comme le montre cette évolution de la nomenclature, l'infection par le PRRSV qui est souvent subclinique peut engendrer des troubles d'avortements tardifs très importants et une baisse de fertilité chez les truies avec une mortalité périnatale ou une faiblesse importante à la naissance chez les porcelets. Les souches les plus virulentes sont à l'origine d'une pneumonie sévère, entrainant une fièvre, une baisse de croissance et un taux de mortalité très élevé chez les porcs sevrés et les porcs à l'engraissement (Brockmeier et al., 2017). Le PRRSV est aussi responsable d'une cyanose au niveau des oreilles qui se manifeste par une coloration bleu-rougeâtre (Keffaber, 1989; Wensvoort, 1993). Des souches peu virulentes, notamment de PRRSV-1, induisent des signes cliniques plus faibles, mais sont soupçonnées de favoriser les infections secondaires par d'autres agents pathogènes, même si cela reste à démontrer.

La circulation de ce virus entraîne des pertes économiques très importantes pour la filière porcine (Holtkamp et al., 2013). Ces pertes ne sont pas uniquement liées au PRRSV, mais aussi aux surinfections et aux co-infections qui peuvent survenir. Une étude néerlandaise estime à 126 euros la perte moyenne par animal suite à une épidémie de PRRSV (Nieuwenhuis et al., 2012). Une autre étude européenne estime les pertes annuelles d'un élevage de 1 000 porcs sévèrement touché par le virus à 650 090 euros (Montaner-Tarbes et al., 2019). Aux États-Unis, les pertes annuelles causées par le PRRSV atteignent 560 millions de dollars américains (Neumann et al., 2005). La Chine a également connu de graves répercussions économiques après l'émergence de la nouvelle souche virulente en 2006. Cette souche se caractérisait par des taux élevés de morbidité et de mortalité allant jusqu'à 100 % (Tong et al., 2007). L'organisation mondiale de la santé animale a enregistré une contamination de 310 000 porcs avec la mort de 81 000 animaux dans 26 provinces, augmentant ainsi les prix de la viande de porc sur le marché chinois. En parallèle, d'énormes budgets ont été dédiés à la campagne de vaccination augmentant ainsi la charge financière sur l'État. Jusqu'à 2007, la Chine a administré plus de 314 millions de doses pour immuniser 100 millions d'animaux.

5. Hôte cellulaire et tropisme tissulaire

Le tropisme du PRRSV est assez particulier avec une gamme de cellules cibles très limitée. Le virus se réplique principalement dans les macrophages alvéolaires pulmonaires (AM) et les macrophages intravasculaires pulmonaires (Meulenberg, 2000; Thanawongnuwech et al., 2000). Il infecte également les macrophages de divers organes tels que les amygdales, les nœuds lymphatiques, la rate, le foie et le placenta (Duan et al., 1997; Meulenberg, 2000). D'autres études confirment la réplication du PRRSV dans les cellules dendritiques dérivées des monocytes (Mo-DCs) (Flores-Mendoza et al., 2008; Wang et al., 2007) mais pas dans les DC conventionnelles (Elise Bordet et al., 2018b).

Le PRRSV possède un tropisme pour les cellules exprimant le récepteur CD163 à leur surface (Van Gorp et al., 2010). Ce récepteur interagit avec les glycoprotéines GP2a et GP4 du virus (Zhang et Yoo 2015). Une réplication du virus au niveau des cellules germinales telles que les spermatides et les spermatocytes a été démontrée également chez les sangliers infectés par le PRRSV (Sur et al., 1997). D'autres part, des tests *in vitro* ont montré la capacité d'une lignée de cellules rénales de singe (*African green monkey kidney cells*, MARC-145) à répliquer le PRRSV-2 (Kim et al., 1993). En conséquence, l'amplification et la production du PRRSV-2 s'effectuent sur la lignée MARC-145 tandis que le PRRSV-1 nécessite une amplification sur des macrophages alvéolaires (Renson et al., 2017).

6. L'infection des cellules hôtes et la réplication virale

Comme tous les artérivirus, le PRRSV pénètre dans la cellule par endocytose médiée par la clathrine (Kim et al., 1993). Quelques heures après l'internalisation du virus, des vésicules à double membrane dérivant du réticulum endoplasmique se forment. Ces vésicules transportent les virions répliqués pour une fusion avec la membrane cytoplasmique menant au bourgeonnement du virus (Meulenberg, 2000; Pedersen et al., 1999).

Le récepteur CD163 est le récepteur le plus étudié et le plus important pour une infection par le PRRSV. L'expression de ce récepteur par des cellules non-permissives au PRRSV assure l'adhésion, l'internalisation et parfois la réplication de ce dernier une fois tous les cofacteurs essentiels pour la réplication présents (Calvert et al., 2007). L'interaction du virus avec le récepteur CD163 est dans ce cas-là assurée par les glycoprotéines virales GP4 et GP2a (Das et al., 2010). D'autre part, cinq autres récepteurs cellulaires au PRRSV ont été identifiés (Zhang and Yoo, 2015).

Ce sont le sulfate d'héparane, la vimentine, le récepteur CD151, le CD169 (aussi appelé sialoadhésine ou Siglec-1) et le DC-SIGN (« *dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin* » ou CD209) (figure 9).

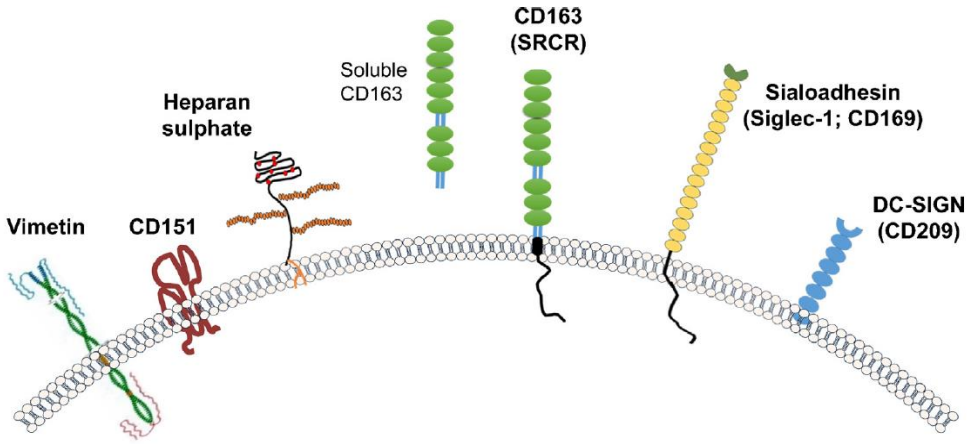

Figure 9. *Les six récepteurs cellulaires du PRRSV (Zhang et al. 2015)*

Chaque récepteur occupe un rôle différent dans le processus d'infection des cellules hôtes par le PRRSV. Le récepteur CD169 se fixe aux acides sialiques sur le complexe formé par la GP5 et la protéine M du PRRSV et contribue à l'adhésion et à l'internalisation de ce dernier dans les AM (Vanderheijden et al., 2003). Le sulfate d'héparane contribue également à la première étape d'adhésion du PRRSV et renforce l'interaction avec le récepteur CD169 (Delputte et al., 2005). Il s'agit donc d'une coopération entre le sulfate d'héparane et la CD169 pour promouvoir l'adhésion et l'internalisation du virus sans que le CD169 soit absolument essentiel pour l'infection des AM (Zhang and Yoo, 2015). Finalement, la vimentine et le récepteur CD151 contribuent à l'infection des cellules MARC par le PRRSV-2 (Huang et al., 2009; Wu et al., 2014). L'interaction de la vimentine avec le virus s'effectue par la protéine N (Huang et al., 2009).

7. La transmission et la persistance du virus

Les échanges de porcs infectés ou d'équipements entre les établissements d'élevage favorisent souvent la circulation du virus (Lunney et al., 2010). Cette transmission est assurée par le sperme et les fluides oraux de l'animal (S. A. Dee et al. 2006). D'autre part, même si la transmission aérogène entre les différents élevages semble très peu probable (Otake et al., 2002; Arruda et al.,

2019), celle-ci reste la seule explication lors d'émergences de la maladie sans preuve d'échanges directs avec des élevages contaminés (Dee et al., 2010; Arruda et al., 2019).

Les amygdales et les nœuds lymphatiques forment les pricipaux sites de cette persistance (Xiao et al., 2004). Le PRRSV peut persister pour une longue durée chez l'animal infecté et peut se transmettre bien longtemps après l'infection. Les études ont montré une transmission du PRRSV jusqu'à 99 jours post-infection et une détection des particules virales allant de 105 à 157 jours (Allende et al. 2000; Horter et al. 2002; Wills et al. 1997). Le mécanisme de persistance inclut une émergence de variants viraux pouvant échapper à la défense de l'hôte. Un phénomène d'échappement aux anticorps neutralisants par glycosylation des protéines virales et masquage des épitopes neutralisants a été décrit (Ansari et al., 2006). Cette persistance est aussi expliquée par une inhibition du développement des anticorps neutralisants (Mulupuri et al., 2008) liée à une prolifération anormale des lymphocytes B (Butler et al., 2014). Les études ont montré également une diminution de l'activité présentatrice des DC suite à l'infection par le PRRSV (Butler et al., 2014). Dernièrement, une hypothèse a été émise et qui associe la baisse de l'activité cytotoxique à la capacité du PRRSV à induire une atrophie du thymus empêchant le développement des thymocytes et causant une déficience en lymphocytes cytotoxiques (Butler et al., 2019). Ces mécanismes varient beaucoup en fonction des différentes souches. Ainsi, les souches virulentes induisent une forte réponse inflammatoire et sont éliminées efficacement, alors que les souches moins virulentes ont tendance à persister et à altérer la réponse immune (Morgan et al., 2013; Weesendorp et al., 2013; Elise Bordet et al., 2018a).

La transmission transplacentaire est possible durant la gestation (Rowland, 2010). La réplication et la persistance du PRRSV chez le fœtus ont été démontrées au niveau du thymus (Rowland, 2010).

8. La gestion de la propagation et la lutte contre le virus

L'utilisation des systèmes de filtration de l'air semble être une technique très efficace pour réduire la transmission du PRRSV par les aérosols dans les élevages (Dee et al., 2010). La décontamination et la désinfection des locaux et des équipements est aussi essentielle pour contrôler la dissémination du virus. Certains éleveurs utilisent la technique du confinement de leurs troupeaux pendant 200 jours après l'exposition de tous les animaux au virus par vaccination ou

contamination. Par conséquent, tout animal introduit au-delà de cette période reste sain (Lunney et al., 2010).

La vaccination reste la mesure la plus efficace pour lutter contre la propagation du PRRSV. Cependant, l'efficacité du vaccin dépend de sa capacité à induire une réponse immunitaire rapide, à ne pas développer d'effets secondaires indésirables et surtout à conférer une protection croisée contre les différentes souches du virus (de Lima et al., 2008; Fang et al., 2008).

Les premiers vaccins inactivés ont été développés au début des années 90, et le premier vaccin vivant atténué a été commercialisé en 1994 (Mengeling, 2005). Depuis ces premiers vaccins, d'autres vaccins ont vu le jour en Europe et aux États-Unis (Mengeling, 2005). Toutefois, aucun vaccin n'a assuré une protection complète et universelle contre le PRRSV (Lunney et al., 2010). Les stratégies de vaccination et les vaccins développés assurent une réduction limitée de la propagation du virus et une certaine amélioration des performances d'élevage. Cette absence d'un système de vaccination solide et fiable est due à l'hétérogénéité des souches du virus. L'importante variabilité au niveau de la glycoprotéine GP5 entre les différentes souches du PRRSV par exemple, peut être à l'origine de l'inefficacité des vaccins pour assurer une protection croisée contre les différentes souches circulant dans les élevages (Lunney et al., 2010). En conséquence, le grand défi pour lutter contre le PRRSV reste à identifier un épitope conservé entre les différents mutants qui puisse jouer un rôle dans le développement d'une protection croisée (Kimman et al., 2009).

Finalement, la combinaison de la vaccination et de la mise en place de protocole d'hygiène et du système de filtration de l'air est primordial pour l'élimination du virus dans une zone géographique donnée (Corzo et al., 2010).

9. La réponse immunitaire de l'hôte porcin à l'infection par le PRRSV

Le PRRSV dispose d'une capacité d'inhibition de la production de cytokines inflammatoires telles que les IFN de type 1 (notamment IFNα et IFNβ), le TNFα et l'IL1 (Thanawongnuwech et al., 2001). Cet effet anti-inflammatoire est dû à la présence de 5 types de nsps trouvées chez le PRRSV. Nsp1β joue un rôle primordial dans l'inhibition de la production des IFN-β en inhibant la voie de signalisation médiée par TLR3 et l'activité du promoteur de TNFα (Beura et al., 2010; Lunney et al., 2010). D'autre part, il a été démontré que nsp2 est responsable de l'inhibition de l'expression des IL1β et TNFα (Chen et al., 2010). Cette inhibition de la production des cytokines

est soupçonnée être à l'origine de la faible réponse immune innée, de la production retardée d'anticorps neutralisants et de la défaillance de la réponse cellulaire cytotoxique face à l'infection par le PRRSV (Costers et al., 2009). En revanche, certaines souches virulentes, telles que la souche Lena (PRRSV-1.3) présentent une forte réponse immune adaptée initiée par les lymphocytes T cytotoxiques qui est à l'origine de la pathogénicité du virus (Elise Bordet et al., 2018a). Cela reste donc très dépendant des différentes souches du PRRSV.

Suite à une infection par le PRRSV, une variété d'anticorps monoclonaux est produite contre les protéines N, M et les glycoprotéines GP3, 4 et 5. Grâce à un domaine antigénique immuno-dominant (acides aminées 55-66 et 80-90), la protéine N constitue la protéine immuno-dominante du virus (Meulenberg et al., 1998a). Par conséquent, la majorité des anticorps monoclonaux produits sont dirigés contre cette protéine structurale (Meulenberg et al., 1998b). D'autre types d'anticorps monoclonaux sont dirigés contre la GP4 et la GP5 et jouent un rôle important dans la neutralisation du virus, suggérant un rôle principal de ces 2 GPs dans l'adhésion du virus à la cellule hôte (van Nieuwstadt et al., 1996; Wieczorek-Krohmer et al., 1996). Cependant, les anticorps anti-GP5 ont montré une meilleure efficacité de neutralisation en comparaison avec les anticorps anti-GP4 (Weiland et al., 1999). La production de ces anticorps neutralisant reste cependant très tardive. Les études montrent une production précoce des anticorps non-neutralisants, alors que la production d'anticorps neutralisants ne s'effectue pas avant quelques semaines ou quelques mois après l'infection (Lopez and Osorio, 2004). Cependant, la persistance de la virémie après l'apparition des anticorps neutralisants remet l'efficacité de ces anticorps en question (Lunney et al., 2010).

D'autre part, les études montrent que la réponse immunitaire cellulaire menée par les lymphocytes T est souvent dirigée vers les protéines M, GP2 et GP5 (Bautista et al., 1999). Cette réponse est aussi induite tardivement au bout de 2 à 8 semaines post-infections avec beaucoup de variabilité entre PRRSV-1 et PRRSV-2, et même entre différentes souches intra-espèce, au niveau du recrutement des cellules T qui ne corrèle pas souvent avec la réplication du virus dans les tissus lymphoïdes (Xiao et al., 2004; Elise Bordet et al., 2018b).

10. Le PRRSV et les voies de signalisation

Parmi les différentes stratégies pour contourner la réponse antivirale de la cellule, le PRRSV est capable d'inhiber la production des IFN en modulant l'activation et la signalisation des

récepteurs tels que RIG1 et MDA5 (Shi et al., 2012) (figure 10). La protéine nsp1 par ces 2 composantes nsp1α et nsp1β affecte directement la phosphorylation et la translocation de l'IRF3 au noyau en empêchant son association à la « *CREB-binding protein, CBP* » et en induisant la dégradation de cette dernière chez les MARC-145 (Beura et al., 2010; Shi et al., 2010) (figure 10). La translocation de l'IRF3 est bloquée par l'inhibition de l'activation de IPS-1 dans la voie de signalisation de RIG1 entrainant une inhibition de la production de l'IFNβ suite à une infection par le PRRSV (Luo et al., 2008) (figure 9). Nsp2 est aussi capable d'affecter la phosphorylation et la translocation nucléaire de l'IRF3 (Li et al., 2010). Elle intervient également dans la voie de signalisation NF-κB en impactant l'ubiquitination de IκB et en empêchant sa dégradation (Sun et al., 2010) (figure 10). Quant à la nsp11, elle effectue le rôle des endoribonucléases et inhibe l'activation du promoteur de l'IFNβ ainsi que l'expression des gènes médiés par l'IRF3 (Shi et al., 2011). Finalement, les protéines non-structurales du PRRSV ne sont pas les seules contributrices à l'inhibition de cette production d'IFN. Il a été démontré que la phosphorylation et la translocation de l'IRF3 est aussi affectée par la protéine N qui contribue à la modulation de l'expression des ARNm de l'IFNβ (Sagong and Lee, 2011).

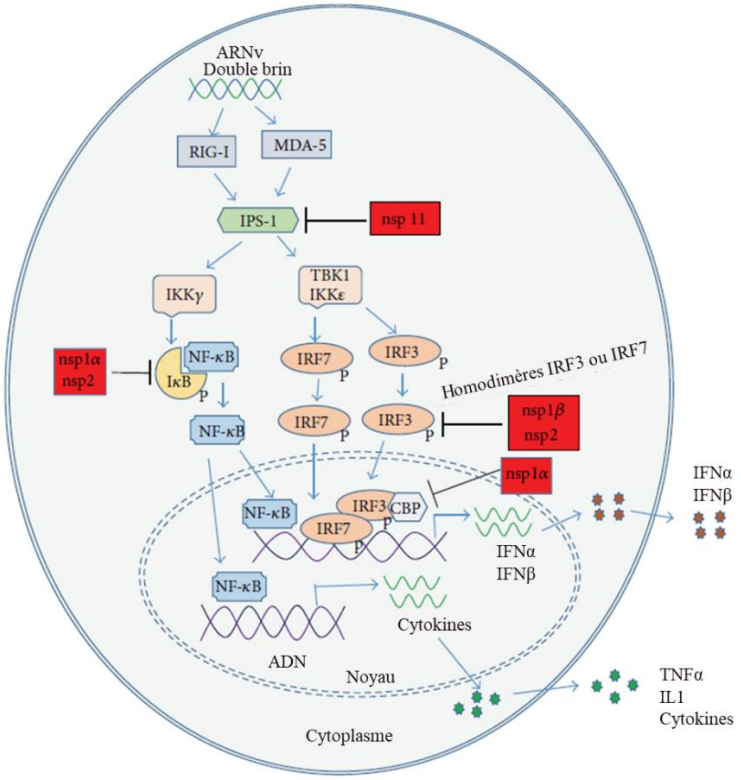

Figure 10. *Interférence des protéines virales du PRRSV avec la production des IFN de type I (traduite de Wang et al. 2014)*

La stratégie du PRRSV ne se limite pas à l'inhibition de la production des IFN, mais aussi à l'inhibition des voies de signalisation induites par ces IFN (Patel et al., 2010a). Le PRRSV est capable de moduler la voie de signalisation JAK/STAT induite par les IFN chez les AM et les MARC-145 (Yang et al., 2017). Cette modulation est associée à une réduction de l'expression des ISG tels que ISG15 et ISG56 (Patel et al., 2010) (figure 11). Il a été démontré que la nsp1 et plus précisément nsp1β induit la dégradation de la Karyophérine alpha 1 (KPNA1) ou l'importine alpha 5 qui contribue à la translocation de l'ISGF3 au noyau (figure 11). La protéine N participe également à l'inhibition de cette importation de l'ISGF3 vers le noyau (figure 10). Par conséquent, l'importation de l'hétérodimère STAT1/STAT2 vers le noyau est bloquée entrainant une réduction de la transcription des différents ISG (Wang et al., 2013b) (figure 11). Le PRRSV dispose également d'un effet antagoniste direct sur les ISG en réduisant par exemple la production et la conjugaison de l'ISG15 grâce à la nsp2 (Sun et al., 2012) (figure 11). Le PRRSV inhibe l'expression du gène de la protéine kinase R (PKR) chez les AM et celui de la protéine transmembranaire 1 induite par les interférons (IFITM 1) chez les MARC-145 (Wang et al., 2014; Xiao et al., 2016).

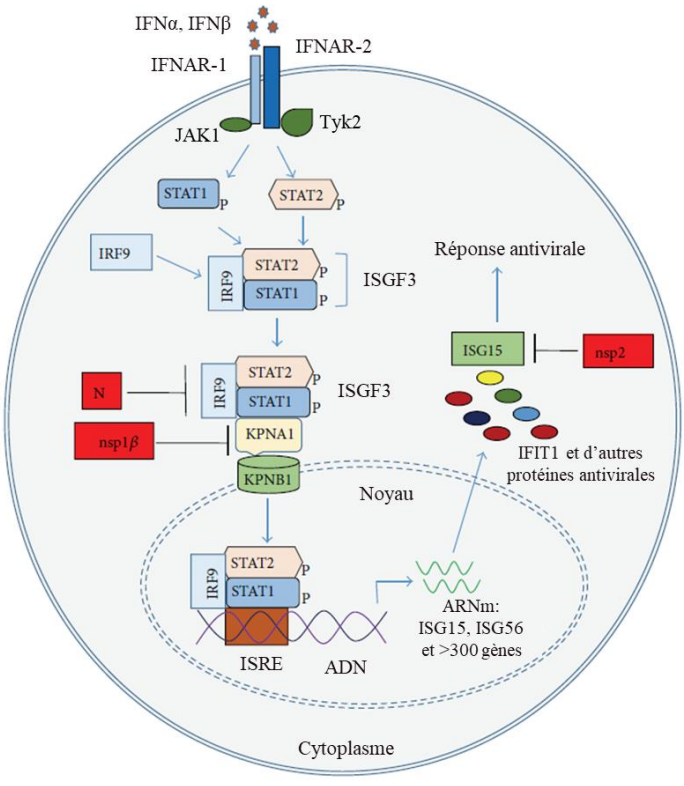

Figure 11. *Interférence des protéines virales du PRRSV avec la voie de signalisation JAK/STAT induites par les des IFN de type I (traduite de Wang et al. 2014)*

D'autre part, le PRRSV utilise une autre technique pour contrôler la voie JAK/STAT. Il s'agit de l'induction de signaux de suppression tels que les protéines suppresseurs de signalisation des cytokines (SOCS) et les micro-ARNs miR-30c (Delgado-Ortega et al., 2011; Wysocki et al., 2012; Zhang et al., 2016). En parallèle, le PRRSV inhibe la production de STAT3 et STAT6 ainsi que l'expression de JAK1. La nsp5 semble jouer un rôle dans l'inhibition de STAT3, cependant le mécanisme derrière cet effet nécessite plus d'investigation (Yang and Zhang, 2017).

À noter qu'il existe, ici encore, une certaine variabilité entre les 2 types et les différentes souches du PRRSV et toutes les souches n'ont pas le même effet sur la voie JAK/STAT et sur la régulation de la production des IFN (Subramaniam et al., 2011; Wang et al., 2013a).

Une autre voie de signalisation occupe une place importante durant l'infection par le PRRSV. Il s'agit de la voie PI3K/Akt qui est activée par le PRRSV chez les MARC-145 et durant la phase précoce de l'infection chez les AM et les Mo-DCs (Zhang and Wang, 2010; Zhu et al., 2013). Une inhibition tardive de cette voie a été notée après infection des Mo-DCs (Zhang and Wang, 2010). Il a été démontré que cette voie joue un rôle très important dans le contrôle de l'expression des gènes viraux et dans l'induction de la réplication et de l'effet cytopathique du PRRSV (Pujhari et al., 2014). L'activation de l'hétérodimère PI3K aboutit à la conversion de la *phosphophatidylinositol 4, 5-bisphosphate* (PIP2) en *phosphatidylinositol-3,4,5-triphosphate* (PIP3). Ensuite, la PIP3 contribue à l'activation de l'Akt qui est responsable du déclenchement d'une cascade de signalisation comprenant les protéines GSK3, p21, Bad, Foxo1 et mTORC1 qui est la cible de la rapamycine chez les mammifères (Pujhari et al., 2014). Cette cascade contrôle plusieurs fonctions cellulaires liées à la synthèse des protéines, à la prolifération cellulaire, au métabolisme, à l'apoptose, à la migration et au trafic vésiculaire (Pujhari et al., 2014). En d'autres termes, le PRRSV se sert de cette voie pour faciliter son internalisation (Ni et al., 2015), induire la traduction de son ARN et augmenter la synthèse des protéines virales essentielles pour sa réplication (Pujhari et al., 2014).

D'un autre côté, les études montrent une activation de la voie p38 MAPK par l'intermédiaire de la GP5 du PRRSV. Cette activation est à l'origine de l'augmentation de la production d'IL10 par les AM suite à l'infection par le PRRSV (Hou et al., 2012). L'inhibition de certains TLRs et du récepteur RIG1 montre clairement que le gène de la réponse primaire de différenciation

myéloïde 88 (MYD88) joue un rôle primordial dans la production d'IL10 (Song et al., 2013) et d'IL1β (Bi et al., 2014) par les AMs.

L'activation de la voie de signalisation des ERK1/2 en phase précoce de l'infection par PRRSV a été liée à l'internalisation du virus par les AMs. L'activation de cette voie assure une induction de la transcription et la traduction de l'ARNv ainsi que la réplication du virus dans un stade plus tardif (Lee and Lee, 2010).

Finalement, la protéine kinase activée par l'adénosine monophosphate (AMPK) qui est connue pour son blocage de la synthèse des acides gras est fortement activée par le PRRSV (Long et al., 2019). Cela entraîne l'inactivation de l'Acetyl-CoA carboxylase 1 (ACC1) qui constitue la première enzyme de la voie de biosynthèse des acides gras. L'ACC1 est considérée essentielle pour la prolifération du PRRSV et son inactivation contribue à la réponse antivirale des cellules (Heaton and Randall, 2011; Long et al., 2019). Ces résultats suggèrent l'activation de la voie AMPK-ACC1 suite à une infection par le PRRSV (Long et al., 2019).

c) La réponse immune suite à la co-infection par le PRRSV et le swIAV

Avant d'évaluer la réponse immune face à une co-infection par le PRRSV et le swIAV chez le porc, il est important de rappeler que ces deux virus ne partagent pas la même cellule hôte principale. Le PRRSV infecte principalement les AM présentant le récepteur CD163 et le swIAV se réplique plutôt dans les cellules épithéliales du tractus respiratoire. Cette différence de tropisme, rend l'évaluation des conséquences moléculaires de ces co-infections plus complexe.

Malgré l'importante contribution du swIAV et du PRRSV au CRP et leur fréquente co-circulation dans les élevages, très peu d'études se sont intéressées à l'évaluation de la co-infection entre ces deux virus et la majorité de ces études ont été faites *in vivo*. Ces études se sont concentrées sur l'évolution des signes cliniques, des lésions macroscopiques et microscopiques et l'excrétion virale chez les porcs (Pol et al., 1997; Van Reeth et al., 1996; Kitikoon et al., 2009; Sangpratum et al., 2019; Pomorska-Mól et al., 2020) (tableau 6). En parallèle, les études *in vitro* évaluant les conséquences moléculaires de la co-infection sur la réponse immune de chacune des cellules cibles restent peu nombreuses (tableau 7).

Une étude épidémiologique a été menée pour étudier l'association entre les infections par le swIAV et le PRRSV et pour évaluer les effets de l'âge et du système d'élevage sur le statut de la co-infection, sur le profile sérologique ainsi que sur les lésions histologiques chez les animaux co-infectés (Dorr, 2007). Il a été montré que les porcs infectés par le PRRSV ont plus de chance de développer une infection par le swIAV. Les porcs infectés par le PRRSV et âgés de 9 ou de 16 semaines ont 15,57 et 5,75 fois plus de chance respectivement d'être surinfectés par le swIAV (Dorr, 2007). Une autre étude montre une corrélation entre l'incidence des co-infections par le PRRSV et le swIAV chez les porcelets non sevrés et le taux de mortalité chez les mêmes porcs une fois sevrés (Alvarez et al., 2015). Une étude américaine estime la séroprévalence pour les 2 infections dans les élevages américains entre 4,9 % et 5,9 % confirmant encore une fois l'importance de ces co-infections chez le porc (Bush, 2003).

Les premières études qui se sont focalisées sur les conséquences cliniques et histopathologiques de ces co-infections, ont bien démontré l'aggravation de l'inflammation au niveau des poumons et des ganglions lymphatiques dans le cas de co-infections en comparaison avec les infections simples (Kitikoon et al., 2009; Pol et al., 1997). Les signes cliniques des porcs co-infectés expérimentalement ont aussi été plus graves que les signes présentés par les porcs mono-infectés (Van Reeth et al., 1996) (tableau 6). Une étude récente effectuée par Czyżewska et

al en 2019 montre bien une augmentation de l'expression des transcrits des IL6 et IL10 chez des porcs co-infectés *in vivo* par les 2 virus. Cette étude confirme encore une fois la sévérité des signes cliniques en cas de co-infections.

Les deux études menées récemment *in vitro* et *ex vivo* ont réussi à évaluer les interactions entre le PRRSV-2 et le swIAV et leurs conséquences sur des AMs, des tranches pulmonaires (*precision cut lung slices, PCLS*) et des cellules épithéliales exprimant le récepteur CD163 (tableau 2). La première étude a bien montré une interférence au niveau de la réplication des virus et un effet synergique concernant l'expression de quelques transcrits tels que ceux de TLR3, RIG1 et IFNβ (Dobrescu et al., 2014). Une infection par le PRRSV-2 3h avant l'infection par le swIAV a réduit la réponse des AM à l'infection par le swIAV, cependant une pré-infection par le swIAV a montré un effet limité sur la réponse au PRRSV-2 (Dobrescu et al., 2014) (tableau 7). La deuxième étude effectuée sur la lignée de cellules épithéliales modifiées exprimant le récepteur CD163, et qui est donc rendue artificiellement permissive aux 2 virus, a montré que les 2 virus sont capables d'influencer la réponse immune des cellules, mais d'une façon différente (Provost et al., 2017). Le PRRSV exerce un effet inhibiteur sur la réponse immune innée alors que le swIAV va entrainer son induction. Cette étude confirme la possibilité d'une interaction d'un virus sur la réplication de l'autre avec des conséquences sur la réponse antivirale, notamment l'expression des gènes codant pour les PRR, les IFN et les ISG comme MX2, OAS et PKR (Provost et al., 2017).

Nombreuses sont les études évaluant les voies de signalisation induites ou inhibées en cas d'infection par le swIAV ou le PRRSV séparément. Ces études se sont intéressées à la réponse de la plupart des cellules cibles en allant des cellules épithéliales aux AM et aux DCs. Cependant et à nos connaissances, il n'y a eu aucune étude qui évalue les voies de signalisation impliquées en cas de co-infections ou de surinfections par ces 2 virus.

Tableau 6. *Description des essaies expérimentaux réalisés* in vivo *pour étudier les co-infections et les surinfections par le PRRSV et le swIAV*

Co-infections	Signes cliniques	Lésions microscopiques	Autres observations	Références
PRRSV swIAV	Signes cliniques plus sévères chez les animaux co-infectés	ND	Excrétion de swIAV retardée en présence du PRRSV (2 jours).	(Van Reeth et al., 1996)
	ND	La co-infection a induit une inflammation plus prononcée de la paroi bronchiolaire.	L'infection par le PRRSV n'a eu aucun effet sur l'infection par le swIAV.	(Pol et al., 1997)
	-Différences négligeables dans les signes cliniques entre les co-infections et les infections simples. Cependant, la prise de poids a tendance à être moins importante chez les animaux co-infectés.	ND	L'intervalle de temps entre les deux infections peut affecter le résultat clinique. Des infections multiples avec un intervalle de 3 ou 7 jours ont entraîné une maladie aiguë tandis qu'un intervalle de 14 jours entre les virus a entraîné une infection subclinique. -Différences négligeables entre les porcs co-infectés et les porcs mono-infectés.	(Van Reeth et al., 2001)
	-Fièvre et réduction de la prise de poids chez les animaux co-infectés -Les animaux co-infectés ont montré la plus grave maladie clinique.	La co-infection a induit une pneumonie interstitielle importante.	-Aucune augmentation de l'excrétion de swIAV -Augmentation de la prolifération des lymphocytes après une infection par le PRRSV -L'infection par le PRRSV a réduit l'efficacité du vaccin swIAV.	(Kitikoon et al., 2009)
	Signes cliniques plus sévères chez les porcs co-infectés	ND	-La charge virale du PRRSV dans le LBA était plus élevée chez les porcs infectés par le PRRSV seul que chez les porcs co-infectés. -Augmentation de l'expression des transcrits des IL6 et IL10 ont chez les porcs co-infectés.	(Czyżewska-Dors et al., 2019)

	Aucune différence majeure dans les signes cliniques. À l'exception d'une fièvre prolongée chez les porcs co-infectés en comparaison aux porcs mono-infectés.	Pneumonie interstitielle était plus sévère après l'infection par le PRRSV. De plus, les animaux co-infectés ont présenté une nécrose épithéliale et une infiltration de cellules mononucléées dans le septum alvéolaire.	- La charge virale H1N1 était plus importante dans le groupe co-infecté que dans le groupe mono-infecté.	(Sangpratum et al., 2019)

Tableau 7. *Description des essais expérimentaux réalisés* in vitro *et* ex vivo *pour étudier les co-infections et les surinfections par le PRRSV et le swIAV*

Co-infections	Cellules utilisées	Interférence virale	Réponse immunitaire	Références
swIAV PRRSV	PCLS AM	- Réplication du PRRSV réduite par swIAV - Le PRRSV-2 n'a pas impacté la réplication de swIAV dans les PCLS, mais a réduit sa réplication dans les AM.	- Le PRRSV-2 a réduit la réponse au swIAV. - swIAV a montré un faible impact sur l'infection par le PRRSV-2. - swIAV affecte l'expression des ISG, mais pas le PRRSV. - Effet synergique pour les expressions de TLR3, RIGl et IFNβ sur les PCLS	(Dobrescu et al., 2014)
	NPTr-CD163	-Le PRRSV-2 a réduit la réplication du swIAV en co-infection et lors de sa surinfection 3h après le swIAV. -swIAV a diminué la réplication du PRRSV lorsqu'il a été inoculé 3h à l'avance.	- Les deux virus à ARN interfèrent l'un avec l'autre. - La pré-infection par PRRSV-2 a moins d'effet que la pré-infection par swIAV. - Aucun effet synergique ou additif évident observé - IFNλ1 sont plus exprimés que les autres IFN.	(Provost et al., 2017)

Chapitre 2

Le virus du syndrome dysgénésique et respiratoire porcin affecte l'infection des cellules épithéliales par le virus de l'influenza A

Chapitre 2 : Le virus du syndrome dysgénésique et respiratoire porcin affecte l'infection des cellules épithéliales par le virus de l'influenza A

a) Introduction des travaux :

Les co-infections par le swIAV et le PRRSV sont très fréquentes dans les élevages de porcs (Fablet et al., 2012b). Différentes études ont évalué ces co-infections *in vivo* pour comprendre l'effet d'une première infection sur une autre ainsi que la réponse immunitaire et la réaction de l'animal qui subissent également des variations en rencontrant les deux virus en même temps ou successivement (Pol et al., 1997; Kitikoon et al., 2009; Sangpratum et al., 2019). Les deux études menées *in vitro* ont été effectuées sur des cellules qui sont susceptibles aux deux virus en même temps (Dobrescu et al., 2014; Provost et al., 2017). Une première a été faite sur les macrophages alvéolaires (Dobrescu et al., 2014) alors que la deuxième a évalué l'interaction entre les deux virus sur des cellules épithéliales exprimant le récepteur CD163 qui est un récepteur primordial pour l'infection par le PRRSV (Provost et al., 2017). Cependant, aucune étude sur les co-infections n'a évalué la réponse des cellules épithéliales au PRRSV, cellules qui constituent la cible principale de l'infection par le swIAV.

