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Étude comparative de la réponse immune innée à une souche porcine d'influenza de sous-type H3N2 et implication potentielle des protéines SOCS

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Résumé

La grippe porcine est une maladie endémique aux conséquences économiques importantes. Le porc constitue un hôte incontournable dans l'écologie des virus influenza. À ce jour, de nombreuses questions relatives à l'immunologie porcine restent à élucider. Parmi les protéines impliquées dans la régulation de la réponse immune, les protéines *suppressors of cytokine signaling* (SOCS) et la *cytokine-inducible SH2 domain containing protein* (CISH) occupent une place centrale. Actuellement, peu d'informations sont disponibles sur ces protéines dans l'espèce porcine.

L'expression des ARNm de SOCS à l'homéostasie a été analysée. Une expression significative des transcrits de SOCS1 a été retrouvée dans le thymus suggérant un rôle de cette protéine dans la différenciation des cellules T comme décrit dans d'autres espèces. De plus, des niveaux d'expression faible de SOCS1 et SOCS3 ont été observés au niveau respiratoire indiquant une expression inductible de celles-ci dans ces tissus.

Afin d'avoir une représentation globale de la réponse immune innée contre une souche de SIV de sous-type H3N2, des cellules épithéliales (NPTr), des macrophages alvéolaires et des explants pulmonaires ont été utilisés. L'expression significative de transcrits impliqués dans la réponse antivirale ainsi que des transcrits de SOCS a été mesurée. Dans les cellules NPTr, l'activation de MAPK et JAK/STAT a été observée après infection. L'expression des transcrits antiviraux et des transcrits de SOCS a été évaluée suite au blocage spécifique des voies. L'inhibition de la voie JAK/STAT réduit significativement l'expression des interférons de type I et III ainsi que l'expression des ARNm de SOCS1. L'ensemble des résultats a contribué à la caractérisation de la réponse immune contre le SIV et pourrait aider à identifier de nouvelles stratégies pour lutter contre l'infection.

Afin de développer un outil alternatif pour l'analyse *in vitro* de la réponse immune innée, la culture en interface air-liquide (ALI) des cellules NPTr a été réalisée. Le but de notre étude a été d'évaluer si les cellules NPTr pouvaient être différenciées dans ces conditions. Des cellules à mucus, des jonctions serrées et une résistance transépithéliale élevée ont été observées après 3 semaines de culture. Cependant, les cellules NPTr n'ont pas développé de cils. La culture des cellules NPTr dans des conditions ALI, a permis une représentation partielle de l'épithélium respiratoire porcin et constitue ainsi une alternative d'étude *in vitro*.

Mots-clés : Influenza, porc, homéostasie, réponse immune innée, SOCS, signalisation, différenciation, interface air-liquide.

Abstract

Swine flu is an endemic disease causing important economic consequences. Pigs play an important role in Influenza ecology. Many questions about swine immunology still have to be answered. Amongst the proteins involved in immune response regulation, suppressors of cytokine signaling (SOCS) and the cytokine-inducible SH2 domain containing protein (CIS) are key regulators of the immune system. Little is known about tissue expression of SOCS and data in pigs are scarce.

Constitutive mRNA expression of SOCS was assessed. The highest mRNA expressions of SOCS1 were observed in thymus suggesting a key role of SOCS1 in T cell differentiation as described in other species. Low levels of expression of SOCS1 and SOCS3 were observed in the respiratory tract suggesting an inducible expression in these tissues.

In order to have a global representation of the immune response to a SIV subtype H3N2, newborn porcine epithelial cell line (NPTr), porcine alveolar macrophages and precision-cut lung slices were used. Significant mRNA expression of genes involved in the antiviral response and SOCS were measured. In NPTr cells, the activation MAPK and JAK/STAT was observed. Fallowing the blocking of each signaling pathways, the expression of antiviral genes and SOCS mRNAs were assessed. Inhibition of JAK/STAT impairs significantly the expression of interferons type I and III and also SOCS1 mRNAs. Together, these results could help to identify new strategies to fight the infection.

In order to provide a practical tool to analyze the immune response of porcine respiratory epithelial cells against different pathogens, we adjusted the air-liquid interface (ALI) *in vitro* culture system to NPTr cells. The purpose of our technical study was the evaluation and characterization of diverse aspects of cell differentiation in different conditions. We evaluated the presence of goblet cells, the epithelium junctional organization and the transepithelial electric resistance. We found in our conditions that the epithelium presents a variable density of mucus cells and a constant transepithelial resistance. We also observed a similar development of the tight junctions during time. This culture system allows a partial *in vitro* representation of porcine upper airway tissue that can be used advantageously to investigate host/pathogen interactions in pigs *in vitro*.

Keywords: Influenza, pig, homeostasis, innate immune response, SOCS, signaling, air-liquid interface.

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Introduction

1. Virus Influenza

1.1. Taxonomie

Les virus influenza appartiennent à la famille des *Orthomyxoviridae* et comprennent différents genres, nommés influenza A, B et C (Webster et al. 1982). Ces virus sont à l'origine de la grippe, manifestation pathologique caractérisée par une haute morbidité et des pertes économiques importantes dans les espèces cibles. Les types B et C sont reliés spécifiquement à l'homme tandis que les types A ont la capacité d'infecter d'autres espèces animales, comme par exemple les porcs, les chevaux, des mammifères marins et des espèces d'oiseaux. Les virus influenza A sont classés en fonction de leur sérotype, basé sur les deux glycoprotéines virales de surface. Seize hémagglutinines (HA) (H1-H16) et neuf neuraminidases (NA) (N1-N9) ont été décrites principalement chez l'avifaune sauvage, mais seulement trois sous types de HA (H1, H2 et H3) et deux NA (N1 et N2) ont été décrits chez l'humain depuis 1918 (Medina and Garcia-Sastre 2011, Nicholson, Wood and Zambon 2003).

1.2. Épidémiologie

Le comportement épidémiologique du virus est déterminé par deux types de variation antigénique des glycoprotéines d'enveloppe. Avec le premier type de variation, la dérive antigénique, de nouvelles souches du virus apparaissent suite à l'accumulation des mutations dans les séquences codant pour les glycoprotéines de surface. Les nouvelles souches sont des variantes antigéniques des souches ayant circulé lors des épidémies précédentes . Cette particularité permet au virus d'échapper à la reconnaissance du système immunitaire ce qui peut entrainer le déclenchement de nouvelles épidémies. La cassure antigénique se produit lors d'une nouvelle émergence, potentiellement pandémique, d'un virus possédant une nouvelle HA, une nouvelle NA ou à la fois une nouvelle HA associée à une nouvelle NA (Kuntz-Simon and Madec 2009, Webster et al. 1982).

Le terme pandémie fait référence à une émergence relativement rapide causant une épidémie de proportions mondiales consécutive à l'absence de protection immunitaire préalable au sein de la population. Chez l'homme, quatre ou cinq pandémies d'influenza ont été décrites pendant le 20ème siècle avec d'intervalles de 9 à 39 ans. La pandémie H1N1 de 1918 communément appelé « grippe espagnole » a été la plus importante avec environ 40-50

millions de morts (Taubenberger and Morens 2008). Les virus A et B circulent chaque année pendant la période hivernale dans les hémisphères nord et sud (McCaughey 2010).

Le suivi épidémiologique de la maladie est relativement difficile car le virus influenza n'est pas associé à des symptômes pathognomoniques et il peut co-circuler en association avec de nombreux autres pathogènes respiratoires. Il est estimé qu'annuellement 10% de la population mondiale est atteinte par la maladie conduisant à la mort d'environ 50000 personnes/an (Rossman and Lamb 2011, WHO 2010)Chez le porc, la maladie est caractérisée par une importante contagiosité qui est à l'origine de pertes économiques importantes dans les élevages affectés (Brown 2000).

1.3. Pathogénie

La transmission de la maladie est liée au mouvement de porcs d'un troupeau infecté vers un troupeau naïf. Elle est d'abord directe par le biais des aérosols formés lors d'épisodes de toux ou d'éternuements, ou indirecte après contact physique. Le virus pénètre dans les cavités nasales et infecte les muqueuses nasale, trachéale et bronchique. Un à trois jours après, il dissémine plus loin dans le tractus respiratoire, jusqu'aux alvéoles pulmonaires. Les signes cliniques, similaires à ceux de la maladie décrite chez l'humain, sont la toux (bien que ça dépende pour beaucoup du sous-type), l'éternuement, le jetage nasal, la fièvre, la léthargie, la dyspnée et l'anorexie avec une morbidité de 100% (Kuntz-Simon and Madec 2009, Madec et al. 1989). Des troubles reproductifs sont également parfois rapportés (Kuntz-Simon and Madec 2009). En l'absence de pathogènes opportunistes, la durée de la maladie est relativement courte. Après le début de l'infection, le virus est excrété pendant sept à dix jours dans la plupart des cas (Brown 2000). Les virus influenza du type A ont été déclarés enzootiques chez le porc et trois sous types (H1N1, H1N2 et H3N2) sont actuellement en circulation dans le monde. En France, les principaux sous-types détectés sont issus de réassortiment entre des souches porcines avec des souches aviaires de sous-type H1N1 et des souches humaines de sous-type H1N2 (Rose et al. 2013).

1.4. Structure

Structurellement, les virus influenza sont des virus enveloppés, dont le génome est formé de huit segments d'ARN monocaténaire de polarité négative qui habituellement codent pour 11 ou 12 protéines virales (Medina and Garcia-Sastre 2011). L'enveloppe virale, formée

Figure 1

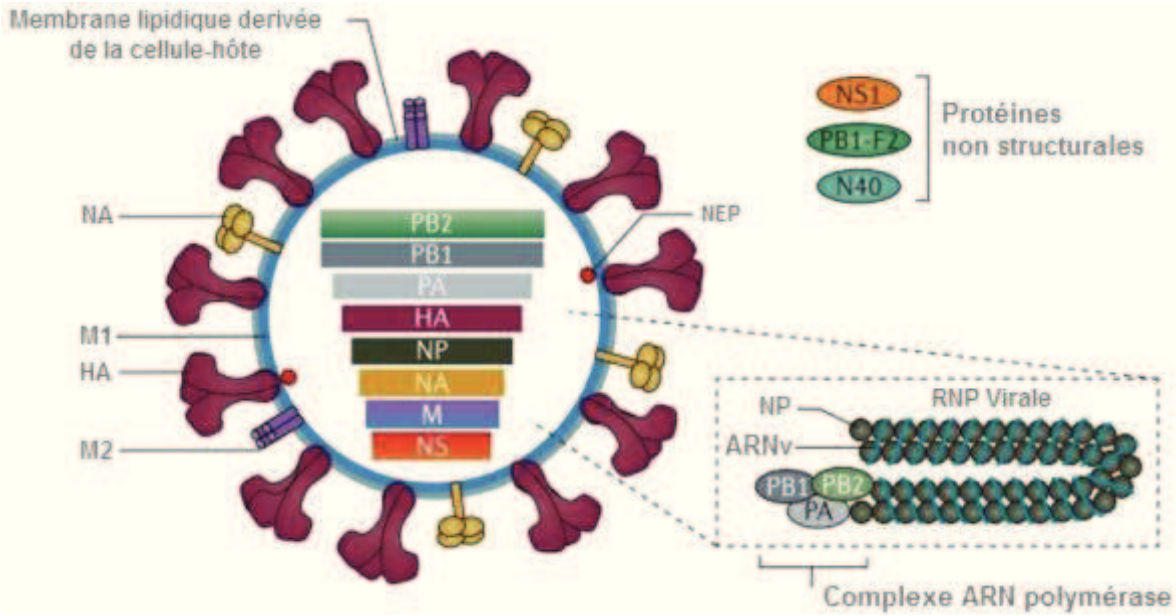

Figure 1. Structure des virus influenza, adapté de Medina, R. A., et Garcia-Sastre, A. (2011). *Nat Rev Microbiol.*

à partir de la membrane plasmique de la cellule hôte est une bicouche lipidique comprenant les glycoprotéines transmembranaires HA, NA et les protéines matricielles M1 et M2 (Webster et al. 1992). Le « corps » viral contient la ribonucléocapside, la nucléo protéine (NP), les protéines non-structurales 1 et 2 (NS1 et NS2) ainsi que les trois polymérases (PB1, PB2 et PA) qui forment le complexe ARN polymérase (Nayak, Hui and Barman 2004, Arranz et al. 2012, Crisci et al. 2013) (Figure 1).

1.4.1. Glycoprotéines d'enveloppe

1.4.1.1. Hémagglutinine

L'hémagglutinine est une protéine présente à la surface du virus (Webster et al. 1992). Elle est encodée par le quatrième segment du génome viral et constitue une des plus importantes protéines d'enveloppe. En effet cette protéine permet au virus de cliver les récepteurs cellulaires de l'hôte constitués par les acides sialiques (AS) (Weis et al. 1988). Cette interaction est essentielle pour la fusion du virus avec la membrane cellulaire. Il existe une grande variété d'AS, définie par le type de liaison entre l'AS et le galactose : α2,6 ou α2,3, mais aussi par la nature de l'AS et celle du sucre sous-jacent (Nicholls et al. 2008). Les HA de virus humains se lient préférentiellement aux ASα2,6Gal alors que les virus aviaires se lient aux ASα2,3Gal. Cela participe à la constitution d'une barrière naturelle inter-espèce limitant le risque d'infection des humains par des virus aviaires. Les virus influenza ont non seulement la capacité de se répliquer dans les cellules épithéliales du système respiratoire supérieur mais également dans les monocytes/macrophages ainsi que dans d'autres leucocytes (Ronni et al. 1995, Roberts and Horan 1985). Chez le porc, les deux types d'AS α2,6 et α2,3 sont présents dans la portion inférieure du tractus respiratoire ce qui explique la susceptibilité de cet animal aussi bien aux virus humains qu'aux virus aviaires (Ito et al. 1998, Punyadarsaniya et al. 2011). Cette particularité du porc favoriserait la transmission et l'adaptation à l'homme de virus porcins contenant des éléments aviaires. C'est pour cela que la notion de « *mixing vessel* » est souvent associée au porc.

1.4.1.2. Neuraminidase

La NA des virus influenza est une glycoprotéine membranaire (Webster et al. 1992). Elle est constituée par quatre polypeptides identiques et est hautement conservée entre les virus influenza de type A et B. La NA facilite la pénétration à travers les mucines riches en

AS présentes dans le tractus respiratoire, augmentant l'infectiosité du virus. De plus, la NA est impliquée dans la libération du virus par l'hydrolyse des liaisons entre un AS et l'hydrate de carbone adjacent de la glycoprotéine de surface (Air 2012). La NA a été identifiée comme cible thérapeutique potentielle depuis les années 70s en raison de son rôle majeur dans la propagation des virus influenza et en raison de son domaine cytoplasmique N-terminal, constitué de six acides aminés (MNPNQK), hautement conservé dans presque 100% des virus influenza de type A et B (Air 2012) (Tableau 1).

1.4.2. Complexe viral ribonucléoprotéique

Le complexe ribonucléoprotéique (RNP) appelé aussi complexe viral ARN polymérase est formé de l'ARN viral (ARNv), d'une polymérase virale hétérotrimérique constitué par (PA, PB1 et PB2) et de nombreuses copies de NP (Arranz et al. 2012). Le RNP du virus influenza joue un rôle clé pendant le cycle d'infection viral. Ce complexe macromoléculaire est responsable à la fois de la réplication du génome viral mais aussi de sa transcription en ARN messagers (ARNm) dans le noyau cellulaire (Nayak et al. 2004). Une fois à l'intérieur du noyau, la RNP sert de matrice pour la synthèse d'ARN viral. Les ARNm viraux produits sont reconnus comme des ARNm endogènes par la machinerie cellulaire et traduits en protéines (Portela and Digard 2002). Dans la phase de réplication, une première copie complémentaire de l'ARNv est synthétisée puis encapsidée par l'ARN polymérase et la NP. Finalement, l'ARN complémentaire (ARNc) sert de matrice pour la synthèse de nouveaux ARN viraux à l'origine des nouveaux virions (Vreede and Brownlee 2007).

1.4.3. Nucléo protéine

Dérivée du complexe viral ARN polymérase, NP n'est pas seulement une protéine structurale du virus, elle est également une protéine multifonctionnelle constituée de 498 acides aminés qui interagit avec d'autres protéines virales (ex : PB1, PB2, MA) et des protéines de l'hôte (ex : l'hélicase BAT1/UAP56 et F-actine) pour réguler la transcription virale, l'importation et l'exportation nucléaire des RNPs (Portela and Digard 2002) (Figure 2).

1.4.4. Protéines matricielles M1 et M2

Codées par le segment 7 du génome viral, les protéines matricielles sont activement impliquées dans le cycle de transcription et réplication viral (Rossman and Lamb 2011). M1

Tableau 1. Protéines codées par les virus influenza A, leurs fonctions et leur sensibilité potentielle.

Protéine	Segment	kDa	Fonction majeure	Cible antivirale
HA	4	64	Attachement à la cellule hôte, assemblage des membranes virale et cellulaire	Inhibiteurs de fusion (ex : Tert-butyl hydroquinone)
NA	6	50	Clivage des acides sialiques, relâchement des virions	Inhibiteurs de neuraminidase (ex : oseltamivir)
PB1	2	87	Composante de la polymérase	Interaction entre PB1-PA
PB2	1	86	Composante de la polymérase	*Cap-binding*
PA	3	83	Composante de la polymérase	Interaction entre PB1-PA, endonucléase
NP	5	56	Encapsidation de l'ARN, composante de la RNP	Ciblage de l'ARN ?
M1	7	28	Protéine matricielle, support structural du virion	Oligomérisation ?
M2	7 (épissée)	11	Canaux ionique, dissociation des composants viraux pendant la décapsidation	Inhibiteurs des canaux ioniques (ex : amantadine)
NS1	8	26	Antagoniste de la réponse immune de l'hôte	Ciblage de l'ARN ciblage de CPSF30
NEP	8 (épissée)	14	Exportation nucléaire des RNPs	Sites d'interaction de RNP ?
PB16F2	2	8	Induction de l'apoptose des cellules immunitaires	Sites d'interaction avec les protéines cellulaires

Hale, B. G., Albrecht, R. A., et Garcia-Sastre, A. (2010), *Future Microbiol* 5, 23-41

est localisée en dessous de la membrane lipidique virale, formant la matrice dans laquelle les ribonucléoprotéines virales du complexe viral ARN polymérase se retrouvent (Nayak et al. 2004). La protéine M2 communément décrite comme un canal ionique, possède au moins trois fonctions grâce à ses segments intra, extra et transmembranaires (Cross et al. 2012). Elle est impliquée principalement dans l'entrée, l'assemblage et le bourgeonnement viral (Rossman and Lamb 2011). Dans l'environnement acide de l'endosome, M2 facilite la conduction de protons dans la membrane virale pour dissocier le complexe viral RNP de la protéine M1. Ensuite, le virion peut aller vers le noyau cellulaire et déclencher la réplication virale (Pinto and Lamb 2006, Rossman and Lamb 2011, Takeda et al. 2002) (Figure 2).

1.4.5. Protéine non-structurale NS1

La protéine non structurale 1 (NS1) est codée par un ARNm dérivé du huitième segment de l'ARNm viral. NS1 n'est pas une protéine structurale du virion mais elle est massivement exprimée dans les cellules infectées (Garcia-Sastre 2001). Elle est constituée par une extrémité N-terminale *RNA-binding* et une extrémité C-terminale *effector*. Cette dernière permet d'interagir avec les protéines de l'hôte et de stabiliser son extrémité N-terminale (Wang et al. 2002). Il s'agit d'une protéine multifonctionnelle qui est impliquée dans divers mécanismes permettant d'améliorer l'efficacité de la réplication virale. Elle intervient dans la régulation de la synthèse, le contrôle de l'épissage et de la traduction de l'ARNm viral, ainsi que dans la régulation de la morphogenèse des particules virales. Chez l'hôte, NS1 est impliquée dans la suppression de la réponse immune et dans l'activation de la voie phosphoinositide 3-kinase (PI3K) ayant pour but de diminuer l'apoptose cellulaire (Hale et al. 2008). Afin de supprimer la réponse immune, NS1 peut se lier aux ARN viraux double-brin et diminuer l'activation des protéines impliquées dans la réponse antivirale comme la *2'5-olygoadenylate synthetase* (OAS) et la protéine kinase R (PKR) (Garcia-Sastre 2001) (Figure 3). De plus, au niveau transcriptionel, NS1 peut inhiber l'expression d'interférons (INF) en se liant avec des facteurs cellulaires impliqués dans la terminaison de la transcription et la polyadénylation tel que *cleavage polyadenylation stimulating factor* (Nemeroff et al. 1998). NS1 peut également se lier aux facteurs d'exportation nucléaire afin d'inhiber la migration des ARNm cellulaires vers le cytoplasme (Satterly et al. 2007).

Figure 2

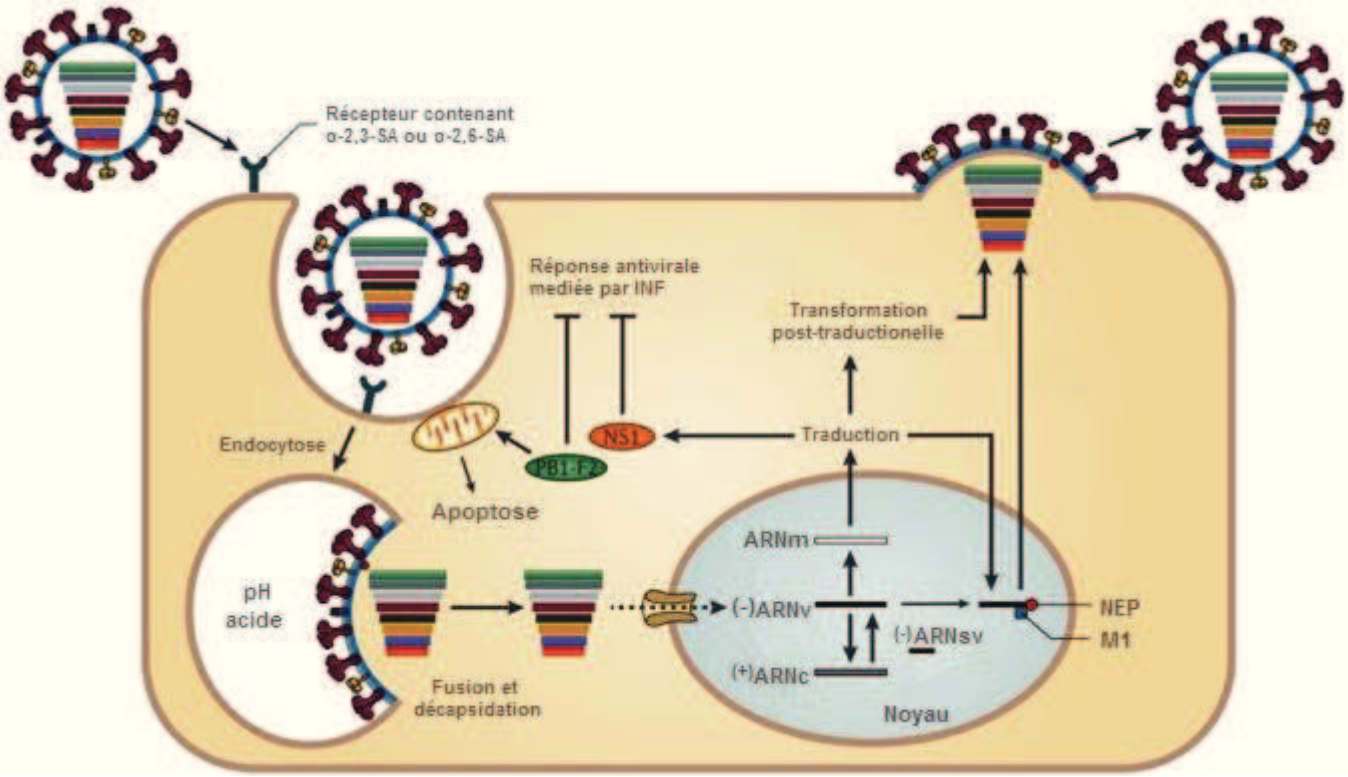

Figure 2. Représentation du cycle de réplication du virus influenza, adapté de Medina, R. A., et Garcia-Sastre, A. (2011). *Nat Rev Microbiol*

2. Importance du porc dans la transmission inter-espèces

La transmission des virus porcins est généralement permise par l'introduction de nouveaux porcs dans un élevage naïf (Brown 2000). Une fois l'élevage infecté, le virus persiste suite à la production de porcelets susceptibles et au renouvellement de l'élevage avec l'entrée de nouveaux animaux (Brown 2000). Habituellement, la maladie se présente simultanément dans plusieurs fermes de la même zone géographique et des épisodes annuels aigus peuvent être observés. La principale voie de transmission est la voie naso-pharyngale après contacts directs entre les animaux. La transmission se fait également, par les secrétions nasales ou par des gouttelettes ou aérosols contaminés. Des facteurs de risque tels que des densités excessives d'animaux, des situations de stress, les changements climatiques ou autres changements environnementaux participent à la diffusion du virus (Brown 2000).

La transmission inter-espèces commence tout d'abord par la transmission par voie oro-fécale du virus entre oiseaux aquatiques sauvages (Munster and Fouchier 2009, Munier et al. 2010). Ces animaux constituent le réservoir naturel où les virus influenza se développent de manière asymptomatique dans le tractus digestif. Apres l'introduction chez une nouvelle espèce animale, les virus subissent une adaptation rapide grâce à la sélection positive de variations génétiques qui ensuite vont favoriser leur transmission et leur adaptation vers d'autres hôtes.

Étant donné sa capacité à être infecté par les virus influenza A humains et aviaires, le porc joue un rôle important dans l'écologie du virus. Des réassortiments génétiques entre des virus grippaux d'origines diverses ont été documentés dans cette espèce. Actuellement les virus influenza circulant chez les porcs sont principalement les sous-types H1N1, H1N2 et H3N2 (Brown 2000, Munier et al. 2010). De multiples introductions de virus humains et aviaires chez le porc et des épisodes de réassortiments génétiques, sont à l'origine de plusieurs lignages différents au sein de chacun des trois sous-types de virus porcins. C'est pour cela que les réassortants identifiés chez le porc sont communément appelés virus *avian-like* ou *human-like* selon leurs fonds génétiques.

Des études séroépidémiologiques indiquent que la transmission des virus porcins à l'homme est plus fréquente chez des personnes travaillant en contact direct avec les porcs (Olsen et al. 2002). Quelques cas sporadiques associés à un syndrome grippal sans gravité et sans transmission inter-humaine efficace ont été observés. Cependant il y a des exceptions tel

Figure 3

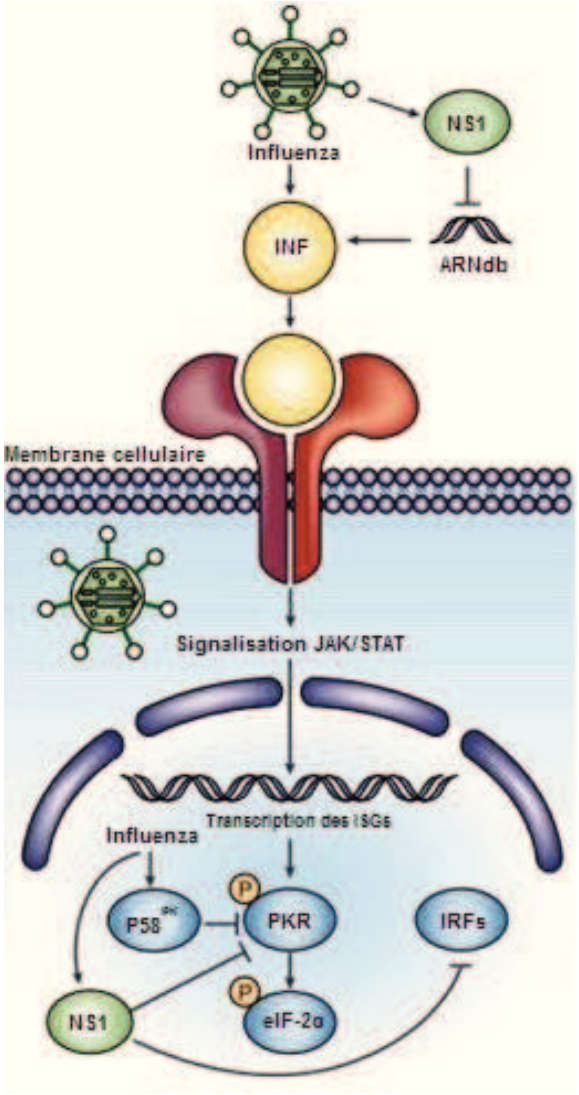

Figure 3. Interaction entre la voie de l'INF de type I et le virus influenza. La protéine non structurale NS1 peut se lier aux ARNdb et aussi diminuer l'activation des protéines impliquées dans la réponse antivirale comme la protéine kinase R. Adapté de Katze, (2002) Nat Rev Immunol

l'épisode grippal de 1976 aux Etats unis (Taubenberger and Morens 2008) et plus récemment en février 2009 la pandémie de la « grippe mexicaine» déclenchée par un triple réassortant constitué d'éléments, aviaires, porcins et humains (Medina and Garcia-Sastre 2011, Itoh et al. 2009). Ce virus a été découvert dans la ville de La Gloria (Veracruz, Mexique) (Fraser et al. 2009). Suite au réassortiment, le virus possédait des gènes d'origine aviaire (PB2 et PA), un gène PB1 d'origine humaine provenant d'un virus H3N2, une HA (H1), NP et NS d'un virus porcin classique H1N2 et finalement une NA (N1) et M provenant d'un virus eurasien *avian-like* porcin (Neumann, Noda and Kawaoka 2009). Caractérisée par une extension géographique rapide, la pandémie grippale de 2009 n'a cependant généralement pas occasionné de manifestations cliniques graves excepté dans certaines populations à risque comme les enfants, les personnes âgées et les personnes immunodéprimées (Medina and Garcia-Sastre 2011).

3. Co-infection

Un scénario pouvant occasionner des problèmes de santé publique ou porcine plus important est la co-infection du virus influenza avec d'autres pathogènes respiratoires. Ainsi, les co-infections avec des bactéries sont à l'origine de nombreux décès particulièrement chez les sujets âgés. Chez l'humain, chaque année les infections par le virus influenza associées à des pneumonies bactériennes sont responsables de la mort de 20000 individus (Pasman 2012, Bakaletz 2004). Suite à l'affaiblissement des défenses de l'hôte, des bactéries appartenant à la flore commensale de la partie supérieure du tractus respiratoire peuvent migrer vers de nouvelles niches écologiques au niveau inférieur du tractus respiratoire dans lequel elles deviennent dangereuses pour l'hôte. Parmi ces bactéries *Staphylococcus aureus*, *Streptococcus pneumoniae* et *Haemophilus influenzae* sont les plus couramment retrouvées (Pasman 2012). Plusieurs facteurs sont favorables aux co-infections. Parmi ceux-ci on retrouve la suppression/modulation de la réponse immune occasionnée par l'infection primaire (notamment par le virus responsable du syndrome dysgénésique et respiratoire porcin, SDRP) ou encore le passage à la chronicité de certaines infections qui favorise à la longue l'établissement d'infections secondaires.

Les co-infections jouent un rôle déterminant lors des épizooties respiratoires porcines. Les virus influenza sont impliqués dans le complexe respiratoire porcin (PRDC), caractérisé par des retards de croissance, de l'anorexie, de la fièvre, des éternuements et de la dyspnée (Deblanc et al. 2012, Hansen et al. 2010). Le PRDC est une maladie multifactorielle du porc qui se caractérise par des épisodes d'infection successifs impliquant des nombreux virus et bactéries respiratoires. Les agents pathogènes les plus fréquemment retrouvés dans ce complexe sont, le virus du SDRP, le circovirus porcin de type 2 (PCV-2), le coronavirus respiratoire porcin (PRCV), *Mycoplasma hyopneumoniae* (Mhp), *Pasteurella multocida*, *Streptococcus suis*, *Haemophilus parasuis* et *Actinobacillus pleuropneumoniae* (Loving et al. 2010, Thacker, Thacker and Janke 2001, Deblanc et al. 2012, Wei et al. 2010). Parmi ces pathogènes, Mhp et le virus influenza porcin (SIV) sont les plus souvent retrouvés chez les porcs entre 10 et 22 semaines d'âge (Thacker et al. 2001). Mhp et SIV affectent de façon similaire les cellules épithéliales et l'appareil muco-ciliaire du système respiratoire, entrainant *in fine* le développement d'une pneumonie suite à l'infection secondaire par les pathogènes opportunistes comme *H. influenzae*, *S. pneumoniae* ou *S. aureus* (Loving et al. 2010, Braciale, Sun and Kim 2012, Pasman 2012). Par ailleurs, l'évolution de l'infection peut différer selon

le pathogène, l'infection par SIV se caractérise par un épisode aigu d'évolution rapide tandis que l'infection par Mhp prend plutôt une forme chronique (Yazawa et al. 2004). Etant donnée les différences de dynamique entre les deux pathogènes, l'infection par SIV survient le plus souvent suite à une primo infection par Mhp, exacerbant les symptômes respiratoires.

4. Réponse immune innée

La réponse immune innée constitue, la première ligne de défense contre les virus, elle est caractérisée par sa rapidité, son caractère non spécifique et l'absence de mémoire. La réponse innée est essentielle pour initier la réponse adaptative et contrôler efficacement l'infection virale. Le mucus et les collectines constituent les premiers éléments de la barrière naturelle empêchant le contact direct entre le virus et les cellules cibles. Parmi les collectines, la protéine surfactante D (SP-D) possède la capacité de se fixer à HA et d'inhiber l'activité de NA. Chez le porc, Hillaire et collaborateurs ont démontré, *in vitro*, la capacité de la SP-D porcine d'empêcher l'adhérence des virus influenza humains saisonniers H1N1 et H3N2 aux récepteurs des cellules épithéliales du système respiratoire supérieur des furets et des humains (Hillaire et al. 2011). Après avoir franchi cette première barrière de défense, le virus atteint les cellules épithéliales respiratoires. Ensuite après infection et lyse des cellules épithéliales, le virus passe ensuite aux macrophages alvéolaires et aux cellules dendritiques résidentes des voies respiratoires.