Dans ce chapitre, nous nous intéressons à la réponse des cellules épithéliales à des souches locales de swIAV et de PRRSV-1. Ces réponses n'ont jamais été étudiées. Les travaux ont été menés dans le but d'investiguer une probable variation de la réponse de ces cellules à une infection grippe en présence du PRRSV-1. Ensuite, l'impact de ce deuxième virus sur la réplication du swIAV a été évalué. Par conséquent, une série d'infection a été menée sur une lignée de cellules épithéliales provenant de trachée de porcelet en faisant varier l'ordre et le délai d'inoculation de ces deux virus.

Dans un premier temps, une évaluation de la mortalité cellulaire et de l'effet du PRRSV sur l'action cytopathique du swIAV a été menée par mesure de l'impédance. Dans un deuxième temps, une étude de l'expression des gènes impliqués dans la réponse immune des cellules épithéliales a été conduite et les expressions des transcrits des gènes des récepteurs, des interférons et des gènes stimulés par ces interférons ont été mesurées par RT-qPCR. Les investigations ont été poussées plus loin en évaluant l'impact du PRRSV actif et inactif sur les voies de signalisation impliquées dans les infections par le swIAV. Les résultats obtenus *in vitro* ont ensuite été confirmés *ex vivo* dans un système plus complexe et plus proche de la situation *in vivo*.

b) Papier n°2 :

vaccines

MDPI

Article

Porcine Reproductive and Respiratory Syndrome Virus Interferes with Swine Influenza A Virus Infection of Epithelial Cells

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Abstract: Respiratory infections are still a major concern in pigs. Amongst the involved viruses, the porcine reproductive and respiratory syndrome virus (PRRSV) and the swine influenza type A virus (swIAV) have a major impact. These viruses frequently encounter and dual infections are reported. We analyzed here the molecular interactions between viruses and porcine tracheal epithelial cells as well as lung tissue. PRRSV-1 species do not infect porcine respiratory epithelial cells. However, PRRSV-1, when inoculated simultaneously or shortly before swIAV, was able to inhibit swIAV H1N2 infection, modulate the interferon response and alter signaling protein phosphorylations (ERK, AKT, AMPK, and JAK2), in our conditions. SwIAV inhibition was also observed, although at a lower level, by inactivated PRRSV-1, whereas acid wash treatment inactivating non-penetrated viruses suppressed the interference effect. PRRSV-1 and swIAV may interact at several stages, before their attachment to the cells, when they attach to their receptors, and later on. In conclusion, we showed for the first time that PRRSV can alter the relation between swIAV and its main target cells, opening the doors to further studies on the interplay between viruses. Consequences of these peculiar interactions on viral infections and vaccinations using modified live vaccines require further investigations.

Keywords: pig; PRRSV; swIAV; coinfection; epithelial cell; interference

1. Introduction

Respiratory infectious diseases are still a major concern in pigs [1]. Most often multiple infectious agents including bacteria, parasites, and viruses are involved in the development of clinical respiratory conditions [2]. Thus, the reductionist approach considering only single infections might miss important interactions [2]. The term "coinfection" describes simultaneous infections of the host with different microorganisms while "superinfection" refers to a succession of infections where one microorganism infects the cell or the host before any infection by a second "superinfecting" microorganism [2,3]. Amongst viruses causing respiratory coinfections and superinfections in pigs, the porcine reproductive and respiratory syndrome virus (PRRSV) and the swine influenza type A virus (swIAV) are of major importance because of their economic impacts and their prevalence worldwide [2]. Indeed, epidemiologic studies in France, Korea, and the USA have observed associations between these two viruses in pigs [4–8]. Both viruses are RNA viruses, PRRSV targeting mostly alveolar macrophages, and swIAV infecting predominantly epithelial cells in the upper and the lower respiratory tracts [9,10]. Two different species, PRRSV-1 (alternatively Betaarterivirus suid 1), from European origin, and PRRSV-2 (alternatively Betaarterivirus suid 2) from North American origin, are described for PRRSV [11]. The three main IAV subtypes encountered in pigs are H1N1, H1N2, and H3N2, however many genetic lineages and antigenic variants within these subtypes are co-circulating [10]. To control PRRSV infections, modified live viruses (MLV) are used as vaccines [9] and both wild type and vaccine strains persist for months in the animal, increasing the risk of dual PRRSV/swIAV infections [9]. In the literature, the clinical outcomes of PRRSV/swIAV coinfections and superinfections varied depending on the experimental settings and the studied viral strains [12,13], and the molecular consequences are still poorly evaluated and understood [2]. In previous studies, we first observed that swIAV H1N1 interfered with PRRSV-2 productive infection of alveolar macrophages (AM) [14]. We secondly demonstrated that PRRSV-2 interfered with swIAV H1N1 infection of genetically modified newborn pig trachea epithelial cells (NPTr) expressing CD163, the main receptor of PRRSV [15]. This interference using NPTr $CD163^+$ cells was not surprising since, in that situation, PRRSV-2 was able to infect the same host cell as swIAV. However, this study did not address the question of interference in physiological conditions of non-modified respiratory epithelial cells.

In the current study, we established protocols to further deciphering viral interactions between PRRSV and swIAV and analyzed the generated data in the context of the available knowledge in the field [2,16]. Viral interference and its impact on both interferon response and induced signaling pathways were assessed in NPTr cells and/or lung slices using local PRRSV-1 and H1N2 swIAV strains that are co-circulating in pigs as previously shown [8].

2. Materials and Methods

2.1. Newborn Pig Tracheal Epithelial Cell Line

NPTr cells [17] were cultured with Dulbecco's modified Eagle medium (DMEM) (Eurobio scientific, Les Ulis, France) supplemented with 10% fetal calf serum (FCS) (Eurobio scientific) and 1% of Streptomycin/Penicillin/Amphotericin (SPA) solution (Eurobio scientific). Flasks were then incubated at 37 °C in a 5% CO_2 humidified environment and sub-passages were carried out once a week to assure the cell line maintenance.

2.2. Alveolar Macrophages

Alveolar macrophages used for the viral titration were obtained from bronchoalveolar lavage (BAL) of lungs collected from 5 to 7-month-old Large White conventionally bred sows. The animals were bred in accordance with European regulations by the experimental unit of animal physiology of Orfasière (UE PAO), Nouzilly, France. They were serologically tested frequently and known to be free of any common viral infection (swIAV, PRRSV, Porcine Circovirus 2, amongst others). In order to reduce the use of animals, the lungs were collected from pigs slaughtered in the course of the regular

management of the experimental unit's herds. As a consequence, no trial number has been attributed since an experimental authorisation was not requested. Once isolated, the lung airways were infiltrated with 250 mL of phosphate buffered saline (PBS) (Eurobio scientific) supplemented with 2 mM EDTA (Sigma-Aldrich, Saint-Quentin, France). The BAL was then collected, centrifuged, and passed through 40 µm cell strainers. After treatment with erythrocyte lysis buffer (10 mM $NaHCO_3$, 155 mM NH4Cl, and 10 mM EDTA), AM were washed with PBS, counted and seeded onto sterile plates and flasks for virus titration and propagation. Roswell Park Memorial Institute medium (RPMI) 1640 medium (Eurobio scientific) supplemented with 10% FCS and 2% of SPA solution was used for AM culture.

2.3. Virus Propagation, Purification and Titration

SwIAV H1N2 strain A/Sw/Ille-et-Vilaine/0415/2011 was selected among the collection of the French National Reference Laboratory for Swine Influenza (ANSES, Ploufragan, France). It was isolated from a nasal swab taken from a pig with acute respiratory disease in a herd located in Brittany, France. It was propagated on Madin-Darby Canine Kidney (MDCK) (ATCC reference CCL-34) cells for 24 hours (h) in DMEM medium supplemented with 10% FCS, 1% of SPA solution, and 2 µg/mL of trypsin TPCK treated (Worthington Biochemical Corp., Lakewood, NJ, USA). The supernatant was then collected, clarified by centrifugation (600× *g*, 20 min) and stored at −80 °C.

PRRSV-1 strain PRRS-FR-2005-29-24-1 (*Finistère* strain; genotype 1.1) (ANSES' collection) was isolated in Brittany, France in 2005 from a herd with abortions. The propagation was performed on AM cultured in RPMI 1640 medium (Eurobio scientific) supplemented with 10% FCS and 2% of SPA solution for 72 h. The DV strain of PRRSV-1 (modified live virus, MLV) used in Porcilis® PRRS vaccine (ANSES' collection—MSD Animal Health) was propagated on the monkey epithelial cell line MARC-145 (ATCC reference CRL-12231).

Aujeszky's disease virus (ADV) strain Kojnok [18] (ANSES' collection) was propagated on NPTr cells for 24 h in a DMEM medium supplemented with 10% FCS and 1% of SPA solution. The supernatant was then collected, clarified by centrifugation (600× *g*, 20 min) and stored at −80 °C.

All viruses were concentrated and purified on Amicon Ultra-15 centrifugal Filters (Sigma-Aldrich – reference number UFC910024 – pore size 100 kDa Nominal Molecular Weight cu-off) after a 20 min centrifugation at 4000× *g* and 4 °C. Titer determinations of swIAV H1N2 and ADV were carried out on MDCK, PRRSV-1 Finistère strain titration was done on AM, and DV strain of PRRSV-1 was performed on MARC-145 using $TCID_{50}$ assay protocol. The viral titers of purified swIAV H1N2 and ADV reached 10^7 $TCID_{50}$/mL and 10^6 $TCID_{50}$/mL, respectively, while PRRSV-1 stock titer was 10^6 $TCID_{50}$/mL.

2.4. Real-Time Monitoring of SwIAV Cytopathic Effects Using Real-Time Cell Analysis

The xCELLigence real-time cell analysis (RTCA) is a recently developed tool allowing the assessment of cell growth and cytopathic effects using impedance in culture wells equipped with gold electrodes [19]. The presence of adherent cells in the wells alters measured impedance. This property can be used to monitor cell viability, migration, growth, spreading, proliferation, and any modification due to viral cytopathic effect [20,21]. Monitoring of real-time cell impedance was performed using the RTCA MP system (ACEA Biosciences, Montigny le Bretonneux, France) provided by LABÉO Frank Duncombe, Saint-Contest, France (https://www.impedancell.fr/). A dimensionless value, called cell index (CI) was measured and represented after derivation of impedance signals registered by the sensor analyzer of the monitor. Assessment of the background reading was carried out by filling all the wells of the E-plate VIEW PET (ACEA Biosciences) with DMEM. NPTr cells were seeded at 3×10^4 cells per well, incubated for 30 min at room temperature as per the manufacturer's recommendations, and finally transferred to the RTCA-MP station placed in the incubator at 37 °C and 5% CO_2. After 24 h, cells were washed twice with PBS and infected with swIAV H1N2, PRRSV-1, or both viruses. The plate was placed back and CI values were measured automatically every 10 min for 72 h. A normalization of the CI values was performed using the RTCA software version 2.0 (ACEA Biosciences) to match the last point before cell infections with the viruses [19].

2.5. Virus Inactivation

After propagation of PRRSV-1 on AM, part of the viral stock was inactivated by adding beta-propiolactone (BPL) (Sigma-Aldrich) (1:4000 dilution) after incubation for 2 h at 37 °C in a 5% CO_2 humidified atmosphere. Inactivated viral stock was clarified, purified (as described above), and stored at −80 °C. The efficiency of the inactivation process was tested by a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) targeting PRRSV-1 (see PCR section) on supernatant obtained after inoculation of the AM with the inactivated virus over three passages.

2.6. Precision-Cut Lung Slices

Lungs were collected from 5- to 7-month-old Large White conventionally bred sows from UE PAO, Nouzilly, France. The diaphragmatic lobes were filled with a 37 °C warm mix of low-melting agarose (Sigma-Aldrich) and RPMI 1640 supplemented with 1% SPA, 10 µg/mL enrofloxacin (Bayer Animal Health, Leverkusen, Germany) and 1 µg/mL clotrimazole (Sigma–Aldrich). The lungs were transported on ice to ensure the solidification of the agarose. Transverse cuts were done and tissue was stamped out as cylindrical portions using a coring tool, then 250 µm slices were prepared in a Krumdiek tissue slicer (model MD4000-01, TSE systems, Chesterfield, MO, USA) at a cycle speed of 60 slices/min. The generated precision-cut lung slices (PCLS) were then incubated at 37 °C in a 5% CO_2 humidified atmosphere, after adding 10% of FCS to the complete RPMI 1640 medium. The medium was changed twice with 1 h interval and twice again after 12 h. At this stage, the viability of the tissue slices was assessed by adding 10^{-4} M methacholine (acetyl-ß-methylcholine chloride, Sigma-Aldrich), and the slices were sorted and prepared for viral infections after observation of the ciliary activity under a light microscope (Olympus CKX31, Tokyo, Japan).

2.7. Virus Infection and Stimulation of Newborn Pig Tracheal Epithelial Cells and Precision-Cut Lung Slices

NPTr cells were cultured in 48 well-plates for 24 h at 1–2 × 10^5 cells per well, washed twice with PBS then infected with swIAV H1N2 at a multiplicity of infection (MOI) of 3 (enabling the infection of all the cells, according to the Poisson distribution) in the absence or presence of inactivated or live PRRSV-1 (at the same MOI). Dual administrations were carried out after variation of the order of administration and the delay between the viruses, going from 0 h to 1 h and finally 6 h (see Figures 1A, 2A, 3A and 4A). In order to assess the importance of the virus species in the interference process and the specificity of PRRSV-1 Finistère strain effects, we also used alternatively the DV strain of PRRSV-1 used in the Porcilis PRRS vaccine (MLV in the manuscript) and ADV strain Kojnok in our dual administration protocol (Figure 1E). Then, to further decipher how PRRSV-1 can interfere with swIAV H1N2, an acid wash (40 mM citric acid, 135 mM NaCl, 10 mM KCl, pH 3.0) was applied to the cells after one-hour incubation with both PRRSV-1 and swIAV H1N2 viruses, or PRRSV-1 alone (Figure 3A). This procedure is commonly used in penetration and growth kinetic assays for enveloped viruses and is known to inactivate non-internalized viruses [22–24].

The same protocol, except acid wash, was used for PCLS infection but without any variation in the order of inoculations with 10^5 $TCID_{50}$/slice/virus (Figure 5A).

NPTr cells were incubated in DMEM while PCLS were kept in RPMI 1640, both supplemented with 1% of SPA solution and incubated at 37 °C and 5% CO_2 before any harvest. Cells were harvested using a special lysis buffer from the RNeasy Mini Kit (Qiagen, Courtaboeuf, France), and PCLS were collected in Trizol reagent (Invitrogen, Cergy Pontoise, France). Supernatants were also collected and stocked at −80 °C.

2.8. Immune Gene Expression Analysis and Virus Detection by Quantitative Real-Time PCR

Total RNA was extracted from NPTr cells and supernatants using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions after cell lysis with RLT buffer (Qiagen). PCLS lysis in Trizol was carried out using FastPrep lysing tubes and the FastPrep homogenizer (MP Biomedicals FastPrep-24™ 5G, Illkirch-Graffenstaden, France). Then, RNA samples were treated with DNAse I Amp Grade (Invitrogen) (1 U/µg of RNA). The absence of genomic DNA contamination was validated by the use of treated RNA as a template directly in PCR. Total RNA quantity and quality were assessed using Nanophotometer (Implen, Munich, Germany). cDNA was generated with a virus reverse transcriptase in the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA) from 100–200 ng of RNA free of genomic DNA per reaction.

The generated cDNA was then diluted (4×) and combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad) following the manufacturer's recommendations. To carry out the qPCR assays the selected conditions were 98 °C for 30 seconds (s), followed by 37 cycles with denaturation at 95 °C for 15 s and annealing/elongation for 30 s at optimal temperature—depending on the chosen target (Table 1). SwIAV viral transcripts, and transcripts associated with genes involved in the innate immune response such as those coding for some pattern recognition receptors, type 1 and 3 interferons, and interferon-stimulated genes were assessed. PRRSV-1 replication was not assessed here since the virus does not infect NPTr cells [25]. Most of the sequences of the primers targeting immune genes used in the study were published previously [14,26–28]. TLR6 and IFNλ3 specific primers were designed specifically for the current study with Clone Manager 9 (Scientific and Educational Software, Cary, NC, USA). qPCR assays were performed on a CFX96 Connect (Bio-Rad). The specificities of the qPCR assays were assessed by analyzing the melting curves of the generated products. The correlation coefficients of the standard curves were always between 0.950 and 0.995 and all the qPCR assays measured efficiencies between 90% and 110% as recommended [29]. Collected samples were normalized internally by simultaneously using the average Cycle quantification (C*q*) of three stable reference genes in each sample [30]. The three reference genes were; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-2-microglobulin (B2M1) and hypoxanthine phosphoribosyltransferase-1 (HPRT-1) [14,26,31]. These selected reference genes were assessed before for their stability as previously described, using geNorm [32]. Then, qPCR data (C*q*) were subjected to Genex macro analysis (Bio-Rad) [30] and expressed as relative values after Genex macro analysis.

Table 1. List of the primers used in the study.

Primer Abbreviation and Full Name	Primer Sequences: Sense (S) and Anti-Sense (AS)	Amplicon Size (bp)	Annealing Temperature (°C)	Accession Number or PMIDs
Viruses				
SwIAV H1N2 M-encoding gene	(S) AGATGAGTCTTCTAACCGAGGTCG (AS) TGCAAAAACATCTTCAAGTCTCTG (P) (6FAM)-TCAGGCCCCCTCAAAGCCGA-(TAM)	100	60	15460317
PRRSV *Finistère* ORF5	(S) AGAACCAGCGCCAATTCAGA (AS) TCTTTTTCGCCTGTCCTCCC (P) (HEX)-AAACACAGCTCCAATGGGGAATGGC-(TAM)	135	60	28241868
ADV gB-encoding gene	(S) GCGGGTACGTGTACTACGAG (AS) GAGGCCCTGGAAGAAGTTGG (P) (6FAM)-ACTACAGCTACGTGCGCATGGTGGAG-(TAM)	287	63	NC_006151
Reference genes				
B2MI *Beta-2-microgobulin*	(S) CAAGATAGTTAAGTGGGATCGAGAC (AS) TGGTAACATCAATACGATTTCTGA	161	58	17697375
HPRT1 *Hypoxanthine phosphoribosyltransferase 1*	(S) GGACTTGAATCATGTTTGTG (AS) CAGATGTTTCCAAACTCAAC	91	60	17697375
GAPDH *Glyceraldehyde 3-phosphate dehydrogenase*	(S) CTTCACGACCATGGAGAAGG (AS) CCAAGCAGTTGGTGGTACAG	170	63	AF017079
Pattern Recognition Receptors				
MDA5 *Melanoma differentiation-associated protein 5*	(S) AGCCCACCATCTGATTGGAG (AS) TTCTTCTGCCACCGTGGTAG	133	60	MF358967.1
RIGI *Retinoic acid-inducible gene I*	(S) CGACATTGCTCAGTGCAATC (AS) TCAGCGTTAGCAGTCAGAAG	126	60	NM_213804
TLR2 *Toll-like receptor 2*	(S) ACGGACTGTGGTGCATGAAG (AS) GGACACGAAAGCGTCATAGC	101	62	NM_213761.1
TLR3 *Toll-like receptor 3*	(S) GACCTCCCGGCAAATATAAC (AS) GGGAGACTTTGGCACAATTC	155	60	NM_001097444
TLR4 *Toll-like receptor 4*	(S) TGTGCGTGTGAACACCAGAC (AS) AGGTGGCGTTCCTGAAACTC	136	62	NM_001293316.1
TLR6 *Toll-like receptor 6*	(S) TCCCAGGATCAAGGTTCTTG (AS) GAGCAGAGTCCCCTTATAAC	370	60	NM_213760.2

Table 1. *Cont.*

Primer Abbreviation and Full Name	Primer Sequences: Sense (S) and Anti-Sense (AS)	Amplicon Size (bp)	Annealing Temperature (°C)	Accession Number or PMIDs
TLR7 *Toll-like receptor 7*	(S) CGGTGTTTGTGATGACAGAC (AS) AACTCCCACAGAGCCTCTTC	174	62	NM_001097434.1
TLR8 *Toll-like receptor 8*	(S) CACATTTGCCCGGTATCAAG (AS) TGTGTCACTCCTGCTATTCG	145	58	NM_214187.1
TLR9 *Toll-like receptor 9*	(S) GGCCTTCAGCTTCACCTTGG (AS) GGTCAGCGGCACAAACTGAG	151	64	NM_213958.1
TLR10 *Toll-like receptor 10*	(S) CTTTGATCTGCCCTGGTATCTCA (AS) CATGTCCGTGCCCACTGAC	51	60	AB_208699,1
Interferons				
IFNα *Interferon alpha*	(S) GGCTCTGGTGCATGAGATGC (AS) CAGCCAGGATGGAGTCCTCC	197	62	JQ839262.1
IFNβ *Interferon beta*	(S) AGTTGCCTGGGACTCCTCAA (AS) CCTCAGGGACCTCAAAGTTCAT	70	60	21645029
IFNλ1 *Swine interferon lamba 1*	(S) GAGGCTGAGCTAGACTTGAC (AS) CCTGAAGTTCGACGTGGATG	115	60	NM_001142837
IFNλ3 *Swine interferon lamba 3*	(S) CCTGGAAGCCTCTGTCATGT (AS) TCTCCACTGGCGACACATT	72	60	29677213
Interferon-stimulated genes				
PKR *Protein kinase R*	(S) CACATCGGCTTCAGAGTCAG (AS) GGGCGAGGTAAATGTAGGTG	166	61	NM_214319
OAS1 *2′-5′-Oligoadenylate Synthetase 1*	(S) CCCTGTTCGCGTCTCCAAAG (AS) GCGGGCAGGACATCAAACTC	303	64	NM_214303
MX1 *Myxovirus resistance protein 1*	(S) AGTGTCGGCTGTTTACCAAG (AS) TTCACAAACCCTGGCAACTC	151	60	NM_214061
MX2 *Myxovirus resistance protein 2*	(S) CCGACTTCAGTTCAGGATGG (AS) ACAGGAGACGGTCCGTTTAC	156	62	AB258432
ISG12 *Interferon-stimulated gene 12*	(S) GTACTTCCTCCCTGATAGG (AS) ACAGCTACAGAAGCCTTG	76	54	NM_001198921.1
ISG15 *Interferon stimulated gene 15*	(S) GATCGGTGTGCCTGCCTTC (AS) CGTTGCTGCGACCCTTGT	176	58	NM_001128469.3

Virus genomes, except for ADV, were amplified using Taqman qPCR assays described previously (Table 1) [33,34]. ADV specific primers were designed specifically for the current study using Clone Manager 9 (Scientific and Educational Software). The cDNA (2 µL—dilution 2×) were combined with 0.3 µL of each primer (10 µM), 0.25 µL of probe (10 µM), 5 µL of Takyon No Rox Probe MasterMix dTTP blue 2× (Eurogentec, Liège, Belgium), and ultra-pure water to reach a final volume of 10 µL. The conditions of the qPCR assays were here 95 °C for 3 min followed by 40 cycles with denaturation at 95 °C for 6 s and annealing/elongation for 15 s at 60 °C. Like the SYBR Green qPCR assays, the Taqman qPCR assays were run on the CFX96 Connect (Bio-Rad). qPCR assays efficiencies were all very close or equal to 100% as recommended in MIQE. Relative viral loads values for swIAV H1N2 in supernatants were calculated by attributing a value of 10 to the highest *Cq* score and then doubling this attributed value for every difference of 1 C*q* since the efficiency for swIAV Taqman assay was reaching 100% in our conditions.

2.9. Western Blotting

NPTr cells were cultured in 48-well plates, then single or dually infected at a MOI of 3 with swIAV H1N2 and/or live or inactivated PRRSV-1. Plates were immersed in liquid nitrogen and the cells were harvested simultaneously at 5 and 10 min before being disrupted with the lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid-EDTA, 1 mM ethylene glycol tetraacetic acid, 1% (*v*/*v*) Triton X-100, 0.5% NP-40), phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate), and protease inhibitors (2 mM phenyl methyl sulfonyl fluoride-PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin) (Sigma-Aldrich and Bio-Rad). Then, equal amounts of proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane after a 30 min incubation on ice and a centrifugation at 12,000× *g* for 20 min at 4 °C. Non-specific sites were saturated by incubating the membranes for 1 h at room temperature (RT) with Tris-buffered saline (TBS, 2 mM Tris-HCl, pH 8, 15 mM NaCl, pH 7.6), containing 0.1% Tween-20 (Bio-Rad) and 5% non-fat dry milk powder (NFDMP). Later on, membranes were incubated with the primary antibodies (working dilution 1:1000, Table 2) in TBS containing 0.1% Tween-20 and 5% NFDMP overnight at 4 °C. Then, the membranes were washed in TBS-0.1% Tween-20 and incubated for 2 h RT with a horseradish peroxidase-conjugated secondary antibody (working dilution 1:10,000). After an additional wash, proteins were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer, Courtabœuf, France) using a G:Box SynGene (Ozyme, Saint-Quentin-en-Yvelines, France) coupled with the GeneSnap software (Syngene UK, Cambridge, UK, release 7.09.17). GeneTools software (Syngene UK, release 4.01.02) was selected for quantification of detected signals. Then, the results were normalized and expressed as the signal intensity in arbitrary units.

Table 2. Antibodies used for western blotting.

Targeted Protein	Specific Antibody
-Phospho-AKT	-Rabbit polyclonal anti-phospho-AKT (Ser473) #9271 (Ozyme)
-AKT	-Rabbit monoclonal anti-AKT (11E7) #4685 (Ozyme)
-Phospho-AMPK	-Rabbit monoclonal anti-phospho-AMPK alpha (Thr172) (40H9) #2535L (Ozyme)
-AMPK	-Rabbit polyclonal anti-Total AMPK #2532L (Ozyme)
-Phospho-ERK1/2	-Rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) #4370 (Ozyme)
-ERK2	-Rabbit polyclonal anti-ERK2 (GTX27948) (Tebu-bio)
-Phospho-JAK2	-Rabbit polyclonal anti-phospho-JAK2 (Tyr1007/1008) #3771 (Ozyme)
-JAK2	-Rabbit monoclonal anti-JAK2 (D2E12) #3230 (Ozyme)

2.10. Immunofluorescence Analyses

Before staining, PCLS were fixed with acetone:methanol (50:50) (VWR International, Radnor, USA) while cells were fixed with 3% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.2% Triton ×-100 (Sigma-Aldrich). SwIAV H1N2 infected cells were identified after using a mouse monoclonal antibodies targeting the viral nucleoprotein (dilution 1/50) (OBT0846, clone: 1341, Bio-Rad) followed by an appropriate goat anti-mouse secondary antibody coupled to Alexafluor555 (dilution 1/100) (Ref: A21121, Invitrogen). Cells infected with PRRSV-1 were identified by using a monoclonal antibody recognizing the viral N protein (dilution 1/100) (mouse IgG1, Ref: BIO 276, Bio-X Diagnostic, Rochefort, Belgium). A goat anti-mouse secondary antibody coupled to Alexafluor488 was then used (dilution 1/100) (Ref: A21137, Invitrogen). Cy3-labeled monoclonal antibody recognizing beta-tubulin (dilution 1/100) (C4585, Clone TUB 2.1, Sigma-Aldrich) was selected as ciliated cell marker, and anti-MHC-II antibody (dilution 1/100) (clone MSA3 from monoclonal antibody center Washington State University—Pullman, WA, USA) was used as a marker for macrophages and other antigen-presenting cells. Cell nuclei were stained after incubation with 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 10 min at room temperature. The cells and the PCLS were finally washed with PBS, mounted in Mowiol 4-88 (Sigma-Aldrich). Tissue sections were covered by micro-cover glass (13 mm) (Dominique Dutscher SAS, Brumath, France). Images were generated using a ZEISS LSM 780 laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany) equipped with solid-state lasers 405, 561, and 633 nm and argon laser 488 nm.

2.11. Statistical Analyses

The expression of viral and cellular transcripts in cells and lung slices was expressed as relative values and data are expressed as mean ± standard deviation (SD). Due to the non-normal distribution, all data were sorted by rank before performing an ANOVA test using GraphPad Prism (GraphPad Software version 7.0, San Diego, CA, USA). Finally, Tukey's test was performed to compare the means of the ranks among different groups. *p*-values less than 0.05 were considered statistically significant. Only significant differences between single swIAV H1N2 condition and other infection conditions as well as differences between the various coincubation conditions were indicated in Figures 1–5. In Figure 6, all the significant differences are shown except those involving the control conditions.

3. Results

3.1. Simultaneous PRRSV-1 Contact with NPTr Epithelial Cells Strongly Decreases swIAV H1N2 Replication and Immune Gene Expression

To assess the impact of PRRSV-1 on infection of epithelial cells by swIAV H1N2, we first monitored the survival of NPTr cells upon inoculation of swIAV H1N2 or PRRSV-1 or both viruses simultaneously using xCELLigence Real-Time Cell Analysis (RTCA) (Figure 1A,B). In the control conditions (grey curve) the normalized cell index (CI_n) increased over time due to cell adhesion and proliferation. In swIAV H1N2 infected culture conditions, the CI_n began to clearly decrease from 8 hours post-infection (hpi) (Figure 1B; pink curve), which corresponds to the induction of the virus-mediated cytopathic effects (CPE). The decrease in the CI_n values intensified to reach zero around 32 hpi (Figure 1B), with no more adherent cells present in the wells at 48 hpi. Conversely, at 72 hpi, in PRRSV-1 inoculated culture conditions (Figure 1B; blue curve), the CI_n values were not statistically different from CI_n values observed in control conditions, in agreement with the known incapacity of PRRSV-1 to infect epithelial respiratory cells. Interestingly, in coinoculated cell cultures, the swIAV H1N2-induced CPE was completely abolished, as the CI_n values remained high over 72 h (Figure 1B; red curve).

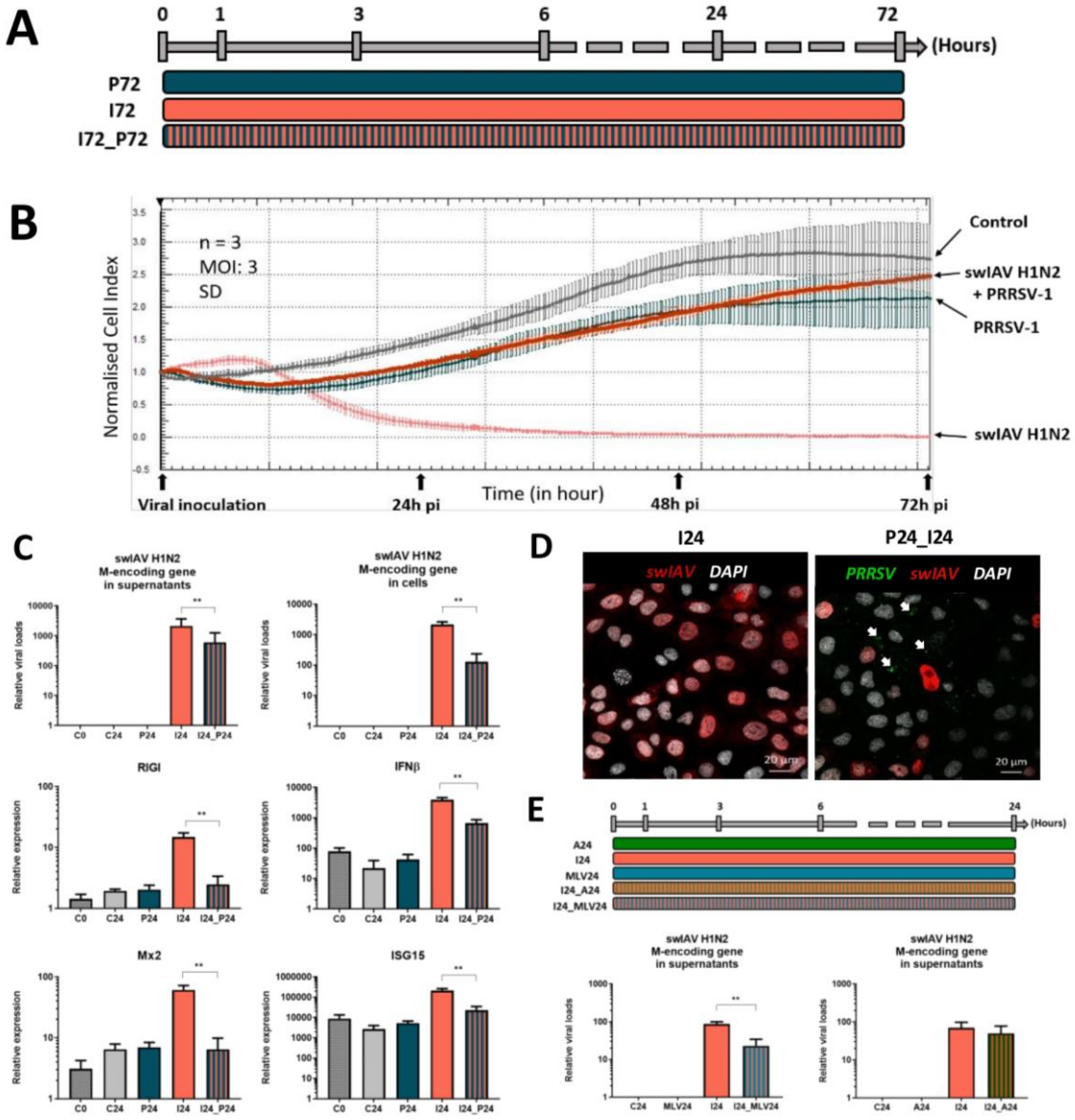

Figure 1. Protocol (**A**), normalized cell index (**B**), relative viral loads and relative expression of cellular transcripts (**C** and **E**), and immunostaining (**D**) of the different conditions in newborn pig tracheal cells showing the negative impact of PRRSV-1 on swIAV H1N2 replication and NPTr cells response. Cells were infected at a MOI of 3, *n*= 6 wells per condition for qPCR and *n*=3 for the impedancemetry. Mean values and standard deviations are represented. C0 and C24 stand for the non-infected conditions over 0 h and 24 h respectively, P24 and P72 stand for PRRSV-1 stimulation over 24 h, and 72 h, I24, and I72 for swIAV H1N2 infection over 24 h and 72 h, A24 for ADV infection over 24 h, MLV24 for stimulation with the DV strain of PRRSV-1 over 24 h, I24_P24, I24_A24, I24_MLV24, and I72_P72 for the coincubation of 2 viruses over 24 h or 72 h. Comparisons were carried out using a one-way ANOVA test and Tukey's post-test. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**). NPTr cells (**D**) were fixed after 24 h and stained with an antibody against viral N protein to detect PRRSV-1 particles and PRRSV-1 infected cells (in green—white arrows) and an anti-nucleoprotein polyclonal antibody to detect swIAV H1N2 particles and infected cells (in red). Cell nuclei were stained (in white) using 4′,6′-diamidino-2-phenylindole (DAPI). Images were generated using a laser-scanning confocal microscope.

In a second experiment, swIAV H1N2 and PRRSV-1 were added simultaneously or separately to NPTr cells for 24 h before the extraction of total RNA from the infected cells. Then, the expression of swIAV viral transcripts and the expressions of genes coding for innate immune mediators were assessed (Figure 1C for the most significantly affected genes and Table S1 for all the data).

The expression of swIAV viral transcripts—namely swIAV M-encoding gene—was significantly reduced in the coincubation condition compared to swIAV H1N2 single infection ($p < 0.01$). Thus, the addition of PRRSV-1 in culture medium resulted in a reduction of swIAV H1N2 replication. These results were further confirmed after titration of the virus in the supernatant of the different conditions using $TCID_{50}$ assay (data not shown). Similarl to what we observed with swIAV viral transcripts, the gene expression of innate immune mediators was significantly reduced in the coincubation condition compared to swIAV H1N2 infection ($p < 0.01$) (Figure 1C and Table S1). Microscopic analysis of cells after 24 h infection confirmed the massive infection of NPTr cells by swIAV H1N2 virus alone. However, coincubation of swIAV H1N2 with PRRSV-1 drastically diminished the number of influenza-infected epithelial cells (Figure 1D). Some PRRSV-1 particles were visible in coincubation conditions (Figure 1D).

We cannot completely rule out that the observed effect of the PRRSV on the replication of swIAV might be related to substances present in the inoculum such as cytokines derived from the infected cells during the amplification process of the virus. However, ultrafiltration and concentration of the viral stocks as well as the dilution of the virus inoculum drastically reduced the concentration of any substances potentially present in the PRRSV-production medium. It is also worth mentioning that PRRSV can inhibit the IFN type 1 protein production and more generally the innate immune response [35]. This feature reduces the chances of having significant concentrations of many cytokines after the amplification of the virus on live cells. On the other hand, the ADV used as a control, is known to induce cytokine production [36], but it did not have any statistically significant impact on the replication of swIAV comparatively to wild-type PRRSV-1. This is additional evidence that the strong reduction of swIAV replication is PRRSV specific.

Moreover, the strain DV of PRRSV-1, an attenuated vaccine strain that is used in PRRSV MLV vaccination protocols, was also capable to affect the replication of swIAV (Figure 1E). Conversely, when PRRSV-1 was replaced by ADV that is able to infect NPTr cells and epithelial cells in general, no reduction of swIAV H1N2 replication was observed (Figure 1E) demonstrating the specificity of the interference we observed.

3.2. The Effect of PRRSV-1 Is till Observed if PRRSV-1 Is Added Shortly before swIAV H1N2 to NPTr Cells

To further analyze the impact of PRRSV-1 exposition on swIAV H1N2 infection, we then carried out PRRSV-1 exposition before (1 to 6 h) or after (1 to 6 h) swIAV H1N2 infection (Figure 2A–C and Table S2). The more PRRSV-1 was added in advance, the less it interfered with swIAV H1N2 infection (Figure 2B). Indeed, the genomic loads of swIAV H1N2 were significantly more reduced ($p < 0.01$) when PRRSV-1 was added 1 h before swIAV H1N2 compared to the condition when PRRSV-1 was added 6 h before. Similarly, when PRRSV-1 exposition took place after swIAV H1N2 infection, the condition of 1 h delay showed a higher impact on swIAV H1N2 genomic loads compared to the condition with a delay of 6 h (Figure 2C). Regarding the genes involved in type I interferon response, their expression tended to decrease more when PRRSV-1 was added a shorter time in advance (Figure 2B,D). Conversely, their expression increased when PRRSV-1 addition was delayed (Figure 2C,D) ($p < 0.05$).

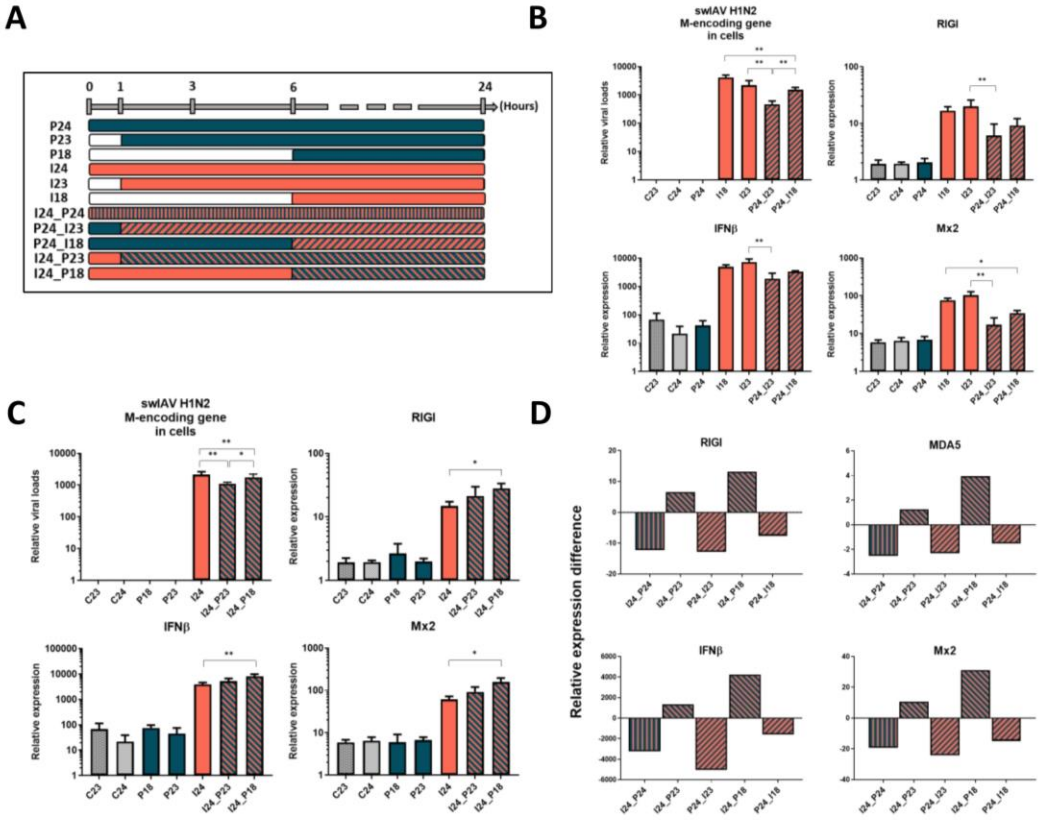

Figure 2. Protocol (**A**) and relative expression of viral and cellular transcripts (**B**,**C**) of the different conditions in newborn pig tracheal cells showing the impact of the delay and the order of the viruses on swIAV H1N2 replication and NPTr cells response. Difference of the relative expression between dual stimulations and influenza single infections is represented in panel (**D**). Cells were infected at a MOI of 3, n= 6 wells per condition for qPCR. C23 and C24 stand for the non-infected conditions over 23 h and 24 h respectively, P24 stands for PRRSV-1 stimulation over 24 h, I24 for swIAV H1N2 infection over 24 h, I24_P24, P24_I23, P24_I18 stand for dual administrations (PRRSV-1 then swIAV) of both viruses with a delay of 0, 1, and 6 h respectively. I24_P23 and I24_P18 stand for dual administration after reversing the viruses (swIAV then PRRSV-1) order with a delay of 1 and 6 h respectively. Mean values and standard deviations are represented. Comparisons were carried out using a one-way ANOVA test and Tukey's post-test after sorting the data by rank. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**).

3.3. Inactivation of Non-Internalized Viruses by Acid Wash Abrogates PRRSV-1 Effect on swIAV H1N2 Infection in NPTr Cells

To further decipher how PRRSV-1 interferes with swIAV H1N2 infection process, we performed acid wash after the incubation of viruses with the cells (Figure 3A). Interestingly, the acid wash which is known to inactivate the non-internalized viruses nearly abrogated the PRRSV-1 effect on swIAV H1N2 infection and on immune gene expression (Figure 3B). Thus, PRRSV-1 impact on swIAV infection occurs mainly from the surface of the epithelial cells and not in intracellular compartments, as expected since PRRSV-1 is described as unable to enter respiratory epithelial cells.

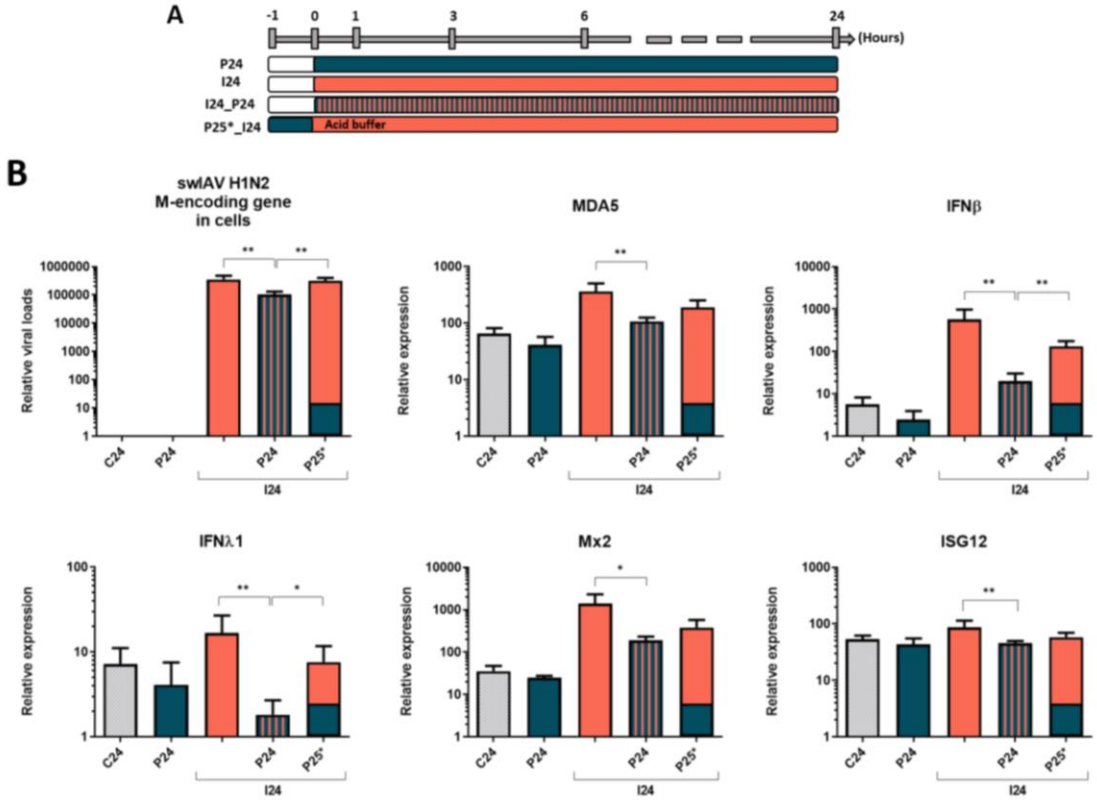

Figure 3. Protocol (**A**) and relative viral loads and relative expression of cellular transcripts (**B**) showing the impact of the inactivation of non-penetrated PRRSV-1 particles by the acid buffer wash on swIAV H1N2 replication and NPTr cells response. Cells were infected at an MOI of 3, n= 6 wells per condition for qPCR. C24 stands for the non-infected condition over 24 h, P24 stands for PRRSV-1 stimulation over 24 h, I24 for swIAV H1N2 infection over 24 h, I24_P24 for the coincubation of both viruses over 24 h, and P25*_I24 for the stimulation with PRRSV-1 for 1 h then infection with swIAV H1N2 for 24 h after an acid buffer wash. Mean values and standard deviations are represented. Comparisons were carried out using a one-way ANOVA test and Tukey's post-test. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**).

3.4. Inactivated PRRSV-1 still Impacts swIAV H1N2 Infection of Tracheal Epithelial Cells

Since PRRSV-1 does not penetrate in epithelial cells, we wanted to determine if PRRSV-1 inhibition of swIAV replication required live viral particles or if it was also possible to observe this inhibition when using inactivated PRRSV-1. As presented in Figure 4 (most significantly affected genes) and Table S3 (all the genes), inactivated PRRSV-1 was still able to interfere with swIAV H1N2 infection of porcine tracheal epithelial cells. However, the inactivated virus seemed less prone than the live virus to alter NPTr-swIAV interactions when added to tracheal epithelial cells simultaneously to swIAV H1N2. Some genes such as MDA5 and IFNβ did not show any significant differences between swIAV H1N2 single infection and coincubation with inactivated PRRSV-1 (Table S3). On the other hand, viral replication and type I interferon response—from the PRR to the ISG—were always significantly lower when live PRRSV-1 was simultaneously added to swIAV H1N2 infected cells instead of inactivated PRRSV-1 ($p < 0.01$).

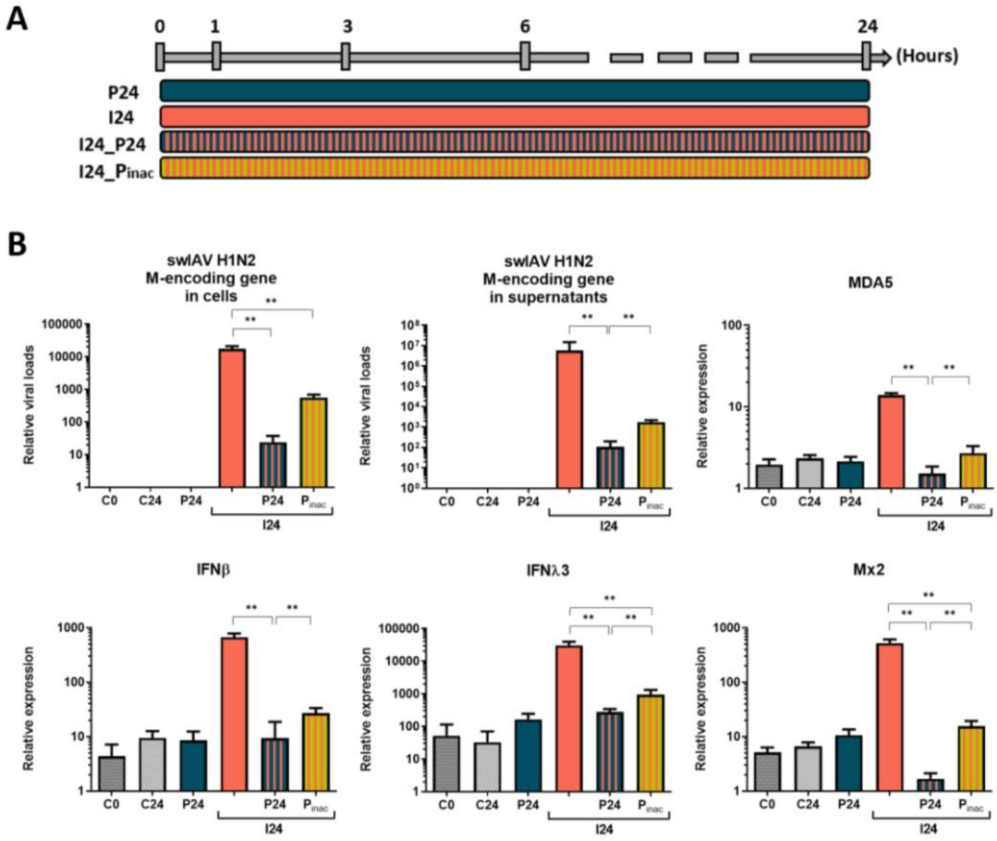

Figure 4. Protocol (**A**) and relative viral loads and relative expression of cellular transcripts (**B**) of the different conditions in newborn pig tracheal cells showing the impact of inactivated PRRSV-1 on swIAV replication and NPTr cells response compared to live PRRSV-1. Cells were infected at an MOI of 3, n= 6 wells per condition for qPCR. C0 and C24 stand for the non-infected conditions over 0 h and 24 h respectively, P24 stands for PRRSV-1 stimulation over 24 h, I24 for swIAV H1N2 infection over 24 h, I24_P24 and I24_Pinac for the coincubation of both viruses over 24 h using live and inactivated PRRSV-1, respectively. Mean values and standard deviations are represented. Comparisons were carried out using a one-way ANOVA test and Tukey's post-test. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**).

3.5. PRRSV-1 Impacts swIAV H1N2 Infection in Primary Tissue Lung Slices

Having observed PRRSV-1 interference on swIAV H1N2 infection in epithelial cell culture, we wondered if this effect could be observed in more physiologic settings. We thus proceeded to PRRSV-1 and swIAV H1N2 infections ex vivo on precision-cut lung slices (PCLS) (Figure 5 and Table S4). Confocal microscopy was used to confirm the host cells of the local virus strains used in this study. We observed actual infection of macrophages (MHC-II-positive alveolar cells) by PRRSV-1 as well as infection of epithelial cells (β-tubulin-positive cells) by swIAV H1N2 (Figure 5B). Swine IAV H1N2 replication detection using RT-qPCR (Figure 5C) confirmed the interference of PRRSV-1 on swIAV H1N2 production in the relevant target tissue. Indeed, swIAV H1N2 replication and type I interferon response were reduced in the presence of live and inactivated PRRSV-1 (Figure 5C), although, as shown with NPTr cells, inactivated PRRSV-1 appeared less effective (except on IFNβ transcript expression where wild-type PRRSV-1 and swIAV H1N2 acted as good inducers unlike inactivated PRRSV-1) and the decrease in the expression was not always significant compared to swIAV H1N2 condition. Thus, in a tissue containing the respective cellular targets of swIAV and PRRSV we made observations similar to what we observed using tracheal epithelial cells that are not susceptible to PRRSV.

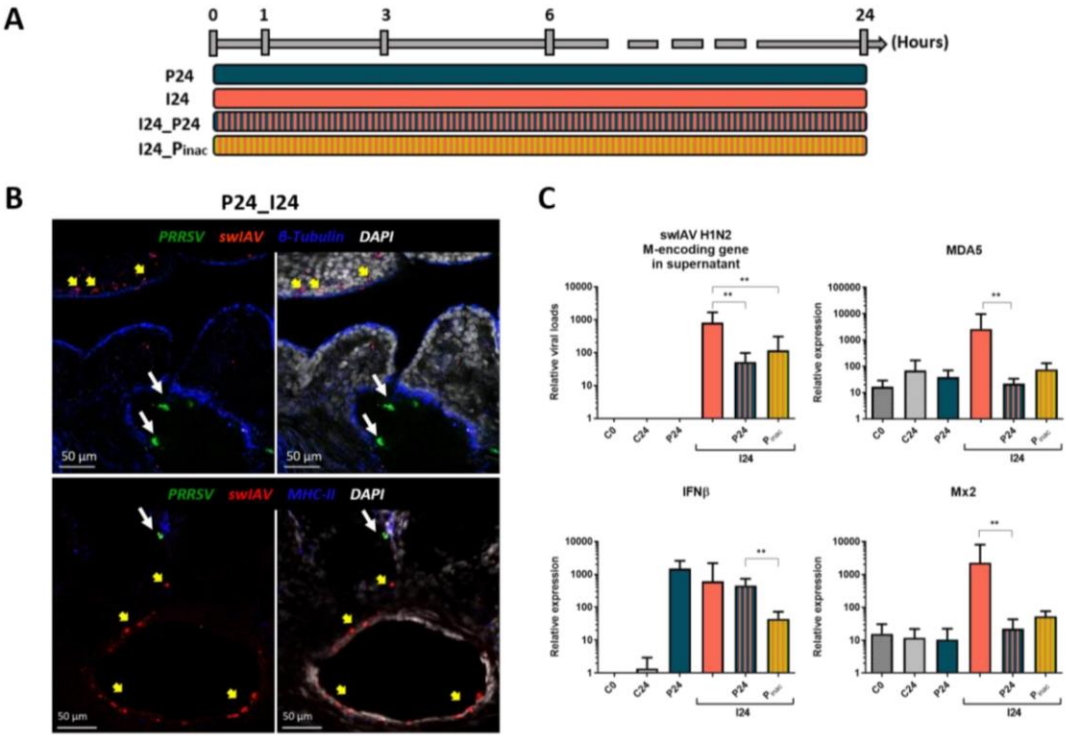

Figure 5. Protocol (**A**), immunostaining (**B**), and relative viral loads and relative expression of cellular transcripts (**C**) of the different conditions in precision-cut lung slices (PCLS) showing the impact of live and inactivated PRRSV-1 on swIAV replication and cell response. PCLS were coinfected with 10^5 particles of swIAV H1N2 and/or 10^5 particles of live or inactivated PRRSV-1. After 24 h, PCLS were fixed and stained with an antibody against viral N protein to detect PRRSV-1 particles (in green) and PRRSV-1 infected cells (white arrows) and an anti-nucleoprotein polyclonal antibody to detect swIAV H1N2 (in red) and infected cells (yellow arrows). Anti-beta-tubulin monoclonal antibody was used for epithelial ciliated cells staining while anti-MHC-II antibody was used for macrophages and other MHC-II presenting cells staining (in blue). Cell nuclei were stained (in white) using 4′,6′-diamidino-2-phenylindole (DAPI). Images were generated using a laser-scanning confocal microscope. n= 10 slices per condition for qPCR. C0 and C24 stand for the non-infected conditions over 0 h and 24 h respectively, P24 stands for PRRSV-1 infection over 24 h, I24 for swIAV H1N2 infection over 24 h, I24_P24 and I24_Pinac for the coinfection with both viruses over 24 h using active and inactivated PRRSV-1 respectively. Mean values and standard deviations are represented. Comparisons were carried out using a one-way ANOVA test and Tukey's post-test after sorting the data by rank. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**).

3.6. PRRSV-1 and swIAV H1N2-Induced Signaling Pathways in NPTr Cells

We then explored (using western blotting) the main signaling pathways known to be involved in virus infections and host's antiviral responses (PI3K/AKT, AMPK, MAPK ERK1/2, and JAK/STAT pathways) using NPTr cells. As presented in Figure 6, AKT, AMPK, and JAK2 phosphorylations were decreased in the presence of live PRRSV-1 at 5 and 10 min post-infection (mpi) (Figure 6A,B,D). This significant decrease was observed with PRRSV-1 alone or PRRSV-1 with swIAV H1N2 compared to swIAV H1N2 single infection ($p < 0.05$). In agreement with the previous experiments (Figure 4B), inactivated PRRSV-1 triggered patterns similar to live PRRSV-1, although with a constantly lower impact (Figure 6A,B,D). Conversely, live PRRSV-1, but not inactivated PRRSV-1, triggered ERK phosphorylation, alone or in the presence of swIAV H1N2 (Figure 6C). SwIAV H1N2 had a limited impact on AKT, AMPK, ERK, and JAK2 phosphorylations at 5 and 10 mpi.

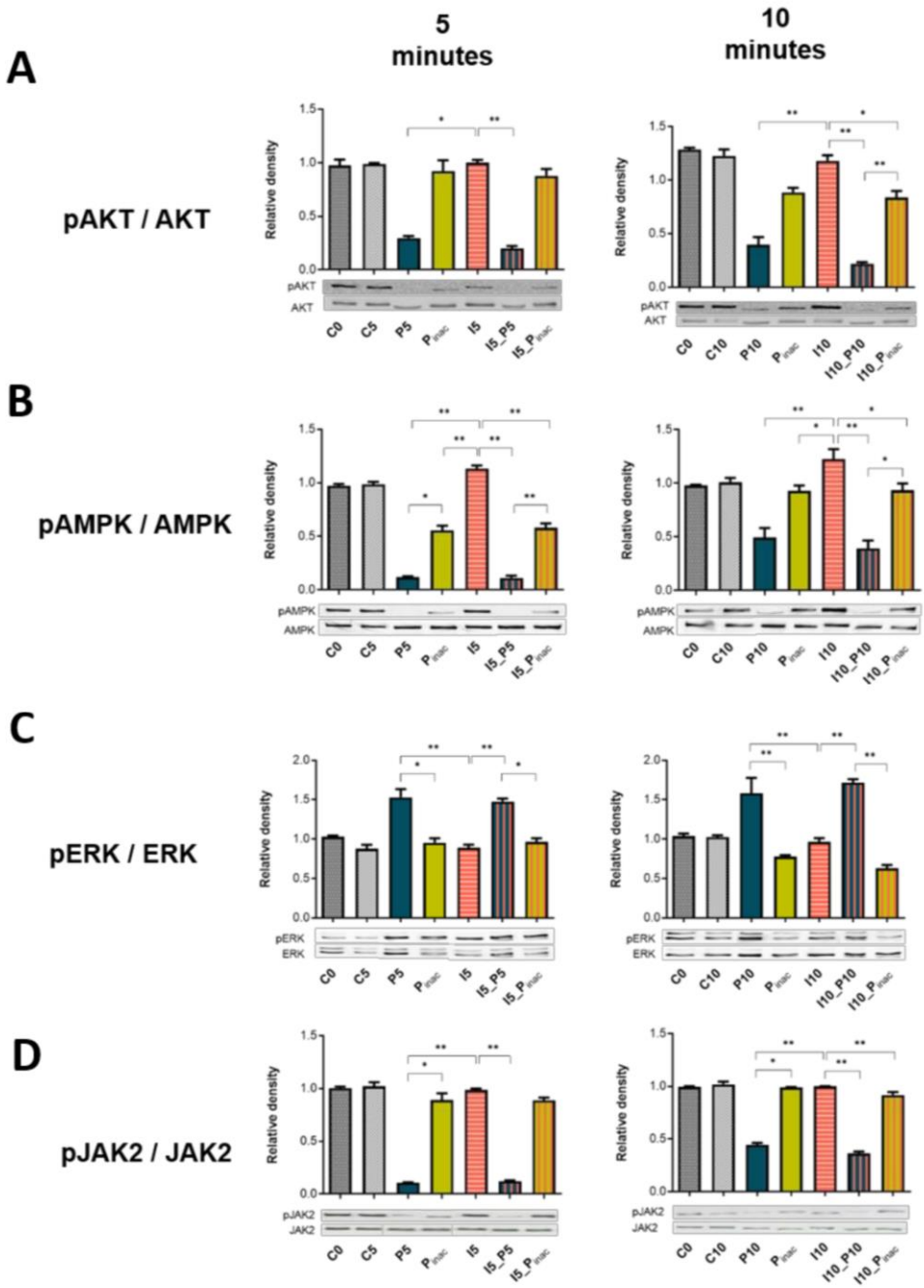

Figure 6. Western blots of phospho-AKT (**A**), phospho-AMPK (**B**), phospho-ERK1/2 (**C**), and phospho-JAK2 (**D**) in newborn pig tracheal cells infected with swIAV H1N2 in the absence or presence of PRRSV-1. Cells were infected at an MOI of 3 and harvested simultaneously at 5 and 10 min, so the results are representative of two independent experiments. C0, C5, and C10 stand for the cells from the non-infected conditions over 0, 5, and 10 min; P5, P10 and Pinac for cells stimulated with active or inactivated PRRSV-1 simultaneously for 5 and 10 min, while I5 and I10 stand for cells infected with swIAV H1N2 for 5 or 10 min. AKT, AMPK, ERK, and JAK2 are shown as loading controls and did not change with each condition over time. Mean values and standard deviations are represented, n= 3 wells per condition. Comparisons were carried out using a one-way ANOVA test and Tukey's post-test after sorting the data by rank. Differences were considered significant when $p < 0.05$ (*) or $p < 0.01$ (**).

4. Discussion

In the current study, we were interested in the molecular impact of PRRSV-1 on swIAV H1N2 infection. Indeed, both viruses are frequently encountered in the porcine respiratory tract [4–8] and their interactions are still poorly understood [2]. Some previous studies assessed the consequences of PRRSV/swIAV coinfections regarding virus replications and innate responses of their target cells and tissues [14,15]. However, in these studies, the interactions between PRRSV and tracheal epithelial cells, the main target of swIAV [10] had not been evaluated whereas these cells meet PRRSV viral particles during the infection process [9]. We observed here that PRRSV-1 interacted with tracheal epithelial cells, triggering ERK signaling protein phosphorylation and inhibiting AKT, AMPK, and JAK2 signaling protein phosphorylation. PRRSV-1 was also shown to inhibit swIAV H1N2 infection of epithelial cells when inoculated at the same time or shortly before or shortly after the swIAV, and this is observed, although at a lower level with inactivated PRRSV-1. To our knowledge, infection of porcine epithelial cells in the porcine respiratory tract by PRRSV has never been reported even if PRRSV-2 (but not PRRSV-1), has been shown in vitro to infect some epithelial cells from other species. St-Jude porcine lung (SJPL) cells originally described as an immortalized porcine lung epithelial cell line have been shown to be permissive to PRRSV-2 [25]. However, SJPL cells were actually not porcine cells but monkey cells as evidenced after karyotype and genetic analyses [37]. Two other monkey cell lines, the MARC-145 and CL2621 cells (subclones of MA104 monkey kidney cell line) allow the full replication cycle of PRRSV-2 and are commonly used for PRRSV-2 in vitro propagation. However, porcine tracheal epithelial cells such as NPTr cells were not permissive to PRRSV-1 or PRRSV-2 [17,25]. Thus, different cell surface molecules are needed to allow PRRSV interaction, entry, and infection of cells: porcine sialoadhesin—also known as sialic acid-binding immunoglobulin-type lectin 1 (Siglec-1) or CD169, Siglec-10, CD151, MYH9, and heparan sulfate [38–40] have been shown to mediate the interaction of PRRSV with cells; however, only CD163 has been demonstrated as essential for PRRSV genome delivery from endosome to the cytosol of the cells [41] and replication in the porcine host [42].

Regarding swIAV, the hemagglutinin (HA) provides the binding site to interact with the epithelial host cell. More specifically HA binds to a host cell receptor that contains terminal α-2,6-linked or α-2,3-linked sialic acid moieties [10]. Then, the cleavage of HA by cellular proteases is required for fusion and viral infection. NPTr cells are fully susceptible to swIAV and the virus can perform its full cycle in these cells [17,26]. Since PRRSV's envelope glycoproteins (GPs) such as GP5 contains sialic acid, involved in the interaction with sialoadhesin on macrophages to allow attachment and internalization, we could postulate possible direct interactions between swIAV particles and PRRSV [43,44]. This phenomenon could explain to some extent the inhibition of the swIAV H1N2 replication. This hypothesis is strengthened by the fact that PRRSV's sialic acid are α-2,3- and α-2,6-linked sialic acids [44]. The probable binding of PRRSV to swIAV particles may lead to the formation of aggregates trapping the swIAV particles and limiting their access to the sialic acids on the cell's surface. This potential competition between virus sialic acids and cellular sialic acids for swIAV HA would need to be further assessed.

We have observed that PRRSV was able to inhibit AKT, AMPK, and JAK2 phosphorylation and enhance ERK phosphorylation in NPTr cells, however its ligand on these cells is still unknown. We can hypothesize that PRRSV is interacting with heparin sulfates that are ubiquitously expressed on epithelial cells [45–47]. Regarding the relation between PRRSV and signaling pathways, it has been shown previously that PRRSV-2 can both activate and inhibit PI3K/AKT pathway in porcine monocyte-derived dendritic cells (Mo-DCs), MARC-145 and AMs depending on the phase of the viral cycle [48,49]. Interestingly, in these studies, heat-inactivated PRRSV-2 failed to inhibit PI3K/AKT in Mo-DCs, indicating that virus replication is essential for this inhibition [48]. Similarly, in our conditions, BPL-inactivated PRRSV-1 failed to inhibit AKT phosphorylation in epithelial cells. Although the inactivation processes were different in these two studies, it can be hypothesized that PRRSV inactivation would still allow the virus to attach to the epithelial cells, trigger receptor-mediated membrane signaling but not replication-dependent signaling. Regarding the AMPK pathway, it has been reported that host cells could antagonize PRRSV-2 infection via the activation of the AMPK-ACC1 signaling pathway [50].