4.1. Cellules épithéliales

Le tractus respiratoire comprend un épithélium de type pseudostratifié (Sanders, Doherty and Thomas 2011). Parmi les différents types cellulaires de l'épithélium on retrouve des cellules ciliées impliquées dans l'évacuation des particules, des cellules caliciformes également appelées *goblet cells,* productrices du mucus ; des cellules de Clara, localisées dans les bronchioles terminales, productrices du surfactant ; des cellules sensorielles neuro-épithéliales et finalement des cellules basales qui donneront naissance aux cellules de remplacement (Morrisey and Hogan 2010). La répartition entre les différents types cellulaire varie en fonction de la localisation dans le tractus respiratoire (Sanders et al. 2011). Au niveau alvéolaire, deux populations cellulaires principales sont présentes, les cellules alvéolaires de type I et celle de type II, *alveolar epithelial type I and II* (ATI et ATII). Les deux populations collaborent au maintien de l'homéostasie dans le poumon face à des agents étrangers comme le virus influenza. Les cellules ATI sont d'apparence fine et elles ont une surfaces apicale importante qui s'étale jusqu'à l'alvéole adjacente. Ces cellules constituent 10% de la population cellulaire alvéolaire et elles représentent 95% de la surface cellulaire en contact avec l'air inspiré (McElroy and Kasper 2004, Johnson et al. 2002). Par leur capacité à produire du surfactant, les ATII jouent un rôle important dans le transport transépithelial

d'ions dans l'alvéole et accessoirement elles servent de cellules souches pour le renouvellement des populations d'ATI et ATII (Sanders et al. 2011). Les cellules ATII constituent quant à elles seulement 5% de la surface alvéolaire et leur rôle principal est la protection de l'alvéole grâce à leur capacité à sécréter des quantités importantes de peptides antimicrobiens (Sanders et al. 2011).

4.1.1. Appareil muco-ciliaire

Ce système de protection est constitué des cils présents sur les cellules épithéliales respiratoires et du mucus secrété par les cellules caliciformes (Voynow and Rubin 2009, Vareille et al. 2011). L'appareil muco-ciliaire est responsable de la protection de la muqueuse par la création d'une barrière semi-perméable mobile qui permet l'échange sélectif de nutriments, eau et gaz, et empêche en même temps l'entrée des pathogènes. L'appareil muco-ciliaire permet aussi d'épurer par clairance muco-ciliaire jusqu'à 90% des particules inhalées grâce au battement des cils (Vareille et al. 2011). Le mucus secrété constamment par les cellules caliciformes et les cellules de la sous-muqueuse épithéliale est composé d'environ 200 protéines comme les mucines, les substances antimicrobiennes (lysozymes et défensines), cytokines et protéines anti-oxydantes (Nicholas et al. 2006). Les mucines sont des glycoprotéines antivirales et anti inflammatoires qui grâce à leur interaction avec les immunoglobulines A (IgAs), les collectines et les défensines, contribuent à la réponse immune innée et dans une certaine mesure à la réponse adaptative. Actuellement chez l'homme, 11 mucines ont été décrites (MUC1, 2, 3, 4, 5AC, 5B, 6, 7, 8 ,13 et 19) (Vareille et al. 2011). Chez le porc, MUC2, MUC5AC et MUC5B ont été décrites dans la portion inférieure du tractus respiratoire (Kim et al. 2011a, Lacunza et al. 2009). Lors d'un fonctionnement normal de l'appareil muco-ciliaire, le mucus est évacué par les mouvements ciliaires (escalator muco-ciliaire). Tout cela demande un équilibre optimal entre la composition du mucus, le volume de liquide péri-ciliaire et la fréquence de battement des cils. Dans les processus infectieux comme celui occasionné par le virus influenza, cet équilibre peut être rompu suite par exemple à l'hyper-sécrétion de mucus. Ce déséquilibre s'accompagne de difficultés respiratoires manifestes suite au blocage partiel des voies respiratoires (Voynow and Rubin 2009, Tamura and Kurata 2004).

4.2. Reconnaissance virale

L'infection par le virus influenza peut être détectée par le système immunitaire de différentes façons. La première étape est l'identification de l'agent pathogène. Pour cela les récepteurs de reconnaissance de motifs microbiens *pathogen recognition receptors* (PRRs) présents dans ou sur les cellules vont reconnaitre l'agent agresseur grâce à ses motifs moléculaires *pathogen associated molecular patterns* (PAMPs). Cette reconnaissance aboutit au déclenchement d'une série d'événements de signalisation cellulaire aboutissant à la sécrétion de cytokines inflammatoires, d'interférons de type I ou III, de chimiokines et de peptides antimicrobiens. Trois catégories de PRRs sont impliquées dans la reconnaissance de l'ARN viral du virus influenza A et dans l'induction d'une réponse interféron : les *Toll like receptors* (TLRs), les récepteurs *retinoic acid inducible gene-I* (RLRs) et le *nucleotide oligomerization domain (NOD)-like receptors* (NLRs) (Blasius and Beutler 2010, Pang and Iwasaki 2011, Takeuchi and Akira 2009).

4.2.1. Toll like receptors

Capables de reconnaitre des motifs dérivés des bactéries, virus, levures et protozoaires, les TLRs sont capables d'identifier la plupart des agents pathogènes (Blasius and Beutler 2010, Medzhitov 2001). Cette famille de récepteurs se localise à la surface et à l'intérieur de la cellule. Lors de l'infection par le virus influenza, les TLRs sont les premiers récepteurs qui reconnaissent des motifs caractéristiques du virus. À cause de sa structure particulière, l'ARN viral est une cible de choix pour les récepteurs localisés au niveau des endosomes (Ehrhardt et al. 2010, Blasius and Beutler 2010, Heil et al. 2004). Ces récepteurs jouent un rôle très important dans la reconnaissance virale. TLR7 est un récepteur intracellulaire spécialisé dans la reconnaissance de l'ARN simple brin résultant de la dégradation du complexe RNP dans les endosmoses acidifiés (Lund et al. 2004). Constitutivement exprimé dans les cellules épithéliales alvéolaires et bronchiques, TLR3 est responsable de la détection de l'ARN double brin (Guillot et al. 2005). Pendant l'infection, la réplication du virus ARN positif simple brin s'effectue à partir d'un ARN double brin intermédiaire qui peut être reconnu tant par TLR7 que par TLR8 (Blasius and Beutler 2010). D'autres TLRs capables de reconnaitre le virus influenza sont les TLR2 et TLR4. Ils sont présents à la surface cellulaire et ils peuvent reconnaitre les glycoprotéines d'enveloppe HA et NA (Takeuchi and Akira 2009). TLR3 reconnait l'ARN double brin dans les cellules dendritiques (DC), tandis que TLR7 et TLR9 sont fortement exprimés dans les DC plasmacytoïdes, un type de cellule dendritique reconnue

Figure 4

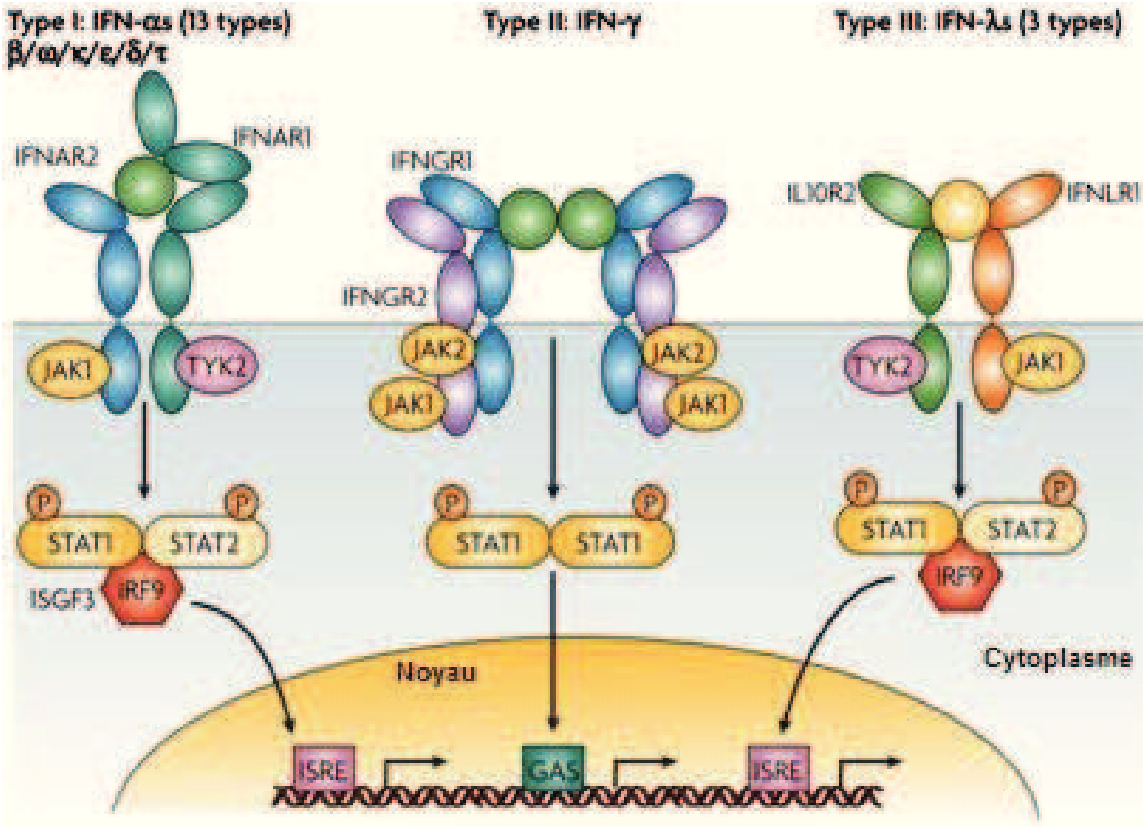

Figure 4. Activation des récepteurs des INF de type I, II et III. Les INF de type I se lient spécifiquement au *receptor complex* de l'INFα/β qui se divise en IFNAR1 et IFNAR2. L'interféron de type II (INFγ) interagit avec le récepteur IFNγ *receptor complex* IFNGR et les interférons de type III se lient au *receptor complex* IFNLR constitué par deux sous unités, IFNλR1 et IL10R2. Adapté de Borden, (2007) *Nat Rev Drug Discov*.

pour produire de grandes quantités d'INF de type I en réponse aux virus (Takeuchi and Akira 2009, Kariko et al. 2004). Récemment, Ioannidis et collaborateurs ont observé *in vitro* la présence de TLR3, TLR7 et TLR9 à la surface de cellules épithéliales différenciées, démontrant que la localisation de ces TLRs n'est pas exclusivement endosomale (Ioannidis et al. 2013).

4.2.2. RIG-I like receptors

Les RLRs sont une famille d'hélicases qui joue un rôle important comme senseurs cytoplasmiques pour initier et moduler la réponse antivirale. Actuellement, trois membres de cette famille ont été identifiés : *le retinoic acid-iducible gene I* (RIG-I), le *melanoma differentiation associated factor 5* (MDA5) et le *laboratory of genetics and physiology 2* (LGP2) (Ehrhardt et al. 2010, Meylan, Tschopp and Karin 2006). Ils sont exprimés dans la plupart des tissus où ils participent à l'induction de la réponse innée. Dans les tissus on les retrouve exprimés notamment dans les cellules myéloïdes, les cellules épithéliales et les cellules du système nerveux central (Loo and Gale 2011). Leur expression reste relativement faible mais elle peut augmenter significativement en présence d'INF suite notamment à une infection virale (Kang et al. 2004, Yoneyama et al. 2004, Loo and Gale 2011). Lors de l'infection par le virus influenza, RIG-I semble extrêmement sensible à la présence d'ARN génomique. La reconnaissance de l'ARN génomique est indépendante de la longueur de celui-ci. Un seul groupement phosphate à l'extrémité 5' de l'ARN suffit pour déclencher la signalisation par RIG-I (Pichlmair et al. 2006). Contrairement à RIG-I, il a été démontré *in vitro* que MDA5 reconnaissait préférentiellement des fragments de hauts poids moléculaire de poly(I:C) (Kato et al. 2006). Peu d'informations sont disponibles à propos des ligands de LGP2 mais étant donné l'absence de la région N-terminale *caspase activation and recruitment domain* (CARD) dans sa structure, LGP2 pourrait avoir des fonctions de régulation de RIG-I et MDA5 en masquant spécifiquement l'ARN cible pour réduire la reconnaissance par RIG-I et MDA5 (Yoneyama et al. 2005). En l'absence de ligands, RIG-I peut aussi auto-réguler son activité par des interactions intramoléculaires entre le domaine CARD et le domaine répresseur localisé en C-terminal *repressor domain* (RD) (Loo and Gale 2011).

4.2.3. NOD like receptors

Impliqué dans la reconnaissance des motifs viraux ADN et ARN, le *nucleotide oligomerization domain (NOD)-like receptors family pyrin domain containing 3* (NLRP3) reste jusqu'à présent le mieux étudié des membres de cette famille de récepteurs (Pang and Iwasaki 2011, Muruve et al. 2008). Il fait partie de l'inflammasome NLRP. Les inflammasomes sont des complexes cytoplasmiques qui jouent un rôle fondamental dans la reconnaissance et la défense contre les infections virales et notamment l'infection par le virus influenza (Pang and Iwasaki 2011, Schroder and Tschopp 2010). Ce sont des complexes multi-protéiques qui agissent comme des plateformes d'activation de la caspase-1, laquelle est impliquée dans la maturation et la sécrétion de cytokines pro-inflammatoires comme l'IL1β et l'IL18 dans l'espace extracellulaire (Schroder and Tschopp 2010). L'activation de l'inflammasome et la sécrétion d'IL1β conduit à la production de chimiokines intervenant dans le recrutement et le maintien dans le poumon de cellules inflammatoires comme les neutrophiles et les monocytes (Pang and Iwasaki 2011). Thomas et collaborateurs ont montré que l'absence de l'activité de NLRP3/caspase-1 dans la production de cytokines et chimiokines augmente la nécrose pulmonaire et réduit la capacité respiratoire, compliquant l'évolution de la maladie dans les premiers jours de l'infection. L'activation de l'inflammasome, augmente le recrutement de neutrophiles et monocytes par la production de davantage de chimiokines, ce qui améliore la réponse antivirale et contribue à réparer le tissu pulmonaire endommagé (Thomas et al. 2009).

4.3. Interférons

La famille des cytokines INF a été décrite par la première fois en 1957 par Isaacs et collaborateurs qui en évaluant l'inactivation du virus influenza à différentes températures dans des membranes chorio-allantoïques, ont observé l'apparition d'un facteur qui inhibait la réplication des virus (Isaacs and Lindenmann 1957). Actuellement, la famille INF est reconnue comme une pièce fondamentale de la réponse innée contre les infections virales. Trois classes d'INF ont été décrites par rapport au récepteur utilisé pour envoyer leur signal à la cellule et par rapport à leur séquence en acides aminés (Ioannidis et al. 2013). Ces trois classes sont connues sous le nom d'INF de type I, II et III (Randall and Goodbourn 2008). Malgré les différences parfois importantes constatées entre ces classes d'interféron, elles partagent néanmoins des voies de signalisation communes comme les *Janus Kinase/signal*

transducers and activators of transcription (JAK/STAT) en réponse principalement à la stimulation des TLRs (Noppert, Fitzgerald and Hertzog 2007).

4.3.1. INF de type I

Particulièrement impliqués dans la réponse antivirale, les INF de type I agissent rapidement en se liant spécifiquement à l'*INFα/β receptor complex* (IFNAR) qui se divise en IFNAR1 et IFNAR2 (Randall and Goodbourn 2008, Borden et al. 2007) (Figure 4). Actuellement, les INF de type I restent les interférons les mieux étudiés. Parmi les interférons de type I on distingue 9 sous-types d'INFα codés par 13 gènes localisés dans le chromosome 9 chez l'humain (Nyman et al. 1998), ainsi que des INFβ, INFκ, INFε, INFo, INFτ et INFδ (Sadler and Williams 2008, Randall and Goodbourn 2008). Actuellement chez le porc, 39 gènes fonctionnels de l'INF de type I on été identifiés. 17 INFα sous-types, 11 INFδ, 7 INFω, INFαω, INFβ, INFε et INFκ (Sang et al. 2010b). Chez le porc, seulement l'INFδ ne possède aucun homologue chez l'homme (Noppert et al. 2007)

Les INF de type I jouent plusieurs rôles à différents niveaux de la réponse immunitaire, d'abord pour contrôler le pathogène au site d'entrée, ensuite pour diminuer sa propagation, et finalement dans le développement d'une protection à plus long terme contre ce même pathogène. Les mécanismes par lesquels les INF entrainent l'inhibition de la réplication virale diffèrent selon le type du virus considéré et selon la population cellulaire impliquée (Sang et al. 2010b). Plusieurs étapes du cycle viral comme l'entrée, la transcription, l'initiation de la traduction, la maturation, l'assemblage et la libération sont contrôlées à différents niveaux par les *IFN-stimulated genes* (ISGs) dont la transcription est induite par les INF. Par ailleurs, les INF de type I sont impliqués dans la prolifération des cellules inflammatoires et la multiplication/survie des cellules parenchymateuses contribuant ainsi à une résolution plus rapide de la maladie (Noppert et al. 2007). D'autre part, dans certaines situations d'infection, les INF peuvent également réguler l'expression des molécules impliquées dans l'apoptose cellulaire comme le *interferon regulatory factor* (IRF1), le *signal transduction and activator of transcription 1* (STAT1), les caspases 4 et 8 et les ligands des récepteurs *tumor necrosis factor* (TNF) (*TNF-related apoptosis-inducing ligand* (TRAIL) et CD95L) pour protéger l'hôte en limitant la dissémination du pathogène (Noppert et al. 2007). En ce qui concerne le recrutement cellulaire, les INF de type I sont responsables de l'induction de récepteurs de chimiokines telles que CXCL10 et CCL12 qui sont impliquées

dans le recrutement des cellules *natural killer* (NK), des macrophages et des lymphocytes T (Salazar-Mather, Lewis and Biron 2002, Theofilopoulos et al. 2005, Noppert et al. 2007).

Les INF de type I sont aussi impliqués dans des nombreux processus liés à la réponse immune adaptative (Noppert et al. 2007). Ils participent à la maturation et à la survie des cellules T, à la génération et à l'activation de cellules dendritiques (CD) myéloïdes immatures ainsi qu'à la différenciation des cellules B par la stimulation de l'IL17 (Rogge et al. 1998).

4.3.2. INF de type II

Les INF de type II sont aussi impliqués dans la réponse antivirale mais également dans la réponse à d'autres types de pathogènes. Les INF de type II sont uniquement représentés par l'INFγ qui interagit avec le récepteur *IFNγ receptor complex* (IFNGR) (Shuai et al. 1992, Borden et al. 2007) (Figure 4). L'INFγ est produit par un groupe réduit de cellules, les cellules NK, *cluster of differentiation* 4 (CD4+) *helper* et les cellules CD8+ cytotoxiques. Cependant il possède principalement, une fonction immuno-régulatrice (Bonjardim 2005, George et al. 2012).

4.3.3. INF de type III

Plus récemment découverte, la famille des INF de type III est composée de trois membres (IFNλ1, 2 et 3) nommés aussi IL29, IL28A et IL28B respectivement. Chez le porc, deux INF de type III ont été identifiés : *Sus scrofa* INF-λ1 et *Sus scrofa* INF-λ3 (Sang, Rowland and Blecha 2010a). Ces interférons interagissent avec le complexe récepteur (IFNLR) constitué par deux sous unités, IFNλR1 et IL10R2 (Kotenko et al. 2003, Sheppard et al. 2003, Borden et al. 2007) (Figure 4). Les INF de type I et III partagent les mêmes activités biologiques que les INF de type I pendant l'infection mais leur distribution et régulation semblent différentes (Ioannidis et al. 2013). D'une part le récepteur pour les INF type I, IFNAR, est présent dans toutes les cellules tandis que l'expression d'IFNLR est limitée aux cellules épithéliales et aux cellules plasmacytoïdes (Ank et al. 2008). Lors d'infections *in vivo* par le virus influenza, Jewell et collaborateurs ont démontré que les INFλ sont produits en quantités plus importantes et plus longtemps par rapport aux INF de type I (Jewell et al. 2010, Ioannidis et al. 2013).

4.4. Gènes stimulés par les interférons

La liaison entre les INF de type I et leur récepteur IFNAR entraine l'activation du récepteur par la phosphorylation du résidu tyrosine présent au niveau de la portion intracellulaire du récepteur (Sadler and Williams 2008). Une fois phosphorylé, le récepteur devient un site de réception pour les facteurs de transcription STAT, qui vont ensuite se phosphoryler eux-mêmes. Ultérieurement, STAT se dissocie du récepteur IFNAR et migre vers le noyau sous forme homo ou hétéro-dimérique. Ensuite, à l'intérieur du noyau cellulaire, STAT se lie aux éléments agissant en *cis* du promoteur des *IFN-stimulated genes* (ISGs) pour induire la transcription de plus de 300 ISGs (Stark et al. 1998, Bonjardim 2005).

La plupart des ISGs ne sont pas directement impliqués dans la réponse antivirale, néanmoins, ils codent notamment pour des PRRs qui vont ensuite contribuer à augmenter la production d'INF grâce à la modification des voies de signalisation et des facteurs de transcription(Sadler and Williams 2008). D'autre part, des gènes stimulés par les INF codent pour des protéines qui induisent l'apoptose, régulent les processus post-transcriptionels (épissage, *editing* de l'ARNm, dégradation de l'ARN et traduction des protéines) (Sadler and Williams 2008). Parmi les ISGs qui jouent un rôle direct dans la réponse antivirale, *IFN-stimulated protein of 15 kilodaltons* (ISG15), la GTPase *myxovirus resistance 1* (Mx1), la ribonucléase L (RNaseL), OAS et PKR sont les mieux caractérisés (Randall and Goodbourn 2008, Sadler and Williams 2008).

4.4.1. IFN-stimulated protein of 15kDa

Nommée d'après la masse moléculaire de la protéine qui en est issue, ISG15 reste un des gènes les plus induits par les INF de type I. La protéine possède une activité ressemblant à l'ubiquitination appelée en anglais « *ISGylation* », mais contrairement à l'ubiquitination, cette activité n'aboutit pas à la dégradation de la protéine cible (Sadler and Williams 2008). En revanche, ISG15 diminue la dégradation de *l'IFN-regulatory factor 3* (IRF3) par le virus et finalement augmente l'expression des INFβ (Loeb and Haas 1992). De plus, lors d'expériences réalisées *in vitro* utilisant des cellules traitées avec des INF de type I, ISG15 était secrétée en grandes quantités et exerçait une activité similaire aux cytokines en modulant la réponse immune. Toutefois les mécanismes d'action précis restent encore à élucider (D'Cunha et al. 1996). Lors de l'infection par le virus influenza B, ISG15 est ciblée par la

protéine virale NS1 qui bloque la liaison covalente entre ISG15 et sa protéine cible et inhibe son activité (Yuan and Krug 2001).

4.4.2. MxGTPases

La famille des GTPases est constituée de MxA et MxB chez l'homme et de Mx1 et Mx2 chez la souris (Ehrhardt et al. 2010, Sadler and Williams 2008, Lindenmann 1962). À ce jour, parmi les virus identifiés comme sensibles à l'activité Mx sont retrouvés des *Orthomyxoviridae*, des *Paramyxoviridae*, des *Rhabdoviridae*, des *Togaviridae* et des *Bunyaviridae* (Sadler and Williams 2008). Les protéines Mx sont exprimées par différents types cellulaires comme les hépatocytes, les cellules endothéliales et dans quelques cellules immunitaires comme les monocytes, les cellules dendritiques plasmacytoïdes et les cellules myéloïdes (Fernandez et al. 1999). Elles sont principalement impliquées dans les phases précoces de l'infection virale. Situées dans le réticulum endoplasmique, les protéines Mx vont détecter la présence d'éléments viraux dans le cytoplasme et prévenir leur transcription (Sadler and Williams 2008). Mx1 inhibe l'activité virale par l'interaction avec la sous-unité PB2 du complexe viral ARN polymérase (Huang et al. 1992, Stranden, Staeheli and Pavlovic 1993). La protéine Mx1 porcine (poMx1) est capable d'intervenir au début de l'endocytose pour bloquer le passage de particules virales vers le noyau et inhiber la transcription virale (Palm et al. 2010).

4.4.3. Protéine kinase R

Pendant la réplication virale, PKR est produite en grandes quantités en réponse aux INF de type I et III induits par les ARNs double brin (Kerr, Brown and Hovanessian 1977, Sadler and Williams 2008). En condition normale, à l'homéostasie, PKR reste sous forme monomérique mais dès que l'infection commence, PKR se dimérise et s'active. PKR est une sérine/thréonine kinase caractérisée par deux activités kinases différentes: l'autophosphorylation pour déclencher la réaction enzymatique et la phosphorylation du *eukaryotic initiation factor 2* (eIF-2α) aboutissant à la diminution de l'activité eIF2 et à l'inhibition de la synthèse des protéines (Garcia et al. 2006). PKR est aussi impliquée dans l'inhibition de la prolifération cellulaire tumorale. Lors de l'infection par le virus influenza, NS1 est capable d'inhiber l'activité de PKR par inhibition de la signalisation antivirale dépendant des ARN double brin grâce à son *dsRNA-binding domain* (Garcia et al. 2006).

4.4.4. 2'5-olygoadenylate synthetase

La combinaison d'OAS et RNAseL permet de lutter efficacement contre l'infection virale. L'activation d'OAS permet la synthèse de liaisons 2'5-phosphodiester pour polymériser l'ATP en oligomères d'adénosine et ensuite activer RNAseL**.** L'augmentation de l'activité RNAseL entraine la dégradation des ARN cellulaires et viraux (Roberts et al. 1976, Rebouillat and Hovanessian 1999). De par leur expression constitutive, les protéines OAS peuvent avoir également une activité PRRs pour la détection d'ARN double brin dans le cytoplasme. L'ARN dégradé et libéré consécutivement à l'action de la RNAseL, constitue un élément activateur des PRRs, tels RIG-I et MDA5 qui vont ensuite encore augmenter l'induction des gènes des INF de type I (Sadler and Williams 2008).

4.5. Macrophages

En réponse à l'infection des alvéoles, les cellules épithéliales vont activer/recruter les macrophages alvéolaires et monocytes grâce à la production de cytokines et chimiokines comme l'IL1β, la *macrophage inflammatory protein-1-α* (MIP-1α)/CCL3, la *monocyte chemoattractant protein 1* (MCP-1)/CCL2 et le *tumor necrosis factor alpha* (TNFα) (Crisci et al. 2013). La deuxième ligne de défense de la réponse immune innée est activée (Kim et al. 2008). Les macrophages alvéolaires recrutés à l'endroit de l'attaque vont être activés et vont éliminer les cellules infectées par le pathogène et ainsi limiter la dissémination de l'infection vers d'autres populations cellulaires. Lors de l'infection par le virus influenza, les macrophages continuent à produire des cytokines pro-inflammatoires comme le TNFα, l'IL1, l'IL6, des INF de type I et des chimiokines (McGill, Heusel and Legge 2009, Damjanovic et al. 2012). D'une part, les macrophages vont présenter des antigènes viraux aux cellules de la réponse immune adaptative et d'autre part, ils vont être aussi impliqués dans l'activation des cellules NK, la régulation de la réponse Th1, la survie des cellules B et la production des anticorps par la production d'IL12 et d'IL10 (Kreijtz, Fouchier and Rimmelzwaan 2011). De plus, les macrophages alvéolaires ainsi que les cellules épithéliales peuvent produire du *nitric oxide synthase 2* (NOS2) qui est impliqué principalement dans la modulation de diverses activités rencontrées dans les voies aériennes comme les tonus musculaire et vasculaire, la motilité ciliaire et la viscosité muqueuse (Message and Johnston 2004).

4.6. Cellules dendritiques

Localisées en-dessous de la barrière épithéliale et au-dessous de la membrane basale, les CD surveillent la lumière du tractus respiratoire grâce à leurs dendrites qui passent à travers les jonctions serrées entre les cellules épithéliales (Kreijtz et al. 2011). En présence de cellules infectées, de virions ou de corps apoptotiques, les CD exercent leur activité de détection et de neutralisation par opsonisation (Tamura and Kurata 2004). Les CD migrent ensuite via le système lymphatique afférent vers le ganglion. Les CD y dégradent les protéines virales et les immuno-peptides résultants sont présentés (GeurtsvanKessel and Lambrecht 2008). Dans le ganglion, les CD peuvent présenter les antigènes viraux grâce au complexe majeur d'histocompatibilité (CMH) aux cellules T et les activer (Crisci et al. 2013). Pour la présentation de molécules via le CMH de type I, les peptides dérivés du virus seront libérés dans le cytosol et transportés vers le réticulum endoplasmique où ils sont associés aux molécules CMH de type I et forment ainsi les complexes CMH/antigène. Ultérieurement, les complexes sont transportés via l'appareil de Golgi vers la membrane plasmique où ils sont reconnus par les cellules T cytotoxiques CD8+. Lors de la présentation des molécules via le CMH de type II, les protéines virales sont dégradées au niveau des endosomes/lysosomes où elles libèrent des peptides qui s'associent aux molécules CMH de type II (Kim, Sun and Braciale 2011b). Les complexes résultants sont ensuite transportés vers la membrane plasmique où ils sont reconnus par les cellules T *helper* CD4+ (Kreijtz et al. 2011).

4.7. Cellules *natural killer*

Les cellules NK sont des effecteurs majeurs de la réponse immune innée qui se retrouvent dans l'ensemble des tissus lymphoïdes et non lymphoïdes de l'organisme. La cytotoxicité et la production des cytokines/chimiokines (INF de type II) sont les principales fonctions de cette population cellulaire (Biron 1997). Ces fonctions sont régulées par le récepteur d'activation NKG2D et le récepteur d'inhibition Ly49 (Lanier 1998). Les cellules NK peuvent reconnaitre des ligands non liés au CMH grâce à leurs récepteurs de cyotoxicité NKp30, NKp44, NKp46 et CD16. Pendant l'infection par le virus influenza, les cellules NK peuvent reconnaitre HA avec leurs récepteurs NKp44 et NKp46 et induire la lyse cellulaire par un processus appelé *antibody-dependent cell cytotoxicity* (ADCC) (Arnon et al. 2001).

5. Réponse immune adaptative

La réponse immune adaptative est déclenchée suite à la reconnaissance de l'élément étranger par le système immunitaire inné. Elle constitue le deuxième versant de la réponse immune aux agresseurs extérieurs, virus et autres (Braciale et al. 2012). Elle est constituée d'une composante humorale avec des anticorps spécifiques du pathogène et d'une composante cellulaire avec l'immunité à médiation cellulaire et principalement les cellules T.

5.1. Immunité humorale

L'infection par le virus influenza conduit finalement à la production d'anticorps spécifiques (IgM, IgG et IgA essentiellement) dirigés principalement contre les glycoprotéines de surface, notamment HA (Gerhard et al. 1997, Mancini et al. 2011). Les anticorps spécifiques de l'HA se fixent à la tête trimérique globulaire de celle-ci et empêchent l'attachement et l'entrée du virus dans la cellule hôte (Mancini et al. 2011). Par ailleurs, ces anticorps facilitent la phagocytose des particules virales par les cellules exprimant le récepteur Fc déclenchant ainsi l'ADCC et l'élimination des cellules infectées. Les anticorps dirigés contre la NA inhibent l'activité enzymatique de celle-ci et empêchent le clivage des acides sialiques présents à la surface cellulaire évitant ainsi la dissémination de nouvelles particules virales (Sylte and Suarez 2009). Similairement aux anticorps dirigés contre l'HA, les anticorps dirigés cotre la NA entrainent l'ADCC (Mozdzanowska et al. 1999, Mancini et al. 2011). Ils sont également impliqués dans l'élimination des cellules infectées (Kreijtz et al. 2011). En plus des anticorps dirigés contre les glycoprotéines de surface, les anticorps dirigés contre M2 et NP confèrent dans une moindre mesure une protection contre le virus (Sukeno et al. 1979, Feng et al. 2006). Néanmoins, grâce à la très faible variabilité de leur structure M2 et NP sont considérées comme des cibles vaccinales prometteuses (Mancini et al. 2011).

À la surface de la muqueuse respiratoire, les isotypes d'immunoglobulines qui confèrent le plus de protection contre le virus influenza sont les IgA, (Mazanec, Coudret and Fletcher 1995). Grâce à leurs caractéristiques conformationnelles d'immunoglobulines polymériques, les IgA sont transportées par le mucus au long du tractus respiratoire pour donner une protection locale des cellules épithéliales. Dans les espaces intracellulaires, le transport des IgA est réalisé par le récepteur polymérique (pIgR) qui est exprimé au niveau baso-latéral des cellules épithéliales de la muqueuse sécrétoire. Les cellules plasmatiques de la *lamina propria* sous les surfaces épithéliales sont engagées dans la production des IgA

(Mazanec et al. 1995, van Riet et al. 2012). L'isotype IgG confère une protection à plus long terme du tractus respiratoire tandis que l'isotype IgM induit la neutralisation par le complément et signe plutôt une primo-infection (Fernandez Gonzalez, Jayasekera and Carroll 2008).

5.2. Immunité cellulaire

Suite à l'infection par le virus influenza des cellules T CD4+, des T CD8+ et également des cellules T régulatrices (Tregs) sont induites et activées. La fonction primaire des cellules T CD4+ *helper* est de diriger et cibler adéquatement la réponse immune afin d'optimiser les défenses de l'hôte en supprimant les réponses immunes non essentielles. Elles sont aussi impliquées dans des maladies auto-immunes, l'asthme, des réactions allergiques et des processus tumoraux (Santamaria 2001). Cette capacité modulatoire des cellules CD4+ est essentielle pour le bon fonctionnement du système immunitaire (Zhu, Yamane and Paul 2010). Suite à la présentation des antigènes viraux par les cellules présentatrices, les CD4+ s'activent pour se multiplier et secréter des cytokines. La différenciation dépend du type d'infection, de la cellule présentatrice ainsi que de l'affinité avec le CMH et les types de cytokines/chimiokines présentes pendant l'activation (Soghoian and Streeck 2010, Feighery and Stastny 1979). Les cellules T CD4+ peuvent se différencier en Th1, Th2, Th17, T *follicular helper* (Tfh) ou *inducible regulatory T* (Tregs) (King 2009). Les cellules CD4+ Tregs sont impliquées dans la régulation de la réponse immune notamment dans les infections virales chroniques. Cette sous-population exprime le *zinc-finger transcription factor forkhead box P3* (FOXP3) et le récepteur de l'IL12 (CD25) (Kaser et al. 2012). Elles sont induites en présence de TGF-β (iTregs) et elles peuvent être sélectionnées dans le thymus dans les états précoces (*natural* Tregs) (Soghoian and Streeck 2010).