Thus, inhibition of AMPK phosphorylation would help the virus in its multiplication cycle. On the contrary, PRRSV-1 was able to activate the MAPK ERK1/2 pathway in epithelial cells. This observation is in line with a previous observation reporting activation of MAPK ERK1/2 signaling pathway in AMs infected by PRRSV-2 later in the infection process [51]. In that study, UV-inactivated PRRSV-2 was sufficient to trigger ERK phosphorylation, suggesting that the viral entry process may be responsible for early ERK activation [51]. Indeed, UV treatment enables receptor binding and internalization but prevents viral gene synthesis. In our study BPL-inactivated PRRSV-1 was unable to induce ERK phosphorylation suggesting that our inactivated virus was unable to enter epithelial cells while the live virus would be able to enter to some extent, possibly staying in endosomal vesicles. The presence of PRRSV-1 particles in some epithelial cells suggested in our study by confocal microscopy would support this hypothesis as well as previous studies reporting the presence of PRRSV antigen in non-macrophage cells [14,52,53]. On the contrary, the results obtained following the acid wash applied after incubation of the cells with the viruses suggest that most of the PRRSV-mediated interference is linked to non-internalized viral particles.

SwIAV needs to activate MAPK ERK1/2 and PI3K signaling pathways to mediate the vacuolar (H^+)-ATPases-dependent intracellular pH change that is required for endosome fusion [54]. We observed induction of this pathway in NPTr cells 60 min post-swIAV-infection and later (data not shown) indicating a reaction of the cells to the swIAV H1N2 infection process. The decreased activation of PI3K/AKT could partially explain the inefficient swIAV H1N2 infection of NPTr when PRRSV-1 was co-applied to the cells. However, AKT phosphorylation inhibition was observed with live PRRSV-1 but not inactivated PRRSV-1, whereas inactivated PRRSV-1 was still able to inhibit swIAV replication as well, indicating that other mechanisms are involved. Indeed, the only signaling pathway commonly impacted by live and inactivated PRRSV was AMPK. We can thus hypothesize that PRRSV-1 ligand on epithelial cells might trigger an AMPK inhibitory signal. Interestingly, it has been recently reported that AMPK mediated autophagy promoted IAV replication [55], thus PRRSV-mediated AMPK inhibition could be responsible for the decrease in swIAV replication.

As per the JAK-STAT signaling pathways, it plays an important role in swIAV infection. Following IFNα/β production and binding to their receptors (IFNAR), JAK-STAT signaling leads to the recruitment and phosphorylation of IRF9 into the STAT1/STAT2 heterodimer to make ISG factor 3 (ISGF3) [56]. Translocation of ISGF3 to the nucleus induces the transcription of the different ISGs contributing to the antiviral defense against swIAV infection [57]. On the other hand, PRRSV has been described to inhibit the IFN-activated JAK-STAT signal transduction and ISG expression in MARC-l45 and AM cells [58]. In order to evade the host antiviral response, PRRSV is capable of inhibiting IFN-activated JAK-STAT signaling by blocking the ISGF3 nuclear translocation [59]. As a consequence, studies showed lower transcript levels of ISG15 and ISG56 and lower protein levels of STAT2 in PRRSV infected cells following IFN stimulation [60]. Our findings confirmed that PRRSV-1 interferes with the JAK-STAT signaling in NPTr cells, by downregulating the phosphorylation of JAK2 and leading to the low expression of ISGs even after swIAV infection.

Overall, PRRSV may interfere with swIAV early infection over two levels, (i) direct inhibition of swIAV infection/replication of epithelial cells; (ii) modification of the host's innate immune response, mainly through AM infection and killing [9]. Herein we have shown that coinoculation with PRRSV-1 might impact swIAV H1N2 capacity of replication in a respiratory epithelial cell line and lung tissue. Conversely, the inhibition of innate immune response by PRRSV and especially its capacity to antagonize antiviral interferon response using its nonstructural proteins [58] would facilitate swIAV replication. We thus propose that the balance between these phenomena might explain the discrepancies observed between different in vivo coinfection experiments, for which PRRSV primary infection increases, decreases or has no impact on swIAV co/superinfections. Finally, it is also known that swIAV infects unproductively alveolar macrophages (for review see [61]), the main target of PRRSV, which raises the possibility of a reverse interference of swIAV on PRRSV replication, adding another level of complexity in the interactions between these two viruses.

5. Conclusions

In conclusion, our study is showing for the first time that PRRSV can alter the relation between swIAV and its main target cells opening the doors to further studies on the interplay between respiratory viruses. Interference between PRRSV and swIAV could also have consequences regarding vaccination, as PRRSV vaccination involves MLV that showed a similar impact as a wild-type live virus. Based on our results, further investigations would be necessary to evaluate how PRRSV vaccination could modulate a porcine host's susceptibility to concomitant swIAV infection, potentially offering indirect and short term heterologous protection to the pigs.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-393X/8/3/508/s1, Table S1: Statistical comparison between mRNA relative expressions in swIAV H1N2 (I) single infections and co-inoculations of NPTr cells with PRRSV-1 (P). The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last column. Comparisons were made using one-way ANOVA test and Tukey's post-test., Table S2: Statistical comparisons between mRNA relative expressions in swIAV H1N2 (I) single infections and co-inoculations of NPTr cells with PRRSV-1 (P) with 1 and 6 hours' delay. The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last four columns. Comparisons were made using one-way ANOVA test and Tukey's post-test, Table S3: Statistical comparisons between mRNA relative expressions in swIAV H1N2 (I) single infections and co-inoculations of NPTr cells with active (P) and inactivated PRRSV-1 (Pinac). The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last three columns. Comparisons were made using one-way ANOVA test and Tukey's post-test, Table S4: Statistical comparisons between mRNA relative expressions in swIAV H1N2 (I) single infections and multiple infections of Precision-Cut Lung Slices (PCLS) with active (P) and inactivated PRRSV-1 (Pinac). The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last three columns. Comparisons were made using one-way ANOVA test and Tukey's post-test.

Author Contributions: F.M. and N.B. conceived and designed the experiments; methodology, G.S. (Georges Saade), D.M., C.H., P.R., E.H., L.D. and J.D.; software, G.S. (Georges Saade), D.M., C.H., E.H., L.D. and J.D.; validation, G.S. (Georges Saade), J.Z., S.P., O.B., G.S. (Gaëlle Simon), J.D., N.B. and F.M.; formal analysis, G.S. (Georges Saade), L.D., J.Z., S.P., O.B., G.S. (Gaëlle Simon), J.D., N.B. and F.M.; investigation, G.S. (Georges Saade), D.M., C.H., P.R., E.H., L.D. and J.D.; resources, G.S. (Georges Saade), N.B., J.D., S.P., O.B., G.S. (Gaëlle Simon) and F.M.; writing—original draft preparation, G.S. (Georges Saade), N.B. and F.M.; writing—review and editing, G.S. (Georges Saade), N.B. and F.M.; supervision, N.B. and F.M.; project administration, N.B. and F.M.; funding acquisition, N.B. and F.M. All authors have read and agreed the published version of the manuscript.

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

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c) Données supplémentaires

Tableau 8. *Statistical comparison between mRNA relative expressions in swIAV H1N2 (I) single infections and co-inoculations of NPTr cells with PRRSV-1 (P). The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last column. Comparisons were made using one-way ANOVA test and Tukey's post-test.*

	Messenger RNA	mRNA relative expression ± SD				I24 *vs* I24_P24
		Control	P24	I24	I24_P24	
Pattern recognition receptors	**RIG-I**	1.94 ± 0.1	2.04 ± 0.3	14.83 ± 2.5	2.48 ± 0.9	<0.0001**
	MDA5	2.09 ± 0.3	1.71 ± 0.2	4.50 ± 0.7	1.97 ± 0.7	0.0009**
	TLR2	3.72 ± 1.6	4.49 ± 2.4	6.99 ± 5.8	3.15 ± 3.1	ns
	TLR3	2.78 ± 0.4	2.23 ± 0.3	1.92 ± 0.3	1.62 ± 0.4	ns
	TLR4	2.45 ± 2.0	4.26 ± 4.9	3.15 ± 4.8	3.51 ± 3.8	ns
	TLR6	6.80 ± 2.0	6.02 ± 1.4	3.42 ± 0.6	3.22 ± 1.4	ns
	TLR7	2.65 ± 0.9	2.01 ± 0.6	2.01 ± 0.6	1.92 ± 0.8	ns
	TLR8	4.90 ± 5.2	9.41 ± 12.6	15.46 ± 22.1	27.56 ± 40.2	ns
	TLR9	9.83 ± 15.9	23.21 ± 16.8	19.31 ± 9.5	20.49 ± 20.7	ns
	TLR10	4.20 ± 1.2	2.68 ± 1.5	1.50 ± 0.3	3.63 ± 2.9	ns
Interferons	IFNα	3.56 ± 1.1	4.25 ± 1.8	3.88 ± 0.8	5.45 ± 4.5	ns
	IFNβ	21.88 ± 17.4	42.25 ± 19.7	3911.05 ± 726.6	673.17 ± 195.4	0.0011**
	IFNλ1	2.27 ± 0.9	2.75 ± 1.1	5.31 ± 1.0	3.53 ± 1.5	ns
Interferon stimulated genes	**PKR**	1.87 ± 0.2	2.23 ± 2.0	4.74 ± 2.1	1.96 ± 0.9	0.0392*
	OAS	59.25 ± 6.9	74.35 ± 21.3	1677.44 ± 373.0	100.67 ± 69.9	0.0015**
	MX1	1.57 ± 0.2	1.49 ± 0.2	22.04 ± 4.6	2.75 ± 0.8	0.0031**
	MX2	6.46 ± 1.4	6.96 ± 1.4	61.01 ± 11.0	6.44 ± 3.4	0.0001**
	ISG12	47.60 ± 15.2	66.88 ± 18.3	111.00 ± 60.1	48.99 ± 43.9	ns
	ISG15	2648.03 ± 1365.5	5241.87± 1400.4	209624.87 ± 54923.2	22576.62 ± 1243.8	0.0003**

Ns: not significant, SD: Standard deviation, ***P*<0.01 and **P*<0.05, Genes showing significant differences are represented in bold.

Tableau 9. *Statistical comparisons between mRNA relative expressions in swIAV H1N2 (I) single infections and co-inoculations of NPTr cells with PRRSV-1 (P) with 1 and 6 hours' delay. The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last four columns. Comparisons were made using one-way ANOVA test and Tukey's post-test.*

	Messenger RNA	mRNA relative expression ± SD									I23 *vs* P24_I23	I18 *vs* P24_I18	I24 *vs* I24_P23	I24 *vs* I24_P18
		Control	P24	I18	I23	I24	P24_I23	P24_I18	I24_P23	I24_P18				
Pattern recognition receptors	**RIG-I**	1.94 ± 0.1	2.04 ± 0.3	16.79 ± 2.87	19.89 ±5.82	14.83 ± 2.5	6.13 ± 3.62	9.18 ± 2.96	21.36 ± 8.4	28.02 ± 5.4	0.0079**	ns	ns	0.0486*
	MDA5	2.09 ± 0.3	1.71 ± 0.2	5.28 ± 0.23	5.75 ± 1.42	4.50 ± 0.7	3.31 ± 0.88	3.78 ± 1.13	5.75 ± 1.9	8.44 ± 1.3	ns	ns	ns	ns
	TLR2	3.72 ± 1.6	4.49 ± 2.4	8.72 ± 3.3	6.51 ± 3.82	6.99 ± 5.8	7.77 ± 3.95	7.45 ± 5.65	6.76 ± 2.5	8.08 ± 5.7	ns	ns	ns	ns
	TLR3	2.78 ± 0.4	2.23 ± 0.3	2.63 ± 0.24	2.13 ± 0.54	1.92 ± 0.3	2.62 ± 0.57	2.94 ± 0.6	2.31 ± 0.2	2.31 ± 0.4	ns	ns	ns	ns
	TLR4	2.45 ± 2.0	4.26 ± 4.9	1.57 ± 2.44	2.08 ± 1.41	3.15 ± 4.8	4.26 ± 6.36	4.79 ± 3.44	6.43 ± 4.5	1.53 ± 1.6	ns	ns	ns	ns
	TLR6	6.80 ± 2.0	6.02 ± 1.4	3.03 ± 1.17	3.52 ± 0.29	3.42 ± 0.6	7.6 ± 1.89	7.05 ± 1.8	4.27 ± 0.6	4.47 ± 1.5	0.0013**	0.0004**	ns	ns
	TLR7	2.65 ± 0.9	2.01 ± 0.6	2.54 ± 0.46	2.01 ± 0.6	2.01 ± 0.6	2.01 ± 0.6	2.01 ± 0.6	2.80 ± 0.3	2.76 ± 0.4	ns	ns	ns	ns
	TLR8	4.90 ± 5.2	9.41 ± 12.6	9.35 ± 6.56	10.96 ± 9.64	15.46 ± 22.1	86.97 ± 62.99	20.39 ± 21.46	14.43 ± 20.1	4.43 ± 8.8	ns	ns	ns	ns
	TLR9	9.83 ± 15.9	23.21 ± 16.8	36.25 ± 13.4	28.91 ± 13.8	19.31 ± 9.5	21.34 ± 8.78	19.87 ± 10.09	9.12 ± 5.9	18.07 ± 12.0	ns	ns	ns	ns
	TLR10	4.20 ± 1.2	2.68 ± 1.5	2.03 ± 1.01	2.28 ± 0.65	1.50 ± 0.3	4.77 ± 1.81	5.15 ± 2.01	2.73 ± 0.6	2.20 ± 1.6	0.0448*	0.0062**	ns	ns
Interferons	**IFNα**	3.56 ± 1.1	4.25 ± 1.8	3.62 ± 0.89	2.97 ± 0.41	3.88 ± 0.8	5.82 ± 1.69	4.47 ± 2.05	2.77 ± 0.5	2.62 ± 0.7	0.0317*	ns	ns	ns
	IFNβ	21.88 ± 17.4	42.25 ± 19.7	4974.1 ± 858.5	7334.6 ± 2013.14	3911.05 ± 726.6	1897.06 ± 1072.05	3384.59 ± 216.18	5237.35 ± 1460.9	8161.37 ± 1662.9	0.006**	ns	ns	0.0097**
	IFNλ1	2.27 ± 0.9	2.75 ± 1.1	5.97 ± 1.92	6.12 ± 1.94	5.31 ± 1.0	4.33 ± 1.10	5.78 ± 1.71	5.68 ± 1.5	8.71 ± 2.2	ns	ns	ns	ns
Interferon stimulated genes	**PKR**	1.87 ± 0.2	2.23 ± 2.0	6.52 ± 0.69	7.72 ± 0.68	4.74 ± 2.1	3.10 ± 1.45	4.84 ± 0.98	5.91 ± 2.2	6.24 ± 3.6	0.0026*	ns	ns	ns
	OAS	59.25 ± 6.9	74.35 ± 21.3	2302.61 ± 442.6	2409.17 ± 625.62	1677.44 ± 373.0	293.18 ± 169.09	640.35 ± 132.64	2034.66 ± 508.0	4931.54 ± 1228.9	0.0004**	0.0046**	ns	0.0067**
	MX1	1.57 ± 0.2	1.49 ± 0.2	27 ± 2.89	32.27 ± 7.22	22.04 ± 4.6	7.28 ± 3.57	12.12 ± 1.95	32.67 ± 7.8	53.08 ± 11.8	0.0054**	ns	ns	ns
	MX2	6.46 ± 1.4	6.96 ± 1.4	76.60 ± 9.57	104.36 ± 24.31	61.01 ± 11.0	17.34 ± 8.96	34.70 ± 6.05	92.21 ± 28.5	159.12 ± 37.5	0.0002**	0.0436*	ns	0.0229*
	ISG12	47.6 ± 15.2	66.88 ± 18.3	138.32 ± 29.34	141.56 ± 26.67	111.00 ± 60.1	74.99 ± 49.32	78.26 ± 38.43	117.07 ± 26.1	147.15 ± 35.6	0.0292*	ns	ns	ns
	ISG15	2648.03 ± 1365.5	5241.87± 1400.4	244759.59 ± 144055.18	272094.50 ± 65321.64	209624.87 ± 54923.2	80336.62 ± 83086.94	98097.28 ± 20568.71	226895.23 ± 163975.2	682603.42 ± 230651.7	ns	ns	ns	ns

Ns: not significant, SD: Standard deviation, ** P<0.01 and * P<0.05, Genes showing significant differences in one of the conditions at least are represented in bold.

Tableau 10. *Statistical comparisons between mRNA relative expressions in swIAV H1N2 (I) single infections and co-inoculations of NPTr cells with active (P) and inactivated PRRSV-1 (Pinac). The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last three columns. Comparisons were made using one-way ANOVA test and Tukey's post-test.*

	Messenger RNA	mRNA relative expression ± SD					I24 *vs* I24_P24	I24 *vs* I24_Pinac	I24_P24 *vs* I24_Pinac
		Control	P24	I24	I24_P24	I24_Pinac			
PRR	**RIG-I**	2.79 ± 0.9	3.91 ± 0.5	105.93 ± 26.1	1.38 ± 0.2	4.70 ± 1.2	<0.0001 **	0.0004**	<0.0001**
	MDA5	2.32 ± 0.2	2.14 ± 0.2	13.87 ± 08	1.51 ± 0.3	2.69 ± 0.5	<0.0001 **	ns	<0.0001**
Interferons	**IFNβ**	9.39 ± 3.2	8.46 ± 3.9	665.89 ± 116.5	9.29 ± 9.3	26.78 ± 6.5	<0.0001 **	ns	0.0006 **
	IFN λ1	225.87 ± 49.1	29.85 ± 21.4	780.17 ± 228.9	27.64 ± 3.9	75.39 ± 62.8	<0.0001**	<0.0001**	ns
	IFNλ3	32.55 ± 37.9	166.08 ± 78.6	30523.76 ± 9265.3	277.79 ± 64.6	938.36 ± 372.4	<0.0001**	0.0074**	<0.0001**
Interferon stimulated genes	**PKR**	5.30 ± 0.8	3.92 ± 0.6	30.70 ± 3.5	1.15 ± 0.1	5.36 ± 0.8	<0.0001**	<0.0001**	<0.0001**
	OAS	1.57 ± 0.5	18.01 ± 3.2	434.40 ± 109.1	4.43 ± 0.9	23.18 ± 8.2	<0.0001**	<0.0001**	<0.0001**
	MX1	2.54 ± 0.6	4.42 ± 0.8	164.11 ± 28.5	1.45 ± 0.3	8.74 ± 2.3	<0.0001**	0.0070**	<0.0001**
	MX2	6.62 ± 1.2	10.64 ± 2.9	527.58 ± 87.1	1.67 ± 0.4	15.54 ± 3.8	<0.0001**	0.0001 **	<0.0001**
	ISG12	2.78 ± 0.3	1.79 ± 0.2	6.32 ± 0.7	1.50 ± 0.2	2.90 ± 0.4	<0.0001**	0.0180*	<0.0001**
	ISG15	1.64 ± 0.3	7.35 ± 1.5	250.15 ± 118.5	8.57 ± 1.6	17.95 ± 5.8	<0.0001**	ns	0.001**

Ns: not significan Ns: not significant, SD: Standard deviation, ***P*<0.01 **P*<0.05, Genes showing significant differences in one of the conditions at least are represented in bold.

Tableau 11. *Statistical comparisons between mRNA relative expressions in swIAV H1N2 (I) single infections and multiple infections of Precision-Cut Lung Slices (PCLS) with active (P) and inactivated PRRSV-1 (Pinac). The mean values and the standard deviations of the relative expression are shown for every condition. P values are presented in the last three columns. Comparisons were made using one-way ANOVA test and Tukey's post-test.*

	Messenger RNA	mRNA relative expression ± SD					I24 *vs* I24_P24	I24 *vs* I24_Pinac	I24_P24 *vs* I24_Pinac
		Control	P24	I24	I24_P24	I24_Pinac			
PRR	**RIGI**	20.23± 29.5	24.55 ± 12.5	21647.0 ± 5685.1	31.14 ± 22.7	49.87 ± 22.4	<0.0001 **	0.0057**	ns
	MDA5	69.12 ± 97.6	39.05 ± 30.4	2634.07 ± 7029.9	21.61 ± 12.1	75.96 ± 54.8	<0.0001 **	ns	ns
Interferons	**IFNβ**	1.37 ± 1.5	1485.37 ± 1068.1	615.66 ± 1558.2	457.14 ± 274.2	44.01 ± 28.6	ns	ns	0.0027 **
Interferon stimulated genes	**PKR**	23.99 ± 34.3	16.43 ± 10.9	837.37 ± 2222.9	11.81 ± 7.4	26.83 ± 15.9	0.0001**	ns	ns
	OAS	15.61 ± 24.8	32.86 ± 33.1	785.82 ± 1158.7	21.19 ± 28.4	51.40 ± 65.6	0.0271*	ns	ns
	MX1	58.92 ± 14.1	104.76 ± 82.8	14702.68 ± 38494.2	283.59 ± 214.5	477.83 ± 168.3	0.0007**	ns	ns
	MX2	11.75 ± 10.1	10.45 ± 11.9	2258.53 ± 5784.8	22.17 ± 20.8	52.90 ± 23.3	<0.0001**	ns	ns
	ISG12	16.77 ± 19.1	73.45 ± 60.9	5583.01 ± 10106.2	869.02 ± 530.3	571.86 ± 265.8	ns	ns	ns
	ISG15	76.74 ± 78.6	204.13 ± 338.9	17267.75 ± 26798.8	182.57 ± 107.4	2508.50 ± 3556.5	0.0055**	ns	ns

Ns: not significant, SD: Standard deviation, ***P*<0.01 **P*<0.05, Genes showing significant differences in one of the conditions at least are represented in bold.

d) Conclusion et discussion complémentaire :

Le PRRSV ne se réplique pas dans les cellules épithéliales, mais certaines études montrent une détection de l'antigène viral au niveau de ces cellules (Xiao et al., 2010; Frydas et al., 2013). La possibilité d'une interaction entre PRRSV et cellules épithéliales n'est donc pas exclue. Par conséquent, l'interférence du PRRSV avec la multiplication d'autres virus ou avec la réponse de ces cellules suite à des infections virales plus fréquentes nécessitait une investigation. Dans ce chapitre l'interférence du PRRSV-1 avec la réplication du swIAV H1N2 sur des cellules épithéliales a été explorée. La réplication du swIAV H1N2 a été réduite en présence du PRRSV-1 et l'expression des transcrits des gènes codant pour les IFN et les ISG a également été modifiée par ce dernier virus. Cet effet a été démontré avec la souche PRRSV-1 Finistère et avec la souche vaccinale MLV. L'utilisation du virus ADV qui n'a montré aucun impact sur la réplication du swIAV H1N2 confirme l'effet spécifique au PRRSV-1 qui n'est vraisemblablement pas lié à l'encombrement stérique entre les deux virus présents dans le milieu de culture.

Cette interférence avec l'infection des cellules par le swIAV H1N2 a été modifiée en changeant l'ordre et les délais d'infection entre les deux virus. Ainsi, l'effet du PRRSV-1 sur la réplication du swIAV H1N2 et la réponse des cellules est plus important en rapprochant les délais entre les deux infections. Cet effet n'est plus observé en déposant le PRRSV-1 bien après le swIAV H1N2. Cela laisse assez de temps au swIAV H1N2 pour infecter les cellules et initier sa réplication sans être affecté par le PRRSV-1.

Dans ces travaux, il a été déduit que le PRRSV-1 affecte la réponse des cellules épithéliales en adhérant à la membrane cytoplasmique. Cependant, son « récepteur » reste à déterminer, mais on peut penser au sulfate d'héparane qui est exprimé d'une façon ubiquitaire chez les cellules épithéliales (Simon Davis and Parish, 2013). La réplication du swIAV H1N2 et l'expression des transcrits cellulaires associés qui n'étaient pas affectées en éliminant les particules de PRRSV-1 non internalisées par les lavages à l'aide d'une solution acide nous laissent penser à une stimulation au niveau de la surface des cellules. Il a été constaté qu'après l'élimination du PRRSV-1, les résultats de la surinfection grippale sont similaires à une mono-infection par le swIAV H1N2.

L'inactivation chimique du PRRSV-1 par la β-propiolactone réduit son effet sur la réponse des cellules à l'infection par swIAV H1N2 sans l'abolir. La réplication du swIAV H1N2 et l'expression de quelques IFN et ISG sont aussi réduites en présence du PRRSV-1 inactivé. Ces

résultats confirment encore une fois que cette interférence avec le swIAV H1N2 ne nécessite pas la réplication du PRRSV-1 ni l'accomplissement de son cycle complet. L'impact du PRRSV-1 est donc, du moins en partie, lié à une signalisation membranaire qui interagit avec les voies de signalisation induites par le swIAV H1N2 au niveau des cellules épithéliales. Les investigations de ces voies de signalisation menées dans ce chapitre montrent un effet spécifique au PRRSV-1 dans le cas de l'infection PRRSV-1 et de la co-incubation. Cet effet est représenté par une baisse de la phosphorylation de l'AKT, de l'AMPK et de JAK2 et par une augmentation de la phosphorylation des ERK 1/2 au bout de 5 à 10 minutes. Il s'agit de voies essentielles au swIAV pour compléter son cycle de réplication. L'inhibition de la voie AKT/PI3K par exemple qui est essentielle pour l'internalisation du swIAV (Ehrhardt et al., 2010) entraîne une baisse de sa réplication. Le PRRSV-1 inactivé qui n'affecte pas la phosphorylation de l'AKT était aussi capable d'inhiber la réplication du swIAV H1N2. Donc il existe d'autres mécanismes qui sont impliqués dans l'effet du PRRSV-1 comme l'inhibition de la phosphorylation de AMPK effectuée aussi bien par le PRRSV-1 actif que l'inactivé. L'activation de la phosphorylation des ERK pourrait aussi jouer un rôle. Cette activation qui, selon Lee *et al.* 2010 dépend de l'internalisation du PRRSV dans les AM, n'est pas induite en présence du PRRSV-1 inactivé dans notre étude. Cela nous laisse soupçonner une internalisation du PRRSV-1 qui est inhibée suite à l'inactivation chimique du virus. En parallèle, nos résultats en microscopie montrent une présence des particules de PRRSV-1 malgré tous les lavages effectués, et donc nous laissent penser à une internalisation du PRRSV-1 par les cellules épithéliales.

L'inhibition de la voie JAK/STAT par le PRRSV-1 dans les cellules épithéliales a également été démontrée chez les cellules cibles de ce virus telles que les AM (Wang and Zhang, 2014). Cette inhibition pourrait être à l'origine de la baisse de l'expression des ISG.

Par ailleurs, l'inhibition par le PRRSV-1 de la réponse antivirale innée représentée par une baisse de l'expression des IFN et des ISG, ainsi que la réponse adaptative (humorale et cytotoxique) pourrait favoriser la surinfection grippale. Cela suggère une augmentation de la réplication du swIAV H1N2 au niveau des cellules épithéliales. Cette hypothèse ne semble pas cohérente avec nos résultats qui montrent une baisse de la réplication du swIAV H1N2 en présence du PRRSV-1. Ces différents phénomènes et hypothèses pourraient être à l'origine de l'hétérogénéité entre les différentes études *in vivo* des co-infections PRRSV/swIAV. Certaines études trouvent qu'une pré-infection au PRRSV réduit la réplication du swIAV (Van Reeth et al., 1996) alors que d'autres

montrent une augmentation ou voire aucun impact sur l'infection par le swIAV (Kitikoon et al., 2009; Sangpratum et al., 2019).

Bien que les cellules épithéliales soient les cellules hôtes du swIAV, ce virus infecte également les AM et pourrait donc interférer avec le PRRSV à plusieurs niveaux. Parmi les travaux effectués, une évaluation de cette co-infection a été conduite sur des tranches pulmonaires fines (PCLS). En cas de co-infection, les résultats de cet essai *ex vivo* montrent aussi une baisse de la réplication du swIAV H1N2 et de l'expression des transcrits des ISG qui est moins importante en présence du PRRSV-1 inactivé. Ces résultats rejoignent les résultats des expérimentations menées sur les cellules épithéliales dans notre étude.

Une hypothèse concernant l'interaction directe entre les particules virales du swIAV H1N2 et du PRRSV-1 a été évoquée dans ce chapitre. L'hémagglutinine du swIAV adhère à la membrane cellulaire en s'accrochant à la liaison α-2,6 et α-2,3 de l'acide sialique avec le galactose (Medina and García-Sastre, 2011). Ce même type de liaison de l'acide sialique au galactose est trouvé au niveau des acides sialiques identifiés dans les glycoprotéines de l'enveloppe du PRRSV (Delputte and Nauwynck, 2004; Li and Murtaugh, 2015). Une forme de liaison entre les particules peut donc être imaginée. Cette interaction avec les particules du swIAV H1N2 aboutirait à la formation d'agrégats qui empêcheraient l'adhésion des swIAV à la membrane des cellules.

Dans le but de confirmer ou infirmer cette hypothèse et d'évaluer les interactions entre les particules virales indépendamment des interactions avec les récepteurs membranaires, une dernière expérience a été menée en mélangeant les deux virus sur glace avant d'inoculer les cellules épithéliales (figure 12A). La réplication de swIAV H1N2 a été davantage inhibée par rapport à la condition où les virus ont été déposés directement sur les cellules (figure 12B). L'expression des gènes impliqués dans la réponse immunitaire a également été réduite ou presque abolie (figure 7B).

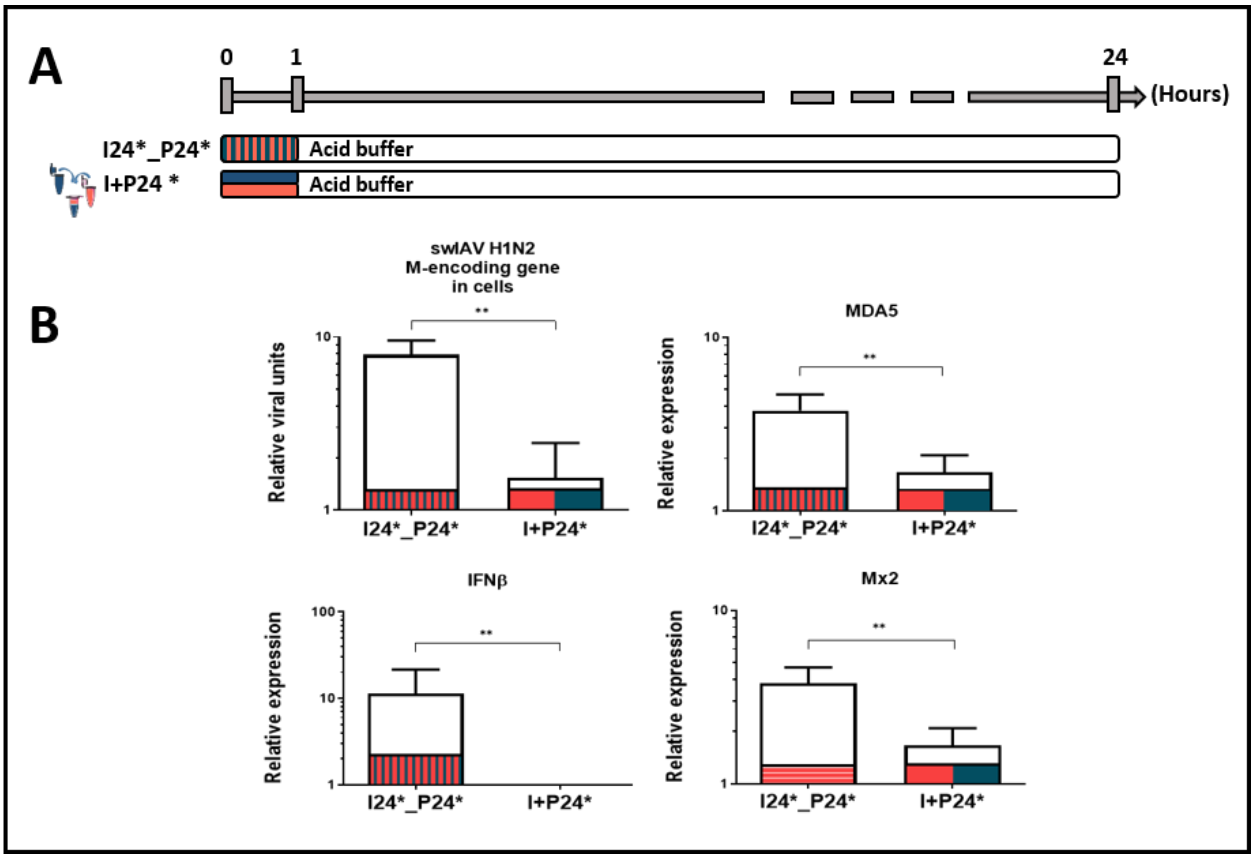

Figure 12. *Protocole (A), expression relative des transcrits viraux et cellulaires (B) montrant l'impact de l'interférence des particules virales sur la réponse des NPTr avant tout contact avec les récepteurs membranaires des cellules. Les cellules ont été infectées à une MOI de 3, n = 6 puits par condition pour la qPCR. I24_P24 désigne la co-administration des deux virus sur 24h et I + P24 désigne la combinaison des deux virus avant d'être déposé sur les cellules. (*) représente le lavage tampon acide des cellules 1h après l'incubation avec les virus. Les valeurs moyennes et les écarts types sont représentés. Des comparaisons ont été faites en utilisant un test one-way ANOVA suivi d'un test de Tukey. Les différences sont considérées significatives lorsque P <0,05 (*) ou P <0,01 (**).*

Ces résultats ajoutent un nouveau scénario à l'interaction entre les deux virus qui limite l'accès des particules virales à la membrane de la cellule et augmente l'impact du PRRSV sur la réplication du swIAV et sur la réponse immune induite.

Cette complexité d'interaction entre les deux virus nécessite encore plus d'investigation pour une meilleure maîtrise des conséquences de ces co-infections sur l'hôte porcin.

Chapitre 3

Sensibilité des macrophages à l'infection par le PRRSV suite à une première infection par d’autres agents pathogènes

Chapitre 3 : Sensibilité des macrophages à l'infection par le PRRSV suite à une première infection d'autres agents pathogènes

A- Prospection de liens potentiels entre la sensibilité à l'infection par le PRRSV des macrophages alvéolaires et d'autres agents infectieux respiratoires présents chez les porcs d'élevage conventionnel

a) Introduction des travaux :

Les interactions entre les différents agents pathogènes responsables du CRP méritent d'être étudiées. Une première infection du tissu respiratoire par un agent pathogène risque de moduler la réponse immune ainsi que la sensibilité de certaines cellules à une deuxième infection par le même agent pathogène ou un micro-organisme différent. La mise en place d'une réponse immune suite à la première infection conduit à l'entraînement des cellules effectrices et peut contribuer à la lutte contre une deuxième infection rendant l'hôte porcin moins sensible à celle-ci. Il s'agit donc de la mise en place d'une immunité entraînée capable de modifier les mécanismes de première défense contre un agent pathogène. Par conséquent, cette immunité entraînée peut aussi avoir un impact sur la réplication des virus ou la multiplication des bactéries responsables des surinfections respiratoires chez le porc.

Les travaux de ce chapitre ont été menés dans le but d'évaluer l'effet d'une première infection par un agent pathogène viral ou bactérien sur la réponse des macrophages à une surinfection par le PRRSV et sur leur capacité à répliquer ce virus. Ces macrophages, qui constituent la cible principale du PRRSV, comportent les AM et les macrophages intravasculaires pulmonaires (*Pulmonary intravascular macrophages*, PIM). Une première étude avait montré plus de variabilité de la sensibilité des AM à l'infection PRRSV en comparaison avec celle des PIM (Elise Bordet et al., 2018b). Cela pourrait-être lié au fait que les AM sont directement exposés aux agents pathogènes respiratoires alors que les PIM sont plutôt protégés par les barrières épithéliales et endothéliales.

Dans un premier temps une étude terrain a été menée à partir d'un élevage de porcs conventionnel en région Pays de la Loire dans le but d'identifier les agents pathogènes les plus fréquents et d'évaluer la possibilité de présence de deux ou de plusieurs infections en même temps. Dans un deuxième temps, l'impact de ces infections sur la réplication du PRRSV-1 et sur la réponse immune innée des macrophages a été évalué. Une comparaison a également été effectuée entre la réponse des PIM et celle des AM provenant d'un élevage conventionnel, EOPS ou à statut contrôlé.

b) Papier n° 3 :

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Short communication

Prospecting potential links between PRRSV infection susceptibility of alveolar macrophages and other respiratory infectious agents present in conventionally reared pigs

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome virus (PRRSV) is one of the main component of the porcine respiratory disease complex (PRDC), which strongly impact the pig production. Although PRRSV is often considered as a primary infection that eases subsequent respiratory coinfections, the possibility that other PRDC components may facilitate PRRSV infection has been largely overlooked. The main cellular targets of PRRSV are respiratory macrophages among them alveolar macrophages (AM) and pulmonary intravascular macrophages (PIM). AM, contrarily to PIM, are directly exposed to the external respiratory environment, among them co-infectious agents. In order to explore the possibility of a co-infections impact on the capacity of respiratory macrophages to replicate PRRSV, we proceed to *in vitro* infection of AM and PIM sampled from animals presenting different sanitary status, and tested the presence in the respiratory tract of these animals of the most common porcine respiratory pathogens (PCV2, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma floculare*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Streptoccocus suis)*. In this exploratory study with a limited number of animals, no statistic differences were observed between AM and PIM susceptibility to *in vitro* PRRSV infection, nor between AM coming from animals presenting very contrasting respiratory coinfection loads.

1. Introduction

Respiratory infections are one of the major cause of disease in pigs, leading to economic losses as well as to antibiotics overuse. Because of its sanitary importance, this condition has been given a name: porcine respiratory disease complex (PRDC). The major PRDC components described so far are swine influenza A virus (swIAV), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine circovirus 2 (PCV2) as well as bacteria such as *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida, Bordetella bronchiseptica* and *Streptoccocus suis* (Opriessnig et al., 2011). Among them, PRRSV, because of the duration of infection (more than a month), its immunomodulatory properties as well as the emergence of highly pathogenic strains, is considering as one of the pillars of PRDC (Montaner-Tarbes et al., 2019).

The pathogens constituting the PRDC are thought to interact with each other as well as with the host tissues, in a complex manner (among others see (Ellis et al., 2004; Lévesque et al., 2014; Thacker et al., 1999) (Saade et al., 2020)). Indeed, whereas the adaptive immune response can largely be considered as pathogen-specific, the immediate inflammatory response and the countermeasures triggered by the pathogens to quench this response may impact the infectious capacities of a second pathogen. Moreover, some infections may trigger trained immunity (especially on monocytes and macrophages, the main targets of PRRSV), and the persistent character of the majority of these microbes may led to long term tissue remodelling.

In a previous study we observed that pulmonary intravascular macrophages (PIM) were equivalently susceptible as alveolar macrophages (AM) to PRRSV infection, and this *in vitro* as well as *in vivo* (Bordet et al., 2018). However, these preliminary data pointed on a lower variability between animals in PIM PRRSV susceptibility compared with AM PRRSV susceptibility. Since AM, seated in the airways, are more exposed to respiratory pathogens than PIM, which are protected from external environment by three tissue layers (epithelium,

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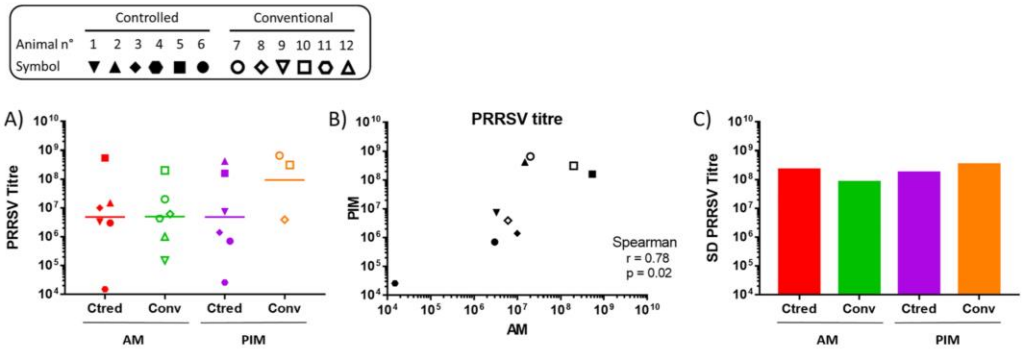

Fig. 1. AM and PIM from different animals' origins were not distinctly susceptible to PRRSV infection.
Gradient and plastic-adherence enriched alveolar macrophages (AM) and pulmonary intravascular macrophages (PIM) were infected by Lena strain PRRSV at an MOI of 10^{-3} for 24 h. PRRSV titre was measured by RT-qPCR. Each symbol represents one animal. Macrophages from controlled animals (Ctred), macrophages from conventional animals (Conv). A) PRRSV titre using GAPDH as reference gene (Genex macro analysis (Bio-Rad)). The correspondence between animal identity numbers (for correspondence with Table 1) and symbols are indicated in the cartouche. B) Correlation plot of AM and PIM PRRSV titre according to data depicted in A) using Spearman test. C) Standard deviations of data depicted in A).

interstitium and endothelium), we postulated that the variability of AM susceptibility to PRRSV infection might be due to factors present in the airways such as coinfectious agents. In order to test our hypothesis, we set up an original approach consisting in monitoring ongoing *in vivo* recurrent respiratory infections and at the same time *in vitro* AM and PIM capacities to replicate PRRSV, in animals presenting different sanitary status. Since this influence between the pathogens and the components of the respiratory immune system might come from interactions lasting several weeks, and that persistent infections are naturally much more susceptible to be involved in coinfections situations, we focused on the following persistent PRDC components: PCV2, *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae* and the related *Mycoplasma hyorhinis and Mycoplasma floculare*; *Pasteurella multocida, Bordetella bronchiseptica*, *Streptoccocus suis* and excluded *a priori* the short term swIAV infection that, moreover has been already shown to down-regulate PRRSV infection *ex vivo* (Dobrescu et al., 2014).

2. Materials and Methods

2.1. Pig lung cells collection

Lung tissue samples were obtained from 5- to 7-month-old Large White conventionally bred sows coming from two origins: 6 animals from the Unité Experimentale de Physiologie Animal de l'Orfrasière (UEPAO, Tours, France) and 6 from PORCI MAUGES slaughterhouse (Beaupréau, France). Finally, 4 supplementary Large White specific pathogen free (SPF) 2 to 3-month-old sows from the highly controlled SPF facility of ANSES, Ploufragan (France) free of numerous respiratory pathogens such as PRRSV, swine Influenza virus, porcine circovirus type 2, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica* and *Haemophilus parasuis* were also tested. In order to reduce animal experiments, all the animals were euthanized in the course of the regular management process of the herds, hence the absence of trial number or experimental authorisation and the age difference according to the origin of the animals. Tracheo-bronchial tissue and lymph nodes were sampled and directly frozen in dry ice for subsequent pathogens detection (Zimmerman et al., 2012). A broncho-alveolar lavage (BAL) procedure was then performed twice on the isolated left lung with 250 mL of PBS supplemented with 2 mM EDTA (PBS/EDTA), to collect AM. Next, a 1-cm slice of external lung parenchyma was dissected from the same lung. Tissues were minced and incubated in nonculture-treated Petri dishes, to avoid plastic adherence of macrophages, for 2 h at 37 °C in complete RPMI, consisting of RPMI 1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, and 10% inactivated foetal calf serum (FCS) (all from Invitrogen, Paisley, UK), containing 2 mg/mL collagenase D (Roche, Meylan, France), 1 mg/mL dispase (Invitrogen), and 0.1 mg/mL Dnase I (Roche). Cells were passed through 40 µm cell strainers and red blood cells lysed with erythrocytes lysis buffer (10 mM NaHCO3, 155 mM NH4Cl, and 10 mM EDTA). Next, cells were washed with PBS/EDTA, counted, and step-frozen in FCS/10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO).

2.2. Cell infection and viral titration

Parenchymal and alveolar macrophages of one conventional and one controlled animal were systematically thawed and infected in parallel. Because of technical reasons, not enough PIM could be retrieved from animals 9, 11, 12 from Beaupréau slaughterhouse (Fig. 1A), leading to only 3 conventional PIM infection tests. Thawed cells were first enriched in macrophages and depleted in dead cells by 1.065 density iodixanol gradient (Optiprep®, Nycomed Pharma, Oslo, Norway). These gradient-enriched mononuclear phagocyte cell preparations were further enriched in macrophages by 2 h plastic adherence, leading, from bronchoalveolar lavage to an AM purity of 86% +/-8%, and for parenchyma to a PIM purity of 51%+/-20%. Enriched macrophages were then cultured in complete RPMI for 24 h in flat-bottom 96-well plates at 3.10^5 cells/well and then infected at a multiplicity of infection (MOI) of 10^{-3} with PRRSV virus Lena strain in complete RPMI. At 24 hpi, plates were centrifuged and pelleted cells were lysed in 350 µL of RNA extraction buffer (RLT Buffer, QIAGEN). These MOI and incubation time have been set to measure the PRRSV titre during the linear phase of the infection, in order to observe weak infectivity differences between the different macrophages. Total RNA were extracted using RNeasy Plus Mini kit (QIAGEN) according to the manufacturer's instructions. RNA were reverse transcribed using oligo(dT) and random primers (BioRad iScript Reverse Transcription supermix). RNA samples were treated with DNAse I Amp Grade (Invitrogen) (1 U/µg of RNA). The absence of genomic DNA contamination was validated by the use of treated RNA as a template directly in PCR. Total RNA quantity and quality were assessed using Nanophotometer (Implen, Munich, Germany). cDNA was generated with a virus reverse transcriptase in the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA) from 100-200 ng of RNA free of genomic DNA per reaction. Diluted cDNA (4X) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's recommendations. Real-time assays were run on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Samples were normalized internally by simultaneously using

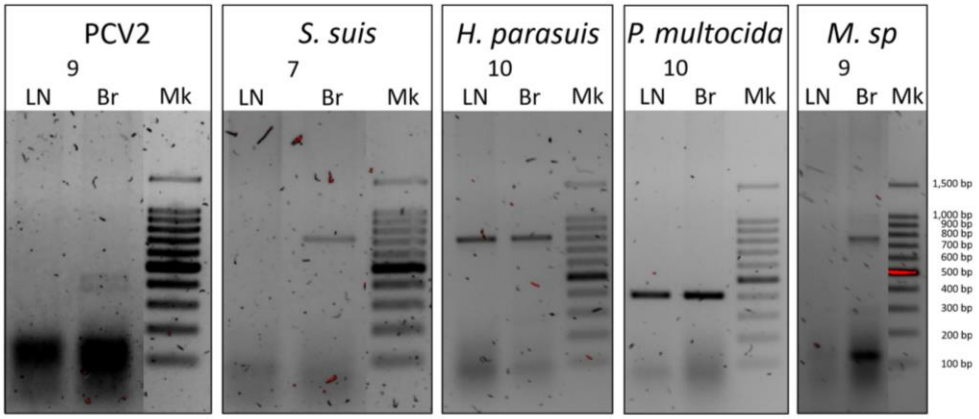

Fig. 2. Detection of respiratory pathogens.
Example of positive samples detected by PCR of PCV2 (PCV2 - 470 bp), *Streptococcus suis* (*S. suis* - 700 bp), *Haemophilus parasuis* (*H. parasuis* - 820 bp), *Pasteurella multocida* (*P. multocida* - 410 bp), and *Mycoplasma* species (*M. hyopneumoniae* - 1,000 bp, *M. floculare* - 754 bp, *M. hyorhinis* - 1,129 bp) from Tracheal epithelium and trachea-bronchial lymph node. The results for all pigs are shown in Table 1.

the average Cycle quantification (C*q*) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference gene in each sample. Then, 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. TTC AGT TCC GGT GA); GAPDH(F: CTT CAC GAC CAT GGA GAA GG, R: CCA AGC AGT TGG TGG TAC AG), TBP1 (F: AACAGTTCAGTAGTTATGAGCCAGA, R: AGATGTTCTCAAACGCTTCG), ActB (F: CACGCCATCCTGCGTCTGGA, R: AGCACCGTGTTGGCGTAGAG), HPRT1 (F: GGACTTGAATCATGTTTGTG, R: CAGATGTTTCCAAACTCAAC), IFN-α (generic see (Sang et al., 2011)) (F: GGC TCT GGT GCA TGA GAT GC, R: CAG CCA GGA TGG AGT CCT CC), IFN-ß (F: GTT GCC TGG GAC TCC TCA A, R: CCT CAG GGA CCT CAA AGT TCA T), Mx1 (F: AGT GTC GGC TGT TTA CCA AG, R: TTC ACA AAC CCT GGC AAC TC), Mx2 (F: CCG ACT TCA GTT CAG GAT GG, R : ACA GGA GAC GGT CCG TTTA C) and PKR (F: CAC ATC GGC TTC AGA GTC AG, R: GGG CGA GGT AAA TGT AGG TG). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene since it was endowed with one of the most stable expression in the whole porcine lung (Delgado-Ortega et al., 2011) as well as specifically in lung macrophages (Maisonnasse et al., 2016). Then, qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) (Vandesompele et al., 2002). In all experiments other reference genes (TBPI or ActB and HPRT1) were used in parallel with GAPDH and gave comparable results. All the AM/PIM were negative for PRRSV before *in vitro* infection.

2.3. PCR for pathogens detection

Tracheobronchial tissue and lymph node were thawed and mechanically (Lysing matrix E FastPrep24, MpBio) disrupted before DNA extraction using QIAamp cador Pathogen kit (INDICAL BIOSCIENCE). A proteinase K enzymatic lysis was then performed. A PCR was then run using previously described primers for PCV2 (F: TTT AGG GTT TAA GTG GGG GGT C, R: CCG GAT CCA TGA CGT ACC CAA GGA GGC G, 470 bp fragment expected) *Pasteurella multocida* (F: AAG GGA TGT TGT TAA ATA GAT AGC, R: GCT TCG GGC ACC AAG CAT AT, 410 bp fragment expected) *Haemophilus parasuis* (F: GTG ATG AGG AAG GGT GGT GT, R: GGC TTC GTC ACC CTC TGT, 820 bp fragment expected), *Streptococcus suis* (F: TTC TGC AGC GTA TTC TGT CAA ACG, R: TGT TCC CTG GAC AGA TAA AGA TGG, 700 bp fragment expected) (Cheong et al., 2017) or for *Mycoplasma* species (*M. hyopneumoniae* F: TTCAAAGGAGCCTTCAAGCTTC, 1,000 bp fragment expected; *M. floculare* F: GGGAAGAAAAAAATTAGGTAGGG, 754 bp fragment expected; *M. hyorhinis*, F: CGGGATGTAGCAATACATTCAG, 1129 bp fragment expected, and a common reverse primer R: AGAGGCATGATGATTTGACGTC) (Stakenborg et al., 2006). The PCR was performed in a 20 µL reaction mixture containing 4 µL of extracted DNA (containing a minimum of 25 ng of DNA), 0,5 µM of each primer, 1,5 to 2,5 mM of MgCl2, 0,1 mM of deoxynucleoside triphosphate and 0,02 U of GoTaq G2 Flexi DNA Polymerase (Promega). The PCR was carried out for 40 cycles consisting of denaturation for 20 seconds at 94 °C, annealing for 30 seconds at Tm and extension for 30 seconds at 72°c using thermal cycle (BioRad). Then, 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.

2.4. Statistical analysis

Data were analysed using Graph Pad Prism (version 6) and Mann-Whitney test was used to compare relative expressions. Non-parametric approaches were chosen due to the few number of samples available. The Mann-Whitney's test was used to compare unpaired samples based on ranking. The AM and PIM from the same animal were considered as paired samples. For paired samples, the Wilcoxon matched-pairs signed rank test was used. When scatter plots are used, the mean is depicted by a horizontal bar. Factor Analysis for Mixed Data (FAMD) was performed using FactoMineR package of RStudio (version 3.6.1) in order to investigate the relation between isolated pathogens and gene expression.

3. Results and Discussion

AM and PIM were first collected from animals coming from 2 facilities presenting distinct health status: the controlled pig breeding facility from the UEPAO (INRA, Nouzilly, France) and the PORCI MAUGES slaughterhouse (Beaupréau, France) which processes animals from the conventional farms of the surrounding area, known from the regional veterinary services as a PRRSV-free area (Blanquefort and Benoit, 2000). The AM and PIM were infected *in vitro* at a multiplicity of infection (MOI) of 0.001. Twenty-four hours later the cellular viral load was measured using RT-qPCR. No significant differences were observed neither between AM from the two different origins nor between AM and PIM from the same animals (Fig. 1A). Conversely, we could observe a correlation ($r = 0.78$, $p = 0.02$) of *in vitro* PRRSV infections between AM and PIM from the same animal (Fig. 1B), in contradiction with the hypothesis that AM exposed to airways co-infections would be differentially susceptible to PRRSV than PIM. Finally, standard deviations of

Table 1
Conventional but not controlled animals presented viral and bacterial respiratory infections

		Controlled												Conventional											
		1		2		3		4		5		6		7		8		9		10		11		12	
		Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN	Br	LN
in vivo infection (whole tissues PCR detection)	PCV2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-
	A. pleuro	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	H. para	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	M. floc	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
	M. hyop	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M. hyorh	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	P. multo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-
	S. suis	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-
in vitro titre (AM)	PRRSV	3,3E+06	1,5E+07	1,E+07	1,5E+04	5,4E+08	3,E+06	2,0E+07	6,1E+06	1,5E+05	4,3E+06	2,0E+08	1,E+06												

Bronchial epithelium (Br) and trachea-bronchial lymph node (LN) of animals from different sanitary status (Controlled, from UEPAO, INRA and Conventional, from commercial slaughterhouse) were tested by PCR for the presence of different respiratory pathogens as illustrated in Fig. 2. The titre of *in vitro* PRRSV-infected AM (MOI 0,01, 24 h post-infection, RT-qPCR-relative expression) from the same animals are depicted in the last row (see Fig. 1). The second row depict the identification number of each animal as identified in Fig. 1).

PRRSV titres presented no differences between AM and PIM from the two origins (Fig. 1C).

Respiratory infections occurring *in vivo* at the time of AM and PIM collection were then detected by PCR on tracheo-bronchial lymph nodes and bronchial epithelium tissue (Fig. 2), using previously described primers (Cheong et al., 2017; Stakenborg et al., 2006). Strikingly, none of the tested pathogens were detected in controlled animals whereas all the conventional animals were positive for at least one of the tested pathogens (Table 1), *P. multocida* (4 animals), *S. suis* (3 animals) and PCV2 (3 animals) being the most frequent infections. One animal was detected positive for 5 pathogens simultaneously (animal n°3, for PCV2, *H. parasuis*, *M. floculare*, *P. multocida* and *S. suis*). However, no link could be inferred between PRRSV *in vitro* titre upon AM infection, and the *in vivo* detection of one or more respiratory pathogens. To note, no animals presented *M. hyopneumoniae* and *M. hyorhinis* infections.

Type I IFNs responses were then evaluated in respiratory macrophages upon *in vitro* PRRSV infection by testing IFNα (generic), IFNβ and three interferon stimulating genes Mx1, Mx2 and PKR transcripts. No differences were observed between PRRSV mock or infected AM or between AM from the two different origins (Fig. 3A), neither in raw transcriptomic expression, nor in induction (fold increase) upon PRRSV infection (data not shown). Once again, no consistent difference could be observed between AM and PIM from the same animals.

Finally, in order to test the possibility of a non-identified respiratory infection occurring in both controlled and conventional animals, we also tested AM from the highly controlled SPF facility of ANSES, Ploufragan (France). The same infections and measures than with controlled and conventional AM were performed. AM from SPF animals were as susceptible to PRRSV infection as AM from controlled and conventional animals (Fig. 3B). We observed, as expected, a clear upregulation of IFNβ upon PRRSV infection in all conditions (Fig. 3C). More globally, the only difference which can be observed for type I IFN and ISG responses (Fig. 3D) was the lower IFNβ transcript level before PRRSV infection in AM from SPF animals, which might be due either to the SPF status of the animals or to their lower ages (3-month-old compared with 5 to 7-month-old for conventional and controlled animals).

In order to globally analyse our quantitative (PRRSV titre, IFN-I related genes fold change upon *in vitro* PRRSV infection) as well as categorical (presence or not of other pathogens in the respiratory tract) data, we proceeded to a Factor Analysis for Mixed Data (FAMD) (Fig. 3E). According to the great variability as well as to the small size of the samples, no significant links between AM origins, type of respiratory infections and IFN-related genes induction could be observed. The analysis of the quantitative variables however showed an interesting segregation on the first and second axis (encompassing respectively 26% and 25% of the total variability of the sample) of PRRSV-Lena, the three ISG transcripts measured (Mx1, Mx2, PKR) and IFNα, but not IFNβ transcriptomic induction. Thus ISG genes expressions correlation with PRRSV titre argues for the induction by PRRSV of type I IFN protein(s) expression.

In conclusion, although the difference of pathogens load from conventional and controlled animals was striking, no difference of respiratory macrophages PRRSV infection susceptibility could be observed, neither according to the pathogens' identities nor to the tissue location of the macrophages (AM and PIM), invalidating our hypothesis of a recurrent airways infection leading to the onset of AM more susceptible to PRRSV infection. One possibility would be that the observed AM susceptibility variation in our preliminary study was due to *M. hyorhinis* infections as reported by others (Lee et al., 2016; Thacker et al., 1999), a pathogen that was not detected in the animal tested in this study.

Despite this negative result, that might be confirmed by a larger scale campaign, our study allowed us to highlight a clear correlation between AM and PIM PRRSV susceptibility *in vitro*, in agreement with their high similarity (Bordet et al., 2018; Maisonnasse et al., 2016). Moreover, we could note that in the *Pays de la Loire* area (France), conventional, commercial pigs presented systematically one or several respiratory

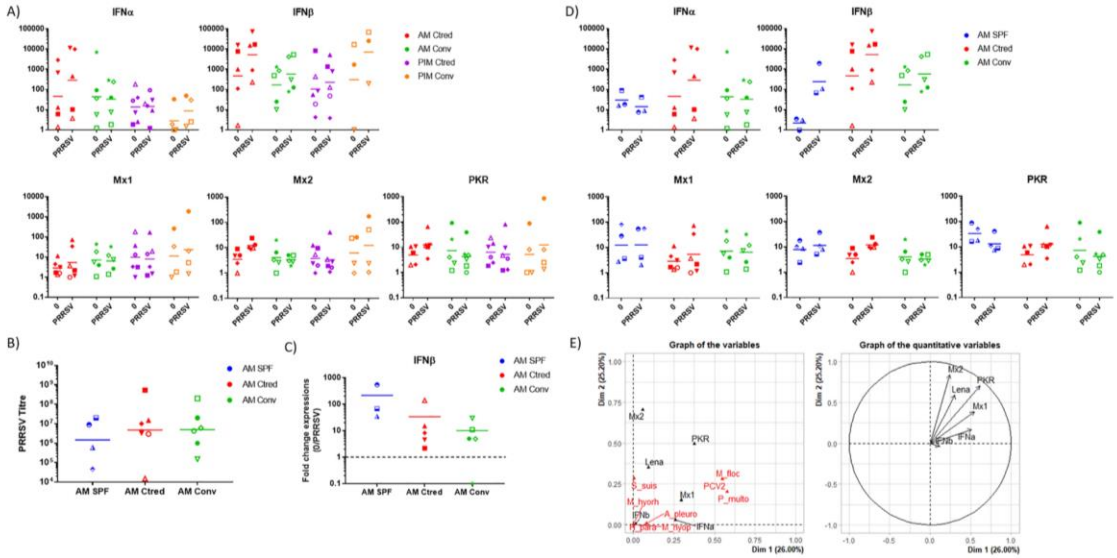

Fig. 3. AM and PIM from different animals' origins did not distinctly respond to PRRSV infection.
A)Transcriptomic expression of type I IFNα and β genes and three representative interferon stimulated genes Mx1, Mx2 and PKR from *in vitro* infected AM and PIM from different origins. The samples are the same, with the same identification as in Fig. 1. B) Comparison of *in vitro* PRRSV infection of AM from SPF animals with the previously depicted (Fig. 1) PRRSV infected AM from controlled and conventional animals. C) Transcriptomic expression of type I IFNα and β genes and three representative interferon stimulated genes Mx1, Mx2 and PKR from *in vitro* infected AM from SPF animals. The samples are the same, with the same identification as in Fig. 3B. D) Factor Analysis for Mixed Data (FAMD) plotting together data from Fig. 1, Table 1 and Fig. 3.

infections in perfect agreement with the concept of CRP, with 4 out of 6 (2/3) of the conventional pigs presenting at least two simultaneous infections, among them 3 out of 4 presented at least 3 pathogens.

This study is a first step showing the feasibility and interest of testing in parallel, on conventional animals, the *in vivo* presence of respiratory pathogens as well as the *in vitro* sensitivity, response and functions of AM in presence of different respiratory mimicking conditions such as PRRSV, PCV2 or Influenza infections as well as TLR-ligand stimuli or oxidative stress, for instance, allowing to explore the possible impact of long term respiratory infections on AM, one of the primary respiratory barrier.

Approval

All authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

Declaration of Competing Interest

None

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c) Ce qu'il faut retenir :

Cette étude apporte des données supplémentaires sur le statut sanitaire des élevages de porcs conventionnels dans la région Pays de la Loire. Nos résultats montrent que tous les animaux sont porteurs d'une infection respiratoire et que pour 2 porcs sur 3 cette infection est composée d'au moins deux agents pathogènes. Ces résultats correspondent bien à une étude préalable menée dans la région du Grand Ouest de la France et qui confirme que la majorité des maladies respiratoires développées dans les élevages français sont liées à une association de plusieurs micro-organismes (Fablet et al., 2012b). Selon les résultats de notre étude restreinte, le PCV2, *A. pleuropneumoniae, H. parasuis, P. multocida, S. suis et Mycoplasma floculare* semblent être les micro-organismes qui circulent le plus dans la région. Toutes ces données renforcent le besoin d'étudier ces co-infections et d'évaluer leurs impacts sur la réponse immune du porc.

D'autre part, les résultats de cette étude ne montrent aucun effet de ces infections virales ou bactériennes sur la réplication du PRRSV-1 par les macrophages. De même, aucun effet n'a été démontré sur la réponse immune notamment la production des IFN et l'expression des gènes stimulés par ces IFN suite à une surinfection *in vitro* par le PRRSV-1. Cette conclusion a été obtenue en comparant la réponse des macrophages provenant de l'élevage conventionnel à celle des macrophages issus de porcs à statut contrôlé et séronégatifs pour toutes les infections investiguées dans l'étude (PRRSV, swIAV, PCV2, *A. pleuropneumoniae, H. parasuis, M. flocculare, M. hyorhinis, M. hyopneumoniae, P. multocida* et *S. suis*). Cette réponse similaire peut être liée à la présence d'un micro-organisme non testé dans notre étude, mais présent dans les deux élevages en même temps. Une deuxième comparaison avec la réponse des AM EOPS ne montre aucune différence vis-à-vis des autres conditions à part une faible expression de départ de l'IFNβ témoignant de l'absence de toutes infections respiratoires. Toutes ces données ne montrent aucun lien entre l'origine des macrophages, le type de pré-infections, la réplication du PRRSV et l'expression des gènes impliqués dans la réponse immune innée. Par conséquent, l'hypothèse de départ qui suggère un lien entre la sensibilité des AM à l'infection PRRSV et les infections récurrentes a été rejetée.

En parallèle, la comparaison entre les AM et les PIM ne montre aucune différence quant à la sensibilité à l'infection PRRSV. Au contraire, une corrélation de la réplication du PRRSV sur les AM avec sa réplication au niveau des PIM a été enregistrée pour chaque animal.

Finalement, cette étude nécessite d'être continuée en élargissant l'échantillonnage et en testant d'autres élevages avec plus d'animaux par élevage. Cela rajoute de la fiabilité aux résultats et permet d'étudier l'effet d'autres agents pathogènes notamment ceux qui n'ont pas été détectés ou testés dans nos travaux. Il s'agit peut-être de certains micro-organismes possédant beaucoup plus d'impact sur la susceptibilité des AM au PRRSV, mais qui ont été négligés.

B- L'entraînement de la réponse antivirale et anti-inflammatoire des AM suite à une infection par le swIAV

a) Introduction des travaux

Les AM effectuent un rôle essentiel dans la réponse immune antivirale chez le porc suite à l'infection du tractus respiratoire inférieur (Heui Man Kim et al., 2008). Ce rôle est représenté par l'élimination des cellules mortes par phagocytose, la présentation des antigènes aux lymphocytes et la production de cytokines anti-inflammatoires dans le but de lutter contre les infections virales, éliminer le virus et préserver la fonction respiratoire. Suite à un premier contact avec un micro-organisme, ces AM peuvent subir une forme de modulation à long terme se manifestant par une mémoire épigénétique responsable d'un entraînement, ou au contraire d'une paralysie face à une infection secondaire (Netea et al., 2020). Les infections induisent une inflammation et une activation des mécanismes effecteurs de la réponse immune. En parallèle de la réponse pro-inflammatoire, des mécanismes anti-inflammatoires sont mis en place pour éviter une inflammation exagérée, la limiter dans le temps et réduire les lésions tissulaires. L'immunité entraînée implique donc une reprogrammation épigénétique et métabolique des cellules immunitaires innées telles que les macrophages, assurant un contrôle qualitatif et quantitatif de la réponse suite à une stimulation retardée ultérieure (Netea et al., 2020). Des réponses immunitaires entraînées mal orientées peuvent contribuer à l'aggravation de l'infection, entraînant un état hyper-inflammatoire chronique ou un état persistant de tolérance immunologique. Ce dernier est un mécanisme qui atténue la réponse inflammatoire de l'hôte pour maintenir l'homéostasie et prévenir les lésions tissulaires et la défaillance des organes. Cela augmente le risque des infections secondaires et favorise la réplication des micro-organismes liée à une diminution de l'activité du système immunitaire (Netea et al., 2020).

Récemment, certaines études se sont intéressées à cette réponse immune entraînée. Une étude menée sur la paroi bactérienne de *Candida albicans* (β-glucanes) a mis en évidence son effet protecteur se reposant sur la mobilisation de la réponse innée via la reprogrammation des monocytes (Quintin, 2019). La stimulation par ces β-glucanes induit un entraînement des monocytes et des macrophages et rend leur réponse à des surinfections hétérologues plus efficace et plus rapide (Quintin, 2019). D'autres travaux effectués sur le vaccin de la tuberculose (*Bacille*

Calmette et Guérin, BCG) ont montré des mécanisme d'immunité entraînée au niveau du tractus respiratoire bovin suite à l'administration nasale du BCG (Guerra-Maupome et al., 2019).

Des travaux menés chez la souris ont montré un entraînement immunitaire local des AM aboutissant à une meilleure protection contre une infection respiratoire par *Streptococcus pneumoniae* suite à une première stimulation par un adénovirus (Yao et al., 2018). Finalement, une autre étude chez la souris, montre qu'une infection par le IAV aboutit au remplacement des AM par des macrophages dérivés de monocyte qui assurent par la suite une réponse pro-inflammatoire plus forte et une meilleure protection contre les infections bactériennes (Aegerter et al., 2020).