Les lymphocytes T CD8+, représentent la quasi-totalité de l'activité lymphocytaire T cytotoxique (Doherty et al. 1997). Les lymphocytes TCD8+ naïfs se différencient, suite à la reconnaissance antigénique, en lymphocytes T mémoires et en lymphocytes T effecteurs ou cytotoxiques. Ces derniers, détruisent les cellules exprimant sur leur surface un CMH de type I associé à un antigène (van Riet et al. 2012). L'interaction entre le CMH de la cellule infectée et le récepteur TCR du lymphocyte entraine l'apoptose cellulaire(Aoshi et al. 2011). Les cellules T CD8 sont essentielles pour l'élimination des virus, mais la dérégulation de leur

activité cytotoxique ainsi qu'une production excessive des cytokines pro-inflammatoires peuvent aussi entrainer des complications et une évolution défavorable de la maladie.

6. Régulation de la réponse immune

Les cytokines appartient a une grande famille des glycoprotéines qui régulent des processus biologiques majeurs comme le développement embryonnaire, l'immunité et l'hématopoïèse (Lotem and Sachs 2002, Yoshimura et al. 2012). Principalement dans les cellules immunitaires, les cytokines sont impliquées dans la survie, la prolifération, la différenciation et la fonctionnalité cellulaire (Kubo, Hanada and Yoshimura 2003). La réponse immune de l'hôte permet de contrôler l'infection virale, une réponse exagérée peut avoir de graves conséquences pour l'hôte. Pour rester efficace, la réponse immune doit être régulée à différents niveaux. Pour cela, des mécanismes d'atténuation de la signalisation cytokinique impliquant diverses protéines sont nécessaires. Trois familles de protéines inhibent spécifiquement différents aspects de la signalisation des cytokines : la *SH2-containing phosphatase* (SHP), les *inhibitors of activated STATs* (PIAS) et les *suppressors of cytokine signaling* (SOCS) (Wormald and Hilton 2004).

6.1. SH2-*containing phosphatase*

Chez les vertébrés et invertébrés, la sous-famille de protéines tyrosine phosphatases SHP est hautement conservée. Elle est constituée par deux membres, SHP-1 et SHP-2 qui ont deux domaines consécutifs N-terminal SH2 et un domaine C-terminal protéine-tyrosine phosphatase respectivement. Les deux membres vont cibler les résidus phosphotyrosines de récepteurs à cytokines grâce à leurs domaines SH2. SHP1 régule négativement la transduction du signal par la déphosphorylation du récepteur d'IL4, le récepteur de l'érythropoïétine et JAK2 (David et al. 1995, Wormald and Hilton 2004) . D'autre part, SHP-2 semble participer à la régulation positive de la signalisation. SHP-2 est aussi impliquée dans l'inhibition de la signalisation via le récepteur gp130 (Neel, Gu and Pao 2003).

6.2. *Mammalian protein inhibitor of activated STAT*

Les membres de la famille des PIAS, PIAS3, PIAx et PIASy ont été identifiés à partir de leur similarité structurale avec PIAS1. Elles ont été initialement reconnues en tant qu'inhibiteurs spécifiques des facteurs de transcription STAT1, STAT2, STAT3 et STAT4 (Chung et al. 1997, Liu et al. 1998). Les protéines PIAS interagissent également avec d'autres facteurs de transcription comme le *nuclear factor-κB* (NFκB) et les *SMA- and MAD-related proteins* (SMADs) (Shuai and Liu 2005). Trois mécanismes généraux d'action de PIAS ont

été décrits. Tout d'abord, PIAS peut bloquer la capacité de liaison du facteur de transcription à l'ADN. Par exemple, PIAS1 bloque la liaison de STAT1 ou NFκB p65 aux promoteurs des gènes endogènes *in vitro* (Liu et al. 1998, Liu et al. 2005). Le deuxième mécanisme décrit un recrutement d'autres co-régulateurs comme les *histone deacetylases* (HDAC) par PIAS, ce qui entraine la régulation de la transcription. Enfin, le dernier mécanisme consiste en l'inhibition de la transcription par l'activité *small-ubiquitin-like-modifier E3 ligase* (SUMO-E3) de PIAS qui permet la sumoylation du facteur de transcription (Shuai and Liu 2005). Le terme sumoylation fait référence à une activité similaire à l'ubiquitination entraînée par SUMO-E3 (Shuai and Liu 2005).

6.3. *Suppressors of cytokine signaling*

Ce chapitre, basé sur un article de revue (Vet Immunol Immunopathol. 2013 Jan 15;151(1-2):1-19.), abordera les aspects les plus importants concernant une famille de régulateurs de la réponse cytokinique, les *suppressors of cytokine signaling* (SOCS) chez les mammifères : leur mode d'action et leur structure, leur participation dans les infections microbiennes et l'utilisation de ceux-ci comme éventuels agents thérapeutiques.

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Review paper

SOCS proteins in infectious diseases of mammals

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ABSTRACT

As for most biological processes, the immune response to microbial infections has to be tightly controlled to remain beneficial for the host. Inflammation is one of the major consequences of the host's immune response. For its orchestration, this process requires a fine-tuned interplay between interleukins, endothelial cells and various types of recruited immune cells. Suppressors of cytokine signalling (SOCS) proteins are crucially involved in the complex control of the inflammatory response through their actions on various signalling pathways including the JAK/STAT and NF-κB pathways. Due to their cytokine regulatory functions, they are frequent targets for exploitation by infectious agents trying to escape the host's immune response. This review article aims to summarize our current knowledge regarding SOCS family members in the different mammalian species studied so far, and to display their complex molecular interactions with microbial pathogens.

Contents

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1. Introduction

Cytokines are essential mediators of cell-to-cell communications. By activating cell surface receptor complexes, they regulate processes that are involved in the growth, the differentiation, and the defense of various cells. Although lacking catalytic domains, members of the cytokine receptor superfamily mediate ligand-dependent activation of protein tyrosine phosphorylation (Yasukawa et al., 2000). Cytokines induce homo- or hetero-dimerization and activation of their cognate receptors thereby triggering the oligomerization and activation of members of the cytosolic janus kinase (JAK) family (Yasukawa et al., 2000). The activated JAKs subsequently phosphorylate further signalling targets including signal transduction and activators of transcription (STAT) proteins.

Each member of the JAK family contains (i) a conserved catalytic kinase domain that can be phosphorylated upon ligand stimulation, and (ii) an enzymatically inactive pseudo-kinase domain at the carboxyl terminus that regulates the activity of the catalytic domain (Shuai and Liu, 2003). Through their amino-terminal regions, activated JAK proteins physically and selectively interact with cytokine receptors (Dalpke et al., 2008) thereby allowing the phosphorylation of specific tyrosine residues in these proteins. The phosphorylated tyrosine residues in turn serve as docking sites for members of the STAT protein family (Darnell, 1997; Vinkemeier et al., 1998). STATs are subsequently phosphorylated by JAKs, triggering thus their dimerization and release from the receptor complex. Next, the STAT dimers translocate to the nucleus where they induce the transcription of cytokine-responsive genes (Darnell, 1997; Levy and Darnell, 2002). The signalling events outlined above were originally found to be initiated by type I interferons (IFNs); however, it is known today that this pathway can be activated by a large number of cytokines, growth factors and hormones.

This signalling response must be tightly regulated, especially with respect to its duration and intensity. Regulation of the innate immune response is critical, since an excessive inflammatory reaction can be deleterious (Liu et al., 1998; Yasukawa et al., 2000). Negative regulation of JAK/STAT signalling is carried out by a number of factors. Among them suppressors of cytokine signalling (SOCS) were shown to play a prominent role (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997; Yoshimura et al., 1995). SOCS 1–7 and cytokine-inducible SH2 protein (CIS) are the eight members of this family of intracellular proteins that are expressed following cellular activation by various molecular stimuli including cytokines and pathogen-associated molecular patterns (Kubo et al., 2003; Yoshimura et al., 2007, 2012).

2. The SOCS family

SOCS proteins range in size from 198 to 581 amino-acids. CIS, SOCS1, and SOCS3 are the best characterized so far (Alexander, 2002). All SOCS proteins share the same basic structure and are composed of three functional domains as depicted in Fig. 1A (Hilton et al., 1998; Yoshimura et al., 2007). The most conserved feature is the central Src homology 2 (SH2) domain, which is involved in both the recognition and binding of cognate phosphotyrosine motifs (Hilton et al., 1998; Yoshimura et al., 2007) (Fig. 1A). This domain specifies the target of each SOCS/CIS protein to execute its regulatory function (Sasaki et al., 2000). Upstream (towards the N-terminus) of the central SH2 domain is a variable region with an extended SH2 subdomain (ESS) that contributes to the physical interaction with the substrate. Located downstream of the SH2 domain is a C-terminal 40-amino-acid domain termed SOCS box (Bullock et al., 2007; Hilton et al., 1998; Yasukawa et al., 2000; Yoshimura et al., 2007) (Fig. 1A). The SOCS box interacts with elongin B, elongin C, cullin 5, and with the RING-finger-domain-only protein to recruit E2 ubiquitin-transferase (Kamizono et al., 2001; Kamura et al., 2004). This functional complex ubiquitinylates the target proteins and hence marks them for proteasomal degradation (Kamizono et al., 2001; Kamura et al., 2004). The SOCS box also stabilizes SOCS proteins, probably by sheltering them from proteasome-mediated turnover (Piessevaux et al., 2009). The later function also provides protection from functional interferences between different SOCS proteins.

Cytokine receptor signalling is controlled by SOCS at three different levels (Fig. 1B and Fig. 2). Firstly, the small N-terminal kinase inhibitory region (KIR) that is specific to SOCS1 and SOCS3 can prevent the action of JAKs. The KIR acts via a competitive mechanism that is due to its sequence homology with the pseudosubstrate inhibitory region of JAKs (Fig. 1B). Interaction of the KIR with the JAK activation loop thus blocks the catalytic activity of JAKs (Kubo et al., 2003; Sasaki et al., 1999; Yasukawa et al., 1999). Secondly, SOCS proteins can suppress cytokine signalling by competing with downstream signal transducers through their interaction with the phosphorylated domains of the receptor (Fig. 1B) (Ram and Waxman, 1999). Thirdly, their

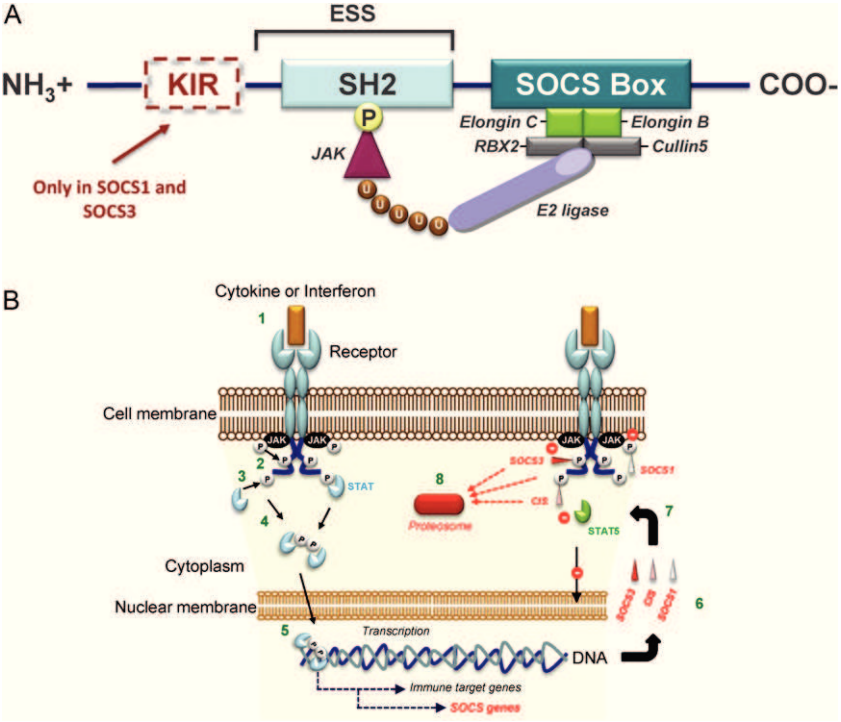

Fig. 1. (A) Structure of SOCS proteins. All SOCS proteins have (i) a central SH2 domain, (ii) an amino-terminal end domain of variable length including an extended SH2 sub-domain (ESS) and (iii) a carboxy-terminal SOCS box. SOCS1 and SOCS3 contain an additional amino-terminal kinase inhibitory region known as KIR. The SH2 domain of each SOCS determines its target-specificity, through binding phosphorylated (P) tyrosine residues that are specific to each substrate, such as JAK proteins. The SOCS box interacts with a complex containing elongin B, elongin C, cullin5, RING-box-2 (RBX2), and E2 ligase (also known as E2 ubiquitin-conjugating enzyme). This complex keeps the bound substrate close to the ubiquitinating machinery, thus facilitating its ubiquitination (U) and driving it towards proteosomal degradation. The KIR domain functions as a pseudosubstrate that inhibits the kinase activity of the SOCS-associated proteins. (B) Mechanism of suppression of the JAK/STAT pathway by SOCS1, SOCS3, and CIS. The cytokine or interferon stimulation of their cell surface receptors (1) activates receptor-associated JAK proteins by their phosphorylation (P). Then, activated JAKs phosphorylate receptor cytoplasmic domains (2). Recruited STATs are consequently activated by JAK phosphorylation (3). This phosphorylation enables their dimerization (4). Dimerized they can enter the nucleus and trigger as transcription factor complex the expression of various target genes including SOCS genes (5). Various SOCS proteins such as SOCS1, SOCS3 and CIS are produced (6). They can (7), as SOCS1 but also SOCS3, inhibit the JAK activity with their kinase inhibitory region. They can also, as SOCS3, compete with recruited STAT proteins for shared phosphotyrosine residues or specifically, as CIS, bind the phosphorylated tyrosine residues of cytokine receptors through the SH2 domain consequently masking the STAT5 docking site. Moreover, their SOCS box mediates ubiquitination and degradation of bound receptor components (8). Consecutively to their actions transcription factor complexes cannot anymore form and access the nucleus.

SOCS box mediates the transfer of their protein targets to the ubiquitination machinery and thus assigns them for proteasomal degradation (Fig. 1B) (Kamizono et al., 2001; Kamura et al., 2004; Verdier et al., 1998). Like other SOCS-box-containing factors, SOCS proteins also function as E3 ubiquitin ligases and directly mediate the degradation of associated proteins (Fig. 1B) (Yoshimura et al., 2007).

Since their first discovery in 1997 (Starr et al., 1997), SOCS proteins have been described as important regulators of the JAK/STAT pathway. Shortly after, they have also been reported to be involved in allergy, tumorigenesis, and inflammatory diseases (Yasukawa et al., 2000). SOCS proteins are generally not highly expressed at homeostasis and have short half-lives (typically 1–2 h) (Delgado-Ortega et al., 2011; Haan et al., 2003). They are encoded by cytokine-inducible genes that are rapidly transcribed after exposure of cells to cytokines (Matsumoto et al., 1999; Naka et al., 1997). As outlined later in this article, SOCS proteins also have the ability to regulate other signal transduction pathways such as the NF-κB, MAPK or JNK/p38 pathways (Frobose et al., 2006; He and Stephens, 2006; Ryo et al., 2003).

SOCS proteins have been found in numerous vertebrate species including man (*Homo sapiens*), chimpanzee (*Pan troglodytes*), Sumatran orangutan (*Pongo abelii*), rhesus monkey (*Macaca mulatta*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), Chinese hamster (*Cricetulus griseus*), Guinea pig (*Cavia porcellus*), dog (*Canis lupus familiaris*), giant panda (*Ailuropoda melanoleuca*), cattle (*Bos taurus*), sheep (*Ovis aries*), pig (*Sus scrofa*), horse (*Equus caballus*), African elephant (*Loxondota africana*), rabbit (*Oryctelagus cuniculus*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), zebrafish (*Taeniopygia guttata*), bony fish (*Osteichthyes* spp.) and even in some invertebrates such as the Chinese mitten crab (*Eriocheir sinensis*) and the fruitfly (*Drosophila melanogaster*) (Bruel et al., 2010; Cheeseman et al., 2008; Rincon et al., 2007; Sandra et al., 2005; Starr et al., 1997; Stec and Zeidler, 2011; Wang et al., 2011; Winkelman et al., 2008; Yan et al., 2011; Zhang et al.,

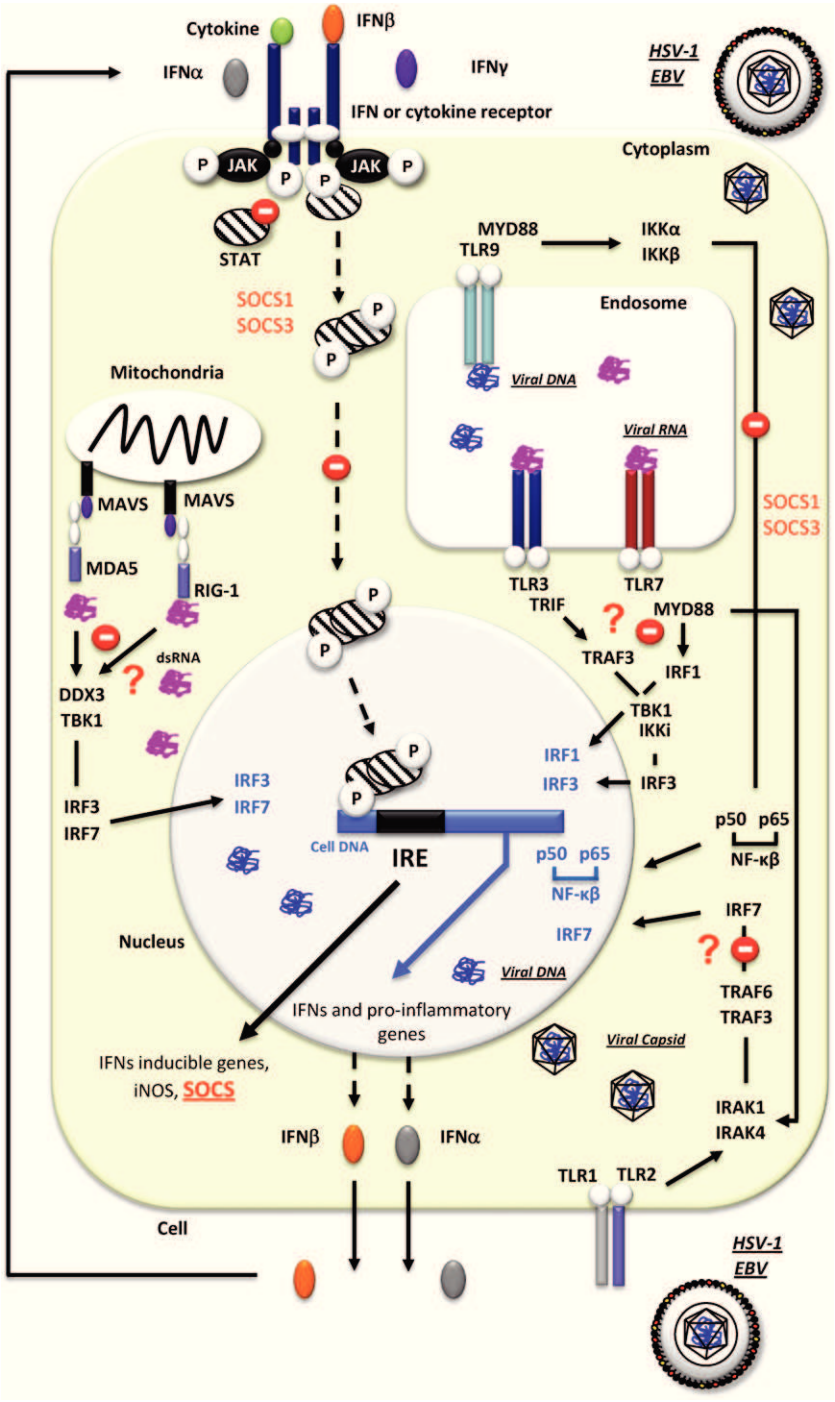

Fig. 2. Overview of established and potential pathways targeted by SOCS proteins in a cell infected by HSV-1 and/or EBV. After HSV-1 infection and the stimulation of several pathogen recognition receptors (MDA5, RIG-1, TLR1/TLR2, TLR3, TLR7, TLR9), IFNβ is transcriptionally activated following the stimulation of various signalling pathways. SOCS proteins can act at different levels (inhibition of the JAK activity, competition with recruited STAT proteins for shared phosphotyrosine residues, phosphorylation of receptor tyrosine residues, ubiquitination and degradation of bound protein components) in the cell as indicated by stop signs. Question marks (?) indicate pathways that are possibly targeted by SOCS to modulate the anti-viral immune response. DDX3, Dead box protein 3; dsRNA, double stranded ribonucleic acid; EBV, Epstein-Barr Virus; HSV-1, Herpes Simplex Virus type 1; IFN, interferon; IKKα, I kappa-B kinase-alpha; IKKβ, I kappa-B kinase-beta; IKKi, kinase I kappa B kinase i; iNOS, inducible nitric oxide synthase; IRE, interferon response element; IRAK, IL1-Receptor-associated kinase; IRF, IFN-regulatory factor; JAK, janus kinase; MAVS, mitochondrial antiviral signalling protein; MDA5, melanoma differentiation-associated gene 5; MYD88, myeloid differentiation primary-response protein 88; NF-kβ, Nuclear factor kappa-B; P, phosphorylated tyrosine; p50, p50 subunit of NF-κB; TBK1, TANK-binding kinase 1; TRIF, TIR-containing adaptator inducing interferon-β;TLR, toll like receptor; TRAF, Tumor necrosis factor receptor (TNFR)-associated factor; RIG-1, Retinoic acid-inducible gene I; STAT, signal transduction and activators of transcription.

2010). The polypeptide sequences of SOCS proteins are highly conserved between the different species, especially among mammals. For example, mouse and rat SOCS1 share 95–99% amino acid (aa) identity with their human orthologue, while human and porcine SOCS4, SOCS5 and SOCS6 share 93, 96.6, and 92.3% aa identity, respectively (Bruel et al., 2010; Starr et al., 1997). In a recent study, Zhang et al. (2010) even showed a significant sequence similarity (56%) between SOCS2 orthologues from species as distantly related as *E. sinensis* and *H. sapiens*. These percentages were even higher when considering only the domains critical for the structure and the function of the protein.

2.1. SOCS proteins and their established immunomodulatory functions

So far, vertebrate SOCS protein functions have only been studied in mammals and bony fishes. In the following, we will focus on the established roles of mammalian SOCS proteins as immunomodulatory factors and their implication in the pathogenesis of infectious diseases (see Section 3 "Role of SOCS proteins during microbial infections"). The current knowledge on SOCS protein functions in bony fishes has recently been summarized in an excellent review article by Wang et al. (2011).

2.1.1. CIS

CIS is a 28.6 kDa protein (referring to the human prototype) which binds through its SH2 domain to phosphorylated tyrosine residues of activated cytokine receptors and does not seem to physically interact with JAKs (Alexander, 2002; Matsumoto et al., 1999). CIS inhibits cytokine signalling by blocking binding sites that are otherwise used to recruit and to activate STATs (Matsumoto et al., 1999). CIS is closely related to SOCS2; it inhibits the signal transduction of erythropoietin, IL2, IL3, growth hormone (GH) and prolactin, by binding the STAT5 sites of the respective receptor (Verdier et al., 1998; Yoshimura, 2005). Transgenic mice over-expressing CIS exhibited growth defects, impaired mammary gland development and reduced numbers of both natural killer (NK) and NKT cells similarly to STAT5a knock-out (KO) and/or STAT5b KO mice (Matsumoto et al., 1999). This phenotype is consistent with the notion that CIS plays a specific role in the regulation of the STAT5-mediated cytokine response (Matsumoto et al., 1999). However, mice lacking the *CIS* gene are phenotypically normal (Marine et al., 1999a).

CIS aa sequences of different mammalian species display a high degree of conservation especially when the two functional regions – SH2 domain and SOCS box – are considered (Fig. 3). The sequence of the SH2 domain is fully conserved between the mammalian species given in Fig. 3, except for the pig where the available aa sequence (ensemble.org ENSSSCG00000011414) appears to be truncated and divergent to the commonly described isoforms one and two. This sequence needs to be confirmed and rapid amplification of cDNA-ends by polymerase chain reaction using the conserved part of the sequence should provide insights. The SOCS boxes of bovine or canine CIS differ by two mismatches from those of primates and the mouse, which are virtually identical to one another. When comparing the aa sequences of mammalian and avian (chicken and turkey) CIS proteins, only slight differences are appreciated in the SH2 domain while their SOCS box sequences are more distantly related (Fig. 3).

It has been shown that variant alleles of multiple CIS polymorphisms impact the susceptibility to major infectious diseases in humans (Khor et al., 2010) suggesting an important role of CIS in immunity against various pathogens. It is likely that this holds true also in other mammals.

2.1.2. SOCS1

SOCS1 is a 23.4 kDa protein (human prototype) that can be induced by various factors acting on or through a signal transduction pathway which is either STATs-dependent or -independent. These factors include cytokines, insulin, LPS, and CpG DNA (Dalpke et al., 2001; Kawazoe et al., 2001; Stoiber et al., 1999). SOCS1 strongly regulates macrophage activation through the JAK/STAT and the TLR/NF-κB pathways (Ryo et al., 2003; Yoshimura et al., 2007). To suppress the TLR/NF-κB pathway, SOCS1 binds to the p65 subunit of NF-κB, thereby facilitating its ubiquitin-mediated proteolysis (Ryo et al., 2003). This mode of action is particularly important for the regulation of inflammatory processes, septic shock and innate/adaptive immune responses. SOCS1 can also block other signalling factors such as the insulin receptor substrate 1 (IRS1) and IL1 receptor-associated kinase (IRAK) (Fujimoto and Naka, 2003; Kawazoe et al., 2001). Moreover, SOCS1 may also inhibit the activity of IL4, IL6, and IFNγ (Fujimoto and Naka, 2003). Observations in mice lacking SOCS1 suggest that this protein plays a key role in the negative regulation of signalling initiated by IFNγ (Morita et al., 2000). SOCS1 also has role in the control of viral and parasitic infections by means of its capacity to modulate the sensitivity of cells to both IFN type I (IFNα and IFNβ) and type II (IFNγ) (Yoshimura et al., 2007; Zimmermann et al., 2006). SOCS1 (as well as SOCS2 and SOCS3) mRNA expression was found to be up-regulated after administration of another type I IFN, IFNτ, in the uterus of cyclic ewes, suggesting an additional role for SOCS proteins in the negative regulation of fertility-related IFNτ signalling in ruminant dams (Sandra et al., 2005). Finally, SOCS1 has a pivotal function in T lymphocyte development and regulation and is therefore highly expressed in the thymus throughout all thymocyte developmental stages (Marine et al., 1999b; Trop et al., 2001).

The SOCS1 aa sequence is highly conserved between different mammalian species (Fig. 4). For example, there is 100% identity between human and porcine SOCS1 aa sequences in all the major functional regions of the protein (KIR, ESS, SH2, and SOCS box). Unsurprisingly, sequence differences between mammal species are most frequently observed outside of the functional regions. More prominent aa sequence differences are observed between galliform birds (chicken and turkey) and mammals, especially regarding the length of the Poly-Ser region and the sequence of the SH2 domain (Fig. 4). The high level of identity between mammalian SOCS1 amino acid

CIS

```
                10        20        30        40        50        60        70        80        90
           ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
Consensus  XXXXXMVLCVQG--PCPLLAVEQIGQRPLWAQSLELPEPAMQPLPAGAFLEEVAEXETPAQPESEXPKVLDPEEDLLCIAKTFSYLRESG
Human      -----.......--.R......RT.......P.....K.V..............-G....T...-.....................
Chimpanzee -----.......--.R......RT.......P.....K.V.........P.....-G....T...-.....................
Orangutan  -----.......--.R......RS.......P.......V..............-G....T...-.....................
Mouse      -----.......--S.........R...........G.......T...P...T.-...V.A.N.-.......G..............
Rat        -----.......--S....V..................G.......T...P...T.-...V.S.N.-.......G..............
Bovine     -----.......--L.....................QQV....S.......AV.-.S.....R.-...V...................
Horse      -----.......--.................................T.....V.-.........-.....................
Dog        -----.......--..S.....R....A.........................T.-....E....-.....................
Panda      -----.......--..S.....R.............................M..-.........-.....................
Elephant   -----.......XI..S...E.....QT.K.R......S......V.V........-.I...L...Q.....................
Pig        -----.......--.........T..............L......V......A..-.S..H....-.....................
Chicken    -------MILCVPG.H....E.K.QRLS.RGIAED.T.HI.....VP..P..P.PTFAAPE.DGSA.QTR.................
Turkey     MINSLRGNNLHQQR.H....E.K.QRLS.RGIAED.T.HI.....VP..P..P.PTFAAPE.DGSA.QTR.................
Consensus  XXXXXMVLCVQG--PCPLLAVEQIGQRPLWAQSLELPEPAMQPLPAGAFLEEVAEXETPAQPESEXPKVLDPEEDLLCIAKTFSYLRESG
```

```
                100       110       120       130       140       150       160       170       180
           ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
Consensus  WYWGSITASEARQHLQKMPEGTFLVRDSTHPSYLFTLSVKTTRGPTNVRIEYADSSFRLDSNCLSRPRILAFPDVVSLVQHYVASCXADT
Human      ..................................................................................T...
Chimpanzee ..................................................................................T...
Orangutan  ..................................................................................T...
Mouse      ..................................................................................A...
Rat        ..................................................................................T...
Bovine     ..................................................................................A...
Horse      ..................................................................................A...
Dog        ..................................................................................AT..
Panda      ..................................................................................A...
Elephant   ..................................................................................A...
Pig        ..........................................................TAVQATHPGLSDVSA--------------
Chicken    ..........K.............................N.............K......Y..K.............I....T..TTES
Turkey     ..........K.............................N.............K......Y..K.............I....T..TTES
Consensus  WYWGSITASEARQHLQKMPEGTFLVRDSTHPSYLFTLSVKTTRGPTNVRIEYADSSFRLDSNCLSRPRILAFPDVVSLVQHYVASCXADT
```

SH2

```
                190       200       210       220       230       240       250       260       270
           ----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|----:----|
Consensus  RSDSPDPAPTPALPMPXKDXXXXXXXXXXPXATAVHLKLVQPFVRRSSARSLQHLCRLVINRLVADVDCLPLPRRMADYLRQYPFQL
Human      ................KE.APSDPALPAP.P.....................................................
Chimpanzee ................KE.APSDPALPAP.P.....................................................
Orangutan  ................KE.APSDPALPAP.L.....................................................
Mouse      ...............SKQ.APSDSVLPIP-V.....................................................
Rat        ..............V.KP.APGDPVLPIP-V.................................T.....................
Bovine     ......L.T.....T.-.EDAPGD---PALP.................T.............V.....................
Horse      ....TE........T.-.EDAPGDPTLPA.A.....................................................
Dog        .G............T.-.EDVPGDAAPPA.AV.....R.........................P.....................
Panda      ..............T.-.EDVPGDPALPA.SV....................................................
Elephant   ..N......A....A.-.EDATSNPVLPA.T.............................V.....................
Pig        ----------------------------------------------------------------------------------
Chicken    K.EA.Y.P.A.LP.VQ-----------KEVAVA......LR.LG..D.IP........R...CTTE.ER.......G...K......
Turkey     K.EA.Y.P.A.LP.VQ-----------KEVAVA......LR.LG..D.IP........R...CTTK.ER....W..G...K......
Consensus  RSDSPDPAPTPALPMPXKDXXXXXXXXXXPXATAVHLKLVQPFVRRSSARSLQHLCRLVINRLVADVDCLPLPRRMADYLRQYPFQL
```

SOCS box

Fig. 3. Alignments of CIS proteins from different mammalian and avian species. Consensus sequences are presented above and below the alignments. Below the bottom consensus sequence are indicated, in blue, the main regions of the CIS protein (SH2 and SOCS box). Alignments were performed using Emma and Showalign in the EMBOSS software suite.

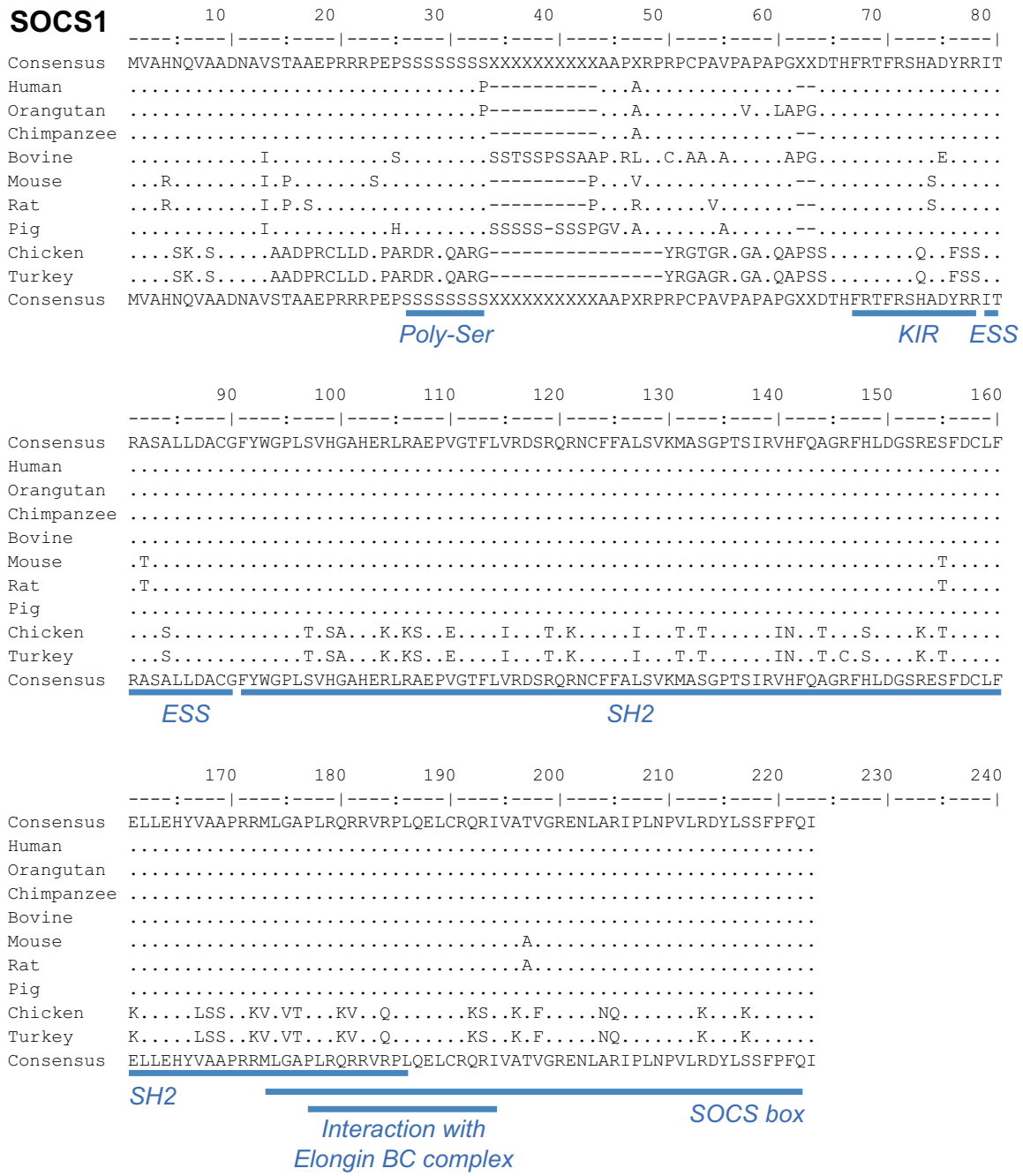

Fig. 4. Alignments of SOCS1 proteins from different mammalian and avian species. Consensus sequences are presented above and below the alignments. Below the bottom consensus sequence are listed, in blue, the main regions of the SOCS1 protein (Poly-Ser, KIR, ESS, SH2, SOCS box and the region interacting with Elongin BC complex). Alignments were performed using Emma and Showalign in the EMBOSS software suite.

sequences suggests a conserved functional mechanism of SOCS1 activity in response to microbial infections. Despite fundamental differences in the aa sequences of mammalian and avian SOCS1, it appears as if a strong cytokine (IFN type I) regulatory activity of SOCS1 is also conserved in the chicken (S. Trapp, unpublished data).