Aucune étude de ce type n'a été publiée chez l'espèce porcine. Il n'existe donc pas de données sur l'immunité entraînée chez le porc suite aux infections virales. Dans le but de poursuivre nos travaux présentés dans la première partie de ce chapitre et d'étudier l'effet de l'infection par le swIAV qui n'a pas été abordée dans ces travaux précédents, une nouvelle série d'expériences a été menée. Cette étude s'intéresse à l'effet d'une infection grippale résolue sur la réponse immune entraînée du porc et sur la réplication du PRRSV et de l'ADV par les AM. Des expérimentations préliminaires ont été effectuées au sein de l'équipe en stimulant les AM par le Poly I:C (PIC) qui est un mimétique du génome de virus à ARN ou par des lipopolysaccharides (LPS) qui miment une infection bactérienne. Les expressions des transcrits des gènes impliqués dans la réponse immune innée tels que les ISG et les gènes codant pour les différentes interleukines et cytokines ont été évaluées. L'activité phagocytaire des AM a été également étudiée. Nos résultats avec les résultats d'autres expérimentations menées au sein de l'équipe seront valorisés dans un seul article que j'ai ajouté en annexe à la fin de ce manuscrit et qui est toujours en cours de rédaction.

b) Matériels et méthodes

i. Production des virus (swH1N2, PRRSV-1, ADV) :

Le swIAV H1N2 (A/Sw/Ille-et-Vilaine/0415/2011) a été isolé en 2011 chez un porc atteint d'une maladie respiratoire aiguë en Bretagne, France (ANSES, Ploufragan, France). Le virus a été propagé sur des cellules Madin-Darby Canine Kidney (MDCK) pendant 24h dans du DMEM (Eurobio Scientific, Les Ulis, France) supplémenté avec 10% de sérum de veau fœtal (SVF) (Eurobio Scientific) et 1% de solution de Stréptomycine/pénicilline/amphotéricine (SPA) (Eurobio Scientific) et 2 µg/mL de trypsine TPCK traitée (Worthington Biochemical Corp., Lakewood, NJ,

USA). Après collecte, le surnageant a été clarifié par centrifugation pendant 20 minutes (min) à 600 x g puis purifié sur des filtres Amicon Ultra-15 (Sigma-Aldrich) après une centrifugation de 20 min à 4000 x g et 4 ° C. Les titres finaux du stock viral ont atteint 10^7 $TCID_{50}$/mL. La souche PRR-FR-2005-29-24-1 du PRRSV-1 a été produite sur des AM cultivés dans un milieu RPMI 1640 (Eurobio Scientific) supplémenté avec 10% de SVF et 2% de solution SPA pendant 72h après avoir été isolé d'un troupeau ayant subi des avortements (Stadejek et al., 2006). Après clarification et purification sur les filtres Amicon, les titres viraux ont atteint 10^6 $DICT_{50}$ /mL. Concernant la souche Kojnok de l'ADV (Kojnok, 1965), la propagation a été réalisée sur des NPTr (Ferrari et al., 2003) dans un milieu DMEM supplémenté avec 10% de SVF et 1% de SPA. Les titres de stocks clarifiés et purifiés ont été estimés à 10^6 $DICT_{50}$ /mL. Tous les virus ont été stockés à -80°C et le titrage a été effectué en $TCID_{50}$.

ii. Les infections *in vivo* et la collecte des AM :

Les AM ont été prélevés sur des porcs EOPS sacrifiés à l'âge de 12 semaines, infectés ou pas 3 semaines plus tôt par voie intratrachéale avec 10^6 TCID50 de swIAV H1N2 (A/Sw/Ille-et-Vilaine/0415/2011) dans 5 mL (figure 13). Les infections *in vivo* ont été effectuées par les collègues de l'ANSES de Ploufragan en respectant les réglementations européennes et françaises sur l'éthique de l'expérimentation animale. La collecte des AM a été réalisée après un lavage bronchoalvéolaire (LBA) avec 250 ml de PBS (Eurobio Scientific) supplémentée avec 2 mM d'EDTA (Sigma-Aldrich, Saint-Quentin, France). Après centrifugation, filtration et application d'un tampon de lyse érythrocytaire (NaHC03 10 mM, NH4Cl 155 mM et EDTA 10 mM), les AM ont été lavés avec du PBS, comptés et conservés dans du SVF avec 10% de DMSO dans l'azote liquide.

iii. Infection des AM par le PRRSV et l'ADV

Après décongélation, les AM ont été cultivés dans des plaques à 96 puits à 2 x 10^5 cellules par puits pendant 1h pour permettre l'adhésion. Les cellules ont été ensuite lavées avec du PBS et infectées pendant 1h avec du PRRSV ou de l'ADV à des MOI de 0,1 et 0,01 pour chaque virus (figure 13). L'inoculum a été retiré après une centrifugation rapide des plaques et les cellules ont

été incubées pendant 24h à 37°C et à 5% de CO2 dans du RPMI 1640 supplémenté avec 1% de SPA (figure 13). Enfin, les plaques ont été centrifugées et les cellules ont été récoltées en utilisant un tampon de lyse spécial du RNeasy Mini Kit (Qiagen). Les surnageants et les cellules lysées ont été stockés à -80°C.

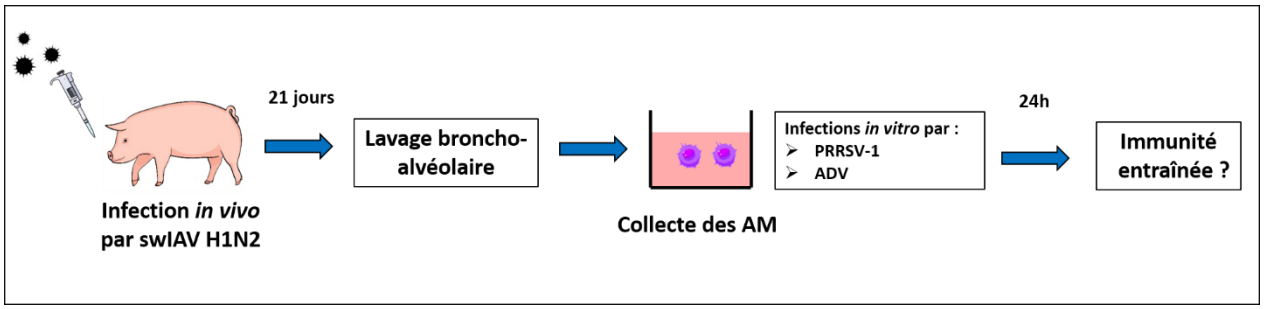

Figure 13. *Protocole d'infections* in vivo *des animaux et* in vitro *des AM collectés à partir de ces animaux*

iv. Statistiques

Un test de Kruskal-Wallis suivi d'un test multiple de Dunn a été appliqué pour comparer les expressions relatives des transcriptions virales de PRRSV et ADV entre AM provenant d'animaux témoins ou infectés par le swIAV. GraphPad Prism a été utilisé à cet effet (GraphPad Software version 7.0, San Diego, CA, USA).

c) Résultats et discussions

Suite à l'infection des porcs EOPS par le swIAV H1N2, et suite à la collecte des AM des poumons de ces animaux 21 jours après les infections, les analyses menées sur ces cellules ont montré une résolution totale de l'infection au bout de ces 21 jours (résultats non montrés). L'absence du virus a également été confirmée après analyses des écouvillons nasaux effectués au moment de l'abattage des animaux (résultats non montrés). Ces résultats ont été attendus, car l'infection par le swIAV H1N2 chez le porc est généralement résolue d'une façon spontanée au bout d'une semaine post-infection (Choi et al., 2004). Cela nous a permis d'étudier l'effet de cette infection grippale résolue sur la fonction des AM et leurs comportements suite à des infections ultérieures**.**

Nous avons exploré le rôle d'une infection par le swIAV dans l'entraînement de la réponse immunitaire innée et donc la protection de l'hôte porcin contre les nouvelles infections virales d'origines différentes. Pour cela, des infections virales par le PRRSV-1 et l'ADV ont été menées sur ces AM collectés 21 jours après une infection *in vivo* des animaux par le swIAV H1N2. Des variations ont été enregistrées en comparant les AM re-stimulés aux AM de la même origine, mais n'ayant pas eu un deuxième contact avec le virus. Cependant, les augmentations des expressions des transcrits viraux et des transcrits des IFN et des ISG qui sont évidentes n'ont pas été représentées sur les figures. Nous nous sommes uniquement intéressés à la comparaison des MA de la même condition de re-stimulation mais d'origines différentes. Contrairement à ce qui était attendu, l'expression des transcrits viraux ne montre aucune diminution dans le cas des AM ayant rencontré le swIAV H1N2 depuis 21 jours (figure 14). En effet, l'infection résolue par le swIAV H1N2 n'a montré aucun impact sur la réplication du PRRSV-1 ou de l'ADV au niveau des MA. De même, l'expression des gènes de l'IL12A et des ISG tels que PKR et Mx2 ne montre aucune variation significative en comparant la réponse des AM issus de porcs infectés une première fois par le swIAV H1N2 aux AM issus de porcs témoins. L'expression des transcrits de l'IFNβ montrent uniquement une différence entre les 2 origines des AM re-stimulés par l'ADV à une MOI de 0,1 seulement (figures 14A) et par le PRRSV-1 à une MOI de 0,01 (figures 14B).

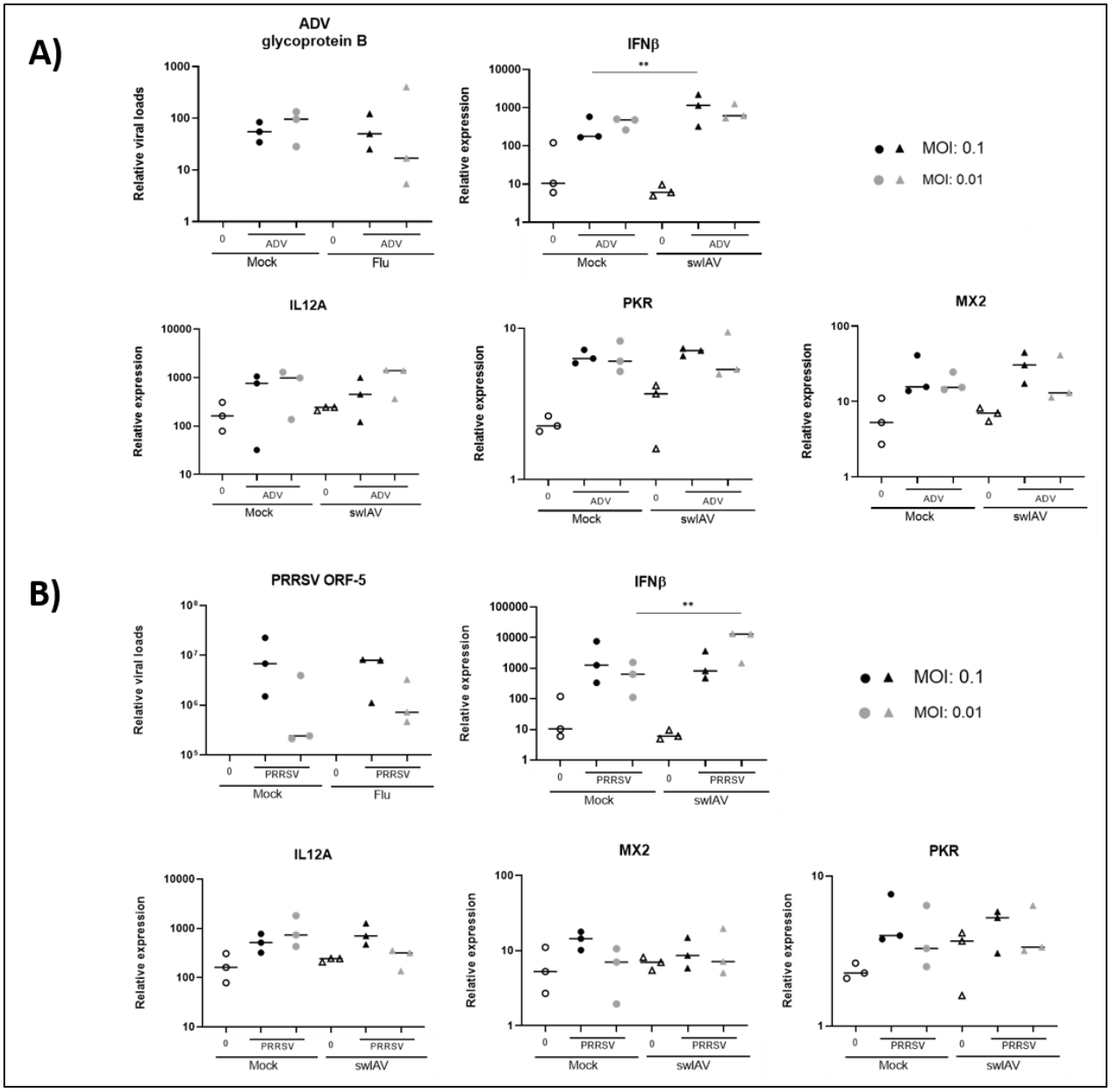

Figure 14. *Les AM entraînés ne sont pas résistants aux infections par l'ADV (A) ou par le PRRSV (B). Les AM prélevés 21 jours après une infection* in vivo *ou pas par le swIAV H1N2 ont été infectés* in vitro *avec le PRRSV ou ADV à MOI 0,1 et 0,01 pendant 24 heures. Les titres de PRRSV et ADV ainsi que les expressions des différents transcrits ont été mesurées par RT-qPCR. La moyenne des triplicats est représentée. (**) représente la différence significative avec p < 0.01.*

Cet effet sur l'expression de l'IFNβ, non observé dans les mêmes conditions mais avec des différentes MOI et qui ne se traduisent pas au niveau de l'expression des ISG demande à être reproduit. Cet effet sporadique peut être également lié aux faibles MOI utilisées qui ne mènent pas

à l'infection de toutes les cellules, surtout que les infections ont été arrêtées au bout de 24h. Ce faible temps d'exposition des cellules aux virus avec ces faibles MOI risque de ne pas être suffisant pour une bonne expression des transcrits en jeu. Cette expérience mérite d'être répétée une deuxième fois en augmentant le nombre de cellules, les MOI et/ou le temps d'infection avant de récolter les cellules. Une augmentation du nombre des échantillons et des réplicats permettra également une meilleure optimisation des résultats.

d) Ce qu'il faut retenir

Les travaux de cette partie de ma thèse ont été effectués dans le but d'évaluer l'effet de l'infection par le swIAV sur la fonction des AM après élimination du virus. Ces AM ont été collectés à partir de deux groupes de trois porcs infectés ou pas par le swIAV H1N2 par voie intratrachéale. Le travail en annexe montre un entraînement de ces AM. Cependant, la réinfection *in vitro* de ces AM par le PRRSV-1 ou l'ADV ne montre aucune résistance des AM entrainés à l'infection par ces virus. La réplication des deux virus s'attaquant aux AM n'a pas été impactée, mais l'entraînement des MA ne se limite pas à la lutte contre ces virus uniquement. Cet entraînement pourrait être efficace en protégeant d'autre types cellulaires contre les infections par d'autres virus n'infectant pas forcément les AM. L'entraînement des MA pourrait par exemple contribuer à l'élimination du PRCoV ou d'autre souche de swIAV qui infectent spécifiquement les cellules épithéliales.

La confirmation de nos résultats nécessite des investigations supplémentaires en répétant les expérimentations et en optimisant toutes les conditions expérimentales.

Ces mécanismes seront capables de révolutionner les pratiques pour contrôler les co-infections respiratoires et pour définir des protocoles de vaccination efficaces. Ces résultats ouvriront les portes au rôle qui peut être joué par certains vaccins à base de virus vivants atténués dans le développement d'une immunité entraînée protectrice contre des infections d'origines différentes. Ainsi, la vaccination annuelle contre le swIAV à base de virus vivants pourrait conférer au troupeau une résistance aux surinfections par le PRCoV ou le PCV2 et donc contribuer à la réduction des répercussions respiratoires liées aux différentes co-infections. Elle pourrait par exemple remplacer la revaccination des truies contre le PRRSV avant la mise à la reproduction.

Discussion générale
et
perspectives

Le complexe respiratoire porcin dans l'état de l'art

Nos travaux ont permis un premier recensement/analyse des études menées sur les co-infections et les surinfections respiratoires d'origines bactériennes et/ou virales chez le porc. Ce recensement est donc effectué pour la première fois avec une approche permettant de comparer les différentes souches et les protocoles utilisés ainsi que les conséquences sur la réplication des agents pathogènes, la réponse immune, les signes cliniques et les différentes lésions macroscopiques et microscopiques.

Avec cette comparaison, une importante variabilité au niveau des résultats pour les mêmes agents pathogènes a été montrée. Cependant, certaines études montrent qu'une association de deux micro-organismes entraîne une réponse anti-inflammatoire, alors que d'autres études rapportent que ces deux agents pathogènes entraînent plutôt une réponse pro-inflammatoire. De même, quand certains chercheurs trouvent une baisse de la réplication d'un virus ou de la multiplication d'une bactérie en présence d'un autre micro-organisme, d'autres chercheurs montrent le contraire. Mes travaux de thèse montrent bien que ces variabilités au niveau des résultats sont la conséquence de différences au niveau des protocoles expérimentaux employés dans les différentes études. La variation du délai et de l'ordre des infections peut changer le résultat des surinfections. La variabilité des souches utilisées entraîne également un grand impact sur les résultats, qui varient en fonction de la virulence des souches d'un même agent pathogène. D'autre part, l'analyse de l'impact des co-infections ou des sur-infections sur les signes cliniques est très intéressante. Malgré les variabilités au niveau des conditions expérimentales et au niveau de la réplication/multiplication virale/bactérienne et de l'inflammation, les *heat maps* générées dans le premier chapitre montrent que la majorité des sur-infections ont tendance à provoquer une augmentation des signes cliniques induits par une première infection. Cela pourrait être expliqué par l'établissement d'une réponse anti-inflammatoire par le premier agent pathogène réduisant l'élimination du micro-organisme co-infectant et donc augmentant son temps d'infection et les signes cliniques associés à ce second agent pathogène. D'autre part, une réponse pro-inflammatoire contribuant dans un premier temps à l'élimination du premier micro-organisme, aggraverait les signes cliniques induits par le second agent pathogène, tel que la toux et la pneumonie en augmentant le recrutement des cellules immunitaires au niveau du tractus respiratoire.

Il est intéressant de signaler que les études ont été effectuées aussi bien sur les bactéries que sur les virus avec une focalisation sur les surinfections bactéries-virus. Les études menées *in vitro* ou *in vivo* sur les co-infections et les surinfections bactéries-virus ont été plus nombreuses que celles menées sur les combinaisons virus-virus ou bactéries-bactéries. Cela met en évidence le manque de connaissances autour des co-infections virus-virus chez le porc et le besoin de les étudier davantage pour une meilleure gestion des infections dans les élevages. D'autre part, les études menées *in vivo* et qui se sont intéressées à l'aspect clinique du CRP sont beaucoup plus nombreuses et donc témoignent du manque de compréhension des interactions moléculaires entre les agents pathogènes et leurs cellules hôtes.

Les facteurs non-infectieux affectant le complexe respiratoire porcin

À toutes les causes infectieuses à l'origine du développement du CRP et associées à la variabilité des résultats entre les différentes études, se rajoutent des facteurs non-infectieux qui impactent le CRP et qui n'ont pas été pris en considération dans nos travaux (figure 15). Il est important de maîtriser les facteurs de gestion (densité animale et conduite en bandes entre autres) et les facteurs environnementaux tels que la température des locaux, l'hygiène, et la ventilation qui contribuent à l'aggravation des infections respiratoires. Une mauvaise gestion d'un troupeau et une absence de maîtrise des facteurs environnementaux peuvent augmenter la transmission des agents pathogènes ou créer un environnement stressant qui entraîne plus de dégâts respiratoires (Martínez et al., 2009).

Ces facteurs non-infectieux peuvent être divisés en trois groupes comprenant les facteurs i) liés à la gestion du troupeau (système d'élevage, taille du troupeau, qualité de l'alimentation), ii) liés à l'environnement (ventilation, humidité, température) et iii) spécifiques aux animaux (âge, race, génétique, immunité) (Opriessnig et al., 2011; White, 2011) (figure 15). Des conditions environnementales stressantes telles que les changements extrêmes de température, l'excès d'humidité et d'ammoniac favorisent le développement des maladies respiratoires. La qualité et la quantité de l'aliment sont aussi importantes, car la poussière et les mycotoxines provenant d'une alimentation de mauvaise qualité peuvent affecter le tractus respiratoire en causant des dégâts au niveau des poumons et en affaiblissant le tonus immunitaire général. Enfin, l'âge et la race des porcs jouent également un rôle important dans la sévérité de la maladie respiratoire. Les études

montrent que certaines races de porcs sont plus sensibles à des infections virales par le PRRSV ou le PCV2 (Halbur et al., 1998; Vincent et al., 2005; Opriessnig et al., 2009), et que les porcelets sevrés ou plus âgés ont plus de chance de développer des infections respiratoires suite à la disparition des anticorps transmis passivement (Cho et al., 2006; Pomorska-Mól et al., 2011).

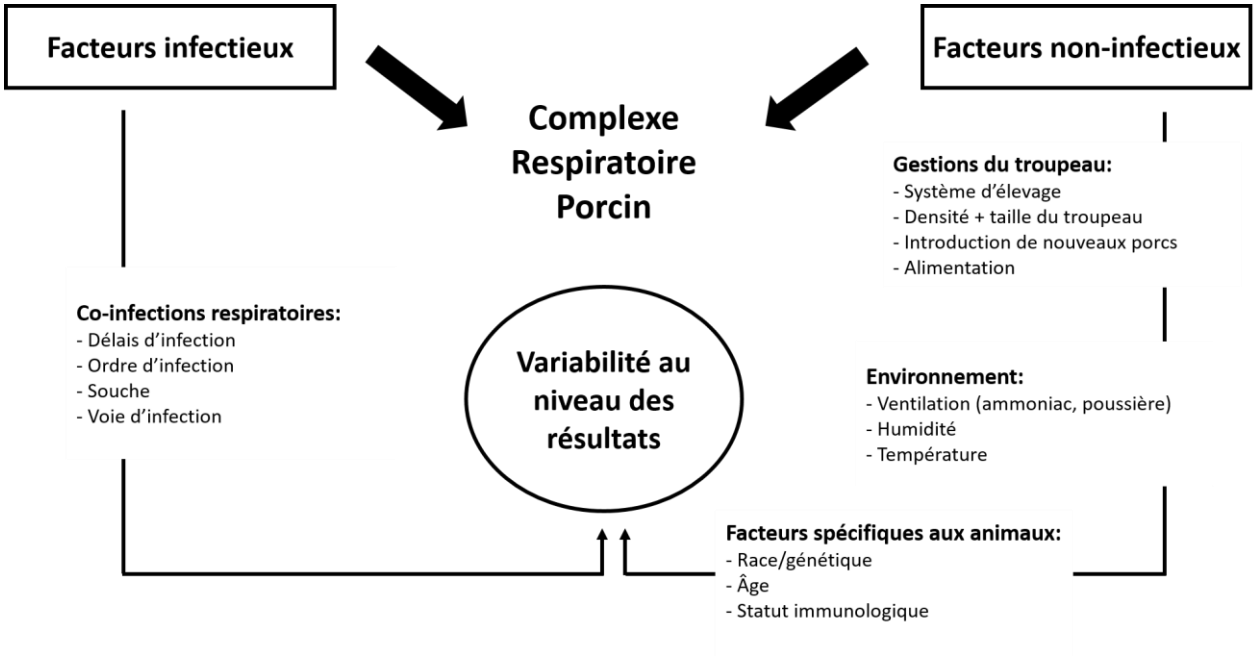

Figure 15. *Les facteurs infectieux et non-infectieux contribuant au CRP et à la variabilité des résultats des différentes expérimentations sur les co-infections respiratoires*

La présence de tous ces facteurs, difficiles à maîtriser, montre la difficulté de reproduire les co-infections ou les surinfections respiratoires dans des conditions expérimentales. Pour conclure, toutes ces différences dans les résultats des expérimentations sur les mêmes combinaisons de micro-organismes peuvent être liées à la virulence des souches utilisées, aux délais entre infections, à l'âge, à la race et au statut sanitaire des porcs qui peuvent altérer leur sensibilité aux infections respiratoires (figure 15).

Réponse de la cellule épithéliale à la co-stimulation par le swIAV et le PRRSV

Dans le but de comprendre l'interaction du swIAV et du PRRSV avec toutes les cellules rencontrées au niveau du tractus respiratoire porcin, nous avons effectué des travaux sur les cellules épithéliales. Nos travaux ont montré une baisse de la réplication du swIAV H1N2 par les cellules

épithéliales en présence du PRRSV-1. Ces résultats correspondent bien aux résultats des travaux précédents menés sur des cellules épithéliales modifiées exprimant le récepteur CD163 du PRRSV (Provost et al., 2017). Il a été montré grâce à ces travaux que le PRRSV-2 réduit la réplication du swIAV H1N1 en situation de co-infection (Provost et al., 2017). Une deuxième étude menée sur les AM réceptifs aux deux virus a montré également une diminution de la réplication du swIAV H1N1 en présence du PRRSV-2 (Dobrescu et al., 2014). D'un autre côté, les études menées *in vivo* ont montré des résultats contradictoires. C'est ainsi que la réplication du swIAV H1N1 a été réduite (Van Reeth et al., 1996; Kitikoon et al., 2009), augmentée (Sangpratum et al., 2019), ou invariable (Pomorska-Mól et al., 2020) en présence du PRRSV selon les études. Cette variabilité peut être expliquée par la différence entre les souches utilisées et le protocole expérimental qui varie entre ces études. Cela a bien été montré dans nos travaux, car en changeant le délai ou l'ordre des infections le PRRSV-1 n'affecte plus la réplication du swIAV H1N2 de la même façon. Sans oublier l'effet de tous les facteurs non-infectieux dont la maîtrise n'est pas toujours évidente dans les conditions *in vivo* comme mentionné précédemment.

En parallèle, cette baisse de la réplication du swIAV H1N2 a été accompagnée par une diminution de l'expression des transcrits des gènes impliqués dans la réponse immune innée des cellules épithéliales. Cela semble très logique, car une baisse de la production du swIAV H1N2 par les cellules, diminue leur stimulation et donc diminue la production des IFN et des ISG.

Cet effet du PRRSV sur la réplication du swIAV H1N2 a été également démontré avec la souche atténuée vaccinale DV du PRRSV-1 utilisée dans le vaccin Porcilis® PRRS.

Les investigations menées dans le but de bien comprendre l'interaction entre le PRRSV et le swIAV d'une part et l'interaction du PRRSV avec la cellule épithéliale d'autre part nous ont permis d'élaborer plusieurs hypothèses pour décrire les différents scénarios d'interaction (figure 16). Une interaction entre les particules virales a été suggérée, car le PRRSV porte au niveau de ses glycoprotéines des acides sialiques branchés en α-2,6 et α-2,3 (Delputte and Nauwynck, 2004; Li and Murtaugh, 2015) qui existent au niveau de la membrane cellulaire et qui constituent des sites d'accrochage pour l'hémagglutinine du swIAV (Medina and García-Sastre, 2011). Les particules virales de deux espèces pourraient finir par s'accrocher (figure 16.1) et former des agrégats empêchant le swIAV d'accéder à ses récepteurs au niveau de la membrane cellulaire

(figure 16.2). Certaines particules du swIAV finissent par adhérer à la cellule en s'accrochant ou pas à des particules de PRRSV (figure 16.3 et 16.4)

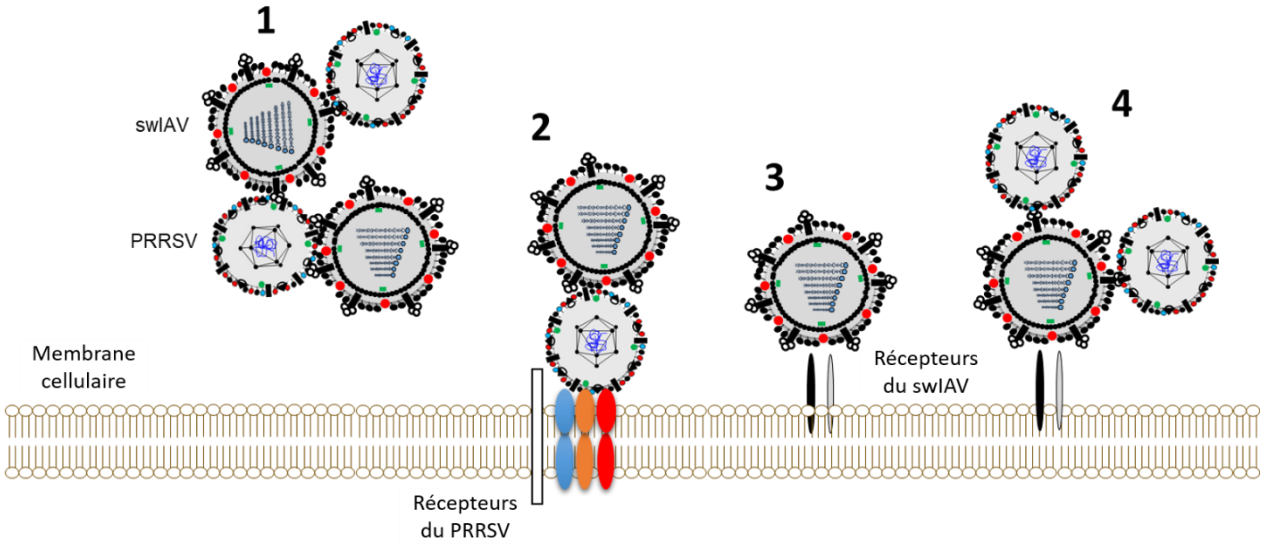

Figure 16. *Les scénarios d'interaction entre les particules virales du PRRSV-1 et du swIAV H1N2 et de leur interaction avec leurs récepteurs à la surface de la cellule épithéliale. (1) représente l'agrégation des particules virales sans interaction avec la membrane cytoplasmique. (2) Adhésion d'une particule de PRRSV-1 à des récepteurs membranaires tout en accrochant une particule de swIAV H1N2 et en l'empêchant d'accéder à ses récepteurs. (3) Adhésion d'une particule de swIAV H1N2 libre à ses récepteurs. (4) Adhésion d'une particule de swIAV H1N2 accrochant simultanément deux particules de PRRSV-1.*

Cette hypothèse a été soutenue par des travaux supplémentaires qui ont montré une inhibition plus importante de la réplication du swIAV et de la réponse immune contre le swIAV après avoir mélangé les deux virus avant l'inoculation des cellules épithéliales. Cela aurait laissé plus de temps aux particules virales pour s'accrocher entre elles avant toute adhésion à la surface de la cellule.

Les résultats après analyse des voies de signalisation induites par le PRRSV-1 seul confirme que le PRRSV-1 est capable d'interagir avec la cellule épithéliale et d'induire l'activation de cascades de signalisation. Cette capacité de « signalisation » qui est perdue après l'inactivation chimique du PRRSV-1 suggère une adhésion du virus ou une éventuelle internalisation dans la cellule. Ces particules de PRRSV-1 inactivées adhèrent moins à la cellule ou n'adhèrent plus du tout. Après l'application d'une solution acide qui interagit avec la capside du virus, modifie sa conformation et entraîne sa dissociation (Mettenleiter, 1989; Koromyslova et al., 2015), toutes les

particules du PRRSV-1 non internalisées ont été détruites. Cette destruction avant l'inoculation par le swIAV H1N2 a montré une disparition de l'effet du PRRSV-1 sur la réplication du swIAV H1N2. Tous ces résultats suggèrent une adhésion du PRRSV à des récepteurs sur la surface des cellules épithéliales, non identifiés précisément à ce stade (figure 16.2). L'inactivation chimique du PRRSV-1 réduit son effet inhibiteur sur la réplication du swIAV H1N2 et sur la réponse immune des cellules épithéliales, cependant cet effet n'a pas été complètement inhibé. Cela peut être expliqué par le fait que le virus inactivé reste capable de former des agrégats avec le swIAV H1N2, mais ne s'accroche peut-être plus à la cellule et n'interfère plus avec les voies de signalisation induites par le swIAV H1N2. Une autre explication peut lier cet effet à la baisse du nombre de particules de PRRSV-1 ou à une altération de leur capacité à adhérer aux cellules consécutive au processus d'inactivation, entraînant une activation moindre des voies de signalisation au niveau des cellules épithéliales.

Nos travaux en microscopie ont mis en évidence la présence de quelques particules de PRRSV-1 « dans » les cellules épithéliales. Malheureusement, nous n'avons pas réussi à identifier la localisation précise de ces particules. Pour cela, des travaux en microscopie avec une technologie d'observation en 3D peuvent être proposés en marquant la membrane cellulaire pour identifier précisément la localisation des particules de PRRSV-1. Un lavage avec la solution acide peut également être effectué avant l'observation en microscopie. Une détection du virus après ce lavage suggérerait son internalisation par les cellules épithéliales.

Tous ces travaux contribuent à la compréhension de l'interaction des souches locales de ces deux virus qui circulent dans la région du Grand Ouest de la France. En plus, ces travaux se rapprochent de la réalité à certains égards et constituent une base pour des investigations *in vivo* chez le porc sachant que nous n'avons pas utilisé des lignées cellulaires modifiées génétiquement ou des cellules provenant d'une autre espèce.

Modulation des voies de signalisation du swIAV H1N2 par le PRRSV-1

Dans le but d'évaluer l'impact du PRRSV-1, quatre voies de signalisation impliquées dans les infections des cellules épithéliales par le swIAV ont été évaluées. L'induction ou l'inhibition de ces quatre voies par les virus entraînent des effets antiviraux contribuant à la lutte contre les

infections ou des effets pro-viraux qui favorisent la réplication de ces virus (tableau 12). L'induction d'une même voie par un même virus peut entraîner simultanément des effets antiviraux et pro-viraux. Un même virus serait aussi capable d'induire une certaine voie sur une cellule et de l'inhiber sur une autre (Silwal et al., 2018). Cela dépend donc de la nature des cellules hôtes, de la souche virale et du stade précoce ou tardif de l'infection (Silwal et al., 2018). L'IAV par exemple, peut induire la voie PI3K/AKT au début de l'infection en augmentant la production des IFN jusqu'à ce qu'il finisse par l'inhiber à un stade plus avancée (Ehrhardt et al., 2006) (tableau 12). Le PRRSV inhibe tardivement cette même voie chez les mo-DC alors qu'il contribue à son induction chez les AM et les MARC-145 pour faciliter l'internalisation, la traduction des ARNv et la synthèse des protéines virales (Zhang and Wang, 2010; Zhu et al., 2013) (tableau 12). La co-infection des cellules épithéliales par le PRRSV-1 et le swIAV H1N2 dans nos travaux montre une inhibition de la phosphorylation de l'AKT. Cela démontre bien l'interaction du PRRSV-1 avec les cellules épithéliales et explique son effet sur la transduction des signaux pro-viraux qui aboutissent à la baisse de la production des IFN induite par le swIAV H1N2.

Tableau 12 : *Modulation des voies de signalisation de l'IAV par le PRRSV-1 au niveau des cellules épithéliales. (↗) signifie une augmentation et (↘) signifie une baisse. (+) représente l'induction d'une voie de signalisation et (-) représente son inhibition par les virus.*

	IAV	PRRSV-1/swIAV	Effets de l'induction
PI3K/AKT	**+** Précoce **-** Tardive	**-**	**Antiviral :** ↗ IFN **Pro-viral :** ↗ Internalisation ↗ Traduction des ARNv ↗ Synthèse des protéines virales
AMPK	**+**	**-**	**Antiviral :** ↗ STING ↗ TNFα ↗ IFN type 1 ↘ Synthèse de l'acide gras

MAPK-ERK1/2	+ Tardive	++	**Pro-viral :** ↗ Transcription ↗ Traduction des ARNv ↗ Export de RNPv ↗ réplication virale
JAK/STAT	+	-	**Antiviral :** ↗ ISG ↘ réplication virale

L'induction de la voie AMPK induit une réponse antivirale basée sur l'activation des stimulateurs des gènes des IFN de type 1 avec pour conséquence une baisse de la synthèse des acides gras. Les études montrent une induction de la voie impliquant AMPK chez les cellules hôtes infectées par le PRRSV ou l'IAV, ce qui permet de lutter contre ces deux infections virales (Silwal et al., 2018; Long et al., 2019) (tableau 12). Fait intéressant, notre étude de co-infection montre une inhibition de la phosphorylation de l'AMPK menant une baisse de la production des IFN et donc une baisse de l'expression des différents ISG (tableau 12). Cela confirme encore une fois que le PRRSV-1 est capable de stimuler les cellules épithéliales d'une façon qui diffère de sa stimulation des AM ou des MARC-145. Cet effet en cas de co-infection, rejoint bien l'inhibition de la voie JAK/STAT par le PRRSV-1 en induisant une réponse antivirale (tableau 12). La voie JAK/STAT est importante pour l'initiation d'une réponse antivirale suite à l'infection par le swIAV (Fensterl and Sen, 2009). Grâce à son inhibition, le PRRSV-1 réduit drastiquement la réponse antivirale de la cellule épithéliale et entraîne une nette baisse de l'expression des ISG.

Finalement, la voie MAPK-ERK1/2 a été induite par la co-infection des cellules épithéliales (tableau 12). Il a été démontré que l'induction de cette voie par l'IAV s'effectue tardivement au moment de l'assemblage et du bourgeonnement du virus (Pleschka et al., 2001). Cela nous laisse penser que cette signalisation précoce est initiée principalement par le PRRSV-1. Les études montrent que ce dernier est responsable de la phosphorylation précoce des ERK1/2 qui est associée à l'internalisation du virus par les AM (Lee and Lee, 2010). Ces résultats nous confortent dans l'idée d'investiguer ultérieurement la possibilité d'internalisation du PRRSV-1 par les cellules épithéliales.

Co-infection des PCLS par le swIAV et le PRRSV

L'utilisation des tranches pulmonaires fines appelées (*Precision Cut Lung Slices*) PCLS gagne de plus en plus en intérêt et en popularité dans le monde de la recherche médicale. Il s'agit d'un modèle *ex vivo* innovant qui s'impose de plus en plus dans les expérimentations en virologie et qui se révèle utile pour préserver la place des cellules dans le tissu d'intérêt et finalement contribuer à réduire l'expérimentation animale. Il s'agit d'un modèle plus proche de la situation *in vivo*, car il montre la réponse de plusieurs types cellulaires, dans du tissu pulmonaire relativement préservé, suite à une infection virale et non pas la réponse d'un seul type cellulaire isolé. Nos travaux en microscopie montrent bien la présence de cellules épithéliales et d'AM dans ces PCLS. Comme pour les cellules épithéliales, la co-infection de ces PCLS par le PRRSV-1 et le swIAV H1N2, réduit la réplication du swIAV H1N2 ainsi que la réponse immune innée induite par celui-ci. Cela ne correspond pas aux résultats trouvés par une autre étude dans laquelle les auteurs ne montrent aucune altération de la réplication du swIAV en présence du PRRSV (Dobrescu et al., 2014). Il est important de signaler, cependant, que cette étude a été menée avec du PRRSV-2, aujourd'hui considéré comme une espèce différente du PRRSV-1, que nous avons utilisé dans nos expérimentations.

Le PRRSV-1 inactivé a eu le même impact sur les PCLS que sur les cellules épithéliales en présence du swIAV H1N2. La réplication du swIAV ainsi que l'expression des transcrits des PRR et des ISG ont été réduites lors de la co-infection avec le PRRSV-1 actif. Cet effet réducteur semble moins important avec le PRRSV-1 inactivé chimiquement (voir figure 5 du 2ème papier, page 101). L'augmentation de l'expression des transcrits des IFN de type 1 au niveau des PCLS suite à l'infection par le PRRSV-1 peut être dûe à la présence d'autres cellules telles que les AM qui sont infectées par le PRRSV-1 et qui répondent différemment à cette infection (figure 5 du 2ème papier). Il a été démontré que l'infection des AM par le PRRSV entraîne une inhibition post-transcriptionnelle des IFN type 1 qui est traduite dans nos travaux par l'inhibition des ISG (Wang et al., 2018). Par conséquent, l'augmentation de l'expression des transcrits des IFN par le PRRSV-1 apparaît compréhensible dans la mesure où elle n'aboutit de toute façon pas à une activation des ISG.

Malgré tous les avantages de ce modèle expérimental qui s'approche de la situation *in vivo* et qui permet d'évaluer la réponse d'une cellule en gardant son interaction avec le reste du tissu

respiratoire, les infections sur les PCLS ne permettent pas de mettre en jeux tous les effecteurs de la réponse immune de l'animal. En effet, les cellules immunes accédant au tissu par voies sanguines ou lymphatiques par exemple ne peuvent pas entrer en lice avec ce système. En plus, une certaine hétérogéinté au niveau des tranches générées et l'incapacité d'estimer précisément le nombre de cellules par tranche, nous empêchent de contrôler certains paramètres notamment la MOI de départ et le nombre de cellules infectées par les virus. Cependant, la cohérence et la reproductibilité de nos données *ex vivo* et *in vitro,* ainsi que la concordance avec les résultats d'une étude *in vivo* menée par des collègues de l'ANSES sur les mêmes souches virales confortent nos données. Cette étude a validé nos résultats et a montré un décalage de l'excrétion du swIAV H1N2 ainsi qu'une réduction de la réponse cellulaire systémique représentée par une baisse de la production des IFN de type 1 en cas de pré-infection des porcs par le PRRSV-1 (données non publiées à ce jour). Ces résultats ont été accompagnés par une atténuation des signes cliniques et de la réponse inflammatoire liés au swIAV H1N2 en présence du PRRSV-1 (données non publiées à ce jour).

Entraînement des AM porcins suite aux infections respiratoires

Les travaux menés dans la première partie du troisième chapitre confirment la possibilité d'étudier l'effet des infections développées *in vivo* sur la sensibilité des cellules immunitaires à des infections *in vitro*. Cette étude, résumée dans le troisième article de ce manuscrit, rapporte des données supplémentaires sur la nature des infections respiratoires présentes dans les élevages de la région des Pays de la Loire. Nos résultats correspondent à ce qui a été décrit dans les deux études effectuées dans le Grand Ouest de la France en 2012 (Fablet et al., 2012b, 2012a). Les acides nucléiques du PCV2, *A. Pleuropneumoniae*, *H. parasuis*, *P. multocida* et *S. suis* ont bien été identifiés. *M. hyopneumoniae* était la seule bactérie identifiée par Fablet et collaborateurs que nous n'ayons pas détectée dans notre étude très restreinte. En revanche, nous avons identifié *M. floculare* chez deux animaux de notre étude.

Suite à la stimulation *in vitro* des AM et des PIM par le PRRSV, aucune différence n'a été notée entre les deux types de cellules provenant du même animal. Cela montre que la susceptibilité des AM et des PIM à l'infection par le PRRSV est affectée de la même façon par les différentes infections respiratoires développées par l'animal au préalable. Ces résultats ne correspondaient pas

à notre hypothèse qui suggérait un effet plus important au niveau des AM qui ont été en contact direct avec les micro-organismes au niveau des poumons.

L'absence de différences entre la réponse des AM des différentes origines était aussi inattendue. Nous nous attendions à voir une baisse de la réplication du PRRSV et une réponse antivirale plus prononcée chez les AM provenant de porcs conventionnels ayant développé d'autres infections respiratoires. Ces résultats négatifs dans notre étude pourraient aussi être expliqués par le faible nombre d'échantillons ou par les variations au niveau du stade de la première infection au moment de la stimulation *in vitro* par le PRRSV-1. Il existe une forte probabilité que les infections primaires *in vivo* soient dans un stade précoce ou très tardif dans lequel la réponse inflammatoire induite n'est pas très efficace. L'impossibilité de déterminer le stade et le début de ces infections constitue le point faible de cette étude, sachant qu'il n'y a pas moyen de savoir à quel moment l'animal a été infecté. Ajoutons, que l'effet des infections résolues, mais non identifiées ne pouvait pas être pris en considération vu que nous nous sommes intéressés uniquement aux infections en cours. Il sera donc intéressant de continuer ces travaux en augmentant le nombre d'animaux testés et de déterminer les infections résolues en identifiant, par exemple, les anticorps à partir des prélèvements de sérum.

La suite des travaux de ce chapitre a été menée sur des AM provenant de porcs infectés expérimentalement par le swIAV H1N2. Cette fois-ci, la date de l'infection est connue et les AM ont été collectés 21 jours après la première infection *in vivo*. Les différentes stimulations *in vitro* ont donc été menées dans le but d'évaluer l'effet de la première infection résolue par le swIAV H1N2 sur la réponse des AM à une deuxième infection bactérienne ou virale. Une première stimulation par les LPS qui est censée mimer une infection bactérienne n'a montré aucune différence sur la réponse immune innée entre les AM de différentes origines. Cela reste à confirmer en réinfectant les AM par des bactéries à tropisme respiratoire.

En revanche, les AM issus de porcs pré-infectés par le swIAV H1N2 ont montré une augmentation de la réponse immune innée suite à une stimulation par le Poly I:C mimant une infection virale. Ce phénomène d'entraînement des MA a été démontré chez la souris suite à une infection par un adénovirus (Yao et al., 2018), ou chez le bovin suite une première stimulation par le vaccin BCG (Guerra-Maupome et al., 2019). Une augmentation de l'efférocytose de ces cellules a également été enregistrée chez les MA entraînés par la première infection swIAV H1N2. Cet

entraînement se représente par une reprogrammation épigénétique et des changements métaboliques des MA qui permettent leur passage vers un état d'entraînement représenté par une réponse antivirale plus forte et plus rapide à des réinfections hétérologues (Netea et al., 2020). À nos connaissances, aucune étude n'a étudié l'effet des swIAV sur l'entraînement de la réponse immune chez le porc, malgré le fait qu'il soit un bon modèle biomédical naturel pour l'étude des IAV (Meurens et al., 2012a).

De façon quelque peu inattendue, le résultat des réinfections par le PRRSV et l'ADV n'a pas été modifié. Aucun effet de l'infection swIAV résolue n'a été constaté sur la réplication de ces deux virus ou de manière plus générale sur la réponse immune innée des MA. Il est important de préciser que la stimulation par le Poly I:C mime une infection par un virus à ARN. L'absence du même effet suite à la stimulation par un virus à ADN comme l'ADV n'est pas surprenante.

Toutes ces expérimentations nécessitent d'être répétées *in vitro* et *in vivo* dans le but de vérifier si l'infection grippale est capable d'assurer une protection contre des infections virales respiratoires hétérologues causées par l'ADV ou le PRRSV (Cox et al., 1990). Dans nos travaux, nous avons évalué l'effet de l'entraînement des AM suite à l'infection grippale sur la réplication de deux virus ayant un tropisme pour les AM. Cependant, les conséquences des infections d'autres cellules par d'autres virus tels que le PRCoV pourraient être aussi impactées suite à l'entraînement des AM. En effet, cet entraînement, qui augmente la capacité de phagocytose des AM, pourrait aussi limiter la propagation d'autres virus. Cela peut être étudié par exemple, en infectant par d'autres virus un système de co-culture de cellules épithéliales et de AM, provenant de porcs pré-infectés par le swIAV ou pas. Ces infections seraient suivies par une évaluation de la réplication virale et des effecteurs de la réponse immune innée.

En se basant sur tous ces résultats et sur la pertinence du modèle porcin pour l'étude du système immunitaire dans son versant respiratoire (Maisonnasse et al., 2016) et les maladies infectieuses humaines (Meurens et al., 2012a), il pourrait être intéressant de promouvoir la vaccination par des IAV atténués comme une pratique pour réduire l'effet des infections virales respiratoires sur la santé humaine. Toutefois, une analyse bénéfice/risque approfondie devrait être menée au préalable par rapport à l'utilisation de vaccins IAV atténués étant donné les phénomènes de dérive et cassures antigéniques associés aux virus influenza de type A.

Contributions à la compréhension du complexe respiratoire porcin

Il a bien été souligné dans ce manuscrit que les co-infections respiratoires chez le porc sont très fréquentes et constituent une vraie menace pour les élevages de porcs dans le monde entier. Les conséquences des co-infections sur la réponse immune du porc et sur la sévérité de la maladie respiratoire sont très complexes et différentes. Elles varient en fonction de la nature virologique ou bactériologique des micro-organismes en jeu, en fonction de leurs cellules cibles et de leurs mécanismes de subversion de la machinerie cellulaire.

Cette complexité dans les interactions entre les différents agents pathogènes et l'hôte porcin varie d'un micro-organisme à un autre. La compréhension de cette complexité nécessite une collaboration entre tous les acteurs de la filière qui s'intéressent à la santé porcine (figure 17). Les analyses sérologiques et les observations des vétérinaires dans les élevages et les abattoirs porcins alimentent les bases de données des études épidémiologiques ayant pour objectif de mener des enquêtes sur l'évolution des maladies respiratoires chez le porc (figure 17). L'identification et l'isolement des agents pathogènes permettent l'élaboration des protocoles expérimentaux et la réalisation des expérimentations aux laboratoires pour mieux comprendre les conséquences des co-infections et les interactions moléculaires qui en résultent (figure 17). Ensuite, les résultats élaborés permettent le développement de modèles informatiques pour évaluer les différents paramètres impliqués et assurer une meilleure surveillance de la circulation des agents infectieux (figure 17). Tous ces travaux conduisent *in fine* à l'élaboration des recommandations pour guider les vétérinaires et les éleveurs dans leur lutte contre les co-infections respiratoires chez le porc (figure 17).

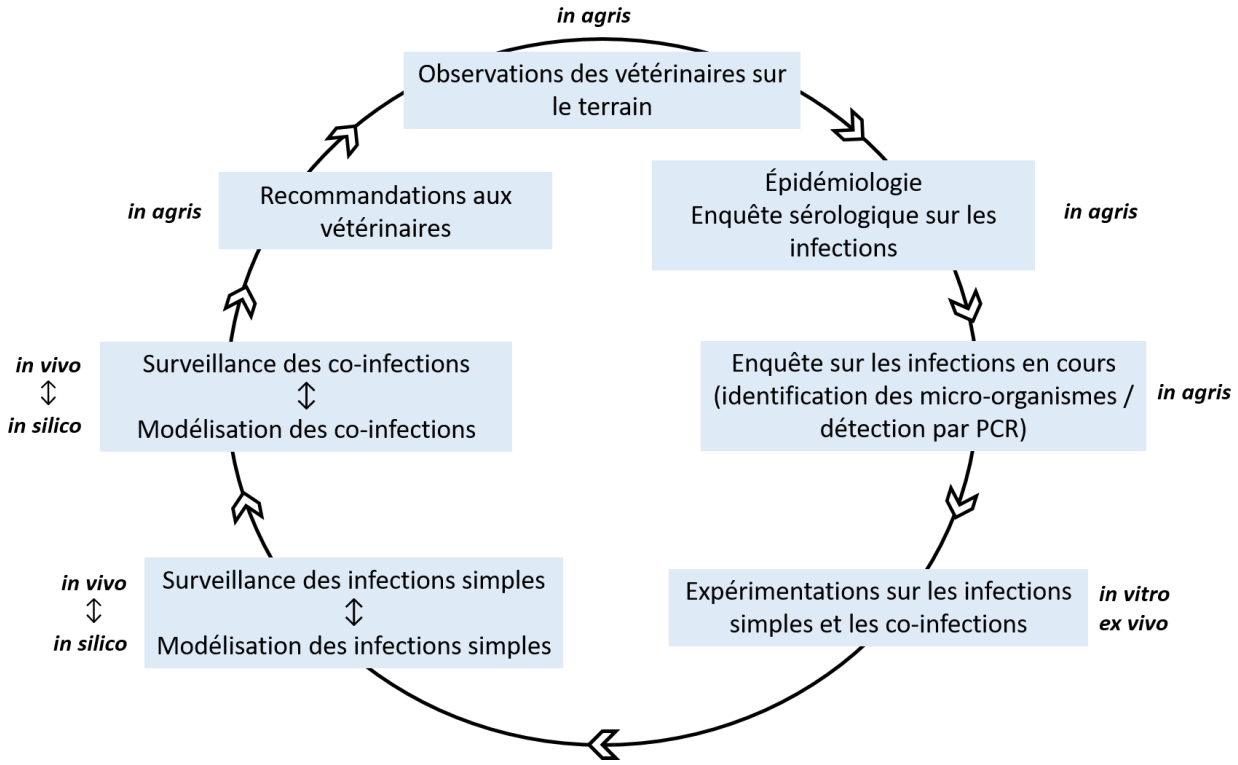

Figure 17. *Cercles de contribution à la compréhension du CRP. Les observations des vétérinaires alimentent l'enquête sérologique sur les infections, qui inspirent les études de laboratoire, qui fournissent des données et des paramètres pour développer des modèles informatiques permettant l'élaboration des recommandations aux vétérinaires et aux éleveurs.*

Mes travaux de thèses ainsi que nos expérimentations *in vitro* et *ex vivo* inspirés des données terrains s'intègrent dans ce cercle de contribution pour améliorer la compréhension du CRP et contribuer à l'élaboration des recommandations émises aux vétérinaires pour assurer une bonne surveillance et un bon contrôle des maladies respiratoires chez le porc. La compréhension de la variation au niveau de la réponse immune en fonction des différents agents pathogènes et de leur combinaison contribue à une optimisation des techniques de diagnostic et de protocoles de vaccination. D'autre part, l'identification de tous les paramètres infectieux et non-infectieux contribuant à l'aggravation des maladies respiratoires sensibilise encore un peu plus les éleveurs et les vétérinaires aux bonnes pratiques pour réduire la dissémination des différents agents pathogènes.

Valorisation et vulgarisation

Communications dans des journées scientifiques et des congrès

- Saade *et al.* 2020. Porcine respiratory cell and tissue co-infections and superinfections with porcine reproductive and respiratory syndrome and swine influenza viruses. *JS 2020 – Journées de Rencontre Scientifique de l'ED EGAAL*, Rennes, France. *2-3 Juillet 2020 (annulée à cause du contexte sanitaire)*

- Saade *et al.* 2020. Co-infections et surinfections du tissu et des cellules respiratoires porcins par le virus du syndrome dysgénésique et respiratoire et le virus de l'influenza A. *XXII^{ème} journée francophones de virologie*, Paris, France. *2-3 Avril 2020 (annulée à cause du contexte sanitaire)*

- Saade *et al.* 2020. Porcine Respiratory Cell and Tissue Co-Infections and Superinfections with Porcine Reproductive and Respiratory Syndrome and Swine Influenza Viruses. *Viruses 2020 — Novel Concepts in Virology*, Barcelone, Espagne. *5-7 Février 2020*

- Saade *et al.* 2018. Porcine reproductive and respiratory syndrome and swine influenza viruses co-infections and superinfections of porcine respiratory cells and tissues. *11ème Symposium du Réseau Français d'Immunologie des Animaux Domestiques*, Tours, France. *26-27 Mars 2018*

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Vulgarisations et activités connexes

- Émission radio : « **Co-infections : quand deux virus valent mieux qu'un** ». *Capsules de science par le Labo des Savoirs*. Radio Prun'. *27 Mai 2020*

- Présentation de l'unité BIOEPAR aux Utopiales : « **Venez incarner un éleveur de bovins !** ». *Festival International de Science-Fiction*, Nantes, France. *31 Octobre 2019*

- Présentation : Co-infections des cellules et des tissus respiratoires porcins par l'influenza A, le Virus du Syndrome Dysgénésique et Respiratoire Porcin (vSDRP) et le virus de la maladie d'Aujeszky. *Info-sciences SECALIM INRA*, Nantes France. *14 Décembre 2018*

- Présentation : « **Why Pigs ? Infections *versus* co-infections** ». *Flashcom Anims Tes Docs*, Nantes, France. *24 Octobre 2018*

- Présentation : Co-infections des cellules et des tissus respiratoires porcins par l'influenza A et le Virus du Syndrome Dysgénésique et Respiratoire Porcin (vSDRP). *Séminaire de l'unité BIOEPAR*, Pornichet, France. *25 Juin 2018*

- Présentation : « Co-infections du virus de l'Influenza A porcin et du virus du syndrome dysgénésique et respiratoire porcin (PRRSV) : Interactions ? réponse immune du porc ? ». *Séminaire de l'unité BIOEPAR*, Nantes, France. *21 Avril 2020*

- Participation au développement d'un jeu de société au sein de l'unité de recherche dans le but de sensibiliser le public à la vaccination et la propagation des maladies infectieuses.

- Engagement associatif au sein de l'association « Anim tes Docs ».

- Adhésion en tant que membre à la Société Française de Virologie (SFV).

- Contrat d'enseignement de 31h avec ONIRIS en 2ème année de thèse pour mener les travaux dirigés des étudiants vétérinaires en premières et deuxièmes années dans les modules suivants :
    - Lactation et production de lait chez les bovins laitiers
    - Reproduction des bovins laitiers ; élevage des jeunes et du pré troupeau

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Annexes

Annexe 1 :

Influenza Infection Induced Antiviral and Anti-inflammatory Training of Alveolar Macrophages

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Abstract
Influenza A represents a major cause of human respiratory infections, however the long term consequences of this usually mild infection are still understudied. Pigs are natural host of *influenza* A viruses and present similar clinical signs as humans. Herein we monitored the impact of a cleared *in vivo influenza* infection on the alveolar macrophages (AM) functions. We observed that 20 days post-infection, AM from *influenza*-infected pigs responded strongly to poly I:C (a mimic of double-strand RNA viruses) but not to LPS (a mimic of gram-negative bacteria) stimulation by upregulating type I IFN genes transcripts, IL12A and IL1β, but not TNFα, IL6 and IL8. Moreover, they presented higher efferocytosis capacities and triggered TGFβ and IL10 expressions in allogeneic responses. These data are in agreement with an innate memory of AM upon *influenza* A infection leading to stronger antiviral and anti-inflammatory responses to viruses, which may result in a more effective and less pathogenic response to secondary viral respiratory infections.

Introduction
Influenza A viruses infect each year 10% of the world population (Weycker et al., 2005). Hence, each of us will be infected many times in our lifetime. Whereas most of the people will heal quickly with minimal care, few will develop serious health complications. With good reasons, most of the *influenza*-related researches focus on these rare life-threatening conditions, and few tackle the consequences of the common, benign *influenza* A infection.
The murine model for *influenza* A infection requires the use of adapted *influenza* strains, that trigger lung infection reaching the lower respiratory tract and lead to fatal illness. This model is thought to correctly mimic highly pathogenic *Influenza* virus infection of humans, however its relevance as a model of the most frequent mild *influenza* infections observed seasonally in humans remains questionable. Conversely, pigs are the natural host of *influenza* strains that occasionally mix with human strains (VanderWaal and Deen, 2018; Chastagner et al., 2019) and usually present a self-limited upper respiratory tract infection leading to mild clinical signs similar to common human infections (Reeth and Vincent, 2019).
AM is an essential component of the anti-viral respiratory immune defense as demonstrated in mouse (Schneider et al., 2014)(Cardani et al., 2017) and pig (H. M. Kim et al., 2008). By

scavenging dead cells, but also by their capacity to produce anti-inflammatory cytokines, AM allow the control of inflammation in the lower respiratory tract, preserving the respiratory function. AM are non-productively infected by *influenza* virus (van Riel et al., 2011b; Meischel et al., 2020b). The ability of some strains to induce AM apoptosis has been hypothesis to be responsible of their higher pathogenicity (Ghoneim et al., 2013) through the triggering of over-inflammation and the facilitation of super-infection. This last point is still a subject to discuss and dispute (Califano et al., 2018).

AM are embryonically derived tissue-resident macrophages (εAM) that self-renew independently from blood monocytes at a steady-state (Guilliams et al., 2013; Schulz et al., 2012). In mice, upon *influenza* infection, depleted AM are replaced by monocytes-derived macrophages (moMΦ) (Aegerter et al., 2020) presenting different features, a stronger response to pathogens and a more important pro-inflammatory profile. These moMΦ occupy the niches freed by AM and eventually (upon 2 months) become indistinguishable from the original εAM.

Trained immunity has been defined as the memory of innate cells, involving mainly monocyte/macrophages. Upon first fungal/bacterial challenges (the best defined stimuli being BCG and β-glucans from different fungi), trained cells are innate cells that will respond stronger to a second related or unrelated challenge. The response involves usually higher pro-inflammatory cytokines release (for review (Netea et al., 2020). Trained innate cells experience metabolism switch increasing aerobic glycolysis and decreasing tricarboxylic acid (TCA) cycle, as well as epigenetic modifications, accountable to the long term memory of these functional modifications. Trained immunity has first been defined as a modification of circulating monocytes (Quintin et al., 2012) and their bone marrow precursors (Cirovic et al., 2020; Kaufmann et al., 2018), leading to a systemic immune upgrade for host defense but also for chronic inflammatory diseases (Netea et al., 2020).

Two recent papers described the local respiratory immune training-like effect of viral infection on murine AM. The first one (Yao et al., 2018) showed that non-replicative adenovirus intranasal instillation, can induce higher antibacterial immunity and an immune training process based on MHC-II upregulation and increased glycolysis of lung resident self-renewable AM. This training has been shown to be CD8 T cells dependent. The second one, using *influenza A* infected mice, demonstrated the replacement of AM by monocyte-derived macrophages (Aegerter et al., 2020) leading to stronger pro-inflammatory response and better anti-bacterial protection.

Herein, using the mild pathogenic porcine model of *influenza A* infection we prospected the long term impact of *in vivo influenza A* infection on AM antiviral and anti-inflammatory functions.

Material and methods

Viruses productions (swH1N2, PRRSV1.1, ADV)

Swine influenza virus H1huN2 (A/Sw/Ille et Vilaine/0415/2011) was isolated in 2011 from a pig with acute respiratory disease in Brittany, France. The virus was propagated on Madin-Darby Canine Kidney (MDCK) cells for 24h in Dulbecco's modified Eagle medium (DMEM) (Eurobio scientific, Les Ulis, France) supplemented with 10% fetal calf serum (FCS) (Eurobio scientific) and 1% of Streptomycin/Penicillin/Amphotericin (SPA) solution (Eurobio scientific), and 2 µg/mL of trypsin TPCK treated (Worthington Biochemical Corp., Lakewood, NJ, USA). After collection, the supernatant was clarified by centrifugation for 20 minutes (min) at 600 x g and then purified

on Amicon Ultra-15 centrifugal Filters (Sigma-Aldrich) after a 20 min centrifugation at 4,000 × g and 4°C. Final titers of the viral stock reached 10^7 TCID50/mL. Porcine Reproductive and Respiratory Syndrome virus type 1 (PRRSV-1) strain PRR-FR-2005-29-24-1 was propagated on AM cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Eurobio scientific) supplemented with 10% FCS and 2% of SPA solution for 72h after being isolated from a herd with abortions (Renson et al., 2017). After clarification and purification on Amicon filters, the viral titers reached 10^6 TCID50/mL. As per the Aujeszky disease virus (ADV) strain Kojnok (Kojnok, 1965), propagation was done on Newborn Pig Trachea (NPTr) cells (Ferrari et al., 2003) in a DMEM medium supplemented with 10% FCS and 1% of SPA solution. Clarified and purified stock titers were estimated at 10^6 TCID50/mL. All viruses were stored at -80°C and titration was performed using TCID50 assay.

In vivo infection and alveolar macrophages collection

AM were collected from sacrificed 12 week-old specific pathogen free (SPF) pigs, infected or not 3 weeks earlier intratracheally with 10^6 TCID50 of swine *influenza* virus $H1_{hu}N2$ (A/Sw/Ille-et-Vilaine/0415/2011) in 5 mL. AM Collection was done after a bronchoalveolar lavage (BAL) with 250 mL of Phosphate Buffered Saline (PBS) (Eurobio scientific) supplemented with 2 mM EDTA (Sigma-Aldrich, Saint-Quentin, France). Following centrifugation, passage through 40 µm cell strainers and application of an erythrocyte lysis buffer (10 mM NaHCO3, 155 mM NH4Cl, and 10 mM EDTA), AM were washed with PBS, counted and conserved in FCS, 10% DMSO in liquid nitrogen.

In vitro re-stimulation

Selection of live and functional cells was done by allowing the adhesion of thawed AM to plastic surface in a 6 well plates at 2.10^6 cells/mL in 800 µL. After washing adherent macrophages were stimulated with 10 ng/mL LPS or Poly I:C (Sigma-Aldrich) for 24h in RPMI supplemented with penicillin, streptomycin and glutamine. Upon stimulation plates were centrifuged (400 g, 4 min) to stick the potential stimulation-resuspended macrophages to the well's bottom, culture media was removed and cells lysed in RLT special lysis buffer from the RNeasy Mini Kit (Qiagen) before processing for RNA extraction and RT-qPCR.

Quantitative Real-Time PCR

Adhesion of thawed AM to plastic surfaces allowed a proper selection of live and functional cells. After adherent cells lysis with RLT buffer (Qiagen), total RNA were extracted using RNeasy Plus Mini kit (QIAGEN) according to the manufacturer's instructions. RNA were reverse transcribed using oligo(dT) and random primers (BioRad iScript Reverse Transcription supermix). RNA samples were treated with DNAse I Amp Grade (Invitrogen) (1 U/µg of RNA). The absence of genomic DNA contamination was validated by the use of treated RNA as a template directly in PCR. Total RNA quantity and quality were assessed using Nanophotometer (Implen, Munich, Germany). cDNA was generated with a virus reverse transcriptase in the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA) from 100-200 ng of RNA free of genomic DNA per reaction. Diluted cDNA (4X) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's recommendations. Real-

time assays were run on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Samples were normalized internally by simultaneously using the average Cycle quantification (C*q*) of three reference genes in each sample (Vandesompele et al., 2002). Then, 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 (Table 1). ActB, HPRT1, RPS24 were used as reference genes since they were endowed with one of the most stable expressions in the porcine lung (Delgado-Ortega et al., 2011; Maisonnasse et al., 2016).

Metabolism

SCENITH^{TM} method (Arguello et al., 2020) measures the level of protein synthesis in response to metabolic pathways inhibitors as commonly used in Seahorse (2-Deoxy-Glucose (2DG), Oligomycin/FCCP and a combination of both). AM from mock or *influenza* infected animals were thawed and cultured in 96 round bottom well plate at 10.10^6 cells/mL in 80 µL complete medium 1h 37°C, 5% CO2 to recover from freezing. Then AM were cultured with nothing, 0.1 M glucose oxidation blocking drug 2-Deoxy-D-Glucose (DG), 1 µM mitochondrial respiration blocking drug (Oligomytcin) or both for 20 min. As a negative control, the translation initiation inhibitor Harringtonine was added 15 min before the addition of Puromycin (Harringtonine, 2 µg/mL; Abcam, cat. ab141941). Then puromycin (10 µg/mL; Sigma-Aldrich, Cat. No. P7255) was added to AM for 20 min before washing in cold PBS. Cells were then permeabilized and intracellularly stained using FoxP3 staining kit (BD Pharmingen) following provider instructions. Translation was measured using anti-Puromycin antibody (1:800, Clone R4743L-E8) coupled to Alexa-647 and AM were identified using anti-CD163 (mouse IgG1, clone 2A10/11, AbD Serotec), and a secondary anti-mouse IgG1 coupled to Alexa-488. Cells were analyzed on a MACSQuant®10 (Miltenyi Biotec) and analyzed using FlowJo 10.6.2.

Flow cytometry

AM were antibody-stained in a blocking solution of PBS, 5 mM EDTA supplemented with 5% horse serum and 5% swine serum (Eurobio scientific). Two independent stainings were applied: i) MHC-II (mouse IgG2b, clone Th21A, Monoclonal Antibody Center Washington State University Pullmann, WA), CD11b (rat antibody, clone M1/70, BD Pharmingen) and Mannose receptor (MR) (mouse IgG1, clone 122D2.8, Dendritics, Lyon), followed by secondary anti-IgG2b antibody coupled to Alexa-488, anti-rat antibody coupled to Alexa-555 and anti-IgG1 antibody coupled to Alexa-647, and ii) CD14 (mouse IgG2a, clone Tuk4 from AbD Serotec), CD163 (mouse IgG1, clone 2A10/11, AbD Serotec), CD172a (IgG2b, clone 74-22-15A, Monoclonal Antibody Center Washington State University Pullmann, WA, followed by secondary anti-IgG2a antibody coupled to Alexa-488, anti-IgG1 antibody coupled to PE and anti-IgG2b antibody coupled to Alexa-647. Cells were analyzed on a MACSQuant®10 (Miltenyi Biotec) and analyzed using FlowJo 10.6.2.

PRRSV and ADV infections of AM

AM were thawed and cultured in 96-well-plates at 2 $x10^5$ cells per well for 1h to allow the adhesion. Cells were washed with PBS and then infected for 1h with PRRSV or ADV at a multiplicity of infection (MOI) of 0.1 and 0.01 for each virus. The inoculum was removed after quick centrifugation of the plates and the cells were incubated for 24h at 37°C and 5% CO_2 in RPMI 1640

supplemented with 1% of SPA. Finally, the plates were centrifuged and the cells were harvested using a special lysis buffer from the RNeasy Mini Kit (Qiagen). Supernatants and lysed cells were stored at -80°C.

Efferocytosis

Confluent NPTr cultured in 6 well plates ($2.4.10^6$ cells/well) were CFSE stained by incubation in 3 mL RPMI, 1 µM CFSE 10 min at 37°C, washed and then triggered to apoptosis by exposing them to a UV lamp for 20 min on ice under the hood. AM from mock or *influenza* infected animals were thawed and deposed at 1.10^6 cells/well on CFSE-stained apoptosis-induced NPTr (ratio AM:NPTr of 1:2.4). Twenty-four hours later, cells were resuspended by flushing twice in ice-cold PBS/EDTA (2 mM). AM were antibody-stained in a blocking solution of PBS/EDTA supplemented with 5% horse serum and 5% swine serum. Anti-CD163 antibody (mouse IgG1, clone 2A10/11, AbD Serotec) was added to the blocking solution at 2 µg/mL for 30 min on ice and then washed in PBS/EDTA followed by the secondary anti-mouse IgG1 Alexa-647 coupled antibodies (anti-mouse IgG1-Alexa647, Invitrogen). Cells were analyzed on a MACSQuant®10 (Miltenyi Biotec) and analyzed using FlowJo 10.6.2.