2.1.3. SOCS3

SOCS3, a 24.8 kDa protein (human prototype), is the second best characterized member of the SOCS family. Through its KIR domain, which is shared with SOCS1 (see Fig. 1), SOCS3 can block the catalytic site of JAKs as a pseudo substrate (Sasaki et al., 1999). In the presence of LPS and following TLR4 stimulation, SOCS3 expression is

strongly induced in macrophages (Yasukawa et al., 2003a). SOCS3 is a key regulator of the divergent activities of pro-inflammatory cytokines such as IL6 and anti-inflammatory cytokines such as IL10. This differential regulation is achieved through its selective binding to the GP130 receptor of IL6, while it does not bind the GP130 receptor of IL10 (Yasukawa et al., 2003a). SOCS3 specifically binds the phosphorylated tyrosine 757 (pY757) of the GP130 receptor (Nicholson et al., 2000; Schmitz et al., 2000), which is also the target of the signalling factor SH2-domain containing protein tyrosine phosphatase 2 (SHP2) (Nicholson et al., 2000; Schmitz et al., 2000). SOCS3 also competes with SHP2 further downstream in the pathway (Alexander, 2002). In some acute or chronic pathological conditions, SOCS3 can also suppress inflammatory responses in which IL6 or other pro-inflammatory cytokines are involved. By reducing the expression of IL6 and IL23, SOCS3 acts as a negative regulator of Th17-cell differentiation, which is involved in inflammatory diseases (Chen et al., 2006).

The aa sequence of SOCS3 is highly conserved in mammals (Fig. 5). Remarkably, only a small number of differences are observed between mammalian species and the chicken (Fig. 5). This high degree of conservation suggests a common immunomodulatory role of SOCS3 in most vertebrate species. This assumption is also supported by a recent study on SOCS3 cytokine regulatory activities in the turbot (*Scophthalmus maximus*) (Zhang et al., 2011a). Therefore, it is likely that most of the observations made in the mouse model are also valid in other mammalian species, including farm and companion animals. Only recently, a link between nutritional Vitamin D deficiency, a down-regulation of SOCS3, and the onset of cardiac hypertrophy and inflammatory processes in epicardial adipose tissue has been established in a Yucatan mini pig model (Gupta et al., 2012). The lesions that developed in the mini pigs receiving a 12-month hypercholesterolemic diet deprived of Vitamin D were clearly associated with a decrease in the (mRNA/protein) expression of the Vitamin D receptor and SOCS3 in cardiomyocytes and epicardial adipose tissue (Gupta et al., 2012). This observation highlights the crucial function of SOCS3 in the development or prevention of pathological conditions in all mammalian species.

2.1.4. Other SOCS family members

The mechanistically actions of the other SOCS proteins have been far less studied. SOCS2 is a 22 kDa protein (human prototype) that regulates the GH signalling pathway through binding the GH receptors and inhibiting the GH-mediated activation of STAT5b (Metcalf et al., 2000; Yoshimura et al., 2005). This is reflected by the observation that SOCS2-deficient mice were 30–40% heavier at 3 months of age, compared with control mice (Metcalf et al., 2000).

SOCS4 and SOCS5, with a size of 50.6 and 61.2 kDa, respectively (human prototypes) share distinct sequence homologies (Hilton et al., 1998). The central SH2 domains of SOCS4 and SOCS5 are virtually identical, while the N-terminal regions and the biological functions of these two proteins are less conserved (Hilton et al., 1998). It has been shown that hyper-methylation in the promoter region of SOCS4 in gastric cancer cells co-regulates other factors involved in tumor growth (Kobayashi et al., 2012).

Being highly expressed in lymphoid organs, SOCS5 appears to prevent Th2 differentiation in mice by inhibiting IL4 signalling (Seki et al., 2002; Yoshimura et al., 2005). It is expressed in Th1 cells, and interacts with the alpha chain of IL4 receptor, irrespective of tyrosine phosphorylation (Seki et al., 2002). By reducing IL4-induced activation of STAT6, this interaction efficiently inhibits Th2 polarization (Seki et al., 2002). Until today, several studies have now demonstrated an implication of SOCS5 and other SOCS proteins in the control of Th polarization and its relevance to disease development (Knosp and Johnston, 2012).

SOCS6, a 59.5 kDa protein (human prototype), binds to insulin receptors and inhibits the activation of ERK1/2, protein kinase B and IRS1 (Fujimoto and Naka, 2003; Mooney et al., 2001). SOCS6 can also bind to IRS2, IRS4 and the p85 subunit of PI3 kinase (Krebs et al., 2002) which are all involved in insulin signalling. SOCS6-deficient mice exhibit slight growth retardation (Krebs et al., 2002). SOCS7 (581 aa in size for the human prototype) is prominently expressed in the mouse brain where it seems to be of major importance. Indeed, mice lacking the gene coding for SOCS7 succumbed to the development of a hydrocephalus after 15 weeks of life (Krebs et al., 2004).

Some evidence suggests that SOCS proteins may also interfere with each-other and compete by triggering their mutual degradation. For example, SOCS2 appears to enhance the degradation of SOCS1 and SOCS3 and possibly CIS as well (Piessevaux et al., 2009). On the other hand, SOCS proteins can also exert synergistic effects that potentiate their different inhibitory activities (Palmer and Restifo, 2009).

3. Role of SOCS proteins during microbial infections

As mentioned earlier, cytokine signalling needs to be finely tuned through a tight control of signal transduction. Taking advantage of the key role of SOCS proteins in the immune responses regulation, a number of microorganisms have developed sophisticated strategies to hijack the SOCS system (Table 1) in order to inhibit immune defense-signalling pathways. Furthermore, it has been shown that immune evasion provided by SOCS functions within specific cell types is critical for determining the susceptibility of a cell to infection (Akhtar and Benveniste, 2011).

3.1. Bacteria

3.1.1. Mycobacterium spp.

Mycobacteria can drive and shape the immune response to establish a persistent host cell infection. The outcome of infection is determined by a critical balance between anti- and pro-inflammatory responses (Kirschner et al., 2010). During *Mycobacterium* spp. infections, pro-inflammatory cytokine signals production is regulated by dendritic cells (DCs) (Flynn and Chan, 2001). Compared to uninfected cells, human macrophages infected with *Mycobacterium avium* showed a reduced response to

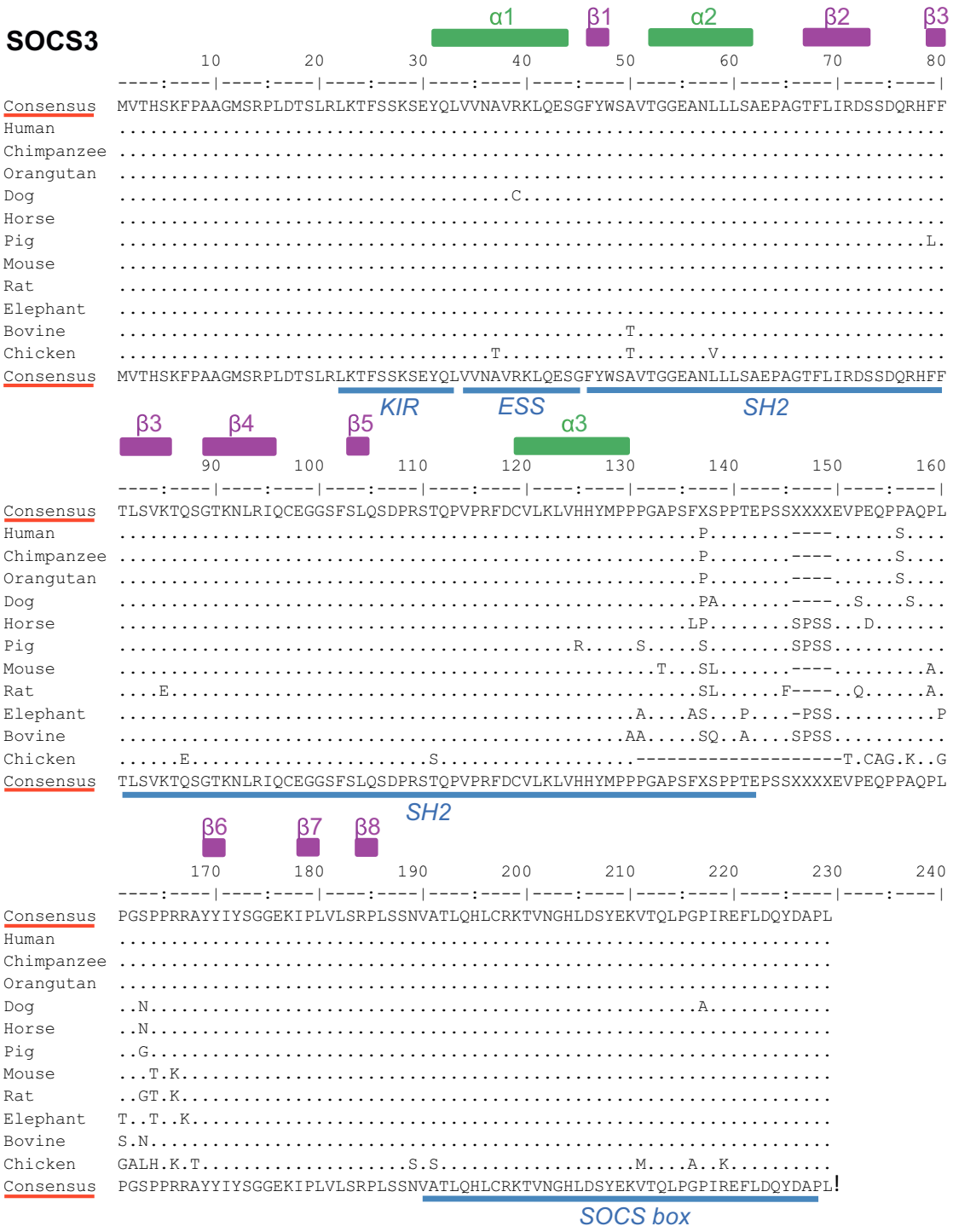

Fig. 5. Alignments of SOCS3 proteins from different mammalian and avian species. Consensus sequences are presented above and below the alignments. Above the top consensus sequence are mentioned the positions of α helix (green) and β sheets (purple). Below the bottom consensus sequence are listed, in blue, the main regions of the SOCS3 protein (KIR, ESS, SH2, and SOCS box). Alignments were performed using Emma and Showalign in the EMBOSS software suite. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

IFNγ with diminished phosphorylation of STAT1 (Vazquez et al., 2006). The expressions of SOCS3 and SOCS1 in the infected macrophages were elevated at both the mRNA and protein levels, an over-expression that was directly correlated with the unresponsiveness of the cells to IFNγ (Vazquez et al., 2006). The up-regulation of SOCS1 triggered by *M. tuberculosis* (*Mtb*) binding to DC-specific ICAM-3 grabbing non-integrin related 1

Table 1
Microorganisms have developed multiple strategies to hijack the SOCS system in order to inhibit immune defense-signalling pathways.

Microorganisms	SOCS proteins	Species involved	Functions
Bacteria			
Chlamydia pneumoniae	SOCS1	Mouse	SOCS1 is induced by infection in a STAT1 and IFNα/β-dependent manner and may protect the host from inflammatory disease (Yang et al., 2008)
Listeria monocytogenes	SOCS3	Human	Decreases IFNγ production (Stoiber et al., 2001)
Lactobacillus rhamnosus	SOCS2/SOCS3	Human	Inhibits JAK2 phosphorylation, alters p38-MAPK signalling (Lee et al., 2010; Latvala et al., 2011)
Mycobacterium spp.	SOCS1/SOCS3	Human/mouse-cattle	Inhibits IL12 production by DCs, inhibits IFNγ signalling (Vazquez et al., 2006; Srivastava et al., 2009, 2011). Up-regulation of IL10 and SOCS3, prevent clearance? (Weiss et al., 2005)
Pasteurella multocida	SOCS1	Human	Increases levels of tyrosine kinase JAK2, hyperactivity of JAK/STAT (Hildebrand et al., 2010)
Streptococcus thermophilus	SOCS2/SOCS3	Human	Inhibits JAK2 phosphorylation, alters p38-MAPK signalling (Latvala et al., 2011)
Protozoa			
Cryptosporidium parvum	CIS/SOCS4	Human	Regulates STAT3–STAT6 phosphorylation, down regulates miR-98 and *let*-7 expression (Hu et al., 2009, 2010)
Entamoeba histolytica	SOCS2	Pig	Potentially regulates IFNγ response (Bruel et al., 2010)
Leishmania donovani	SOCS3	Human	Inhibits IFNγ signalling (Nandan and Reiner, 1995; Ray et al., 2000; Bertholet et al., 2003)
Leishmania major	SOCS1	Mouse	Inhibits IFNγ signalling (Alexander et al., 1998; Bullen et al., 2003)
Toxoplasma gondii	CIS/SOCS1/SOCS3	Mouse	Impairs macrophage activation by IFNγ, inhibits the up-regulation of MCH-II and ICAM1 and reduces iNOS induction, impairs IL12 production (Zimmermann et al., 2006; Mirpuri and Yarovinsky, 2012; Stutz et al., 2012)
Virus			
Coxsackievirus	SOCS1/SOCS3	Human	Impairs IFNβ and IFNγ, impairs CT-1 signalling through gp130 receptor (Yasukawa et al., 2003b; Yajima et al., 2006)
EBV	SOCS1/SOCS3	Human	Alters NF-κB signal cascade and p38-MAPK signalling (Lo et al., 2006)
HBV	SOCS1/SOCS3	Human	Suppression of STAT1, impairs IFNα signalling by suppression of STAT1 and blocking the TLR9/IRF-7 pathway (Xu et al., 2009; Koeberlein et al., 2010)
HCV	SOCS1/SOCS3/SOCS7	Human	Regulates T and B cell functions, impairs production of IL12, inhibits phosphorylation and nuclear translocation of STAT1, degrades insulin receptor substrate 1 (Bode et al., 2003; Yao et al., 2008; Frazier et al., 2010; Pazienza et al., 2010; Ni et al., 2011; Zhang et al., 2011b)
HIV-1	SOCS1/SOCS2/SOCS3	Human	Impairs IFNγ signalling and IL12 production. Attenuates IFNβ signalling (Ryo et al., 2008; Cheng et al., 2009; Yadav et al., 2009; Akhtar et al., 2010; Miller et al., 2011)
HSV-1	SOCS1/SOCS3	Human/mouse	Inhibits IFNα, IFNβ, and IFNγ signalling (Sato et al., 2000; Yokota et al., 2001, 2004, 2005; Li et al., 2011)
IAV	SOCS1/SOCS3	Human/mouse/pig	Inhibits IFNα and IFNβ signalling through RIG-1/MAVS/IFNAR1 pathway (Pothlichet et al., 2008; Jia et al., 2010; Huang et al., 2011). SOCS3 decreases susceptibility of pigs to H5N1 avian influenza virus (Nelli et al., 2012)
MuHV-4	Viral SOCS-box (ORF73)	Mouse	Inhibition of NF-κB pathway (Rodrigues et al., 2009)
PRRSV	SOCS1	Pig	Potentially regulates IFNγ response (Wysocki et al., 2012; Zhou et al., 2011)
RSV	CIS/SOCS1/SOCS3	Human	Impairs type I and type II IFNs inhibiting STAT1 and STAT2 phosphorylation (Zhao et al., 2007; Moore et al., 2008)
SARS Co-V	SOCS3	Human	Enhancement of IL6 signalling by a lower induction of SOCS3 and dysfunction of STAT3 (Okabayashi et al., 2006)
TBEV	SOCS1/SOCS3	Mouse	Potentially limits cytokine response (Mansfield et al., 2010)
WNV	SOCS1/SOCS3	Mouse	Potentially limits cytokine response (Mansfield et al., 2010)

(DC-SIGNR1) protein in DCs and peripheral blood monocytes was also reported to be different in healthy and diseased patients (Srivastava et al., 2009). Patients with a productive form of tuberculosis showed an increased expression of SOCS1, which was reduced after chemotherapy (Srivastava et al., 2009). SOCS1 expression was also shown to result in a blockage of IL12 secretion by infected DCs during *Mtb* infection (Srivastava et al., 2009). In a further study, Srivastava et al. (2011) demonstrated that SOCS1 knock-down in murine T cells significantly improved

the ability of *Mtb*-infected macrophages to clear the infection.

For farm animal species, it has been shown that monocytes obtained from dairy cows that were subclinically infected with *M. avium* subsp. *paratuberculosis* (MAP) had up-regulated expression levels of IL10 and SOCS3 within the first 2 h after exposure to bacteria (Weiss et al., 2005). Although the clinical relevance of this observation was not clear, it was postulated by the authors that an up-regulation of IL10 and SOCS3 might have prevented MAP clearance in the infected animals (Weiss et al., 2005).

3.1.2. Other bacteria

The *Pasteurella multocida* toxin can regulate the JAK/STAT pathway, in an oncogene-like fashion by hijacking host cell pathways and avoiding the host immune defenses. The induction of the STAT-dependent Pim-1 proto-oncogene and subsequent threonine phosphorylation of SOCS1 increases the protein levels of JAK2 (Hildebrand et al., 2010). This disequilibrium leads to a hyperactivity of the JAK/STAT pathway and potential cell transformation (Hildebrand et al., 2010). Similarly, another study has demonstrated a p38 MAPK dependent up-regulation of SOCS3 mRNA/protein levels in murine macrophages in response to continuous stimulation with *Listeria monocytogenes* that eventually resulted in a decreased IFNγ production (Stoiber et al., 2001). In a murine model of *Chlamydia pneumoniae* infection, a STAT1 and IFNα/β-dependent induction of SOCS1 has been observed (Yang et al., 2008). The induction of SOCS1 was linked to a moderate inflammatory response and $RAG1^{-/-}/SOCS1^{-/-}$ mice died early after infection showing severe pulmonary inflammation suggesting a pivotal role of SOCS1 in the control of the host's response (Yang et al., 2008).

In another study aiming to explore how various probiotic or non-pathogenic bacteria strains can modulate the immune system, Latvala et al. (2011) showed that *Lactobacillus rhamnosus* and *Streptococcus thermophilus* can up-regulate SOCS3 gene expression in human macrophages, both directly via the p38-MAPK signalling pathway in the absence of protein synthesis, and indirectly via bacteria-induced IL10 production. Similarly, in a previous study designed to assess the anti-inflammatory effects of *L. plantarum*, *L. rhamnosus* and *L. acidophilus* against *Helicobacter pylori*-associated gastritis, the authors assigned a beneficial role to SOCS2 and SOCS3 in the control of the infection (Lee et al., 2010). In fact, the authors demonstrated that a co-culture of *H. pylori* and probiotic bacteria with a human gastric carcinoma cell line induced the expressions of SOCS2 and SOCS3 with subsequent anti-inflammatory effects through STAT1/STAT3 activation and JAK2 inactivation (Lee et al., 2010). To summarize, there is accumulating evidence that bacterial pathogens takes advantage of the powerful SOCS protein functions to modulate the immune and/or inflammatory response of the respective host. Importantly, this biological feature bears the potential to be targeted by pro- or metaphylactic treatments.

3.2. Parasites

3.2.1. Cryptosporidium parvum

Cryptosporidium parvum is a major cause of acute gastrointestinal diseases in a wide range of mammalian hosts (Petry et al., 2010). In man, the outcome of intestinal cryptosporidiosis largely depends on the immune status. In immuno-compromised patients, gastrointestinal symptoms progressively worsen until the patient's death (Colford et al., 1996). Despite the low tissue-invasive potential of *C. parvum*, both humoral and cell-mediated responses are activated by the host to control the invasion, the reproduction and/or the survival of the parasite (Deng et al., 2004; Kasper and Buzoni-Gatel, 2001). The constitutive expression of TLRs and intracellular Nod-like receptors by intestinal epithelial cells permit the recognition of the parasite and the immediate activation of the innate immune response (Petry et al., 2010). In a study aiming to decipher the regulation of TLR/NF-κB signalling in human cholangiocytes exposed to *C. parvum*, Hu et al. (2009) found that two small endogenous microRNAs, miR-98 and *let*-7, were involved in the regulation of CIS protein expression via translational suppression. *C. parvum* infection in turn increased CIS expression in a TLR4/MyD88 dependent manner and down-regulated both miR-98 and *let*-7 expression. Consequentially, this down-regulation resulted in a decrease of CIS translational suppression (Hu et al., 2009). Furthermore, the same group found that *C. parvum* induced the expression of SOCS4 in human biliary epithelial cells to regulate the phosphorylation of STAT3 and STAT6 (Hu et al., 2010).

3.2.2. Leishmania donovani

Caused by the protozoan *Leishmania donovani*, leishmaniasis threatens millions of people living in or traveling to tropical and subtropical regions (den Boer et al., 2011). *L. donovani* adopts an intracellular life-cycle, which allows it to escape the humoral antibody response. However, Leishmania parasites lack the machinery required for active cellular invasion, and therefore infections are preferentially restricted to macrophages (Rittig and Bogdan, 2000). Inside its host cell, *L. donovani* exists in a non-flagellated amastigote form and needs to protect itself from toxic antimicrobial molecules produced by activated macrophages (Bogdan et al., 1996). Infection with *L. donovani* promastigotes was shown to impair IFNγ mediated tyrosine phosphorylation and subsequently influence the JAK/STAT pathway in both human mononuclear phagocytes and human promonocytic U937 cells (Nandan and Reiner, 1995; Ray et al., 2000). In another study, it has been demonstrated that SOCS3 functions provide potential means for the suppression of macrophage activation during the initiation of the intracellular infection by down-regulating the expression of the IFNγRα chain (Bertholet et al., 2003). The obvious link between SOCS3 over-expression and IFNγRα down-regulation could be explained by the fact that SOCS3 can interact with members of the ubiquitinylation protein family and thus may target receptors to proteosomal degradation (Bertholet et al., 2003).

In agreement with these data, an earlier study demonstrated that killing of the closely related parasite *L. major*

by SOCS1-knockout macrophages was improved upon IFNγ and LPS stimulation (Alexander et al., 1998). Moreover, mice possessing only one copy of the SOCS1 gene endured a worse clinical outcome with increased lesion severity and an overwhelming cytokine activity (Bullen et al., 2003).

3.2.3. *Toxoplasma gondii and Entamoeba histolytica*

Toxoplasma gondii, an obligate intracellular parasite from the *phylum Apicomplexa* (Munoz et al., 2011) invades nucleated cells of warm-blooded vertebrates, in which it multiplies. Its multiplication in both phagocytic and non-phagocytic cells induces the production of IL12 and a strong IFNγ cell-mediated immune response (Sacks and Sher, 2002). In macrophages, *T. gondii* has developed different mechanisms to inhibit both the primary macrophage activation and the antiparasitic actions of type II IFNs. One of these mechanisms is the inhibition of IFNγ signal transduction, as determined by the reduction of IFNγ-mediated MHC class II up-regulation and reduced expression of the inducible nitric oxide synthase (iNOS) (Luder et al., 2003, 2001). *T. gondii* suppresses the IFNγ-mediated activation of murine macrophages, through the induction of SOCS1 and CIS and subsequent impairment of STAT1 tyrosine phosphorylation (Zimmermann et al., 2006). It was further shown that a virulent genotype I strain of *T. gondii* inhibited the up-regulation of MHC-class II and ICAM1 after IFNγ stimulation and reduced the production NO radicals, by inducing both SOCS1 and CIS expression (Stutz et al., 2012). In the latter study, SOCS1 induction was dependent on p38-MAPK signalling, egr2 and egr1 transcription factors activity together with an active cell penetration. Only recently, a role for STAT3 and SOCS3 in the context of *T. gondii* infection has also been evidenced (Mirpuri and Yarovinsky, 2012). The authors showed here that an abolishment of SOCS3 feedback regulation of IL6 signalling resulted in higher susceptibility of the respective KO mice to *T. gondii* infections due to an impaired IL12 production by inflammatory cells (Mirpuri and Yarovinsky, 2012).

A recent study from the group of F. Meurens demonstrated an induction of SOCS2 mRNA in porcine intestinal cells that were co-cultured with the human parasite *Entamoeba histolytica* (Bruel et al., 2010). This gastro-intestinal parasite drives the T auxiliary response towards Th2 and/or Th17 orientations, which are less suitable to allow full recovery from the infection (Guo et al., 2008). The SOCS2 induction could be associated with a subsequent inhibition of the Th1 response consequently helping the parasite to better resist to the host's immune response.

3.3. Viruses

3.3.1. *Hepatitis C virus*

Early after their discovery, SOCS proteins revealed their capacity to inhibit the signal transduction of type I IFNs (Song and Shuai, 1998). Hepatitis C virus (HCV) was the first virus for which the exploitation of SOCS protein functions was demonstrated (Akhtar and Benveniste, 2011; Bode et al., 2003). The flavivirus infects hepatocytes and may eventually cause liver cirrhosis and the onset of hepatocellular carcinoma (Alter, 1997). With its capacity to establish persistent, lifelong infection, HCV has been identified as an effective combatant of the host antiviral immune response (Choo et al., 1989). Like many other RNA viruses, HCV has developed strategies to impair the host's antiviral type I IFN response (Raglow et al., 2011). In chronically HCV-infected patients, Th1 and cytotoxic responses are decreased in the liver and peripheral blood (Thimme et al., 2001). Some studies revealed functional interactions of the HCV core protein with gC1qR, a complement receptor (Frazier et al., 2010; Ni et al., 2011; Yao et al., 2008). Triggered by this interaction, HCV differentially regulates T and B lymphocyte functions through exploitation of programmed death-1 (PD-1) and SOCS1 functions (Frazier et al., 2010; Ni et al., 2011; Yao et al., 2008). The cross talk between SOCS1 and PD1 in human blood derived monocytes/macrophages was further shown to suppress expression of IL12 through the JAK/STAT pathway (Zhang et al., 2011b). The HCV core protein also induces expression of SOCS3 mRNA in human hepatoma cells and inhibits activation, tyrosine phosphorylation, and nuclear translocation of STAT1, thereby counteracting the antiviral activity of IFNs (Bode et al., 2003). Furthermore, it has been demonstrated that the HCV core protein (genotype 3a) can modulate the expression of SOCS7, which is involved in the development of insulin resistance (Pazienza et al., 2010). This expression appeared to be STAT3 independent and to be regulated by peroxisome proliferator-activated receptor gamma activity (Pazienza et al., 2010). In conclusion, HCV employs several strategies to escape the host's immune response by hijacking SOCS functions. Importantly, this effective immune evasion strategy may explain the non-responsiveness of a substantial number (20–50%) of HCV-infected patients to antiviral treatments with pegylated interferon (PEG-IFN).

3.3.2. *Herpesviridae*

Members of the *Herpesviridae* family have developed various mechanisms to escape host defenses. They can selectively block the synthesis and the functions of cellular factors, degrade cellular proteins, and abrogate signalling of the host immune response (Roizman and Taddeo, 2007). Herpesviruses can accomplish a lifelong infection by establishing latency upon primary infection (Roizman, 1996).

During primary infection, Herpes Simplex Virus type 1 (HSV-1) productively infects muco-epithelial cells of the respiratory or genital tract (Whitley and Roizman, 2001). Subsequently, the virus establishes latency in trigeminal or sacral ganglia until possible re-activation. Upon HSV-1 infection, IFNβ is transcriptionally activated following the stimulation of several pathogen recognition receptors and various signalling cascades including TLR3/IFN-regulatory factor 3 (IRF-3) and NF-κB pathways (Paludan et al., 2011) (Fig. 2). IFNβ then triggers the activity the of JAK/STAT pathway with a subsequent type I IFN and SOCS induction (Sato et al., 2000). One strategy used by HSV-1 to escape host defenses and increase its capacity to replicate and/or to persist in the host is the induction of SOCS3 expression, which in turn suppresses the signal transduction activated by IFNβ (Yokota et al., 2001, 2005, 2004) (Fig. 2). SOCS3 expression reaches maximal levels at 1–2 h post-HSV-1 infection, and its induction is determinant to allow robust viral replication during acute infection.

Indeed, viral replication was found to be reduced in cells unable to express SOCS3 in response to HSV-1, or when SOCS3 expression was inhibited through addition of the JAK2 inhibitor WHI-P131 (Yokota et al., 2005). A similar mechanism of resistance to IFN involving the induction of SOCS protein expression was observed in a keratinocyte cell line treated with pJAK2, a peptide inhibitor of SOCS1. This cell line, originally refractory to IFNγ anti-herpesviral treatment, started to respond to the treatment after the application of a SOCS1 antagonist indicating that HSV-1 induction of SOCS1 is one of the mechanisms of its resistance to an IFNγ-induced antiviral state (Frey et al., 2009). More recently, IFNλ-induced suppression of SOCS1 was shown to diminish HSV-1 replication in astrocytes and neurons, highlighting the requirement of SOCS1 for HSV-1 replication in cells from the central nervous system (Li et al., 2011).

Epstein-Barr virus (EBV), a lymphotropic human gammaherpesvirus, is also able to induce SOCS1 and SOCS3 expression after activation of STAT and NF-κB signalling cascades in EBV-transformed nasopharyngeal epithelial cells (Lo et al., 2006). In addition, EBV latent infection induced the suppression of p38-MAPK activities (Lo et al., 2006). Altogether, these findings suggest that EBV can manipulate several anti-viral signalling pathways in a SOCS1/3-dependent fashion. Murid herpesvirus-4 (MuHV-4) was recently shown to inhibit NF-κB activity in latently infected centroblasts of the germinal centre (Rodrigues et al., 2009). Remarkably, this inhibition, which was critical for the establishment of the persistent infection, involved the action of an unconventional SOCS box motif present in an MuHV-4-encoded protein instead of an exploitation of endogenous SOCS protein functions, illustrating yet another viral strategy to employ SOCS-related activities (Rodrigues et al., 2009).

3.3.3. Human immunodeficiency virus

Human immunodeficiency virus (HIV) encodes 15 distinct proteins (Frankel and Young, 1998) among which are two regulatory proteins: transcriptional transactivator (Tat) and the regulator of virion gene expression (Rev) (Peterlin and Trono, 2003). The tropism of HIV for Th cells, macrophages, DCs and microglial cells is determined at the level of viral entry by the concomitant use of CD4 as a primary receptor and co-receptors that are both strain- and target-specific (Peterlin and Trono, 2003). In order to suppress antiviral innate immunity, the HIV Tat protein induces the expression of different members of the SOCS protein family (Akhtar et al., 2010; Miller et al., 2011; Ryo et al., 2008; Yadav et al., 2009). Tat was shown to induce SOCS3-mediated antagonism of IFNβ signalling in macrophages both upstream, at the level of STAT1 and STAT2 activation, and downstream, at the level of expression of the type I IFN effector proteins, dsRNA protein kinase and interferon-induced exonuclease IGS20 (Akhtar et al., 2010). In addition, Tat was shown to induce SOCS2 to subvert type II IFN signalling in human monocytes (Cheng et al., 2009). Strikingly, over-expression of SOCS1 mRNA was also observed in PBMCs after HIV infection (Miller et al., 2011; Ryo et al., 2008). Ryo et al. (2008) also showed that, by physically binding to HIV Gag and facilitating the intracellular trafficking and stability of this viral protein, SOCS1 acts as a crucial host factor for productive HIV replication.

3.3.4. Influenza A virus

Influenza viruses have developed several strategies to down-regulate type I IFN signalling. One of these involves NF-κB-dependent activation of SOCS3 expression, which negatively affects STAT phosphorylation (Pauli et al., 2008). Influenza virus infection activates anti-viral signalling primarily through retinoic acid-inducible gene I (RIG-I), an intracellular sensor of viral RNA genomes and replication (Kato et al., 2006). IAV was shown to induce, in human respiratory epithelial cells, the expression of SOCS1 and SOCS3, and both proteins seemed to differentially regulate type I IFN signalling (Pothlichet et al., 2008). The IAV-induced up-regulation of SOCS1 and SOCS3 appears to be TLR3-independent, but requires a RIG-I/mitochondrial antiviral signalling protein (MAVS)/IFNAR1-dependent pathway (Huang et al., 2011; Pothlichet et al., 2008). Pothlichet et al. (2008) proposed three potential mechanisms by which SOCS1 and SOCS3 may counteract anti-IAV signalling: (i) modulation of JAK activity, (ii) competition with STAT for binding to IFNAR1, and/or (iii) proteasomal degradation of SOCS-flagged antiviral cellular proteins including RIG/MAVS/IFNAR signalling elements.