Direct allogeneic stimulation

Peripheral blood mononuclear cells (PBMC) from Large White SPF sows were collected at ANSES Ploufragan on Ficoll-Paque density gradient (Amersham Biosciences, Uppsala, Sweden) and frozen in FCS, 10% DMSO in liquid nitrogen. PBMC were thawed and mixed with AM from mock or *influenza* infected animals at a AM: PBMC ratio of 1:6 in round bottom 96 wells plate using RPMI medium supplemented with 10% FCS and 1% SPA and L-glutamine at a final concentration of 3.5×10^6 cells per mL. After 3 days of co-culture, total RNA was extracted and genes expression was analyzed by RT-qPCR. Three cytokine (IFNγ, TGFβ and IL10) transcript expressions were measured.

Allogeneic response biases

Following BAL procedure (see above *In vivo* infection and alveolar macrophages collection) peripheral parenchymal (PAR) tissue from diaphragmatic lobes were sampled, minced and incubated in complete RPMI medium supplemented with SPA, L-glutamine and 10% FCS. Tissue digestion was performed by adding 2 mg/mL collagenase D (Roche, Meylan, France), 1 mg/ml dispase (Invitrogen) and 0.1 mg/mL Dnase I (Roche). Digested parenchyma was crushed and filtered on 100µm cell strainers. Red blood cells were lysed using erythrolysis buffer (10mMNaHCO3, 155mMNH4Cl, and 10mMEDTA) for 10 min at 37◦C. PAR cells were then enriched in antigen-presenting cells by 1.065 density iodixanol gradient (Optiprep®, Nycomed Pharma, Oslo, Norway) as previously described (Marquet et al., 2011). The parenchymal cells enriched on allophycocyanin (PAR-APC) were kept on ice overnight before proceeding to allogeneic culture. Allogeneic response was then performed as previously described (E. Bordet et al., 2018). Peripheral blood mononuclear cells (PBMC) from Large White SPF sows were collected at ANSES Ploufragan as above. PBMC were thawed and mixed with PAR-APC at a 1:3 PAR-APC:PBMC ratio selected after different ratio test. PAR-APC:PBMC final co- culture, with final total cell concentration of AM from mock or *influenza* infected animals were then added to the

allogeneic culture at a AM: PAR-APC ratio of 1:2 and poly I:C was added at 10 ng/mL. Final cell concentration was of 2×10^6 cells per mL in RPMI, 10% FCS, 2% SPA, and L-glutamine.
After 5 days of co-culture, total RNA was extracted and genes expression was analyzed by RT-qPCR. Four transcriptions factors (T-bet, GATA3, RORγT, and FOXP3) and 4 cytokines (IFNγ, IL13, IL17, and IL10) expression were chosen as indicators of T-helper polarization (respectively Th1, Th2, Th17, and Treg). IL13 has been chosen instead of IL4 because IL13 has been reported as a better porcine Th2 marker than IL4 (Murtaugh et al., 2009)

Statistics
Due to the low number of replicates (3 per condition), no statistics were used for the comparison of *in vivo* mock or *influenza*-trained AM. The symbol "#" was used in case the values related to AM from infected animals were all upper or lower than those of AM from the mock-infected controls.
Kruskal-Wallis test followed by a Dunn's multiple test were applied to compare the relative expressions of PRRSV and ADV viral transcripts between AM from mock or influenza infected animals. GraphPad Prism was used for this purpose (GraphPad Software version 7.0, San Diego, CA, USA).

Results:
Trained response
To probe the possibility that *influenza A* infection would impact AM functions long after the elimination of the virus, we infected intratracheally 2 groups of 3 animals with 5 ml 10^6 TCID50 of swH1huN2 (A/Sw/Ille et Vilaine/0415/2011) *influenza A* virus or mock preparation. Twenty days post-infection, no more *influenza* virus could be detected in nasal swabs, BAL supernatants and AM (data not shown). Moreover, no difference in AM percentage or absolute count was observed between the two conditions (data not shown). AM extracted from mock or *influenza* infected animals were re-stimulated *in vitro* using low LPS dose (10 ng/mL). AM coming both from mock and *influenza* infected animals responded to LPS by upregulating transcript expression of the pro-inflammatory cytokines TNFα, IL6 and IL8 (Fig. 1A). Since the potential training inducer (*influenza A* virus) was from viral origin we also tested antiviral responding genes expression. IFNβ and IFNλ1 genes showed no modification upon LPS stimulation, although interestingly interferon-stimulated genes (ISG) PKR and Mx2 as well as the Th1-related gene IL12A, presented a higher basal level of transcript expression in AM from *influenza* infected animals than the expression level in AM from Mock animals (Fig. 1A).
Due to the viral origin of the potential training inductor, we then tested the response of AM to poly I:C that mimics double-stranded viral RNA such as *influenza* genome. AM from *influenza* infected animals responded to Poly I:C by a stronger transcript expression of IL1β, IFNβ, IFNλ1 and ISG PKR, Mx2 and IL12A (Fig. 1B), in support of an immune training of AM upon *in vivo influenza* infection. To explain this stronger AM response to Poly I:C we tested if these AM expressed higher levels of dsRNA receptors. Neither TLR3, MDA5 nor RIGI presented any differential expression when comparing AM from mock or *influenza* infected animals (data not shown).

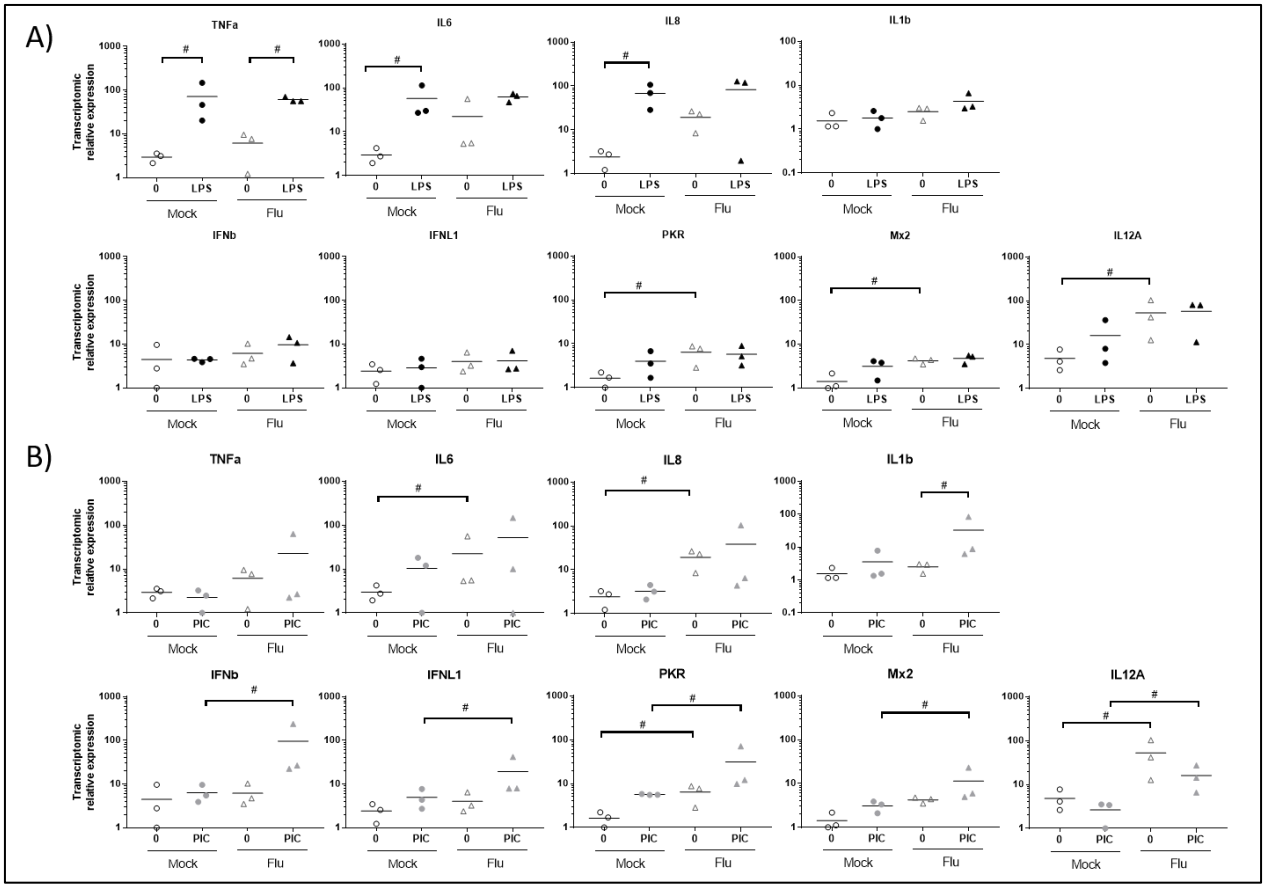

Figure 1 : *AM from influenza-infected animals present an increased antiviral response.*
AM collected 20 days after mock or influenza infections were re-stimulated in vitro using low dose (10 ng/ml) of LPS (A) or Poly I:C (B) for 24h. Transcriptomic expression of TNFα, IL6, IL8, IL1β, IFNβ, IFNλ1, PKR, Mx2 and IL12A.

AM Phenotype and Origin

This differential response of AM from *influenza* infected animals might be a CD8 T cells/IFNγ mediated AM training leading to MHC-II upregulation on AM, as described by Yao et al. (Yao et al., 2018) in mouse. We thus checked MHC-II expression as well as AM count and phenotype. No differences can be observed between AM from mock or *influenza* infected animals (Fig. 2A). A depletion of embryonic-derived AM upon *influenza* infection and their replacement by monocytes-derived macrophages has been observed by Aegerter et al. (Aegerter et al., 2020) in mouse. AM newly derived from monocyte-derived macrophages but not self-renewable resident AM expressed CD11b and CD14 in pig (Maisonnasse et al., 2016) like in mouse (Misharin et al., 2013). We thus checked the expression of these antigens on $CD163^{pos}$ BAL macrophages from mock or *influenza* infected animals. Above 15% of $CD11b^{pos}$ or $CD14^{pos}$ macrophages were detected in both conditions, with no differences according to the *influenza* infection status (Fig. 2A). We further validated the absence of replacement of embryonically-derived AM by monocyte-derived

macrophages upon *influenza* infection by testing a set of genes which have been observed as differentially expressed in monocytes-derived and embryonically-derived macrophages, namely CX3CR1, CCR2, C1QB, CDK1 and CDC20 overexpressed in moMΦ, and MertK, Marco, CD64, HDAC10, CyclinB2 and Topoisomerase III, overexpressed in εMΦ. None of these genes presented a differential expression according to the origin of the AM (Fig. 2B). In agreement with these results, *in vitro* infection with swIAV H1N2 at a MOI of 3 did not trigger porcine AM apoptosis (data not shown).

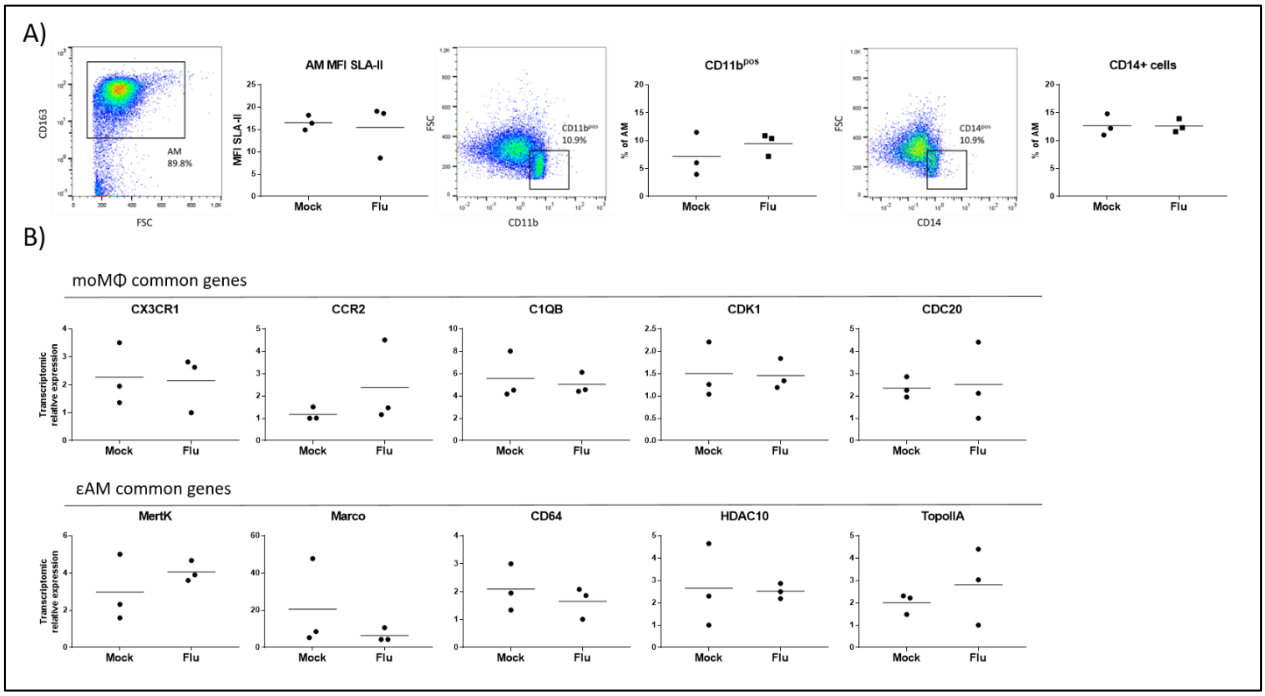

Figure 2 : *Trained AM did not differentially express moMΦ and AM markers*
AM collected 20 days after mock or influenza infections were A) stained with anti-CD163, SLA-II, CD11b and CD14 antibodies, gated on CD163 and the SLA-II expression, mean fluorescence intensity (MFI) as well as the % of $^{CD11bpos+\ and}$ *CD14pos+ cells were measured, B) enriched in macrophages by plastic adherence, their RNA was extracted and moMΦ and εAM-related genes were measured by RT-qPCR.*

<u>Metabolism</u>

Since trained immunity is associated with metabolic modifications, we then tested the expression of different glycolysis related genes (Cheng et al., 2014), namely GAPDH, ALDH1A1, ACSS1, GPI and LDHA, none of them presented differential expression according to the origin of the AM (Fig. 3A). We then tested the preferentially used metabolic pathways of the AM using SCENITH protocol (Arguello et al., 2020), no differences in preferential metabolism pathways could be observed according to the origin of the AM (Fig. 3B).

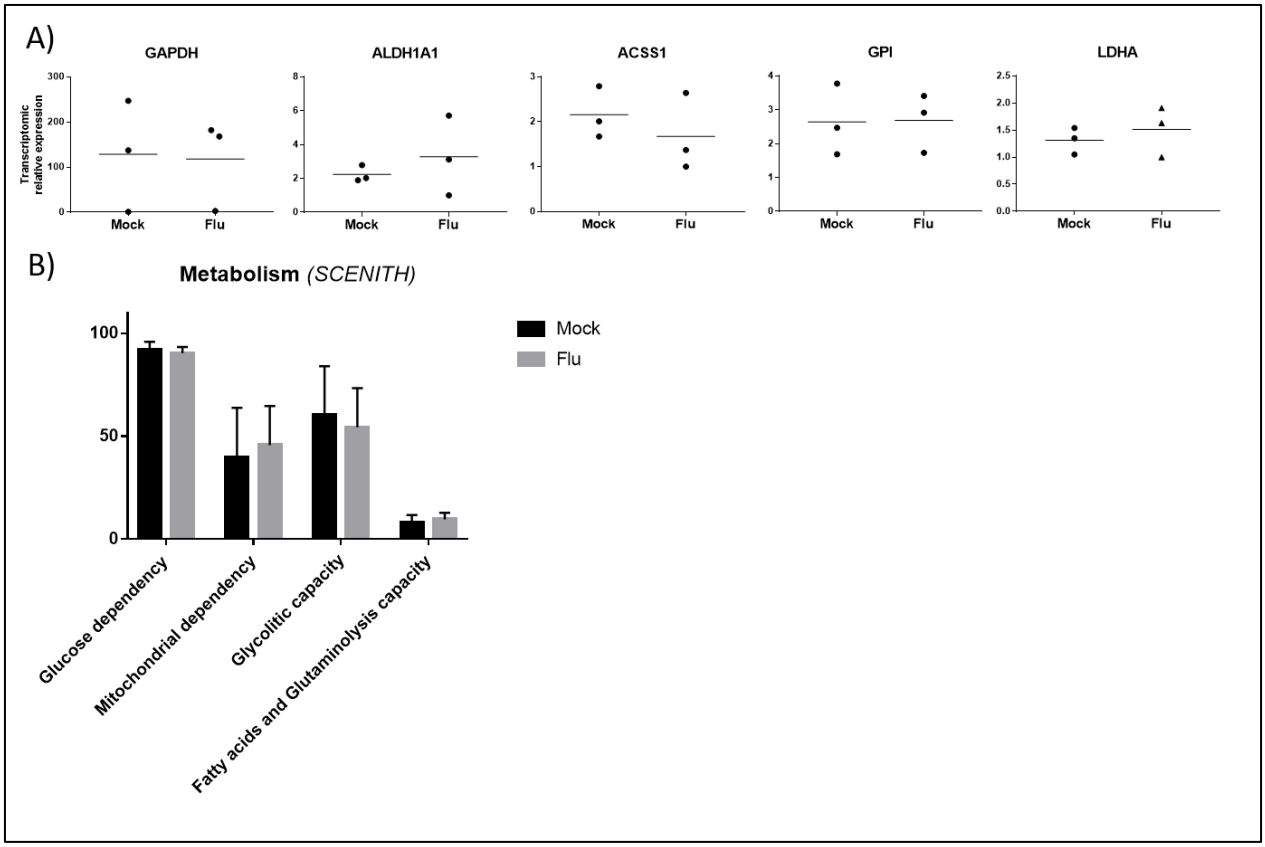

Figure 3 : *AM metabolism is not modified by influenza virus infection.*
AM collected 20 days after mock or influenza infections were A) enriched in macrophages by plastic adherence, their RNA was extracted and metabolism-related genes were measured by RT-qPCR. B) cultured in presence of glucose oxidation blocking drug 2-Deoxy-D-Glucose (DG), and/or mitochondrial respiration blocking drug (Oligomytcin), the translation was measured by puromycin incorporation measure by cytometry and the different metabolic pathways were determined using the Scenith method (Arguello et al., 2020).

Viral infections susceptibility

We then tested if *influenza*-trained AM presented higher resistance to direct infection by viruses with myeloid tropism. PRRSV is an arterivirus with a positive sense RNA genome that targets AM. It is responsible for highly contagious infection and huge economic losses in the pig industry (Lunney et al., 2016). Aujesky disease virus (ADV), also known as *Suid herpesvirus 1* (SuHV-1) or pseudorabies virus, is a herpesvirus that is still present in many countries in domestic pigs and wild boars (Müller et al., 2010). Its genome consists of a double-stranded DNA genome. ADV targets numerous cell types, such as neurons, epithelial cells and macrophages (Nauwynck et al., 2007). Using two different MOI we tested if AM from *influenza* infected animals were more resistant to PRRSV and ADV infections. No consistent difference could be observed in the expression of viral transcripts (Fig. 4).

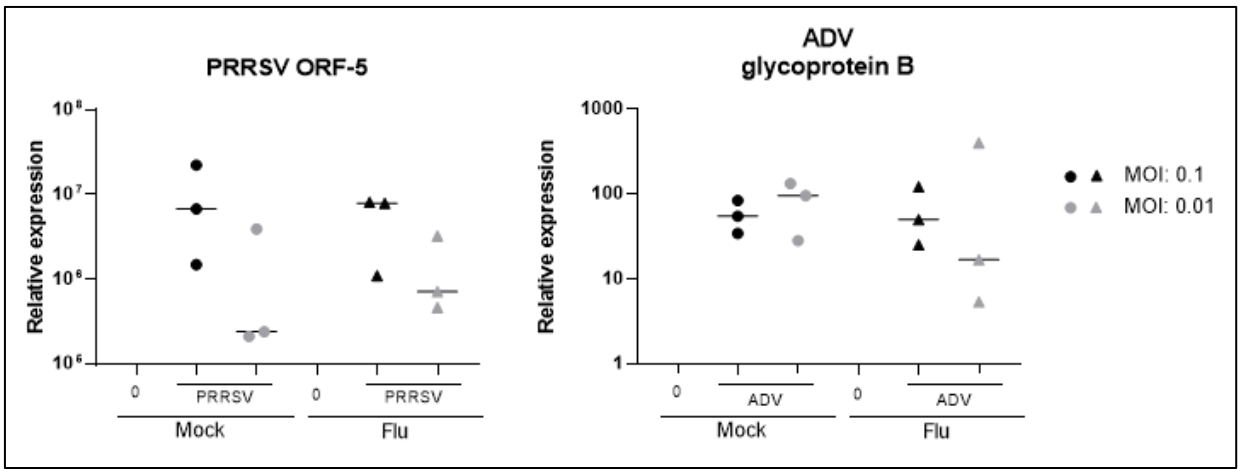

Figure 4 : *Trained AM are not resistant to viral infections.*
AM collected 20 days after mock or influenza infections were infected in vitro with PRRSV or ADV at MOI 0.1 and 0.01 for 24h. PRRSV and ADV titers were measured by RT-qPCR. The mean of triplicate is depicted

Antiviral immune function

A phagocytic paralysis of AM has been recently described upon sepsis, which was linked to CD172a down-regulation (Roquilly et al., 2017), we thus tested the expression of this membrane protein and observed few macrophages presenting a low expression of CD172a, linked with the expression of CD14, however, no differences between the AM from both origins were observed (Fig. 5A). We then wondered if the observed cytokine antiviral response of *influenza*-trained-AM was paralleled with functional antiviral capacities. We first tested the efferocytosis capacity of AM by culturing them with CFSE-labelled porcine epithelial cell line NPTr, triggered to apoptosis by UV treatment. *Influenza*-trained-AM presented a higher capacity to phagocytose apoptotic epithelial cells (Fig 5B).

AM are known to be weak antigen-presenting cells, however they are able to communicate with innate and adaptive immune cells, leading mostly to down-modulation of the immune response (Blumenthal et al., 2001). We thus first cultured AM from mock and *influenza* infected animals directly with allogeneic PBMC to measure the impact of AM viral training on this interaction. AM training had no effect on IFNγ and IL10 transcript production although it triggers a 2.8-fold increase of INFα and a 2.4-fold increase of TGFβ transcriptomic expressions (Fig. 5C). We then interrogated the capacity of trained-AM to skew the adaptive immune response triggered by allogeneic professional APC on peripheral blood T cells and leading to their differentiation toward the different T helper (Th1, Th2, Th17) or regulatory (Treg) responses. AM from mock or *influenza* infected animals were added to culture composed of allogeneic PBMC and APC-enriched parenchymal cells preparation (E. Bordet et al., 2018). As expected the addition of AM from both origins down modulates the allogeneic responses (data not shown), however, AM from *influenza* infected animals showed no differences for Th1, Th2 and Th17 responses compared to mock condition. Conversely, although no consistent FoxP3/Treg signal was observed, the presence of *influenza*-trained AM increased the production of IL10 in the allogeneic culture (Fig. 5D).

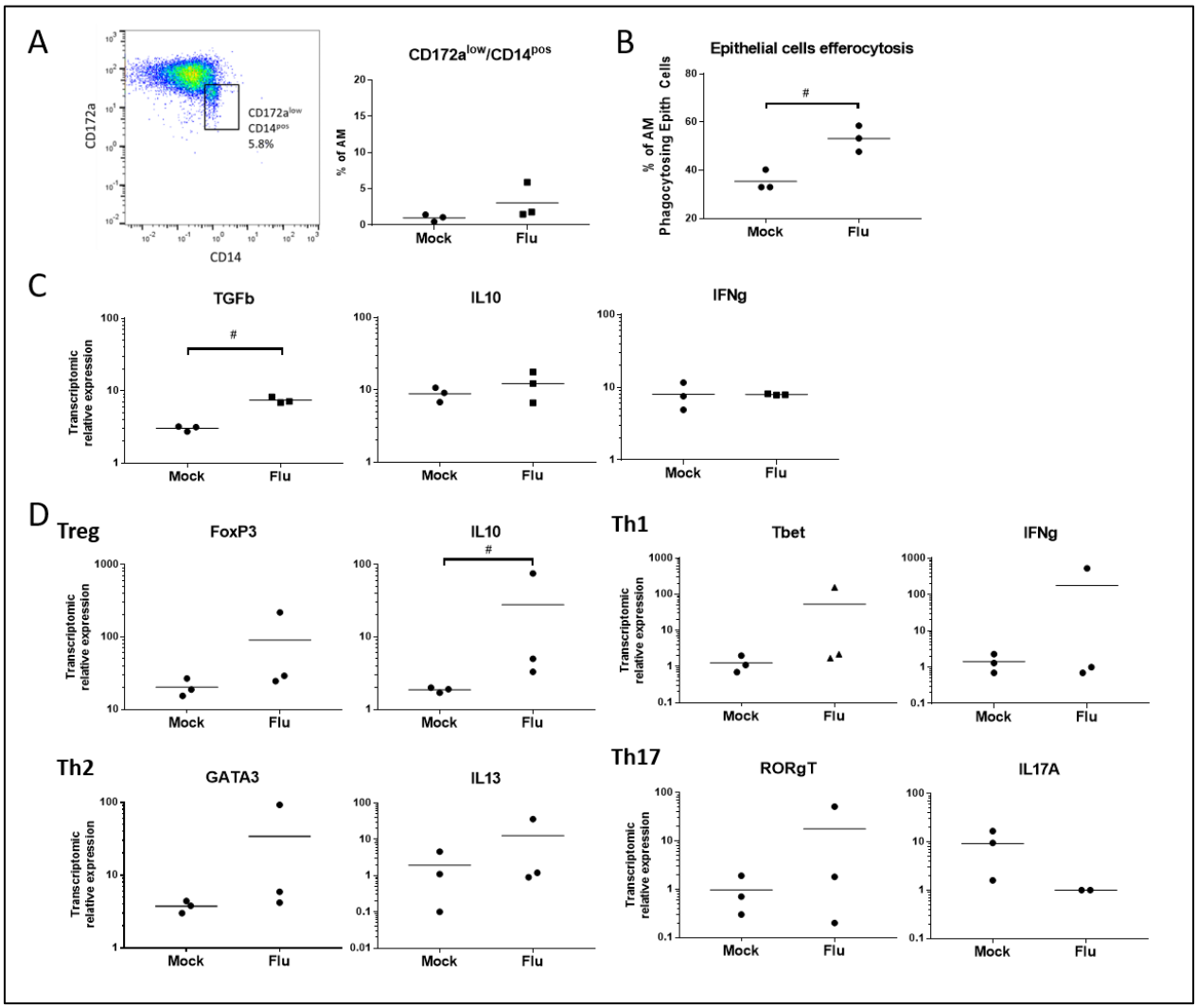

Figure 5 : *AM from influenza-infected animals promote anti-inflammatory responses*
AM collected 20 days after mock or influenza infections were A) stained with anti-CD163, anti-CD172a and anti-CD14 and the percentage of CD172a^{low} cells was measured by flow cytometry. B) cultured 24h with apoptotic, CFSE-stained, epithelial cell line NPTR at a ratio of 1:2.4 before CD163 staining in order to gate on AM and measured the percentage of CFSE^{pos} AM having phagocytosed apoptotic epithelial cells. One experiment representative of 2 independent experiments. C) cultured 3 days in presence of allogeneic PBMC at a ratio of 1:6 before mRNA extraction and RT-qPCR to measure TGFβ, IL10 and IFNγ transcriptomic expression. D) cultured 5 days in presence of allogeneic PBMC and allogeneic lung APC at a ratio of 1:6:2 (AM:PBMC:APC) before mRNA extraction and RT-qPCR to measure FoxP3, IL10, Tbet, IFNγ, GATA3, IL13, RORγT, IL17A transcriptomic expression. One experiment representative of 2 independent experiments.

Discussion

Herein, we observed that a resolved *influenza* A infection can imprint AM for a higher induction, following viral-related but not bacterial related stimulus, of type I IFN pathways, increased expression of Th1 response-related gene IL12A and production of the pleiotropic IL1β cytokine, known to be essential in anti-viral training (Arts et al., 2018).
Interestingly, similarly to BCG induced trained immunity, *influenza*-mediated training also triggers a boost in AM efferocytosis capacities (Mukherjee et al., 2017). However, conversely to classically trained immunity induced by BCG (Kleinnijenhuis et al., 2012) or yeast-extracted β-Glucans (Quintin et al., 2012), we observed here that *influenza*-mediated trained immunity did not modify the expressions of pro-inflammatory cytokines TNFα, IL6 and IL8 upon LPS or poly I:C stimuli.
This imprinting is not associated with metabolic change nor with MHC-II expression upregulation conversely to adenovirus-mediated AM training in mouse (Yao et al., 2018), neither it is associated with the replacement of AM by moMΦ as observed upon *influenza* A infection in mice (Aegerter et al., 2020). We can postulate that this AM training might rely on the property of the majority of *influenza* A virus strains (among them the strain used here) to infect AM unproductively (Meischel et al., 2020b), leading to the presence of viral genome in the AM up to 1 week post-infection (data not shown). So far our attempts to induce *influenza*-mediated training using *in vitro* infection of isolated AM have been unsuccessful (data not shown).
Interestingly, we did not observe desensitization of AM against LPS stimulus as observed in mice upon *influenza* infection (Didierlaurent et al., 2008) nor phagocytic defect as observed in case of murine and human sepsis (Roquilly et al., 2020). These differences rely probably on the variation in the intensity of the induced inflammation, since swine and human *influenza A* viruses do not reach the deep respiratory tract nor trigger cytokine storm despite their pathogenicity.
Although we could not detect direct protection of *influenza*-trained AM for heterologous viral infections (PRRSV and ADV) nor a clear promotion of Th1 response of trained AM, their higher antiviral response upon Poly I:C stimulation, associated with their efferocytosis capacities as well as their TGFβ and IL10 productions upregulation in PBMC are in agreement with a better capacity to control both viral titers and viral-mediated over-inflammation. These results following PRRSV or ADV stimulation could be related to some of our experimental conditions. The low MOI applied in the infections may have not been enough to trigger infections of all the cells, especially on a short duration of 24h. Working with higher MOI, and maybe longer infection time could be essentiel to demonstrate the effect of swIAV on the réplication of PRRSV and ADV.
However, it remains to be demonstrated *in vivo* that a previous *influenza A* infection may protect the host against an unrelated viral infection mainly transmitted through the respiratory tract such as the PRRSV or the porcine coronavirus (PRCoV) (Cox et al., 1990). If effective, and according to the relevance of the porcine model for respiratory immunity (Maisonnasse et al., 2016) and human infectious diseases (Meurens et al., 2012b), it would be interesting to promote *influenza A* attenuated virus vaccination as a practice to decrease the global burden of respiratory viral infections in human health.

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DOCTORAT ECOLOGIE BRETAGNE GEOSCIENCES LOIRE AGRONOMIE ALIMENTATION

Oniris ÉCOLE NATIONALE VÉTÉRINAIRE, AGROALIMENTAIRE ET DE L'ALIMENTATION SANTÉ ET ALIMENTATION AU CŒUR DE LA VIE

Titre : Co-infections des cellules et des tissus respiratoires porcins par le virus de l'influenza A et le virus du syndrome dysgénésique et respiratoire porcin

Mots clés : Porc, co-infections, swIAV, PRRSV, interférence, immunité innée

Résumé : Les co-infections respiratoires chez le porc sont plus fréquentes que les infections causées par un seul micro-organismes. Dans un premier temps, un recensement des études sur les co-infections respiratoires porcines a permis de mettre à jour les connaissances sur ces co-infections virales et/ou bactériennes et de détailler les probables conséquences moléculaires sur l'hôte porcin. Le virus du syndrome dysgénésique et respiratoire porcin (ou *Porcine Reproductive and Respiratory Syndrome Virus*, PRRSV) et les virus responsables de l'influenza porcin A (*swine Influenza A Virus*, swIAV), sont des acteurs majeurs du complexe respiratoire porcin. Le swIAV infecte principalement les cellules épithéliales alors que le PRRSV infecte des cellules exprimant CD163 comme les macrophages alvéolaires (MA). Dans le but d'évaluer la réponse antivirale de l'hôte porcin et d'étudier l'effet d'une pré-infection par le PRRSV sur la réplication du swIAV, une série de co-infections et de surinfections a été effectuée sur des cellules épithéliales trachéales et sur des tranches pulmonaires fines. Les résultats montrent que le PRRSV est capable d'interférer avec l'infection par swIAV et d'altérer la réponse antivirale cellulaire sans infecter les cellules épithéliales. Cet effet du PRRSV parait moins important en augmentant le délai entre les inoculations virales. Finalement, une série d'expérimentations nous a permis d'identifier les agents pathogènes circulant chez des porcs provenant d'un abattoir local et d'évaluer l'effet des différentes infections bactériennes et virales résolues ou pas sur l'immunité entraînée des macrophages alvéolaires et leur capacité à répliquer les virus suite à une surinfection. Ces travaux contribuent à la compréhension de la réponse immune porcine suite aux co-infections respiratoires, pour une meilleure gestion des maladies respiratoire chez le porc.

Title: Co-infections of porcine respiratory cells and tissues with the influenza A virus and the Porcine reproductive and respiratory syndrome virus

Keywords: Pig, coinfections, swIAV, PRRSV, interference, innate immunity

Abstract: Respiratory co-infections in pigs are more common than infections caused by a single pathogen. First of all, we identified the viral and bacterial porcine co-infections studies and we detailed the possible molecular consequences on the porcine host. The porcine reproductive and respiratory syndrome virus (PRRSV) and the swine Influenza A Virus (swIAV), are major contributors to the porcine respiratory disease complex. SwIAV primarily infects epithelial cells while PRRSV infects cells expressing CD163 such as alveolar macrophages (AM). In order to evaluate the antiviral response of the porcine host and to study the effect of a pre-infection with PRRSV on the replication of swIAV, a series of co-infections and superinfections were carried out on tracheal epithelial cells and precision-cut lung slices. The results showed that PRRSV can interfere with swIAV infection and alter the cellular antiviral response without infecting epithelial cells. This effect of PRRSV appears to be less important following an increase in the delay between viral inoculations. Finally, a series of experiments enabled us to identify the pathogens circulating in pigs from a local slaughterhouse and to assess the effect of the various bacterial and viral infections, on the alveolar macrophages trained immunity and their ability to replicate viruses in case of superinfection. This study contributes to the understanding of porcine immune response to respiratory coinfections for a better management of respiratory diseases in swine.