The non-structural protein NS1 of an H5N1 avian influenza virus was shown to reduce the IFN-inducible phosphorylation of STAT proteins, resulting in decreased formation of downstream STAT/DNA complexes (Jia et al., 2010). NS1-mediated inhibition of IFN-inducible signalling involved a reduction of both IFNAR1 and IFNAR2 gene expression, which was likely responsible for the observed decrease in IFN-inducible STAT phosphorylation and DNA binding (Jia et al., 2010). Strikingly, NS1 expression also induced an up-regulation of SOCS1 and SOCS3 (Jia et al., 2010) leading to an efficient abrogation of type I IFN signalling. Very recently a study aiming at elucidating the mechanisms behind the differential susceptibility of pigs and humans to a highly pathogen strain of H5N1 avian influenza virus (humans developing cytokine storm or hypercytokinemia when infected while pigs had barely no symptoms) has shown a potential central role of SOCS3 in this difference of susceptibility between mammal hosts (Nelli et al., 2012). Authors concluded that SOCS3 in pig cells is able to confer resistance against the establishment of hypercytokinemia by moderating the pro-inflammatory response (Nelli et al., 2012).

3.3.5. Respiratory syncytial virus

Respiratory syncytial virus (RSV) is a paramyxovirus causing important respiratory pathologies in young children (Falsey and Walsh, 2000; Staat, 2002). RSV infects and replicates in the airway epithelium and in lung alveolar macrophages (Mellow et al., 2004; Wang et al., 2003). Infection of epithelial cells leads to the expression of host genes involved in the establishment of an interferon antiviral state. RSV infection has been shown to inhibit the type I IFN-JAK/STAT pathway (Ramaswamy et al., 2004). RSV nonstructural proteins NS1 and NS2 prevent the antiviral

effect of type I IFN by specifically inhibiting the phosphorylation and activation of STAT2 (Bossert et al., 2003; Spann et al., 2004). A study using a type II alveolar cell line suggested an important role of SOCS1 in the regulation of the type I IFN response to RSV infection, highlighting the possibility that NS1/NS2 could in part mediate type I IFN antagonism through the induction of SOCS1 (Moore et al., 2008). In macrophage-like U937 cells, RSV also induced rapid expression of SOCS1, SOCS3 and CIS mRNA, which were associated with the inhibition of IFNα-induced STAT1 and STAT2 phosphorylations (Zhao et al., 2007).

3.3.6. Other viruses

Studies were conducted in various viral infection models to clarify the link between SOCS proteins and the inflammatory responses regulation during viral infection. Caco2 (human colorectal adenocarcinoma) cells infected with severe acute respiratory syndrome-coronavirus (SARS-CoV) were found to express lower levels of SOCS3 mRNAs than after RSV infection, along with higher levels of IL6 expression (Okabayashi et al., 2006). The reduction of SOCS3 expression in SARS-CoV infected Caco2 cells allowed prolonged and increased IL6-signalling when compared to RSV-infected, and may thus explain the increased severity of inflammation in SARS-CoV infections (Okabayashi et al., 2006).

Regarding the *Picornaviridae* family members, it was shown that increased levels of SOCS1 and SOCS3 expression in mice have severe effects on the development of coxsackievirus-mediated cardiac injury by favoring viral replication (Yasukawa et al., 2003b). Furthermore, SOCS3 was shown to prevent STAT3 phosphorylation in murine cardiomyocites infected with coxsackievirus B3, resulting in the impairment of the protection through CT-1 signalling via the gp130 receptor (Yajima et al., 2006). Next to HCV, other flaviviruses, namely the arthropod-borne viruses West Nile virus (WNV) and tick-borne encephalitis virus (TBEV), were shown to up-regulate SOCS1 and SOCS3 expression, notably in brain tissues from experimentally infected mice (Mansfield et al., 2010). Here, SOCS1 and SOCS3 may play a role in the pathogenesis of flavivrus induced encephalitis by temporarily preventing neurotoxic cytokine signalling and eventually favoring viral spread and the onset severe neurological disorders (Mansfield et al., 2010).

Hepatitis B virus (HBV) was also described to induce the expression of SOCS proteins (Bock et al., 2008; Ko et al., 2008). SOCS3 over-expression, detected in liver biopsies of chronically HBV-infected patients, was accompanied by a significant suppression of STAT1 (Koeberlein et al., 2010). Despite the over-expression of SOCS3, the same authors also observed a constitutive activation of STAT3 that clearly contributed to the development of severe inflammatory liver diseases (Koeberlein et al., 2010). Another study, in which plasmacytoid DCs were treated with the HBV surface antigen (HBsAg), showed that HBsAg also impairs IFNα signalling by blocking the TLR9/IRF-7/IFNα pathway through an up-regulation of SOCS1 (Xu et al., 2009).

Recently an induction of SOCS1 in response to porcine reproductive and respiratory syndrome virus (PRRSV) infection of porcine alveolar macrophages and lung tissue has been observed suggesting also a role for SOCS in PRRSV infection (Wysocki et al., 2012; Zhou et al., 2011).

Collectively, these studies show that numerous phylogenetically distinct viruses take advantage of SOCS protein functions to warrant sufficient replication in their respective hosts, and it seems likely that this strategy is also adopted by a wide array of veterinary viruses, notably those that lack the coding capacities to harbor various pathogenicity factors in their genomes.

4. SOCS proteins as therapeutic targets

Given the close relationships of SOCS proteins with several infectious agents, the manipulation of SOCS functions might be useful to set up new pro- or metaphylactic approaches for the prevention and control of infectious and inflammatory diseases. Three strategies involving SOCS proteins have been proposed in the literature such as: (i) over-expression of SOCS proteins, (ii) small-molecule antagonists of SOCS signalling, and (iii) down-regulation of SOCS gene expression (Yoshimura et al., 2007).

In the first approach, exogenous expression of SOCS1 using viral vectors has been successfully used to limit host inflammatory response (Mahller et al., 2008; Sakurai et al., 2008). In one of these studies using mice with experimentally-induced arthritis, periarticular injection of a recombinant adenovirus carrying the SOCS3 cDNA dramatically reduced the severity of arthritis and joint swelling compared with control groups (Shouda et al., 2001).

The second approach is based on the development and the use of small-molecule SOCS antagonists. Waiboci et al. (2007) have designed a peptide mimicking the phosphorylated JAK2 activation loop (synthetic peptide pJAK2[1001–1013], LPQDKEYYKVKEP) that exhibits anti-infectious activity (Lucet et al., 2006). Peptide pJAK2[1001–1013] increased IFNγ activity and activation site promoter activity, blocked SOCS1 induced inhibition of STAT3 phosphorylation in IL6-treated cells, and enhanced Ag-specific proliferation (Waiboci et al., 2007). In another study, Frey et al. (2009), in an effort to find a therapeutic alternative to conventional HSV-1 drug treatments, evaluated the synergy between IFNγ and peptide pJAK2[1001–1013] in keratinocytes infected with HSV-1. The competition for SOCS1 binding between pJAK2[1001–1013] and its natural counterpart induced a strong antiviral state against HSV-1 in a dose-dependent manner. In a follow-up study, the same peptide was shown to inhibit the replication of vaccinia virus and encephalomyocarditis virus in cultured cells, and to protect mice challenged with a lethal dose of both viruses (Ahmed et al., 2010).

Using the third approach, Song et al. (2006) showed that SOCS1-silencing in DCs by siRNAs allowed these cells to better evoke anti-HIV-1 antibody and T cell responses in mice. Knock-down of SOCS1 also dramatically

enhanced the ability to generate HIV-1-envelope-specific memory T cell and B cell responses (Song et al., 2006). Furthermore, in order to improve therapeutic and prophylactic vaccine efficiencies, a co-immunization approach with vectors encoding HIV gp140CF and SOCS1-specific siRNA was applied. The authors found that SOCS1-silencing enhanced the effectiveness of DNA vaccination as evidenced by an increased production of gp120-specific antibodies, and better CTL and $CD4^+$ T cell responses in immunized mice (Song et al., 2006). Moreover, Subramanya et al. (2010) reported that targeting the SOCS1 siRNA to DCs through their linkage to a DC-specific fusion peptide enhanced the induction of co-stimulatory molecules and DC production of cytokines in HIV-infected individuals. The SOCS1-silenced DCs stimulated primary $CD8^+$ T cell responses against various antigens in vitro (Subramanya et al., 2010). A similar approach was tested to improve anti-tumor therapy strategies (Shen et al., 2004). In this study, the authors showed that a vaccination protocol using SOCS1-silenced DCs strongly increased antigen-specific, anti-tumor immunity (Shen et al., 2004). SOCS1 silencing probably allowed antigen-presenting immunogenic DCs to persistently stimulate antigen-specific T cells in the murine vaccine recipients (Shen et al., 2004). The authors of this study postulated an inactivation of T regulatory cells by the SOCS1 silenced DCs through an enhancement of DC maturation and the production of pro-inflammatory cytokines (Shen et al., 2004). Only recently, it has also been demonstrated that treatment with IL7 could improve the immune response to persistent infections caused by HIV, HBV and HCV by targeting SOCS3 (Pellegrini et al., 2011). While SOCS3 impaired T cell functions and promoted T cell exhaustion favoring viral persistence, IL7 treatment decreased the amount of SOCS3 within T cells and enabled early virus clearance (Pellegrini et al., 2011; Rincon et al., 2007). For the future, it would be interesting to further evaluate the experimental approach of down-regulating SOCS in order to improve the host's response to antimicrobial vaccines. This approach would be particularly relevant in large animal species which have been demonstrated as valuable models for the study of human infectious diseases, such as the pig (flu, tuberculosis and chlamydiosis) (Meurens et al., 2012) and cattle (RSV infections).

5. Conclusion

SOCS proteins are key regulators of the host immune response, and their functions are hijacked by several pathogens in order to promote their survival and/or increase their propagation. There is no doubt that in the near future, investigators will discover additional roles for SOCS proteins and further implications of these fascinating proteins in the pathogenesis of other infectious diseases. Further studies on SOCS proteins are particularly required in animal species, notably in farm and companion animals, because of the paucity of data outside the field of human health. Due to their central regulatory role, SOCS proteins also appear as ideal targets for the development of novel therapeutic approaches and vaccines both in animal and human health.

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7. Modèles *in vitro* et *ex vivo* pour l'étude des virus influenza

En infectiologie, le développement d'un modèle animal a pour objectif de reproduire le plus fidèlement possible les conditions propres au développement de l'infection dans l'espèce cible. Plus particulièrement, le modèle porcin à été décrit comme un modèle pertinent pour l'étude de pathogènes respiratoires humains. Plusieurs études *in vivo* ont été réalisées ces dernières années en utilisant ce modèle pour mieux comprendre *entre autres* la pathogenèse de *Bordetella pertussis, Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* et *Staphylococcus aureus* (Elahi, Holmstrom and Gerdts 2007, Gil et al. 2010, Luna et al. 2009, Elahi et al. 2005). Concernant l'étude des virus influenza, le porc constitue un modèle de choix en raison des nombreuses ressemblances en termes de pathogenèse et de tropisme tissulaire avec l'humain (Meurens et al. 2012). De plus, le porc joue un rôle fondamental dans l'écologie, l'épidémiologie et l'évolution des virus influenza (Medina and Garcia-Sastre 2011, Ito et al. 1998). Des études *in vivo* ont également été réalisées afin de mieux comprendre la pathogenèse des infections influenza « simples » ou lors d'association avec d'autres agents pathogènes (Khatri et al. 2010, Deblanc et al. 2012, Van Reeth, Van Gucht and Pensaert 2002). Afin de comprendre plus finement les mécanismes mis en œuvre lors de la réponse immune au niveau des populations cellulaires cibles il convient de disposer de systèmes *in vitro* et *ex vivo* adéquats. Les cellules épithéliales constituent la première barrière de défense et elles représentent la population cible majeure des virus influenza. Des nombreux modèles *in vitro* utilisant des lignées cellulaires ou des cellules épithéliales primaires redifférenciées ou non ainsi que des systèmes de culture *ex vivo* avec des explants de tissus respiratoires ont été développés.

7.1. Modèles *in vitro*

Traditionnellement, l'étude de l'interaction entre les virus influenza et l'hôte cellulaire *in vitro* a été réalisée en utilisant des cellules issues d'autres espèces animales. Ces études ont étaient focalisées principalement sur l'évaluation de la capacité du virus à infecter et à se répliquer dans ces cellules. Les lignées cellulaires de mammifères disponibles actuellement sont : la lignée *Madin-Darby canine kidney* (MDCK) (Tobita et al. 1975), la lignée *African Green Monkey Kidney* (VERO) (Genzel et al. 2010, Govorkova et al. 1996) et des monocytes/macrophages humains (Bussfeld et al. 1998, Hofmann et al. 1997).

À ce jour, de nombreuses études utilisant des cellules épithéliales porcines primaires ont été réalisées pour analyser la réponse immune innée en présence du virus influenza. Dans une première étude, afin de définir le rôle des facteurs génétiques de HA et NA dans la capacité d'infection de plusieurs souches de SIV (souches du groupe Sw/MN – souches du groupe Sw/ONT), Busch et collaborateurs ont développé un modèle *in vitro* utilisant des cellules primaires épithéliales porcines *swine respiratory epithelial cells* (SRECs) (Busch et al. 2008). Dans une autre étude, Khatri et collaborateurs ont isolé des cellules souches pulmonaires pour développer un système de culture qui permettrait la différenciation en pneumocytes de type I et II capables d'exprimer des récepteurs d'ASs et supporter la réplication de SIV (Khatri, Goyal and Saif 2012).

Afin de permettre la différenciation ou la redifférenciation *in vitro* de cellules épithéliales respiratoires, la technique de culture en interface air-liquide (ALI) a été développée et décrite dans plusieurs études (Stewart et al. 2012, Sachs, Finkbeiner and Widdicombe 2003, Prytherch et al. 2011, de Jong et al. 1993). Cette technique permet de reproduire plus fidèlement les conditions naturelles rencontrées par les cellules épithéliales étant donné l'absence de milieu du côté apical et la présence du milieu uniquement du côté basal. Dans le contexte de l'infection par le virus influenza, Bateman et collaborateurs ont développé une méthode de culture ALI pour différencier et/ou redifférencier des cellules primaires épithéliales porcines (Bateman, Karasin and Olsen 2013). Afin d'entraîner la différenciation cellulaire, différentes concentrations d'acide rétinoïque et d'*epidermal growth factor* (EGF) on été utilisées, ce qui a pour effet de stimuler la ciliogénèse. Les cellules différenciées exprimaient des ASα2,3 et des ASα2,6 sur leur surface permettant au virus de se répliquer adéquatement (Bateman et al. 2013).

7.2. Modèles *ex vivo*

La culture d'explants de tissus respiratoires offre une alternative attrayante pour l'étude de la réponse immune contre le virus influenza. De nombreuses études ont été réalisées afin d'offrir un système plus complexe reprenant les différents types cellulaires rencontrés *in situ*. Afin d'évaluer la réplication virale lors d'infections successives, Nunes et collaborateurs ont utilisé des explants de trachée appelés *ex vivo organ culture*. Les explants ont montré des mouvements ciliaires pendant 4 jours et des changements histologiques similaires à ceux observés *in vivo*, suite à l'infection par un SIV H1N1. Des lésions comme la

perte des cils et la destruction des cellules épithéliales ont été observées (Nunes et al. 2010). Une autre étude a développé un modèle *ex vivo*, en utilisant des explants issus de différentes portions de l'appareil respiratoire (nasale, trachéale, bronchique et pulmonaire) afin d'évaluer la capacité d'infection de plusieurs virus influenza et la localisation des récepteurs viraux (Van Poucke et al. 2010). Elle a montré que les virus influenza aviaires ont un tropisme faible pour les cellules du tractus respiratoire supérieur et que les virus influenza humain ont un tropisme similaire à celui des SIV (Van Poucke et al. 2010). Une méthode de culture *ex vivo*, appelée *precision-cut lung slices* (PCLS) a été développée (Krumdieck, dos Santos and Ho 1980, Krumdieck 2013). Cette technique consiste à la production d'un grand nombre de fines (250µm) tranches de tissu pulmonaire en conservant intacte l'architecture du tissu (Fisher and Vickers 2013). Peu utilisée dans les études d'infectiologie (Goris et al. 2009, Abd El Rahman et al. 2010), cette technique a été utilisée pour une première fois par Punyadarsaniya et collaborateurs, pour évaluer les intéractions hôte/pathogène dans le cadre de l'infection par les virus influenza aviaires H9N2 – H7N7 et le virus porcin H3N2 (Punyadarsaniya et al. 2011). Dans cette étude, l'infection de cellules épithéliales respiratoires par un SIV et deux sous-types de virus influenza aviaire H9N2 – H7N7 a été évaluée. Les auteurs ont montré que les trois virus sont capables d'infecter et se multiplier dans les cellules ciliées. De plus, le SIV H3N2 a également présenté un tropisme pour les cellules à mucus contrairement aux virus humains qui ont un tropisme pour les cellules non ciliées (Matrosovich et al. 2004).

Objectifs

Chez le porc, les virus influenza, seuls ou en association avec d'autres pathogènes à tropisme respiratoire, sont très répandus dans toutes les régions du monde à forte densité d'élevage (Brown 2000, Crisci et al. 2013). Généralement peu diagnostiqués, ils sont impliqués dans le développement de syndromes respiratoires complexes pouvant être à l'origine de pertes économiques importantes (Brown 2000, Kuntz-Simon and Madec 2009). L'espèce porcine, réceptive à la fois aux virus influenza aviaires et humains, constitue un maillon important dans les transmissions inter-espèces par la génération de virus réassortants. Étant donné le rôle de « *mixing vessel* » du porc lors des infections influenza et les similarités existant entre cet animal et l'Homme, l'espèce porcine constitue un modèle d'étude très pertinent de l'infection grippale humaine (Meurens et al. 2012).

À ce jour, de nombreuses questions relatives à l'immunologie porcine restent en suspens et beaucoup reste à découvrir. La réponse immune de l'hôte dans ses composantes innée et adaptative doit être finement régulée afin de rester bénéfique. Parmi les protéines impliquées dans la régulation de la réponse immune et plus particulièrement dans les réponses cytokiniques, les protéines SOCS occupent une place centrale. Les protéines SOCS et CISH constituent une famille de huit protéines intracellulaires impliquées dans de nombreux processus physiologiques et pathologiques (Yoshimura, Naka and Kubo 2007, Akhtar and Benveniste 2011). Elles ont été décrites notamment chez la souris et dans des cellules humaines comme des régulateurs majeurs de l'expression et de l'action des cytokines en agissant particulièrement sur la voie de signalisation cellulaire JAK/STAT (Endo et al. 1997, Naka et al. 1997, Starr et al. 1997, Yoshimura et al. 1995).

Les SOCS et la plupart des protéines régulatrices de la réponse antivirale sont toujours méconnues dans l'espèce porcine. Le travail de thèse présenté ici **s'inscrit dans le cadre de l'étude de la réponse immune antivirale et de son contrôle dans l'espèce porcine**, une espèce d'intérêt agronomique et médical majeur.

La première étude a été consacrée à l'obtention d'informations préliminaires concernant les SOCS porcines. Dans un contexte non infectieux, **l'objectif a été de**

caractériser l'expression des ARNm des SOCS1-7 et CISH dans différents tissus après identification et sélection préalable des gènes de référence les plus stables dans chaque tissu.

La deuxième étude à été réalisée afin d'évaluer, dans un premier temps, la réponse antivirale face à une souche de virus influenza porcin de sous-type H3N2, et ensuite de commencer à déterminer l'implication des protéines SOCS dans la régulation de cette réponse. L'analyse a été réalisée en utilisant des cellules épithéliales et des macrophages alvéolaires *in vitro* ainsi que des explants de poumon *ex vivo*. **L'objectif de cette étude a été d'abord d'analyser l'expression de transcrits impliqués dans la réponse antivirale et des transcrits de SOCS** (les SOCS étant potentiellement impliqués dans la régulation de la réponse). **Ensuite, l'activation de différentes voies de signalisation (MAPK -ERK 1/2 et p38, PI3K/Akt et JAK/STAT) impliquées dans la réponse antivirale et leur relation avec les transcrits des SOCS ont été évaluées.** Pour ce faire, des inhibiteurs spécifiques de chaque voie ont été utilisés et l'impact de l'inhibition sur l'expression de divers transcrits a été mesuré.

La troisième étude ambitionnait de développer un outil alternatif de culture cellulaire plus pertinent pour l'analyse *in vitro* de la réponse immune innée des cellules épithéliales respiratoires porcines. **L'objectif était donc d'évaluer la capacité de différenciation des cellules épithéliales porcines NPTr en utilisant une méthode de culture en interface air-liquide**. Pour ce faire, différents aspects de la différenciation cellulaire comme l'apparition de cils, la sécrétion de mucus, le développement de jonctions serrées et l'expression de transcrits impliqués dans la différenciation cellulaire ont été évalués.

Étude 1

Expression des ARNm de SOCS1-7 et CISH dans les tissus porcins

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Expression of SOCS1-7 and CISH mRNA in porcine tissues

Les protéines SOCS et la *cytokine-inducible SH2 domain containing protein* (CISH) constituent une famille de huit protéines intracellulaires impliquées dans de nombreux processus physiologiques et pathologiques (Yoshimura et al. 2007). Elles ont été décrites principalement comme des régulateurs de l'expression et de l'action des cytokines en agissant notamment sur la voie de signalisation cellulaire JAK/STAT (Endo et al. 1997, Naka et al. 1997, Starr et al. 1997, Yoshimura et al. 1995). Actuellement, peu d'informations sont disponibles chez le porc. Afin d'accroître les connaissances relatives aux SOCS dans l'espèce porcine, l'expression constitutive de transcrits des SOCS (CISH et SOCS1-7) a été mesurée dans les tissus de 10 porcs *Large White* sains âgés de deux mois. Pour ce faire, des gènes de référence ont été sélectionnés sur la base de leur stabilité en utilisant l'application geNorm qui permet le calcul de la valeur M (Vandesompele et al. 2002). Le gène de référence *beta-2-microglobulin* (B2MI) à été le plus stable dans nos conditions. Des différences significatives pour l'expression des transcrits de SOCS1, SOCS3, SOCS4, SOCS6 et CISH ont été observées au niveau du gros intestin. Dans l'intestin grêle, une expression constitutive des transcrits de SOCS2, SOCS3, SOCS5, SOCS7 et CISH a aussi été identifiée. Dans le thymus l'expression de transcrits de SOCS1 a été également observée. Cette étude apporte un complément d'informations nécessaires à la mise en place de prochaines études sur le rôle des protéines SOCS dans la régulation de la réponse immune innée et adaptative.

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Short communication

Expression of SOCS1-7 and CIS mRNA in porcine tissues

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ABSTRACT

The Suppressor Of Cytokine Signaling (SOCS) proteins are key physiological regulators of the immune system. Little is known about tissue expression of SOCS and data in pigs are extremely scarce. In order to further study SOCS in pigs, preliminary data must be collected. In the current report, we first identified the three most suitable reference genes in ten porcine tissues. The beta-2-microglobulin (B2MI) reference gene was most often particularly suitable in our conditions. Then, using three reference genes we determined the mRNA expression of SOCS1-7 and CIS in every selected tissue. Constitutive mRNA expression was identified for all the members of the SOCS family in the ten tissues. Interestingly, the constitutive mRNA expression of SOCS1, SOCS3, SOCS7 and CIS was rather heterogeneous between tissues while for SOCS2, SOCS4, SOCS5 and SOCS6 differences of expression were less obvious. Highest CIS and SOCS mRNA expressions were observed in large intestine (SOCS1, SOCS3, SOCS4, SOCS6, and CIS), small intestine (SOCS1, SOCS4, SOCS6, and CIS), spleen (SOCS2, SOCS3, SOCS5, SOCS7, and CIS), trachea (SOCS3) and thymus (SOCS1, SOCS2, SOCS4, SOCS7, and CIS). These data will help for further studies about the role of SOCS proteins in the control of porcine innate and adaptive responses.

1. Introduction

The Suppressor Of Cytokine Signaling (SOCS) proteins are key physiological regulators of the immune system that can be exploited by pathogen to circumvent host response in some cases (Akhtar and Benveniste, 2011; Yoshimura et al., 2007). These proteins belong to the major classes of modulators of cytokines signaling as well as protein inhibitors of activated signal transducer and activator of transcription (STAT) (PIAS) and protein tyrosine phosphatases (PTPs) (Greenhalgh and Hilton, 2001). The SOCS family consists of 8 proteins: SOCS1 to SOCS7 and cytokine-inducible SH2-containing protein (CIS). All these proteins contain a variable N-terminal region, a central Src homology 2 (SH2) (Babon et al., 2006) and a conserved C-terminal domain designated as the SOCS box (Hilton et al., 1998). They can act as a pseudo-substrate for Janus Kinase (JAK) *via* a small kinase inhibitory region (KIR) (Sasaki et al., 1999). They can also compete for receptor motif (SH2-dependent) and inhibit STAT binding (Ram and Waxman, 1999). Additionally, *via* the SOCS box, the family members regulate the half-life of a wide range of proteins by promoting the ubiquitination machinery (Callus and Mathey-Prevot, 1998). Ubiquitination of protein *via* the SOCS box can lead to its proteasomal degradation. Previous studies have shown that SOCS proteins are key regulator of immune processes (Croker et al., 2008; Dalpke et al., 2008; Yoshimura et al., 2007). Among the SOCS family members, SOCS1, SOCS3 and CIS are the best characterized so far. In macrophages SOCS1 and SOCS3 are thought to direct effects on MAPK, PI3K and NFκB pathways (Baetz et al., 2004; Nakagawa et al., 2002) in response to a variety of stimuli including lypopolysaccharide, tumor necrosis factor-α (TNF-α), isoproterenol and statins (Ehlting et al., 2007). In pigs, SOCS2 and SOCS3 were identified in 2005 (Du et al., 2007; Piper et al., 2005; Zhao and Tuggle, 2005) while porcine SOCS1, SOCS4, SOCS5 and SOCS6 encoding sequences were

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described more recently (Bruel et al., 2010). With more than 92% of identity, porcine SOCS predicted proteins were very similar to their human counterparts suggesting similar mechanisms of action (Bruel et al., 2010). Regarding the expression of SOCS in porcine tissues there are only a few data in the literature and articles about SOCS and their roles in pigs are extremely scarce (Bruel et al., 2010; Wu et al., 2007; Zhang et al., 2008). In the current study, we aimed to assess CIS and SOCS transcript expressions in various porcine tissues–kidney, large intestine, liver, lung, mesenteric lymph node, small intestine, spleen, stomach, trachea, and thymus. In order to get reliable results, we first determined in every selected tissue the three most stably expressed references genes from a panel of eight commonly used reference genes.

2. Materials and methods

2.1. Animals and sample collections

Selected tissues (kidney, large intestine, liver, lung, mesenteric lymph node, small intestine, spleen, stomach, trachea, and thymus) were taken from a total of twenty two-months-old healthy piglets provided by INRA experimental unit (Nouzilly, France). Then, small pieces of the tissue (3 mm × 3 mm) were immediately snap-frozen in liquid nitrogen and stored at −80 °C. Animals were cared for in accordance with the guidelines of the Institutional Animal Care and Use committee at INRA.

2.2. Real-time PCR assays and validation of reference genes

Quantitative real-time PCR (qPCR) was performed using cDNA synthesized as previously described (Meurens et al., 2007). Primers were designed using Clone Manager 9 (Scientific & Educational Software, Cary, NC, USA) and were purchased from Eurogentec (Liège, Belgium) (Table 1). Diluted cDNA (10×) was combined with primer/probe sets and Mesa Green mix (Eurogentec) according to the manufacturer's recommendations. The qPCR conditions were 95 °C for 5 min, followed by 38 cycles with denaturation at 95 °C for 15 s and annealing/elongation for 45 s (annealing temperature, Table 1). Real time assays were run on a Bio-Rad Chromo 4 (Bio-Rad, Hercules, CA, USA). The specificity of the qPCR reactions was assessed by analyzing the melting curves of the products and size verification of the amplicons. To minimize sample variations, we used identical amount of tissue and high quality RNA. The quality of RNA was assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Massy, France) and RNA integrity numbers (RIN) were calculated. RIN were always ≥6. Samples were normalized internally using simultaneously the average *Cycle quantification* (*Cq*) of the three most suitable reference genes in each sample to avoid any artifact of variation in the target gene. These three most suitable reference genes were selected among eight commonly used reference genes which were investigated in each tissue using qPCR with SYBR green. The genes included beta-actin (*ActB*), beta-2-microglobulin (*B2MI*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribosyltransferase-1 (*HPRT-1*), ribosomal protein L-19 (*RPL-19*), succinate dehydrogenase complex subunit A (*SDHA*) and TATA box binding protein 1 (*TPB-1*). The stability of these reference genes in all the selected tissues was investigated using the geNorm application (Vandesompele et al., 2002). Threshold for eliminating a gene was $M \geq 1$ as recommended (Hellemans et al., 2007). The correlation coefficients of the standard curves were >0.995 and the concentration of the test samples were calculated from the standard curves, according to the formula $y = -M \times Cq + B$, where M is the slope of the curve, Cq the first positive second derivative maximum of amplification curve calculated using PCR Miner (http://www.ewindup.info/miner/version2/data_submit.htm) (Zhao and Fernald, 2005) and B the y-axis intercept. All qPCRs displayed efficiency between 90% and 110%. Expression data are expressed as relative values after Genex macro analysis (Bio-Rad, Hercules, CA, USA) (Vandesompele et al., 2002).

2.3. Statistical analysis

Data for the comparison of differences in relative mRNA expression between tissues were expressed as relative values. Independent and non-normally distributed data (mRNA relative expression) were analyzed using the Kruskal–Wallis test and Dunn's post-test to compare piglet groups (using Graphpad Prism® 4, GraphPad Software Inc., USA). Differences between groups were considered as significant when $p < 0.05$.

3. Results and discussion

Many studies have shown that SOCS proteins are important regulators for cytokine signaling. However, very limited information is available about SOCS in swine and there is currently no data concerning the constitutive expression of SOCS in the different tissues. After the validation of the three most stable reference genes in every tissue we performed RT-qPCR analysis of the eight SOCS proteins in various unstimulated porcine tissues.

Regarding the stability of reference genes, B2MI (7/10), RPL-19 (6/10) and HPRT-1 (5/10) were most often the most stable reference genes when all the tissues were considered while ActB and GAPDH, excepted in thymus, were usually not stably expressed (Fig. 1). Highest M values were observed in liver, lung and stomach (Fig. 1). In the kidney, the large intestine, the MLN, the small intestine, the spleen, the trachea, and the thymus *M* values were most often very close to 0.5 (Fig. 1). In a recent report about stability of reference genes in porcine peripheral blood mononuclear and dendritic cells, RPL-19 was identified as highly stable too (Facci et al., 2011) highlighting the interest of using this gene to standardize RT-qPCR results. In contrast, in another report about the selection of reference genes in pig tissues (Nygard et al., 2007), ranking of reference genes was quite different excepted for HPRT-1 and RPL-4 (instead of RPL-19 in our conditions), which were also usually stable. These discrepancies could be related to the quality of extracted

Table 1
Primer sequences, annealing temperatures of primer sets (°C), expected PCR fragment sizes (bp) and accession numbers or references. Reference genes are underlined.

Primer name	Primer sequence	Annealing temperature(s) (°C)	PCR product (bp)	Accession number or reference
ActB	CACGCCATCCTGCGTCTGGA AGCACCGTGTTGGCGTAGAG	63	100	Nygard et al., 2007
B2MI	CAAGATAGTTAAGTGGGATCGAGAC TGGTAACATCAATACGATTTCTGA	58	161	Nygard et al., 2007
CIS	GGGAATCTGGCTGGTATTGG CCGACAGTGTGAACAGGTAG	62	126	BE014034
GAPDH	CTTCACGACCATGGAGAAGG CCAAGCAGTTGGTGGTACAG	63	170	AF017079
HMBS2	AGGATGGGCAACTCTACCTG GATGGTGGCCTGCATAGTCT	58	83	Nygard et al., 2007
HPRT-1	GGACTTGAATCATGTTTGTG CAGATGTTTCCAAACTCAAC	60	91	Nygard et al., 2007
RPL-19	AACTCCCGTCAGCAGATCC AGTACCCTTCCGCTTACCG	60	147	Meurens et al., 2009
SDHA	CTACAAGGGGCAGGTTCTGA AAGACAACGAGGTCCAGGAG	58	141	Nygard et al., 2007
SOCS1	CGCCCTCAGTGTGAAGATGG GCTCGAAGAGGCAGTCGAAG	62	110	EW101597
SOCS2	CTGCGCATCGAATACCAAG TGTAGAGCGGTTTGGTCAG	58	190	NM_001097461
SOCS3	CACTCTCCAGCATCTCTGTC TCGTACTGGTCCAGGAACTC	62	105	NM_001123196
SOCS4	TCCTGGGACAGGCTCTATG GGTACTTGGGAGGTGTTTC	59	170	ES445034
SOCS5	ACGCTGTGTTTGCAGTCTC ACTTTCCAAGCTCCCTGTC	58	89	DB784235
SOCS6	ATCTCTAGCCGGTGACTTCG GCCCTTCTGCTTCTGTTTCG	62	178	XM_001926570
SOCS7	CACTTGTGGACGTGGACATC GGAAAGACTGCAGGGAAGAC	62	162	ENSSSCT00000019652
TBP-1	AACAGTTCAGTAGTTATGAGCCAGA AGATGTTCTCAAACGCTTCG	60	153	Nygard et al., 2007

RNA which is difficult to compare between studies. In the current report, the stability of references genes was also evaluated in additional tissues such large intestine, MLN, spleen and trachea with B2MI and RPL-19 showing a stable expression (Fig. 1).

Fig. 2 shows that SOCS1-7 and CIS were constitutively expressed in all the selected tissues. This result is in accordance with previous studies (for reviews see (Krebs and Hilton, 2000; Yoshimura et al., 2007)). SOCS2, SOCS4, SOCS5 and SOCS6 presented the lowest mRNA expression variations between tissues. Interestingly, constitutive mRNA expression of SOCS1, SOCS3, SOCS7, and CIS was rather heterogeneous between tissues (Fig. 2) as observed, excepted for CIS, in another study in unstimulated human bronchial epithelial BEAS-2B cells (Pothlichet et al., 2008). SOCS1 mRNA was particularly expressed in the thymus and the large intestine with level of expression significantly higher in these tissues than in the stomach and the trachea ($p < 0.05$ and <0.01). The high expression of SOCS1 mRNA in thymus is in accordance with previous studies in mice and humans showing high expression of SOCS1 in the thymus where this protein plays a critical role in the differentiation of $CD4^+$ T cells (Catlett and Hedrick, 2005; Chong et al., 2003; Marine et al., 1999; Starr et al., 1997, 1998). SOCS2, SOCS4, SOCS7 and CIS mRNA were also usually more expressed in the thymus than in the other porcine tissues suggesting roles for these proteins in T cells differentiation. However, differences between tissues were not always statistically significant. For SOCS2, highest levels of expression were detected in small intestine, spleen and thymus. In a recent report, SOCS2 was induced in response to an intestinal pathogen highlighting the involvement of this SOCS in the response of the intestinal mucosa (Bruel et al., 2010). SOCS3 was more expressed in large intestine ($p < 0.05$) than in kidney. High expressions of SOCS3 mRNA were observed in spleen and trachea but with an important variation between animals. The high mRNA expression of SOCS3 in the trachea, which presents a typical pseudo-stratified respiratory epithelium, correlated well with the identification of an important induction of this particular SOCS in response to influenza virus (Pothlichet et al., 2008). Highest SOCS4 mRNA expressions were detected in the small intestine, large intestine and thymus (Fig. 2). SOCS4 mRNA expression was significantly higher in the small intestine than in the liver ($p < 0.01$). Regarding SOCS5, highest mRNA expressions were detected in the spleen and lowest expression in the liver. Highest mRNA expressions for SOCS6 were observed in the small and the large intestines where a diversified microflora is observed. Concerning SOCS7, highest mRNA expressions were identified in the thymus with higher mRNA expression in this tissue than in the liver ($p < 0.001$), the lung ($p < 0.05$) and the MLN ($p < 0.01$). However, for this SOCS, *Cq* were always around 30 indicating a low

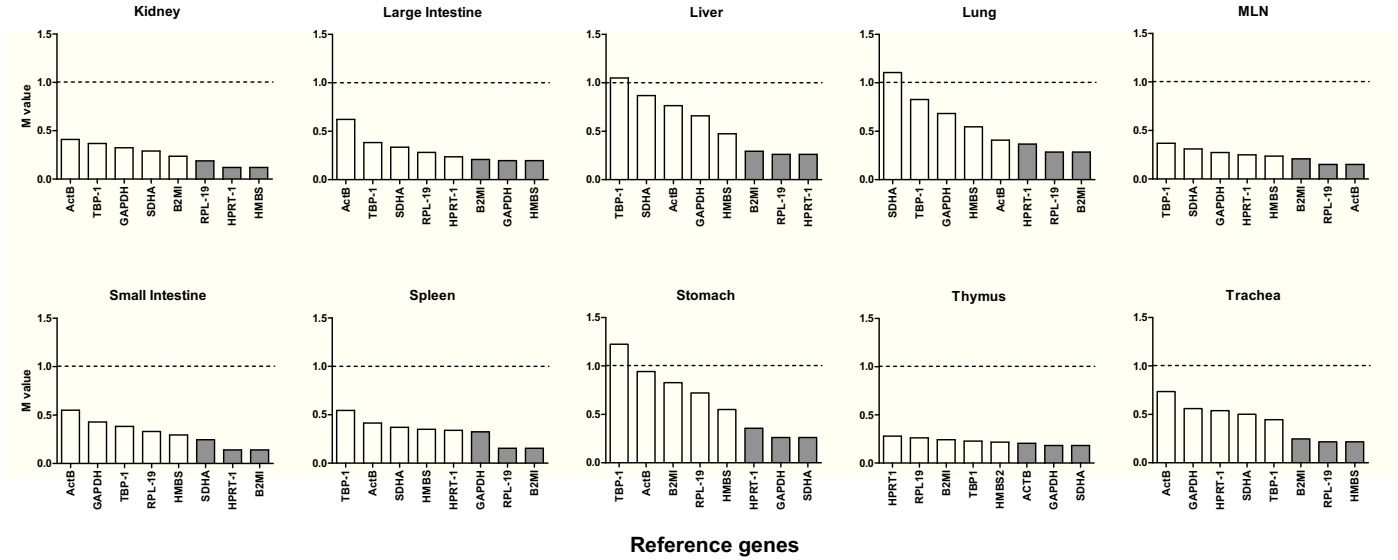

Fig. 1. Selected candidate reference genes and their expression stability for each tissue. Gene expression stability of candidate reference genes was analyzed by the geNorm application. Threshold for eliminating a gene was $\geq$1.0. In grey the three most stable reference genes.

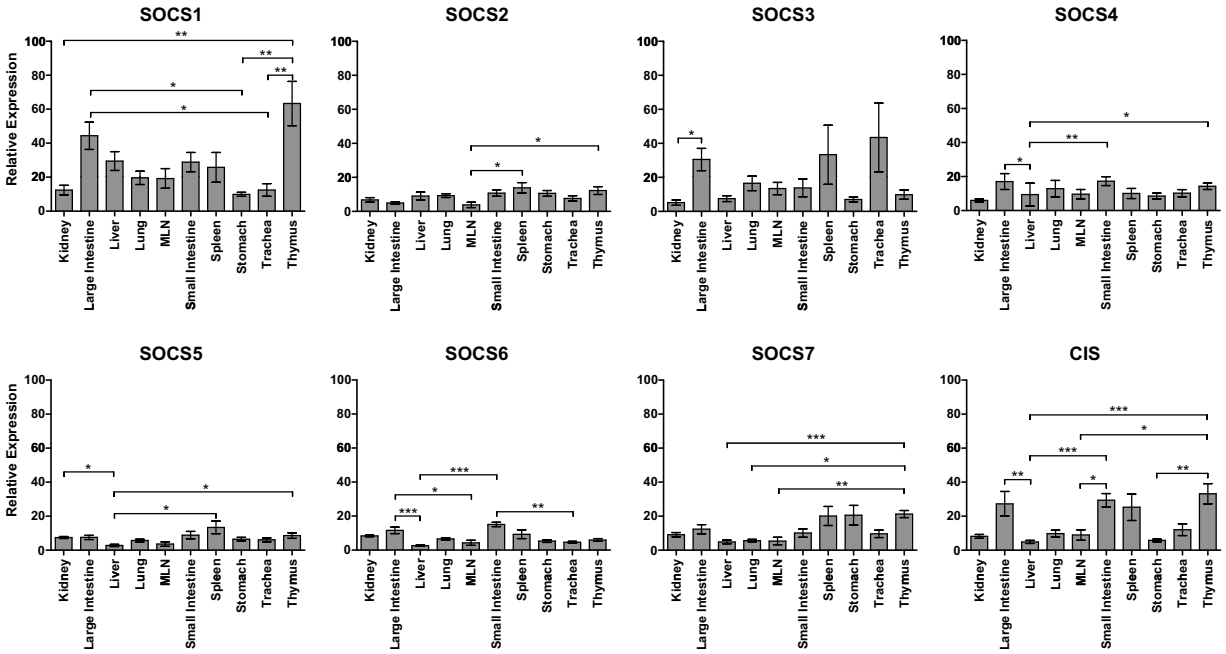

Fig. 2. Relative mRNA expression of SOCS proteins in different tissues from healthy piglets ($n = 10$ minimum for each tissue from a total of twenty piglets). Total RNA was extracted from tissues, complementary DNA was synthesized and transcript expression of SOCS (1–7) and CIS, was assessed using real-time PCR. Comparisons were carried out using non parametric Kruskal–Wallis test and Dunn's post-test. Differences were considered significant when $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

expression of the gene (data not shown). CIS mRNA were more expressed in the thymus, the intestine and the spleen with highest expression in the thymus (for all the significant differences see Fig. 2).

In the current study, we have further investigated the stability of reference genes in porcine tissue. B2MI was most often particularly suitable in the selected tissues in our conditions. Using three optimized reference genes, we assessed mRNA expression of SOCS and CIS in ten porcine tissues. Constitutive mRNA expression was identified for all the members of the SOCS family in the ten selected tissues. Highest mRNA expressions were observed in thymus (SOCS1, SOCS2, SOCS4, SOCS7, and CIS), large intestine (SOCS1, SOCS3, SOCS4, SOCS6, and CIS), small intestine (SOCS1, SOCS4, SOCS6, and CIS), spleen (SOCS2, SOCS3, SOCS5, SOCS7, and CIS), and trachea (SOCS3). These data will help for further studies about the role of SOCS protein in the control of porcine innate and adaptive responses.

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Étude 2

Étude de la réponse immune innée à un virus influenza porcin de sous-type H3N2 au moyen de différents systèmes *in vitro* et *ex vivo*

M. Delgado-Ortega, S. Melo, M. Olivier, J. Dupont, C. Ramé, P. Darsaniya, G. Herrler, D. Soubieux, D. Marc, G. Simon, M. Berri and F. Meurens. *Vet Res* 45(1): 42.

Study of the innate immune response to a H3N2 subtype strain of swine influenza virus in different in vitro *and* ex vivo *systems*

Les maladies respiratoires constituent une problématique importante dans l'élevage porcin, avec des conséquences variables selon la sévérité des lésions, l'état sanitaire du troupeau et les conditions d'élevage. Parmi les virus respiratoires, les virus influenza porcin (SIV) seuls ou en combinaison avec d'autres pathogènes, contribuent très significativement au développement du complexe respiratoire porcin. La réponse immune innée contre les SIV a été décrite dans de nombreuses études (Brookes et al. 2010, Crisci et al. 2013). Cependant, la plupart d'entre elles se focalisaient sur une seule population cellulaire ou étaient directement réalisées dans l'animal, ce qui limitait ou compliquait l'analyse des résultats. Dans notre étude, la réponse immune innée contre une souche de SIV européenne de sous-type H3N2 à été évaluée parallèlement dans la lignée cellulaire *newborn pig trachea* (NPTr), dans des macrophages alvéolaires et dans des explants pulmonaires (*precision-cut lung slices*, PCLS). L'expression de transcrits impliqués dans la reconnaissance du virus, les réponses interféron de type I et III ainsi que dans la régulation de la réponse immune (*suppressors of cytokine signaling*, SOCS) a été évaluée par PCR quantitative à différentes moments. Ensuite, dans les cellules NPTr, l'activation de différentes voies de signalisation impliquées dans la réponse antivirale a été évaluée après infection. En présence du SIV H3N2, l'activation de MAPK (ERK 1/2 et p38) et JAK/STAT a été observée alors que la voie PI3K/Akt ne l'était pas. Afin d'obtenir plus d'informations, l'expression des transcrits impliqués dans la réponse antivirale et des transcrits de SOCS a été évaluée suite au blocage spécifique de chacune des voies. En particulier, l'inhibition de la voie JAK/STAT réduisait significativement les réponses interféron de type I et III ainsi que l'expression des transcrits de SOCS1. L'ensemble des résultats contribue à la compréhension de la réponse immune contre le SIV H3N2 et pourrait aider à termes à identifier de nouvelles stratégies pour lutter contre l'infection.

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VETERINARY RESEARCH

RESEARCH Open Access

Innate immune response to a H3N2 subtype swine influenza virus in newborn porcine trachea cells, alveolar macrophages, and precision-cut lung slices

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Abstract

Viral respiratory diseases remain of major importance in swine breeding units. Swine influenza virus (SIV) is one of the main known contributors to infectious respiratory diseases. The innate immune response to swine influenza viruses has been assessed in many previous studies. However most of these studies were carried out in a single-cell population or directly in the live animal, in all its complexity. In the current study we report the use of a trachea epithelial cell line (newborn pig trachea cells – NPTr) in comparison with alveolar macrophages and lung slices for the characterization of innate immune response to an infection by a European SIV of the H3N2 subtype. The expression pattern of transcripts involved in the recognition of the virus, interferon type I and III responses, and the host-response regulation were assessed by quantitative PCR in response to infection. Some significant differences were observed between the three systems, notably in the expression of type III interferon mRNA. Then, results show a clear induction of JAK/STAT and MAPK signaling pathways in infected NPTr cells. Conversely, PI3K/Akt signaling pathways was not activated. The inhibition of the JAK/STAT pathway clearly reduced interferon type I and III responses and the induction of SOCS1 at the transcript level in infected NPTr cells. Similarly, the inhibition of MAPK pathway reduced viral replication and interferon response. All together, these results contribute to an increased understanding of the innate immune response to H3N2 SIV and may help identify strategies to effectively control SIV infection.

Introduction

Viral respiratory diseases are still a major health issue in pigs reared under confined conditions on intensive breeding farms worldwide. Currently the most common viral pathogens are porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies virus, and porcine circovirus type 2 [1-3]. In the field, these viruses are usually found in association with each other or with bacteria such as *Actinobacillus spp.*, *Bordetella bronchiseptica*, *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, and *Streptococcus suis* [1,3-5]. In particular, influenza A viruses are a major cause of acute respiratory disease on pig breeding farms [6]. Usually, the disease is characterized by depression, loss of appetite, abdominal breathing, tachypnea, fever, and less often, coughing. Morbidity associated with the virus is high and mortality is often very low [6]. Influenza A viruses are classified into subtypes based on the antigenicity of their hemagglutinin (HA) and their neuraminidase (NA) surface proteins [6]. In pigs, three subtypes are described worldwide: H1N1, H1N2, and H3N2 [7,8], however genetic lineage may vary within each subtype depending on the geographical location (North America, Europe, and Asia). Viruses of the three subtypes have been reported frequently in European pigs, often associated with clinical disease [9,10].

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The innate immune response is crucial in the fight against respiratory viruses. It controls viral invasion and replication while the adaptive response is effective for viral clearance through the activity of adaptive immune cells such as lymphocytes [11,12]. Among innate immune cells, epithelial cells, alveolar macrophages, and dendritic cells provide the first line of defense [11-15]. They rapidly recognize pathogens through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), intracellular viral sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), and the nucleotide-binding domain, leucine-rich repeat-containing proteins (NLRs) [16,17]. The innate immune response to SIV has been assessed in many previous studies (for review see [6,16]). However, most of these studies were carried out in a single-cell population, sometimes isolated from other species, or directly in the pig in all its complexity making the establishment of clear conclusions difficult.

Newborn porcine trachea (NPTr) cells [18], porcine alveolar macrophages (PAMs), and precision-cut lung slices (PCLS) may constitute informative and complementary models to study the innate immune response to SIV. Respiratory epithelial cells and PAMs are the main target of the virus in vivo and have been successfully used for in vitro infection studies [6,15,16,19]. PCLS have previously been used for infection studies in birds [20], cattle [21], and pigs [22,23]. The PCLS culture system has several advantages over other systems: the slices can be obtained in large numbers and the general architecture of the tissue is preserved. Thus, differentiated epithelial cells, which are the main target cells of SIV, are maintained in situ and the slices remain viable for more than 7 days [23]. Thus, NPTr cells, PAMs and PCLS together enable the study of host/pathogen interactions in single-cell type populations as well as a multicellular tissue, granting more accurate analysis of the contribution of epithelial cells and macrophages to the global disease response. Use of lung explants (ie PCLS) to study SIV infection has only been reported in a few cases [23-27]. However, the focus of these studies was not the characterization of the innate immune response to the virus. Here, we report the use of NPTr cells in comparison to PAMs and PCLS for the study of the innate immune response to a European H3N2 SIV. Transcripts involved in the recognition of viral patterns, interferon (IFN) type I and III responses, and regulation of the host response (suppressor of cytokine signaling, SOCS) were assessed by qPCR for their expression in response to the infection at different time points. Some significant differences were observed between the three systems, notably in the expression of type III IFN mRNA. For instance, in NPTr cells and PCLS we observed mostly IFNβ and IFNλ1 transcript expression in response to the virus while in PAMs, IFN type III was not significantly induced. The involvement of different signaling pathways such as janus kinase (JAK)/signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2), and phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt) was also evaluated in NPTr cells. Our results show a clear induction of JAK/STAT and MAPK (ERK1/2, p38) signaling pathways while the H3N2 SIV did not induce PI3K/Akt activation in infected NPTr cells. The inhibition of the JAK/STAT pathway using JAK Inhibitor I (420099) in infected NPTR cells had a clear impact on the response of IFN types I and III and the induction of SOCS1 at the transcript level. All together, our data contribute to an increased understanding of the innate immune response of pigs to SIV.

Materials and methods

Ethics statement

Pigs used for this research were kept in the clinic for swine and small ruminants for demonstration and veterinary student training (approval number 33.9-42502-05-09A627) or were obtained from the INRA experimental unit (Nouzilly, France). A total of 12 eight-week-old pigs (German Landrace and Large White) were used. Pigs were healthy and showed no clinical symptoms or serological evidence of influenza and other respiratory or systemic diseases. All studies were carried out in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Treaty Series, nos. 123 [28] and 170 [29] and in accordance with the guidelines of the Institutional Animal Care and Use committee at INRA (France). The protocol was approved by the national and local permitting authorities (animal welfare officer of the University of Veterinary Medicine, Lower Saxony State Office for Consumer Protection and Food Safety). All experimental measurements were in accordance with the requirements of the national animal welfare law. Euthanasia and tissue sampling were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering of the animals.

Epithelial cell line culture

The newborn pig trachea (NPTr, purchased from *Istituto Zooprofilattico Sperimentale, della Lombardia e dell'Emilia Romagna*, Brescia, Italy) cells [18] (between 30 and 50 passages) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Saint-Quentin, France), 20 IU/mL of penicillin, and 20 mg/mL of streptomycin (Invitrogen). Cells were plated onto sterile 24-well plastic plates (Greiner bio-one, Courtabœuf, France) and incubated at 37 °C in a 5%

CO_2 humidified atmosphere. Sub-passages were made when cells reached 100% confluence.

Porcine alveolar macrophages

Porcine alveolar macrophages (PAMs) were obtained from broncho-alveolar lavage (BAL) fluid from a total of 7 eight-week-old pigs and maintained in DMEM supplemented with 2% FCS, 14 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco-Life Technologies SAS, Saint-Aubin, France), 0.1 mM non-essential amino acids (Gibco), 100 μg/mL gentamycin (Gibco), 20 IU/mL of penicillin, and 20 mg/mL of streptomycin (Invitrogen). Red blood cells were eliminated and PAMs were isolated by multiple washes and low-speed centrifugations. PAMs were plated onto sterile 24-well plastic plates (Greiner bio-one) and incubated at 37 °C and 5% CO_2 for 3 h to allow macrophages to adhere. Non-adherent cells were eliminated. PAMs represented > 90% of cells in broncho-alveolar lavage fluid as previously reported [30].

Precision-cut lung slices

Precision-cut lung slices (PCLS) were prepared from the lungs of 5 eight-week-old pigs (minimum one slice/pig for each time point). Immediately after euthanasia, lungs were carefully removed and the left cranial, middle, and caudal lobes were filled with 37 °C low-gelling temperature agarose (GERBU Biotechnik GmbH, Gaiberg, Germany) followed by polymerization on ice. Tissue was excised in cylindrical portions (8-mm tissue-coring tool) and roughly 200 slices/pig approximately 250 μm thick were prepared by using a Krumdieck tissue slicer (model MD4000-01, TSE systems, Chesterfield, MO, USA) with a cycle speed of 60 slices/min. PCLS were incubated in 1 mL of Roswell Park Memorial Institute medium (RPMI) 1640 medium (Gibco), supplemented with 1% antibiotic/antimycotic liquid (100X Antibiotic-Antimycotic, Gibco), 1 μg/mL clotrimazole (Sigma–Aldrich), 10 μg/mL enrofloxacin (Bayer Animal Health, Germany), and 80 μg/mL kanamycin (Gibco) in a sterile 24-well plate at 37 °C and 5% CO_2. The medium was changed every hour during the first 4 h and once after 24 h, prior to infection. Viability was analyzed by observing ciliary activity under a light microscope (Olympus CKX31, Tokyo, Japan). In selected samples, slices were analyzed for bronchoconstriction by addition of 10^{-4} M methacholine (acetyl-ß-methylcholine chloride, Sigma-Aldrich), as previously described [31].

Virus strain and propagation

The swine influenza virus strain A/Swine/Bissendorf/IDT1864/2003 of the H3N2 subtype was isolated from a pig with acute respiratory syndrome in a German herd and kindly provided by Ralf Dürrwald, IDT Biologika GmbH (Dessau-Rosslau, Germany). It was further propagated in the porcine epithelial NPTr cell line by infection at a low multiplicity of infection (MOI) (0.001 plaque forming unit/cell). NPTr cell line has been demonstrated suitable for the culture of SIV [18]. Forty-eight hours post-infection, supernatants were clarified by low-speed centrifugation (2000 × *g*, 5 min), then stored at -80 °C until use. The viral titer of this stock reached 3.3×10^8 plaque forming units (pfu)/mL as determined by a plaque assay on NPTr cells, as described previously [32].

Virus infection

NPTr cells and PAMs were seeded onto sterile 24-well plates at $2\text{-}4 \times 10^5$ cells per well and infected with H3N2 at an MOI of 1 (enabling, according to the Poisson distribution, the infection of 63.2% of the cells with at least one single particle). After 1 h of incubation at 37 °C and 5% CO_2 to allow virus adsorption, cells were washed once with phosphate buffered saline (PBS) and further maintained at 37 °C and 5% CO_2 in 1 mL of medium. For PCLS infection, the procedure was nearly identical except that 10^6 pfu of virus/slice were used since it was not possible to determine the number of target cells in a single slice. The cells and lung slices were incubated at 37 °C and 5% CO_2 for the different time points before collection for RNA extraction or staining.

Real-time PCR assays and validation of reference genes

NPTr cells and PAMs were lysed in RLT lysis buffer (Qiagen, Courtabœuf, France). Precision-cut lung slices were lysed and homogenized in Trizol reagent (Invitrogen) using ceramic beads (BioSpec Products, OK, USA) and the FastPrep FP120 cell disrupter (Qbiogene, Illkirch, France). Total RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations. Quantitative real-time PCR (qPCR) was performed using cDNA synthesized as previously described [33,34]. Primers to assess transcript expression and viral replication were already published or were designed using Clone Manager 9 (Scientific & Educational Software, Cary, NC, USA) and were purchased from Eurogentec (Liège, Belgium) (Table 1). Diluted cDNA (4×) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. The qPCR conditions were 98 °C for 30 s, followed by 37 cycles with denaturation at 95 °C for 15 s and annealing/elongation for 30 s (annealing temperature, Table 1). Real time assays were run on a Bio-Rad Chromo 4 (Bio-Rad, Hercules, CA, USA). The specificity of the qPCR reactions was assessed by analyzing the melting curves of the products and verifying the amplicon sizes. To minimize sample variation we used identical amounts of high quality RNA from cells and tissue. The RNA quality was assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Massy, France) and RNA integrity numbers

Table 1 Primer sequences, annealing temperatures of primer sets (°C), expected PCR fragment sizes (bp) and accession numbers or references

Primer name	Primer sequence	Annealing temperature (s) (°C)	PCR product (bp)	Accession number or reference
ActB	CACGCCATCCTGCGTCTGGA	63	100	[38]
Beta actin	AGCACCGTGTTGGCGTAGAG			
B2MI	CAAGATAGTTAAGTGGGATCGAGAC	58	161	[38]
Beta-2-microgobulin	TGGTAACATCAATACGATTTCTGA			
CISH	GGGAATCTGGCTGGTATTGG	62	126	BE014034
Cytokine-inducible SH2-containing protein	CCGACAGTGTGAACAGGTAG			
GAPDH	CTTCACGACCATGGAGAAGG	63	170	AF017079
Glyceraldehyde-3-phosphate dehydrogenase	CCAAGCAGTTGGTGGTACAG			
HMBS-2	AGGATGGGCAACTCTACCTG	58	83	[38]
Hydroxymethylbilane synthase 2	GATGGTGGCCTGCATAGTCT			
HPRT-1	GGACTTGAATCATGTTTGTG	60	91	[38]
Hypoxanthine phosphoribosyltransferase 1	CAGATGTTTCCAAACTCAAC			
IL-1β	AGAAGAGCCCATCGTCCTTG	62	139	NM_001005149
Interleukine 1 beta	GAGAGCCTTCAGCTCATGTG			
IL-6	ATCAGGAGACCTGCTTGATG	62	177	NM_214399
Interleukine 6	TGGTGGCTTTGTCTGGATTC			
IL-8	TCCTGCTTTCTGCAGCTCTC	62	100	NM_213867
Interleukine 8	GGGTGGAAAGGTGTGGAATG			
IFNα (1-17)	GGCTCTGGTGCATGAGATGC	64	197	[39]
Interferon alpha (Type I)	CAGCCAGGATGGAGTCCTCC			
IFNβ	ATGTCAGAAGCTCCTGGGACAGTT	62	246	[39]
Interferon beta (Type I)	AGGTCATCCATCTGCCCATCAAGT			
IFNγ	GCTCTGGGAAACTGAATGAC	60	167	NM_213948
Interferon gamma (Type II)	TCTCTGGCCTTGGAACATAG			
IFNλ1/IL-29A	GAGGCTGAGCTAGACTTGAC	60	115	NM_001142837
Interferon lambda1 (Type III)	CCTGAAGTTCGACGTGGATG			
IFNλ3/IL-28B	GGCTCCTTGGCGAACTCATC	62	173	GQ996936
Interferon lambda3 (Type III)	TCCTTCTTCTGGGCCTCCTG			
iNOS	GAGAGGCAGAGGCTTGAGAC	62	178	BI344008
Inducible nitric oxide synthase	TGGAGGAGCTGATGGAGTAG			
Mx1	AGTGTCGGCTGTTTACCAAG	60	151	NM_214061
Myxovirus resistance 1	TTCACAAACCCTGGCAACTC			
Mx2	CCGACTTCAGTTCAGGATGG	62	156	AB258432
Myxovirus resistance 2	ACAGGAGACGGTCCGTTTAC			
OAS1	CCCTGTTCGCGTCTCCAAAG	64	303	NM_214303
2′-5′-Oligoadenylate synthetase 1	GCGGGCAGGACATCAAACTC			
SIV M protein	AGATGAGTCTTCTAACCGAGGTCG	62	100	[40]
	TGCAAAAACATCTTCAAGTCTCTG			
PKR	GACATCCAAAGCAGCTCTCC	62	365	NM_214319
Protein Kinase RNA-dependent	CGCTCTACCTTCTCGCAATC			
RPL-19	AACTCCCGTCAGCAGATCC	60	147	[41]
Ribosomal protein L19	AGTACCCTTCCGCTTACCG			

Table 1 Primer sequences, annealing temperatures of primer sets (°C), expected PCR fragment sizes (bp) and accession numbers or references *(Continued)*

RIG-I	CGACATTGCTCAGTGCAATC	60	126	NM_213804
Retinoic acid-inducible gene I	TCAGCGTTAGCAGTCAGAAG			
SDHA	CTACAAGGGGCAGGTTCTGA	58	141	[38]
Succinate dehydrogenase complex subunit A	AAGACAACGAGGTCCAGGAG			
SOCS1	CGCCCTCAGTGTGAAGATGG	62	110	EW101597
Suppressor of cytokine signaling 1	GCTCGAAGAGGCAGTCGAAG			
SOCS3	CAGCTCCAAGAGCGAGTACC	61	179	NM_001123196
Suppressor of cytokine signaling 3	TGACGCTGAGCGTGAAGAGG			
TBP-1	AACAGTTCAGTAGTTATGAGCCAGA	60	153	[38]
TATA box binding protein 1	AGATGTTCTCAAACGCTTCG			
TLR3	CCTGCATTCCAGAAGTTGAG	60	152	NM_001097444
Toll Like Receptor 3	TGAGGTGGAGTATTGCAGAG			
TLR7	TCAGCTACAACCAGCTGAAG	60	140	NM_001097434
Toll Like Receptor 7	CAGATGTCGCAACTGGAAAG			
TLR8	AGCGCGGGAGGAGTATTGTG	62	118	NM_214187
Toll Like Receptor 8	GCCAGGGCAGCCAACATAAC			
TNFα	CCAATGGCAGAGTGGGTATG	62	116	X54859
Tumor Necrosis Factor alpha	TGAAGAGGACCTGGGAGTAG			

Reference genes are in italic.

(RIN) were calculated. RIN were always ≥ 8.1 for tissue and ≥ 9.5 for cells. Samples were normalized internally by simultaneously using the average Cycle quantification (*Cq*) of the three most suitable reference genes in each sample to avoid any artefact of variation in the target gene. These three most suitable reference genes were selected among eight commonly used reference genes. The genes included beta-actin (ActB), beta-2-microglobulin (B2MI), glyceral dehyde-3-phosphate dehydrogenase (GAPDH), hydroxy-methylbilane synthase (HMBS), hypoxanthine phosphori bosyltransferase-1 (HPRT-1), ribosomal protein L-19 (RPL-19), succinate dehydrogenase complex subunit A (SDHA) and TATA box binding protein-1 (TPB-1) (Table 1). The stability of these reference genes was investigated simultaneously in control and infected (1 to 24 h post-infection) NPTR cells, PAMs, and PCLS using the geNorm application [31,35,36]. The threshold for eliminating a gene was $M \geq 1$ for tissue samples and $M \geq 0.5$ for cells as recommended [36]. The correlation coefficients of the standard curves were > 0.995 and the concentration of the test samples was calculated from the standard curves, according to the formula $y = -M^{*}Cq + B$, where M is the slope of the curve, *Cq* the first positive second derivative maximum of amplification curve calculated using PCR Miner [37] and B the y-axis intercept. All qPCRs displayed efficiency between 90% and 110%. Expression data were expressed as relative values after Genex macro analysis (Bio-Rad, Hercules, CA, USA) [35].

Cryosections and immunofluorescence analysis

Infected and non-infected PCLS were mounted on small pieces of filter paper with tissue-freezing medium (Jung, Heidelberg, Germany), then frozen in liquid nitrogen and kept at -80 °C prior to cutting. Ten μm-thick slices were cut by a cryotome (Reichert-Jung, Nußloch, Germany). The sections were dried overnight at room temperature and then kept frozen at -20 °C until staining.

The sections were fixed with 3% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 5 min followed by three washing steps with PBS. All antibodies were diluted in 1% bovine serum albumin (Sigma-Aldrich) and incubated with the sections for 1 h at room temperature (RT) in a humid incubation chamber. After the final incubation step, the sections were washed three times with PBS and once with distilled water. The slices were embedded in Mowiol 4-88 resin (Sigma-Aldrich), covered by no. 1½ circular micro-cover glass (12 mm) (Electron Microscopy Sciences, Hatfield, PA, USA), and stored at 4 °C until examination under the confocal microscope. For detection of infected cells, a monoclonal antibody (IgG2a) against the influenza A virus nucleoprotein (NP) (Clone AA5H, AbDSeroTec MCA400, Düsseldorf, Germany) was used at a 1:750 dilution followed by incubation with an anti-mouse IgG (Sigma-Aldrich) secondary antibody. To visualize cilia, samples were treated with a Cy3-labeled monoclonal antibody recognizing beta-tubulin (dilution 1/600) (Sigma-Aldrich). Nuclei were stained by incubating sections for

15 min at 37 °C in 4′,6′-diamidino-2-phenylindole (DAPI) (Life Technologies Inc., Darmstadt, Germany).

Western blotting

NPTr cells (2-4 × 10^5 cells/well) were virus-infected at an MOI of 1, then incubated for 5, 10, 30, 60 or 240 min. Cells were then disrupted using the lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1 mM ethylene diamine tetraacetic acid-EDTA, 1% (v/v) Triton ×-100, 0.5% NP-40), protease inhibitors (2 mM phenyl methyl sulfonyl fluoride-PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate) (Sigma-Aldrich) (Bio-Rad, Marnes-la-Coquette, France). Lysates were incubated on ice for 30 min and then centrifuged at 12 000 × *g* for 20 min at 4 °C. Equal amounts of proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. Membranes were then incubated for 1 h at RT with Tris-buffered saline (TBS, 2 mM Tris-HCL, pH 8, 15 mM NaCl, pH 7.6), containing 5% non-fat dry milk powder (NFDMP) and 0.1% Tween-20 (Bio-Rad) to saturate non-specific sites. Then membranes were incubated overnight at 4 °C with appropriate primary antibodies (final dilution 1:1000, see Table 2) in TBS containing 0.1% Tween-20 and 5% NFDMP. The membranes were washed in TBS-0.1% Tween-20 and incubated for 2 h at RT with a horseradish peroxidase-conjugated secondary antibody (final dilution 1:10 000). After washing, 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) with the GeneSnap software (Syngene UK, Cambridge, UK, release 7.09.17). Detected signals were quantified with the GeneTools software (Syngene UK, release 4.01.02). The results are expressed as the signal intensity in arbitrary units after normalization as indicated in the figure legends.

Antibodies and chemical inhibitors

Antibodies to phospho-Akt (Ser 473), phospho-ERK1/2 (Thr 202/Tyr 204), phospho-JAK2 (Tyr 1007/1008), and phospho-p38 (Thr180/Tyr182) were purchased from Ozyme. Monoclonal and polyclonal antibodies to Akt, ERK2, p38, and JAK2 were obtained from Tebu Bio (Le Perray-en-Yvelines, France) and Ozyme. All antibodies were used at a 1:1000 dilution in assays. Stock solutions of pharmacological inhibitors such as inhibitor U0126 (inhibiting MEK1/2 and ERK1/2), p38/SAPK2-specific inhibitor SB 202190, and JAK Inhibitor I (420099) (Millipore, Molsheim, France) were all prepared as 1000-× concentrated stocks in dimethyl sulfoxide (DMSO), in order to ensure that the final concentration of DMSO in the culture medium did not exceed 0.1%. Starting one hour before infection, NPTr cells were treated for the whole procedure with each inhibitor at a concentration of 10 μM to block the signaling. The blockage of the cascades was verified at 30 min post-infection.

Statistical analysis

Expression of mRNA in cells and tissues was expressed as relative values. All statistical analyses were done using Prism 5 computer software (Prism 5 for Windows; GraphPad Software, San Diego, CA, USA). One-Way ANOVA was used to detect differences between groups. To account for the non-normal distribution, all data were sorted by rank prior to performing the ANOVA. Tukey's test was used to compare the means of the ranks among the groups. *P* values less than 0.05 were considered significant.

Table 2 Antibodies used for Western blotting

Target	Antibody	Dilution
Phospho-Akt	Rabbit polyclonal anti-phospho-Akt (Ser473) #9271 (Ozyme)	1/1000
Phospho-ERK1/2	Rabbit monoclonal anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) #4370 (Ozyme)	1/1000
Phospho-JAK2	Rabbit polyclonal anti-phospho-JAK2 (Tyr1007/1008) #3771 (Ozyme)	1/1000
Phospho-p38	Rabbit monoclonal anti-phospho-p38 MAPK (Thr180/Tyr182) (12 F8) #4631 (Ozyme)	1/1000
Akt	Rabbit monoclonal anti-Akt (11E7) #4685 (Ozyme)	1/1000
ERK2	Rabbit polyclonal anti-ERK2 (GTX27948) (tebu-bio)	1/1000
JAK2	Rabbit monoclonal anti-JAK2 (D2E12) #3230 (Ozyme)	1/1000
p38	Rabbit polyclonal anti-p38 MAPK (GTX50566) (tebu-bio)	1/1000

See also Additional files 1 and 2.

Results

Reference gene selection

In order to characterize the immune response against our strain of SIV in vivo and ex vivo using qPCR assays, validation of the three most stable reference genes in NPTr cells, PAMs, and PCLS was required (Figure 1). Eight previously described reference genes [33,38,42] were selected for assessment of their stability in our conditions. The selection was based on the M value using geNorm. The threshold was set at 0.5 for homogenous samples (e.g. NPTr cells and PAMs) and 1 for heterogeneous samples (PCLS) [36]. The geNorm analysis showed that HPRT-1, HMBS-2, and RPL-19 were the three most stable genes in NPTr cells with an M value of 0.119, 0.119, and 0.117, respectively (Figure 1). All of the reference genes tested had M values below the threshold, except ActB (0.501). In PAMs the situation was roughly similar and again ActB was the only gene with an M value above the elimination threshold (0.523); the three most stable genes were SDHA, RPL-19, and TBP-1 (0.293, 0.232, and 0.232, respectively) (Figure 1). The selected genes for PCLS were TBP-1, HPRT-1 and HMBS-2, having an M value of 0.305, 0.211, and 0.211, respectively (Figure 1). In the three systems, RPL19 was considered the most stable reference gene. ActB was the most variable reference gene with an M value equal or higher than the threshold.

Viral replication in newborn pig trachea cells and alveolar macrophages

In order to verify the capacity of our SIV strain to infect NPTr cells and PAMs, we assessed viral replication through quantification of the viral M-segment RNA (M-vRNA) in both cell types (Figures 2 and 3). M gene quantification was below the experimental background in both control groups (Figures 2 and 3). In infected NPTr cells, M-vRNA was detected ($P < 0.001$) at 1 h post-infection (hpi) and reached its highest levels 8 and 24 hpi ($P < 0.001$) (Figure 2). Similar results were obtained in PAMs, with detection of M-vRNA as early as 1 hpi (Figure 3); however its level remained slightly lower than that observed in NPTr cells (*Cq* 28 at 1 h for both cell types, and 24 and 18 at 8 h for PAMs and NPTr, respectively). The highest levels of M-vRNA were also observed 8-24 hpi in PAMs (Figure 3).

Innate immune response evaluation in epithelial cells and alveolar macrophages

Respiratory epithelial cells and PAMs are the first cells encountered by the influenza A virus during an infection in the pig. To explore the innate cellular response, the expression of various transcripts was assessed (Table 1 and Figures 2 and 3). The virus induced the expression of RIG-I mRNA as early as 3 hpi reaching the highest levels of expression at 24 h in NPTr cells (Figure 2). In PAMs, a significant increase ($P < 0.001$) of RIG-I mRNA was observed only after 24 h of infection (Figure 3). Regarding TLR3, TLR7, and TLR8 no statistically significant differences were observed between control and infected NPTr cells (data not shown). However in PAMs, TLR3, TLR7, and TLR8 mRNA expressions were significantly up-regulated by the viral infection (Figure 3). Expression of IFNβ mRNA was clearly increased from 3 hpi in NPTr cells ($P < 0.05$), and from 8 hpi in PAMs ($P < 0.001$) (Figures 2 and 3), and reached its highest level at 24 hpi in both cell types ($P < 0.001$). No significant differences in IFNα mRNA were observed between controls and infected cells, whatever the cell type (Additional file 1). For IFN type III such as IFNλ1, statistically significant increases were observed after 8 and 24 h in NPTr cells but not in PAMs (Figures 2 and 3) while no significant differences were observed for IFNλ3 (data not shown). Regarding the interferon-stimulated genes (ISGs) (Figures 2 and 3), virus-induced mRNA over-expressions

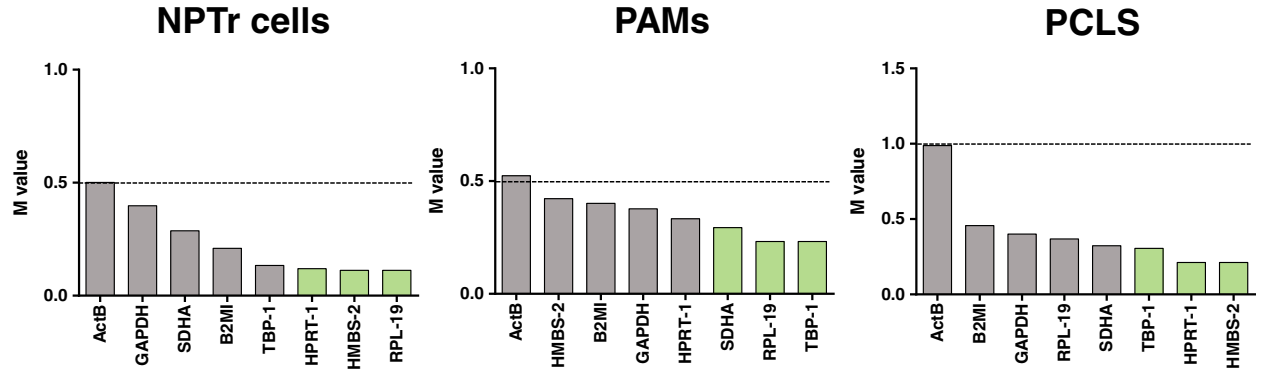

Figure 1 Selected candidate reference genes and their expression stability. The stability of the reference genes has been assessed in a mix of non-infected and infected newborn pig trachea cells (NPTr), porcine alveolar macrophages (PAMs) and precision-cut lung slices (PCLS) at the different time points. Gene expression stability of candidate reference genes was analyzed by the geNorm application. Threshold for eliminating a gene was ≥ 1.0 for PCLS and ≥ 0.5 for NPTr cells and PAMs. The three most stable reference genes are depicted in green.

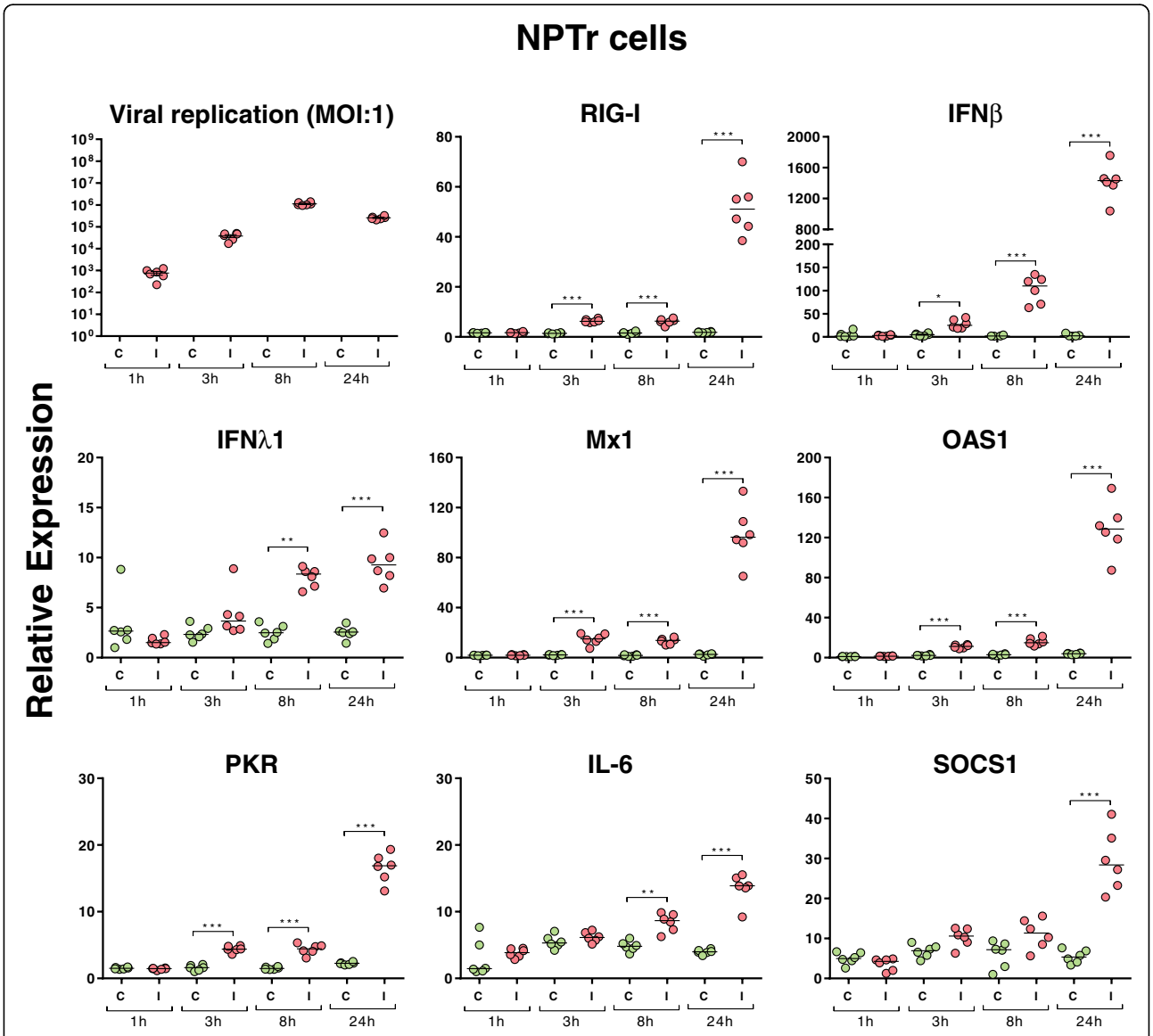

Figure 2 Relative expression of M-viral RNA and of host transcripts in infected newborn pig trachea cells. The NPTr cells were infected with the H3N2 SIV strain at different time points (1 h, 3 h, 8 h, and 24 h). Green dots stand for non-infected NPTr cells and red dots for infected cells (individual values (dots) and median value (bar), $n = 6$ wells per condition). Comparisons were made using one way ANOVA test and Tukey's post-test. Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

($P < 0.05$ and 0.001) were observed for myxovirus resistance 1 (Mx1), and Mx2 (data not shown), 2′-5′ oligoadenylate synthetase 1 (OAS1), and protein kinase R (PKR) from 3 hpi in NPTr cells and from 8 (Mx1) or 24 hpi (Mx2 – data not shown) in PAMs ($P < 0.001$). The pattern of expression for these genes was similar to that observed for IFNβ transcripts. Among all analyzed SOCS transcripts, only SOCS1 mRNA showed a statistically increased expression after 24 h in both NPTr cells and PAMs ($P < 0.001$). IL-6 (Figure 2) and IL-8 (data not shown) mRNAs were also up-regulated in response to the infection in NPTr cells at 8 and 24 hpi but not in PAMs. Regarding IL-1β and TNFα transcripts, we did not detect any significant differences between conditions in NPTr cells even though some trends of induction by the virus were observed (data not shown), while TNFα mRNA expression was significantly increased ($P < 0.001$) in response to the virus in PAMs after only 3 h of infection (Figure 3).

Viral infection of precision-cut lung slices

The innate response of PCLS infected with SIV was also analyzed. A minimum of one slice/pig ($n = 5$) was generated at each time point. The viability of the PCLS was verified prior to infection: the ciliary activity of the bronchial epithelium was maintained when analyzed at 24, 48, and 96 h after their preparation (data not shown).

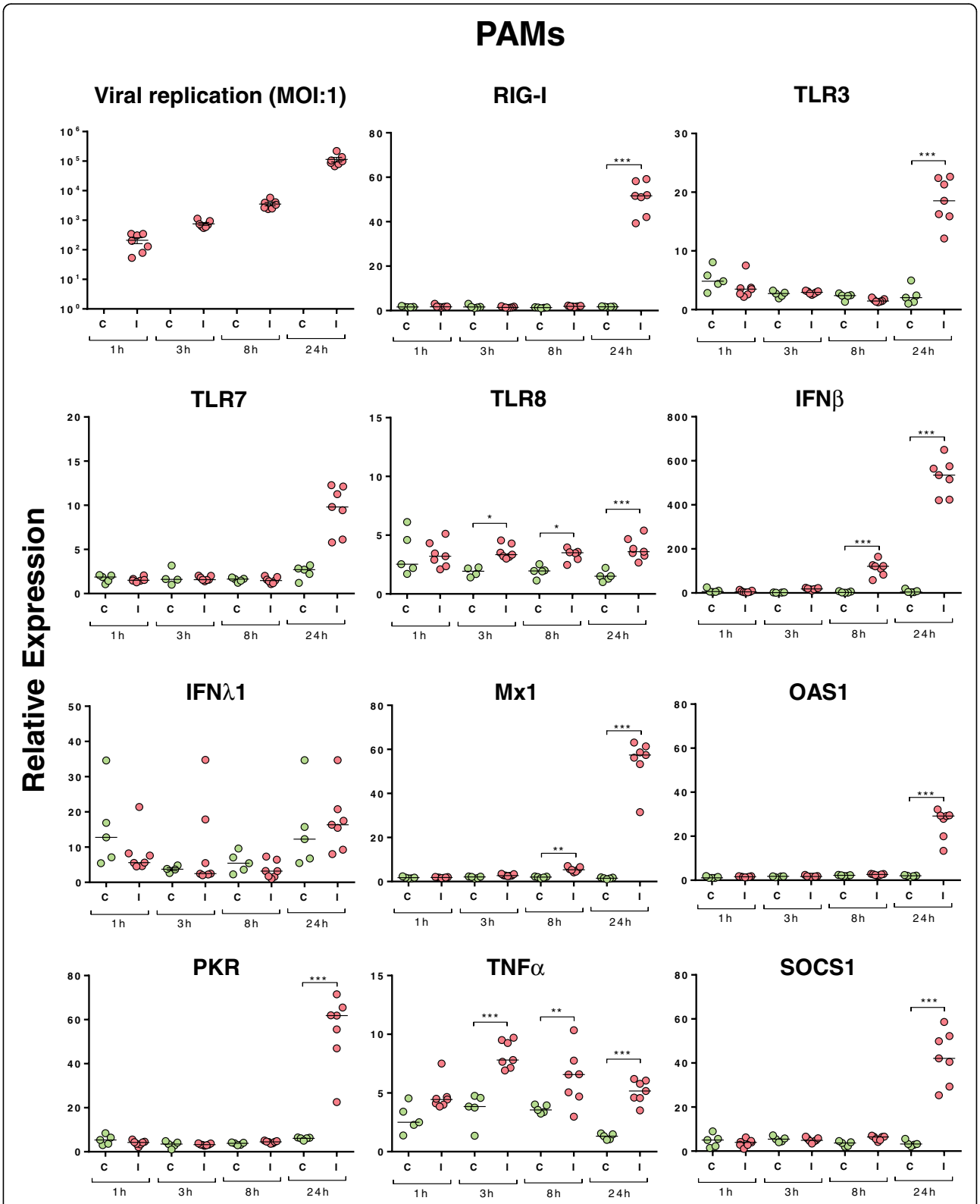

Figure 3 Relative expression of various viral and host transcripts in infected porcine alveolar macrophages (PAMs). The cells were infected with the H3N2 SIV strain at different time points (1 h, 3 h, 8 h, and 24 h). Green dots stand for non-infected PAMs and red dots for infected PAMs (each individual value ($n = 7$) represents the data obtained with PAMs prepared from one pig, bar represents the median value). Comparisons were made using one way ANOVA test and Tukey's post-test. Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

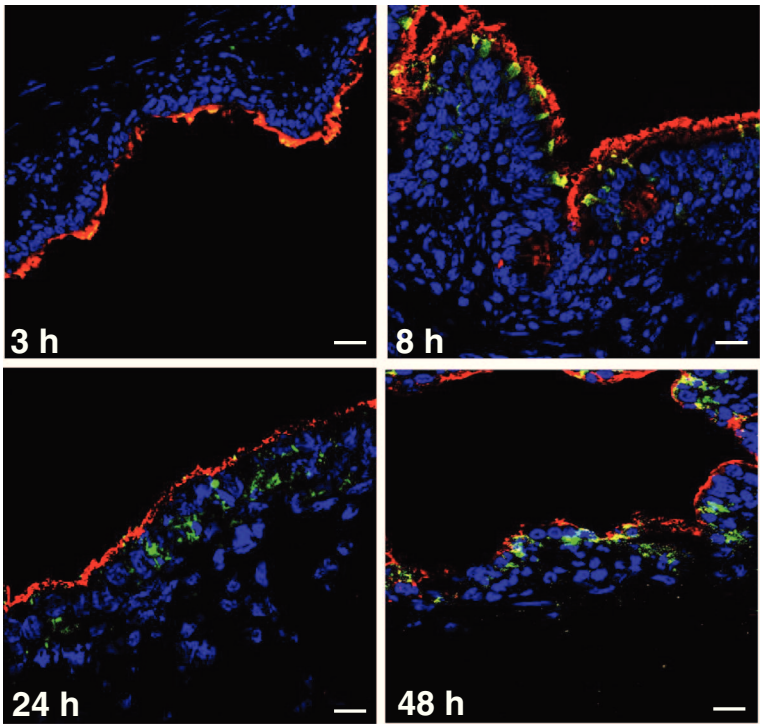

Figure 4 Immunostaining of infected Precision-cut lung slices (PCLS). The slices were infected by the H3N2 SIV strain. Cryosections were prepared after 3, 8, 24, and 48 h of infection and image data were collected using a laser-scanning confocal microscope. Infected cells were stained with an anti-nucleoprotein polyclonal antibody (green) for the detection of SIV. Ciliated cells were stained using an anti-beta-tubulin monoclonal antibody (red). Cell nuclei of prepared slides were stained by incubation with 4′,6′-diamidino-2-phenylindole (DAPI, blue). Scale bar = 20 μm.

Additionally, bronchoconstriction could be triggered by the use of 10^{-4} M methacholine in the four days following slice preparation (data not shown) and subsequently reversed by removal of the drug. These observations provided evidence that porcine PCLS remained viable for up to 96 h under the incubation conditions described in the material and methods section. To monitor viral infection of the slices, cryosections of PCLS were stained with an antibody-targeting SIV nucleoprotein. Only a few infected cells were observed after 3 h of culture with the virus (Figure 4), while the number was considerably increased at 8 hpi. The general architecture of the tissue was progressively altered by the viral infectious process, and after 24 and 48 h a significant lysis of the epithelium was observed (Figure 4). In parallel, viral replication was assessed in the PCLS by qPCR assays targeting the viral M gene segment (Figure 5). M-vRNA was detected only in infected groups, reaching its maximum at 24 hpi (average *Cq*: 22).

Host transcript expression in precision-cut lung slices

Lung slices offer a more realistic system than cell lines to study the interaction between host and pathogen. To better characterize the innate immune response toward the SIV strain, PCLS were infected and the expression of several host genes involved in the antiviral response was assessed at different time points using qPCR assays (Table 1 and Figure 5). An increase in RIG-I transcripts was observed at 8 h, but it did not become significant until 24 hpi ($P < 0.001$). We did not detect any statistically significant increase in expression of TLR3, TLR7, and TLR8 transcripts post-infection, however some trends potentially indicating a viral induction were observed (data not shown). In response to the virus, IFNβ and IFNλ1 mRNA expression were significantly increased ($P < 0.001$) at 24 hpi (Figure 5) while no significant increase was observed for IFNα and IFNλ3 (Additional file 1 and data not shown). Along with the increased expression of IFN types I and III transcripts, mRNA expression of ISGs Mx1, Mx2, and PKR was also significantly elevated relative to controls after 24 h of infection ($P < 0.001$) (Figure 5 and data not shown). The mRNA expression of OAS1 was significantly increased ($P < 0.05$) after only 8 h of infection (Figure 5). Unlike IL-1β, IL-8, and TNFα, IL-6 transcription was significantly up-regulated 8 h after infection (Figure 5). Overall, except for IFNλ1 and IL-6, similar patterns of transcript expression were observed in vitro in the epithelial cells and alveolar macrophages and ex vivo in the PCLS. Regarding the mRNA expression of SOCS (CISH, SOCS1, and

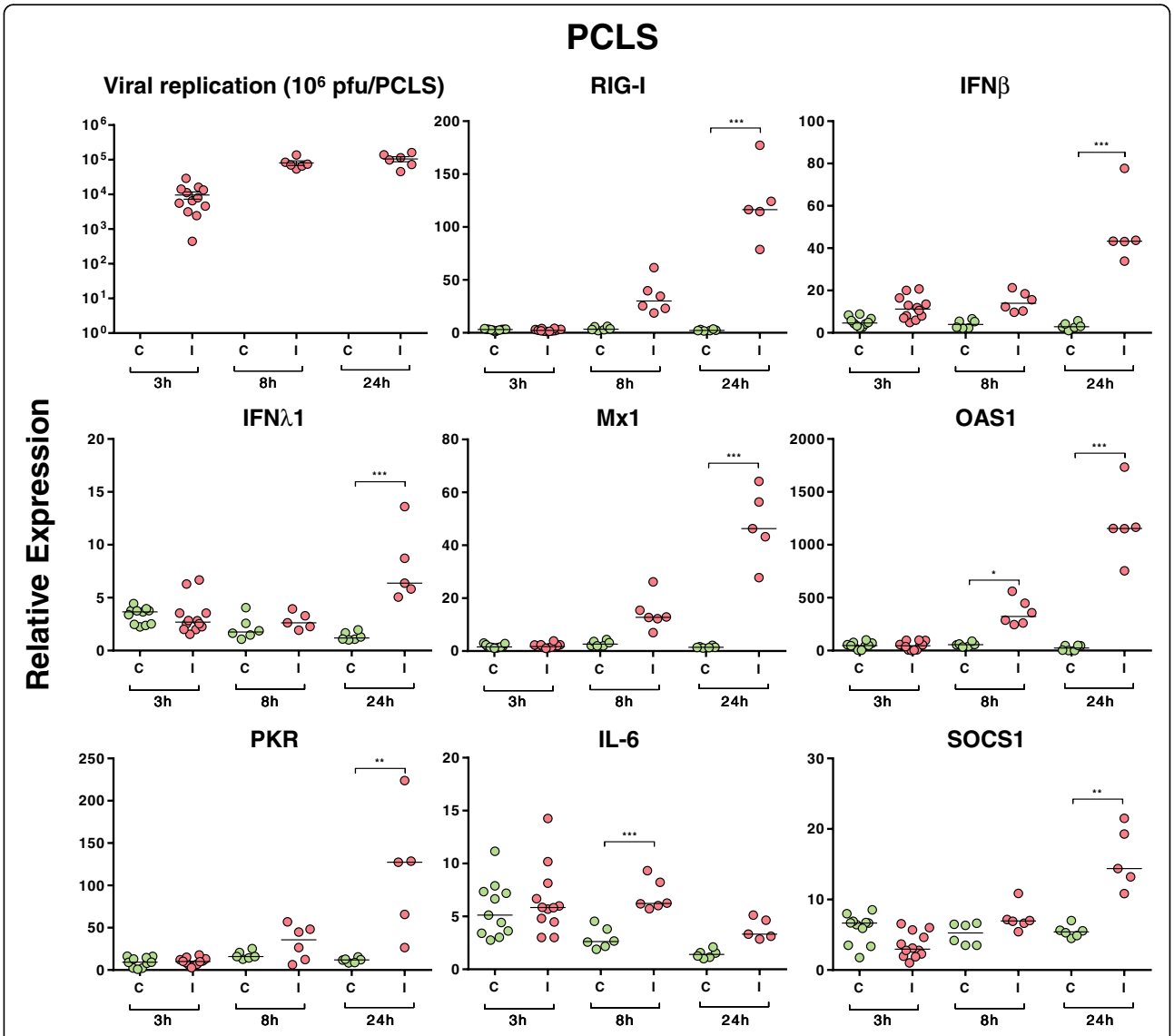

Figure 5 Relative expression of M-vRNA and of host transcripts in infected precision-cut lung slices (PCLS). The slices were infected with the H3N2 SIV strain at different time points (3 h, 8 h, and 24 h). Green dots stand for non-infected PCLS and red dots for infected PCLS (minimum one slice/pig for each time point, total 5 pigs, $n = 5$-12 and median value). Comparisons were made using one way ANOVA test and Tukey's post-test. Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

SOCS3), only SOCS1 transcripts were significantly increased at 24 hpi in PCLS ($P < 0.01$) (Figure 5). These findings are similar to those observed with NPTr cells and PAMs and suggest an involvement of SOCS1 in the immune response against SIV.

Activation of MAP kinases and JAK/STAT pathways in virus-infected epithelial cells

During influenza A infection, various signaling cascades are triggered to counteract pathogen replication and spreading. Many of these signaling pathways are controlled to a certain extent by SOCS proteins [43]. We examined the activation of several signaling pathways involved in pro-inflammatory and antiviral gene expression in response to SIV infection in NPTr cells. MAPK pathways, both ERK1/2 and p38, showed phosphorylations after 5 min of contact with the virus while phosphorylation-mediated activation of JAK2 appeared between 30 and 60 min post-infection (Figures 6A, B, and C). In contrast, activation of the PI3K/Akt signaling pathway was not observed at any time points (Figure 6D); in general, a peak of phosphorylation was observed after 30 to 60 min of infection, followed by a decrease 4 hpi (Figures 6A, B, and C). These results showed that our SIV strain efficiently and rapidly activated MAPK (ERK1/2, p38) and JAK/STAT pathways in NPTr cells.

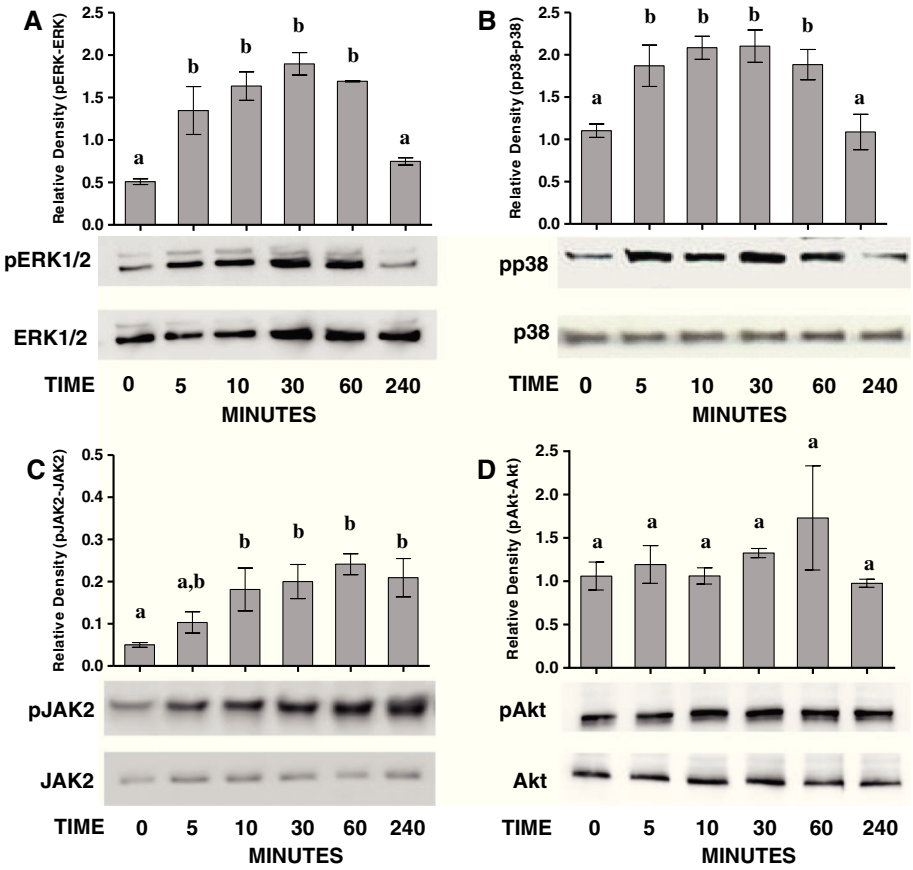

Figure 6 Western blots in infected NPTr cells. Western blots for phospho-ERK1/2 (pERK1/2) **(A)**, phospho-p38 (pp38) **(B)**, phospho-JAK2 (pJAK2) **(C)**, and phospho-Akt (pAkt) **(D)** were performed in newborn porcine trachea (NPTr) cells infected with H3N2 SIV at different time points (0, 5, 10, 30, 60, and 240 min). Total ERK1/2, p38 and JAK2 are shown as loading controls and did not change with each condition over time. Data are presented as means ± SEM, ($n = 3$). Results are representative of three independent experiments. Different letters indicate significant differences (one way ANOVA, $P < 0.05$) as compared to control (time 0).

Specific inhibition of pathways activated in epithelial cells

To investigate whether the MAPK and JAK/STAT pathways are involved in the regulation of SOCS1 mRNA expression in porcine epithelial cells, NPTr cells were pre-treated with chemical inhibitors targeting these pathways, and the expression of SOCS1 transcripts was assessed at 24 hpi (Figures 7, 8 and 9). One h after the pre-treatment, NPTr cells were infected with the H3N2 strain and phosphorylation was evaluated at 30 min post-infection, corresponding to the peak of activation (Figure 7). NPTr cells pre-treated with inhibitors of ERK1/2 (U0126), p38 (SB 202190) and JAK/STAT (JAK Inhibitor I) in the absence of a virus infection showed basal levels of phosphorylation similar to those observed in control groups. By contrast, pre-treated infected cells displayed a complete abolition of phosphorylation in ERK1/2, p38 and JAK/STAT pathways (Figure 7) lasting more than 24 h (data not shown). In order to determine whether these signaling pathways are involved in the regulation of SOCS1 mRNA expression, the assessment of SOCS1 mRNA expression after inhibitor treatments was performed following the same timing (Figure 8) as in the previous experiment (Figure 2). It was observed that the blockade of ERK1/2 and p38 did not significantly affect SOCS1 mRNA expression after 24 h of infection (Figure 8). However, a statistically significant decrease of SOCS1 mRNA expression was observed in NPTr cells treated with JAK Inhibitor I demonstrating an association between activation of JAK/STAT pathway and SOCS1 mRNA expression in porcine respiratory epithelial cells. To further assess the impact of JAK/STAT pathway-inhibition on virus replication and innate response, the relative expression of M-vRNA and mRNAs for several host proteins was assessed (Figure 9). Viral replication was not significantly modified by JAK Inhibitor I except after 24 h of infection ($P < 0.001$) (Figure 9). Similarly the transcript expression of RIG-I, IFNβ, IFNλ1, PKR, and Mx1 was significantly decreased ($P < 0.001$) under JAK Inhibitor I treatment after 24 h of infection (Figure 9). The inhibition of ERK1/2 and p38

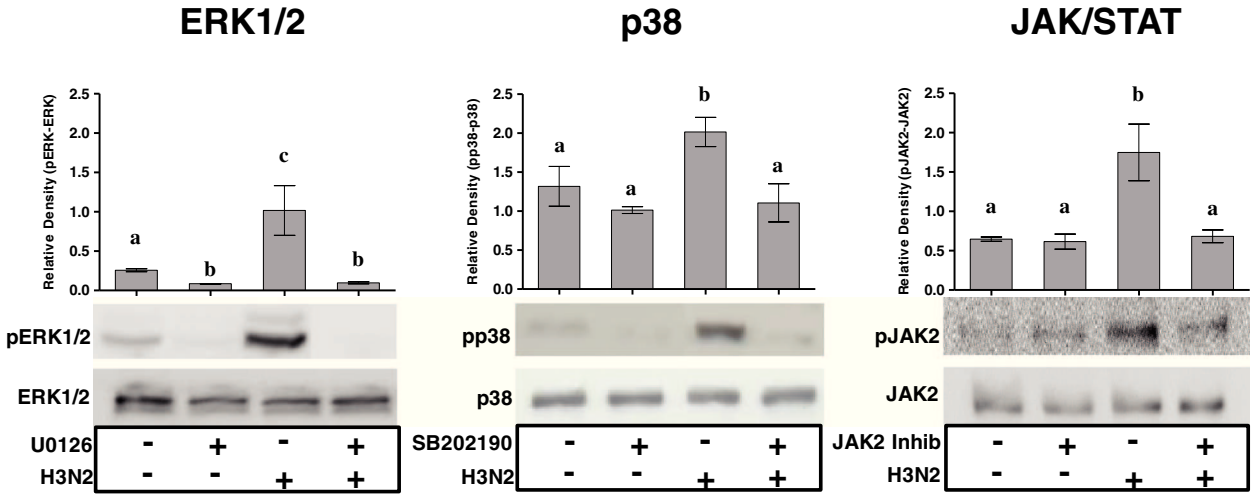

Figure 7 Western blots in infected NPTr cells in presence or absence of chemical inhibitor. Western blots for phospho-ERK1/2 (pERK1/2), phospho-p38 (pp38) and phospho-JAK2 (pJAK2) in newborn porcine trachea (NPTr) cells infected with H3N2 SIV at 30 min in presence or in absence of chemical inhibitors UO126 (ERK1/2), SB202190 (p38), and JAK inhibitor (JAK Inhibitor I). The control group was cultured in presence of DMSO, data are represented as means ± SEM, ($n = 3$). Results are representative of three independent experiments. Different letters indicate significant differences (one way ANOVA, $P < 0.05$) as compared to control (without inhibitor and virus).

had less impact on viral replication and interferon response (Additional file 2). Viral replication was significantly reduced ($P < 0.001$) at 8 (p38) and 24 hpi (ERK1/2) and OAS1 transcript expression was significantly reduced ($P < 0.001$) with both inhibitors 24 hpi. All together these results demonstrated a link between the JAK/STAT pathway and SOCS1 in pigs.

Discussion

The innate immune response to swine influenza viruses has been assessed in many previous studies [6,16]. However, to our knowledge most of these studies were carried out in single-cell populations or directly in an animal host. In this study we compared an epithelial cell line (NPTr), alveolar macrophages (PAMs), and

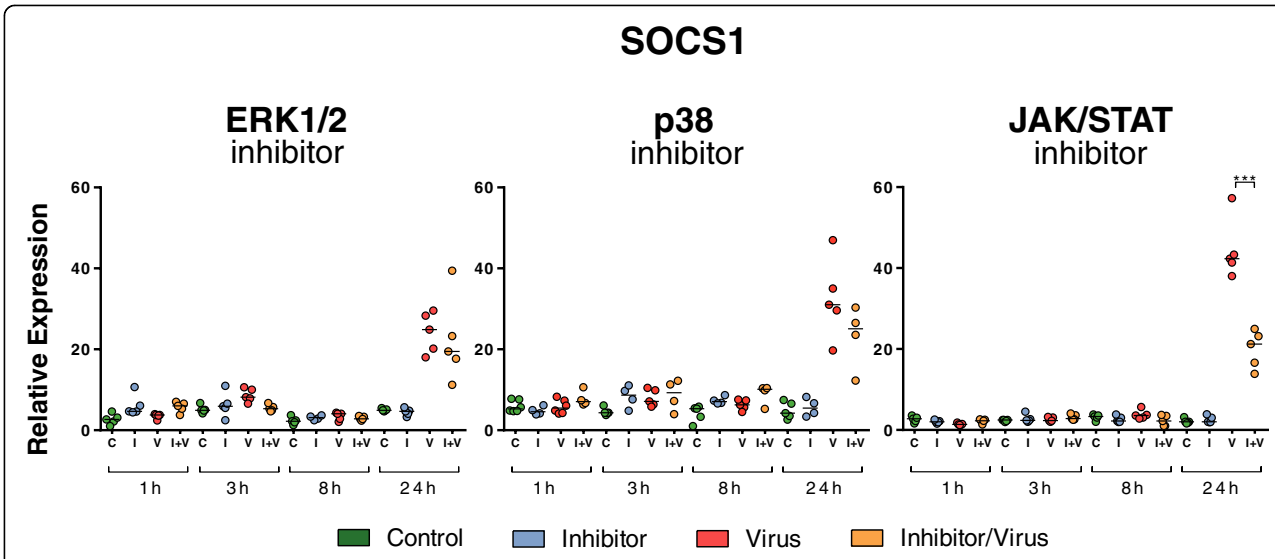

Figure 8 Relative expression of SOCS1 transcripts in infected NPTr cells in presence of chemical inhibitors. The relative expression of SOCS1 transcripts was measured in newborn porcine trachea (NPTr) cells infected with H3N2 SIV in presence of chemical inhibitors UO126 (ERK1/2), SB202190 (p38), and JAK Inhibitor I (JAK/STAT) at different time points (1 h, 3 h, 8 h, and 24 h). The control groups were cultured either in the presence of 0.1% DMSO (C, green) or in the presence of inhibitor (I, blue). Infected cells were either untreated (V, red) or inhibitor-treated (I + V, orange). Comparisons were made using one way ANOVA test and Tukey's post-test ($n = 5$, mean ± SEM). Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

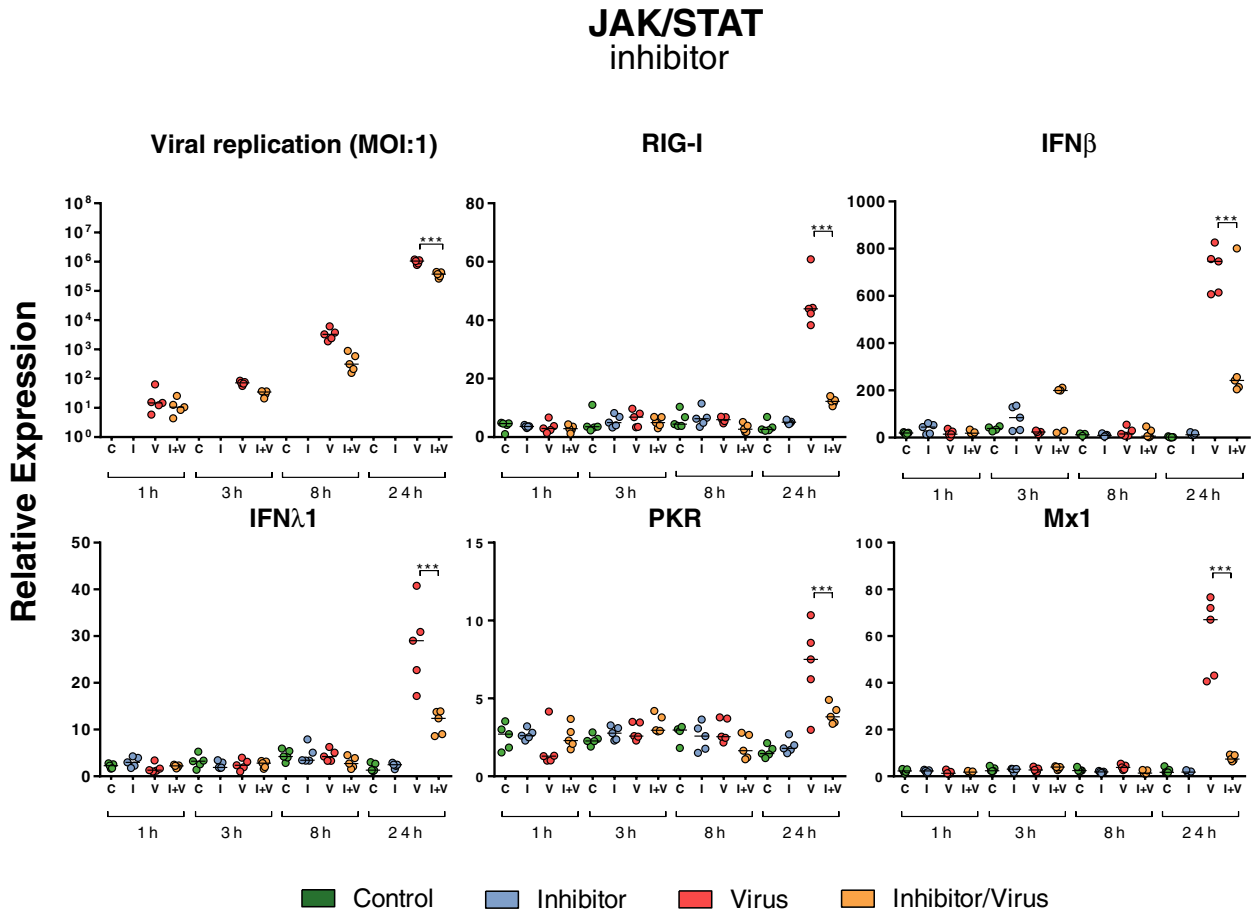

Figure 9 Relative expression of various viral and host transcripts in infected NPTr cells in presence of JAK Inhibitor I. The relative expression of various viral and host transcripts was measured in newborn porcine trachea (NPTr) cells infected with H3N2 SIV in presence of JAK Inhibitor I at different time points (1 h, 3 h, 8 h, and 24 h). The control groups were cultured either in the presence of 0.1% DMSO (C, green) or in the presence of inhibitor (I, blue). Infected cells were either untreated (V, red) or inhibitor-treated (I + V, orange). Comparisons were made using one way ANOVA test and Tukey's post-test ($n = 5$, mean ± SEM). Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

precision cut lung slices (PCLS) for their innate immune response to strain A/Swine/Bissendorf/IDT1864/2003 (H3N2). Then, we assessed several signaling pathways and their relation to the innate immune response observed in the NPTr cells.

Viral replication was higher in NPTr cells than in PCLS and PAMs. This is in agreement with the current knowledge of influenza pathogenesis. Indeed, it is well known that the main target of the virus is epithelial cells [12,15] and NPTr is a cell line consisting of tracheal epithelial cells [18]. This was also confirmed by the immune-staining of the PCLS, where many ciliated epithelial cells were observed in the associated bronchi. In the three systems used in this study, a strong and progressive innate immune response was triggered by the virus. The response in NPTr cells and PCLS was very similar except for a trend toward TLR induction in the latter. This could be explained by the presence of various cell types such as macrophages and/or dendritic cells in this tissue [6,14]. Moreover, observed discrepancies between NPTr cells and PCLS may also be related to the less controlled percentage of cells infected in the PCLS (MOI cannot be determined) and to the potential resistance of a significant proportion of the various cell types to SIV infection. Our study further demonstrated the value of using PCLS in the study of pig/SIV interactions. RIG-I transcription was induced by the virus in NPTr cells, PAMs, and PCLS while TLR3, TLR7 as well as TLR8 transcription were significantly induced only in PAMs. This result was a little bit surprising since it has been observed recently that airway epithelial cells mostly use surface and endosomal TLR3 to detect influenza virus and to initiate IFN production [44]. Following recognition of the virus, interferon responses were triggered. In NPTr cells and PCLS we observed mostly IFNβ and IFNλ1 transcript expression in response to the virus while in PAMs, IFN type III was not significantly induced. Regarding the expression of IFNα transcripts,

no significant differences between infected and non-infected cells/tissues were observed. This result is surprising as IFNα transcripts were previously detected in PAMs infected by another strain of SIV [45]. In that study IFNα mRNA was detected earlier than IFNβ mRNA. The use of qPCR primers targeting IFNα 1-17 may have masked differences in the expression of transcripts associated to a specific IFNα gene. It is known that significant variations in the expression of the various IFNα transcripts upon infection can be observed [46]. Recently, it has been demonstrated that type III IFN is the predominant IFN produced by the airway epithelium [44]. Our data showed the increased production of mRNAs encoding both type I and type III IFNs in epithelial cells. The absence of type III IFN induction in PAMs supports the idea of a predominant role of type III IFN in a specific cell type such as epithelial cells as suggested by Ioannidis et al. [44]. In response to IFN, ISG (Mx1, Mx2, OAS1, PKR) transcripts were identified in the three systems. This observation was particularly clear with the NPTr cells probably because of their nature (epithelial *versus* alveolar macrophages) and their high purity in comparison to primary epithelial cells located in the slices. These two features also probably account for the quicker and higher response observed in NPTr cells when compared to PAMs and PCLs. Because of their role in the regulation of immune response [47,48] and in influenza pathogenesis [49], we looked at the expression of SOCS transcripts in response to SIV in the three systems. Twenty-four hpi SOCS1 transcripts were up-regulated in the three systems. This observation confirmed our previous study postulating the inducible nature of SOCS1 in porcine lungs [33]. Instead of SOCS1, a role for SOCS3 has been recently identified in pigs [50]. The authors showed that the lower susceptibility of pigs to contemporary Eurasian highly pathogenic avian influenza (HPAI) H5N1 virus infection is linked to SOCS3 induction. Under their conditions, swine epithelial cells were expressing SOCS3 proteins in response to both human H1N1 virus and HPAI H5N1 virus [50]. In another study, it was demonstrated that influenza A virus induced NF-κB dependent SOCS3 gene expression in human epithelial cells, suppressing type I IFN response through the JAK/STAT pathway [51]. In our conditions, SOCS3 was never significantly induced in response to H3N2 SIV. It remains to be shown whether these differences are due to different experimental parameters (e.g. virus strain and timing of measurements) or potential discrepancies between RNA levels and protein expression.

In NPTr cells, the Akt signaling pathway was not significantly induced, while we did observe the triggering of MAPK (ERK1/2 and p38) and JAK/STAT pathways. The absence of Akt activation was unexpected, since this signaling pathway was shown to be activated by influenza A virus, probably favoring viral replication [32,52]. However, we assessed different pathway activations early in the infection process, and the protocol used in our study did not allow for the observation of Akt activation. The activation of JAK/STAT pathway has been reported in other species [13]. This pathway controls type I IFN response. JAK/STAT signaling induced formation of a trimeric transcription factor ISGF3, consisting of STAT1, STAT2, and interferon regulatory factor 9 (IRF-9), which regulates the expression of several ISGs [13]. Four MAPK cascades have been described including two isoforms of the ERK1/2, the Jun-terminal kinase (JNK), p38, and ERK5 [52,53]. The signaling pathways convert extra- and intracellular signals into cellular responses, regulating cell activation, differentiation, immune responses, and proliferation [52,53]. In our study we assessed the activation of ERK1/2 and p38, and activation of both cascades was observed. p38 predominantly responds to stress conditions and pro-inflammatory cytokines, whereas ERK1/2 senses mitogenic stimuli [52,53]. MAPK signaling pathways and their role in the regulation of innate response and viral replication have not been well studied in porcine cells infected by influenza virus. However, a recent study showed a robust activation of ERK1/2 in influenza virus-infected swine macrophages [54] and demonstrated that the induction of RIG-I and MDA-5 depends on the activation of ERK1/2 and JNK in these cells. Interestingly and similar to our findings, these authors did not observe any IL-1β induction in response to the tested influenza virus [54]. Unlike IL-1β expression, TNFα was clearly induced early at 3 hpi.

We also investigated to what extent inhibitors of the different pathways could impact SOCS1 transcription. Only JAK/STAT pathway inhibition was accompanied by a significant decrease in the expression of SOCS1 transcripts. This observation confirms the strong association of SOCS (particularly CISH, SOCS1, and SOCS3) with the JAK/STAT pathway. Nevertheless, in our conditions the association was only clear for SOCS1 and not for the two other SOCS family members. To further assess the impact of JAK/STAT pathway inhibition, we then looked at the expression of transcripts accounting for viral replication, viral recognition and interferon response. The treatment with JAK Inhibitor I was accompanied by a significant decrease in viral replication (as assessed by quantification of M-vRNA) and by a diminished expression of RIG-I, IFNβ, IFNλ1, PKR, and Mx1 transcripts 24 h post-infection. The decrease in viral replication remains unexplained, although it could be linked to the presence of more IFN in the culture supernatant consecutive to an inhibition of SOCS1. However, the lower level of expression of IFN transcripts argues against this hypothesis. In another set of experiments using another

SIV strain (unpublished results) we used pJAK2(1001-1013) inhibitor [55,56] and obtained similar results in both epithelial cells and macrophages. The viral replication was significantly reduced, as were the transcriptions of IFN and ISGs. Regarding the inhibition of MAPK pathways, we observed a decrease in viral replication as well as a decrease in the interferon response. Similarly, a study by Pinto et al. [57] demonstrated in human epithelial cells that the inhibition of MEK in the Raf/MEK/ERK pathway resulted in reduced virus titers and lower expression of pro-inflammatory cytokines [57,58]. In another study on HPAI H5N1 virus in mice [59] it was also shown that the inhibition of p38 greatly reduced virus-induced cytokine expression concomitant with reduced viral titers.

In conclusion, our study showed the advantage of using various cellular and tissue systems in the study of the innate immune response to influenza virus. The kinetic approach enabled a comparative assessment of the innate immune response in the main targets of the virus and brought insights to viral triggering of major signaling pathways in NPTR cells. Our data suggests a link between SOCS1 and the JAK/STAT pathway in porcine respiratory epithelial cells. The inhibition of the JAK/STAT pathway using JAK Inhibitor I clearly reduced the response of IFN types I and III and the induction of SOCS1 at the transcript level in NPTr cells. Similarly the inhibition of MAPK pathway reduced viral replication and interferon response. Further investigations are required to understand precisely the role of SOCS in pigs. A better understanding of the host innate immune response and its underlying regulation mechanisms will help identify strategies for effective control of SIV.

Additional files

Additional file 1: Relative expression of IFNα (1-17) transcripts in infected NPTr cells, PAMs and PCLS. The cells and tissue were infected with the H3N2 SIV strain at different time points (1 h, 3 h, 8 h, and 24 h). Green dots stand for non-infected cells and tissue. Red dots stand for infected cells and tissue (NPTR cells: individual values (dots) and median value (bar), $n = 6$ wells per condition; PAMs: minimum one slice/pig for each time point, total 5 pigs, $n = 5$-12 and median value; minimum one slice/pig for each time point, total 5 pigs, $n = 5$-12 and median value). Comparisons were made using one way ANOVA test and Tukey's post-test. Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

Additional file 2: Relative expression of various transcripts in infected NPTr cells in presence of MAPK inhibitors. The relative expression of various viral and host transcripts was measured in newborn porcine trachea (NPTr) cells infected with H3N2 SIV in presence of ERK1/2 and p38 inhibitors at different time points (1 h, 3 h, 8 h, and 24 h). The control groups were cultured either in the presence of 0.1% DMSO (C, green) or in the presence of inhibitor (I, blue). Infected cells were either untreated (V, red) or inhibitor-treated (I + V, orange). Comparisons were made using one way ANOVA test and Tukey's post-test ($n = 5$, mean ± SEM). Differences were considered significant when $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

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

Authors' contributions
MDO carried out most of the experiments, participated in the analysis of the data and drafted the manuscript. SM helped MDO to carry out the experiments. DP participated actively to PCLS experiment. CR was involved in the Western blot preparation and analysis. MO and DS helped for the preparation of the virus, titration and cell culture. DM, GS, GH, MB, and JD participated in the design of some experiments and helped to draft the manuscript. FM conceived the study, actively participated in its design and coordination, analyzed the data, drafted and revised the manuscript. All authors read and approved the final manuscript.

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Étude 3

La lignée cellulaire *Newborn pig trachea* cultivée en interface air-liquide permet une représentation partielle *in vitro* du tissu respiratoire supérieur porcin

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Newborn pig trachea (NPTr) cell line cultured in air-liquid interface conditions allows a partial **in vitro** *representation of the porcine upper airway tissue*

Grâce à des similarités anatomiques, génétiques et physiologiques avec l'humain, le porc (*Sus scrofa domesticus*) représente un bon modèle animal pour l'étude de nombreuses maladies infectieuses chez l'Homme. Afin de développer un outil alternatif pour l'analyse *in vitro* de la réponse immune innée des cellules respiratoires porcines, nous avons testé la culture en interface air-liquide (ALI), de la lignée cellulaire épithéliale *newborn pig trachea* (NPTr). Cette lignée cellulaire, établie à partir d'un élevage indemne de la plupart des pathogènes porcins, est sensible à un large spectre de micro-organismes. Le but de notre étude a été d'évaluer et de caractériser divers aspects de la différenciation cellulaire après culture des cellules dans plusieurs milieux de culture différents. En système ALI, lorsque les cellules NPTr atteignent la confluence, le milieu de culture de la portion supérieure est enlevé pour nourrir les cellules uniquement du côté basal durant quelques semaines. Ensuite à la fin de la période de culture, la capacité de différenciation et de polarisation des cellules a été évaluée. Par immunofluorescence et microscopie électronique la présence des cellules à mucus, de cellules ciliées et de jonctions serrées a été déterminée. Nous avons ainsi observé que la couche cellulaire présentait une densité variable de cellules productrices de mucus et une résistance épithéliale mesurable. Le développement de jonctions serrées entre les cellules a également été observé. Finalement, l'expression de plusieurs transcrits impliqués dans la différenciation cellulaire a été mesurée. La culture des cellules NPTr dans des conditions ALI, permet une représentation partielle du tissu respiratoire supérieur porcin. Ce modèle pourrait être utilisé pour l'étude des interactions hôte/pathogène dans le modèle porcin.

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METHODOLOGY ARTICLE Open Access

Newborn pig trachea cell line cultured in air-liquid interface conditions allows a partial in vitro representation of the porcine upper airway tissue

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Abstract

Background: The domestic pig is an excellent animal model to study human microbial diseases due to its similarity to humans in terms of anatomy, physiology, and genetics. We assessed the suitability of an *in vitro* air-liquid interface (ALI) culture system for newborn pig trachea (NPTr) cells as a practical tool for analyzing the immune response of respiratory epithelial cells to aggressors. This cell line offers a wide microbial susceptibility spectrum to both viruses and bacteria. The purpose of our study was to evaluate and characterize diverse aspects of cell differentiation using different culture media. After the NPTr cells reached confluence, the apical medium was removed and the cells were fed by medium from the basal side.

Results: We assessed the cellular layer's capacity to polarize and differentiate in ALI conditions. Using immunofluorescence and electronic microscopy we evaluated the presence of goblet and ciliated cells, the epithelial junction organization, and the transepithelial electrical resistance. We found that the cellular layer develops a variable density of mucus producing cells and acquires a transepithelial resistance. We also identified increased development of cellular junctions over the culture period. Finally, we observed variable expression of transcripts associated to proteins such as keratin 8, mucins (MUC1, MUC2, and MUC4), occludin, and villin 1.

Conclusions: The culture of NPTr cells in ALI conditions allows a partial *in vitro* representation of porcine upper airway tissue that could be used to investigate some aspects of host/respiratory pathogen interactions.

Keywords: Pig, Epithelial cell, Differentiation, Air-liquid interface, Trachea

Background

The domestic pig represents an excellent animal model to study a wide range of human microbial diseases due to its similarity to humans in terms of anatomy, genetics, and physiology [1-4]. Because of this, there is an increasing need for the development of new biomedical tools in this species. The newborn pig trachea (NPTr) cell line was established from a 2-day-old piglet obtained from a specific pathogen free herd at the *Instituto Zooprofilattico Sperimentale* in Brescia [5]. The NPTr cells are non-carcinoma and non-transformed cells offering a wide microbial susceptibility spectrum which includes not only viruses [5] but also bacteria [6]. They can be used to study host/respiratory tract pathogen interactions at the cellular level. NPTr cells can also replace Madin-Darby Canine Kidney Epithelial Cells [7] for the production of viruses such as porcine influenza viruses [5]. Recently, air-liquid interface (ALI) culture of primary tracheal epithelial cells has been implemented with success in pigs [8,9]. ALI culture conditions allow a more realistic development of epithelial cells *in vitro* [10]. For instance it was shown that the pattern of expression and polarization of Toll-like receptors (TLRs) 3, 7, and 9 in cells cultured in those conditions mirrored that of the airways *ex vivo* [11] with a surface expression of these TLRs. Furthermore, ALI culture can enable the *in vitro* reconstitution of an epithelium presenting many features of the pseudo-stratified epithelium observed in the upper respiratory tract [12]. However, the use of primary epithelial cells for the ALI technique can be challenging. Contamination with fibroblasts and micro-organisms is common, requiring additional purification steps and the use of large amounts of

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antibiotic and anti-mycotic drugs. This creates complications in specific conditions where the use of antibiotics is not possible. Moreover, culture of primary cells requires the sacrifice of more animals than the use of a well-established and easily available cell line. Cell lines have several advantages over primary cells including their low cost, longer life span, and lower variability between passages and experiments [13]. In addition, they are generally easier to transfect and manipulate than primary cells [13]. Epithelial cells, primary cells and cell lines are usually cultured in submerged monolayers on a conventional plastic support. One of the major disadvantages of monolayer culture is the potentially irreversible and total loss of ciliated cells [12,14-16] although there are exceptions such as hamster cells which can develop cilia and goblet cell phenotypes in submerged culture [17,18]. Many studies show that ALI culture conditions are valuable to drive a differentiated phenotype [8,9,12,13,19-22] to an extent similar to that observed in native pseudo-stratified epithelium. This could be due, partially at least, to the thin layer of apical medium minimizing the diffusion barrier and resulting in an enhanced oxygen supply to a level which better meets the requirement of airway epithelial cells. Conversely it has been shown that when the cells are maintained submerged instead of at an air-liquid interface, the differentiation of epithelial cells into ciliated cells was strongly suppressed [19]. Authors showed that the removal of some substances such as epidermal growth factor, cholera toxin, and bovine pituitary extract from the media resulted in up to 4-fold increases in the number of ciliated cells detected [19]. Thus, the selection of the culture conditions has tremendous effects on the morphology and function of epithelial cells *in vitro* [23]. For all these reasons we aimed to assess the differentiation of NPTr cells cultured under ALI conditions. The ability of NPTr cells to differentiate was evaluated by light, fluorescence, transmission, and scanning electron microscopy as well as real time quantitative polymerase chain reaction (RT-qPCR). Expression of tight junction protein zonula occludens-1 *(*ZO-1) and the development of transepithelial electrical resistance (TEER) were also assessed.

Results

Morphological analysis of the epithelial cell layer

Cellular morphological changes were observed first by conventional light microscopy (Figure 1). Prior to confluence, NPTr cells were maintained with medium in the two chambers. After reaching full confluence, NPTr cells were cultured in ALI conditions in DMEM complemented with 10% FCS and antibiotics (Table 1) for a total of twenty-two days. At the beginning of the ALI culture NPTr cells appeared to be a homogenous population of epithelial cells with oval nuclei (Figure 1A). The confluent cells formed a monolayer of tightly packed cells. Over the subsequent days, the culture displayed darker areas, probably of stratified cells (Figure 1B), and lighter areas corresponding to less dense regions. After two and three weeks of culture, NPTr cells created multiple layers and the population seemed less homogenous with apparent increased mucus secretion (Figure 1C-D). In order to evaluate the expression of differentiation markers such as apically expressed β-tubulin (marker of ciliated epithelial cells) and mucin 5 AC (marker of goblet cells), frozen sections of NPTr cells culture were fixed and immuno-stained at the beginning (day 0) and the end (day 22) of the culture (Figure 1E-F). After seven days in ALI conditions (Figure 1E), the culture revealed a monolayer of confluent cells and the presence of multiple cell attachments suggesting the development of an increased internal complexity. After twenty-two days, the culture displayed continuous and robust cellular layers with the presence of more mucin-positive stained cells and a slightly more defined border of β-tubulin-positive cells (Figure 1F and Table 2). As a control, in respiratory pseudo-stratified epithelium collected from a two-month-old healthy pig, mucin-positive stained cells (Figure 1G) and tubulin-positive cells (Figure 1H) were easily observed. The goblet cells, mainly located in the cryptic areas of the epithelium, were capable of producing significant amounts of mucus (Figure 1G). Scanning electron microscopy confirmed the presence of some mucus particles and numerous microvilli at the apical surface at the beginning of the culture (Figure 1I). After twenty-two days of culture under ALI conditions, the surface topography was more complex showing a stratified structure covered by a mucus layer (Figure 1J). The staining for β-tubulin was globally diffused, even if some cells seemed to present a more apical staining, suggesting that villi or cilia had not developed (Figure 1F). Thus, despite the presence of microvilli, there was no evidence of cilia development at the apical surface (Figure 1J). Cell cultures using DMEM medium were monitored up to nine weeks without significant mortality of the cells. No significant differences were observed in terms of cell mortality between the second and the third week of culture where TEER was maximal.

In the experiments where conventional media was replaced by serum-free AECM media or DMEM/HAMF12 supplemented with serum, dexamethasone, and retinoic acid, the cellular layer gradually contracted, never fully covering the insert surface (data not shown), and progressively died preventing any further analyses. When serum-free supplemented DMEM/HAMF12 medium was used the cellular layer developed better (data not shown). However the culture displayed an irregular apical surface with a few mucus cells and low tubulin staining, suggesting poor cellular differentiation.

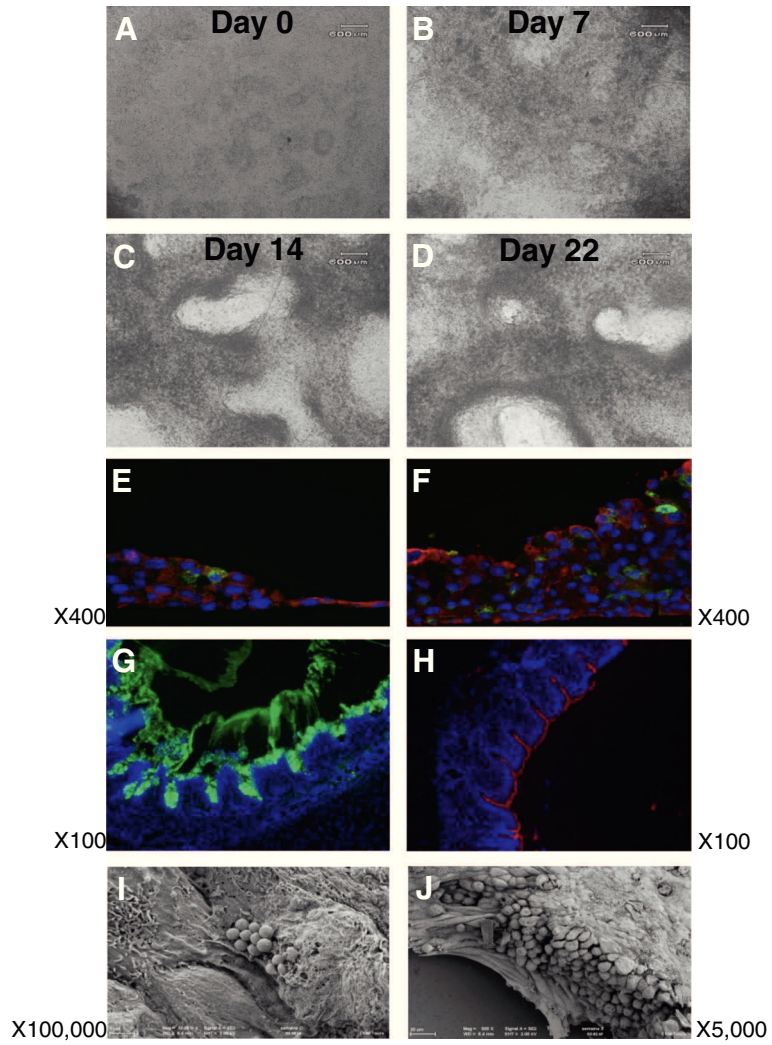

Figure 1 Evolution of NPTr cells in ALI conditions over the twenty-two days of culture. A-D: morphological evolution, representative images of two independent experiments. **E-J:** immunostaining and scanning electron microscopy assessment. NPTr cells were cultured in ALI conditions over twenty-two days and the aspect of the cellular layer was assessed by immunofluorescence and scanning electron microscopy. **E:** After reaching confluence, NPTr cells were cultured in ALI conditions for one week. **F:** After twenty-two days in ALI conditions, NPTr cells showed a thick cellular layer with the presence of numerous mucin positive cells and a delineated border of tubulin positive cells. **G** and **H:** immunostaining of the bronchial epithelium. Tissue was stained with an antibody recognizing mucin 5 AC (green, **G**) or with an antibody recognizing β-tubulin (red, **H**). **I** and **J:** Using scanning electron microscopy, a cellular layer showing a more complex topography and covered by a thick mucus layer was observed.

Table 1 List of the different media used in the study

1) **DMEM** + *10% FCS* + **PS**
2) **DMEM/HAMF12** + Dexamethasone + **PS**
3) **DMEM/HAMF12** + Epidermal growth factor + Insulin + **PS** + Selenium + Transferrin
4) **AECM** + Bovine pituitary extract + Epidermal growth factor + Epinephrine + Hydrocortisone + Insulin + **PS** + Retinoic acid + Transferrin holo
5) **DMEM/HAMF12** + Dexamethasone + *5% FCS* + **PS** + Retinoic acid

FCS: Fetal Calf Serum; PS: Penicillin/Streptomycin.

Transepithelial electrical resistance integrity assessment of the cellular layer

NPTr cells cultured with DMEM complemented with 10% FCS and antibiotics developed progressive TEER along the culture on the transwell (Figure 2A). TEER data throughout the cell culture development displayed quite stable values in the first two weeks of ALI culture (around 150 Ω cm^2). Then, after twenty-two days TEER rose up to 350 Ω cm^2 (Figure 2A), suggesting the formation of stronger cellular junctions. NPTr cells cultured with supplemented AECM medium failed to form a solid structure (Figure 2B). The cellular structure totally

Table 2 Antibodies used for immunofluorescent staining of cultured cells

Target	Antibody	Dilution
β-tubulin	Monoclonal anti-beta-tubuline-Cy3 clone TUB 2.1 Sigma C4585	1/500
Mucin	Monoclonal anti-human gastric mucin 5 AC clone 45 M1 Sigma M5293	1/200
Z0-1	Purified mouse anti-human ZO-1 clone 1/ZO-1 610966 BD Biosciences	1/100
Mouse IgG1	Goat anti-mouse IgG1 AF488 A21121 Molecular Probes™ Invitrogen	1/600
Control	Mouse IgG1 negative control X0931 Dako	1/50

disintegrated after 14 days of ALI culture in these conditions (Figure 2B). In contrast, NPTr cells cultured with serum-free DMEM/HAMF12 medium supplemented with dexamethasone showed TEER values fluctuating around 200 Ω cm^2 (Figure 2C) and the formation of a structure apparently more solid than the ones formed using DMEM. However, the immunostaining showed irregular apical surface with few mucus cells and low tubulin staining suggesting poor cellular differentiation (data not shown). Cells cultured with other media (Table 1) also failed to form a solid structure (data not shown).

Zonula occludens-1 protein expression and ultrastructural analysis of newborn pig trachea cells in air-liquid interface conditions

Complementary immunofluorescence analysis was undertaken to evaluate the establishment of intercellular junctions by NPTr cells cultured with supplemented DMEM in ALI conditions. *Zonula occludens-1* protein (ZO-1) was identified (Figure 3). At the beginning of the culture (day 0), NPTr cells did not display any evidence of positive ZO-1 staining (Figure 3). After 14 days in ALI conditions, NPTr cultures showed positive ZO-1 spots throughout the cytoplasm of most of the cells. The staining intensified at week 3 (day 22) of culture (Figure 3). This general upward trend was correlated with the TEER findings and suggested migration of the tight junction proteins to the cell periphery. However the staining intensity was slight and ZO-1 protein did not seem to reach the cell membrane/cell-cell junctions as expected. This last observation could be linked to the use of an upright fluorescence microscope instead of a confocal microscope. Using transmission electron microscopy, well-developed cellular junctions (tight junction and desmosome) were observed after three weeks of culture under ALI conditions (Figure 4A-B). Moreover, using that technique, microvilli at the surface of the cells were identified (Figure 4C-D). However, no cilia were observed, confirming previous results.

Expression of differentiation marker transcripts from newborn pig trachea cells cultured under two different conditions

To investigate the capacity of NPTr cells to differentiate in ALI conditions, the expression of differentiation and tight junction markers was analysed by RT-qPCR in cells cultured in supplemented DMEM. To normalize the mRNA relative expression, the most stable reference genes were selected among eight commonly used reference genes (Table 3). HPRT1, RPL-19, and GAPDH were the most stable genes with M values under 0.5 for cell samples (0.11, 0.11, and 0.12, respectively). In order to compare the influence of ALI conditions in cellular differentiation, a parallel experiment was performed using conventional plastic supports and again DMEM medium. Cells cultured in ALI conditions showed a significant increase in the mRNA expression of mucin 1 (MUC1), MUC2, occludin (OCLN), and keratin 8 (KRT8) ($p < 0.05$) while a significant decrease in the transcript expression of MUC4 and ZO-1 was observed ($p < 0.05$) (Figure 5A). The profile of mRNA expression in cells cultured on conventional plastic support

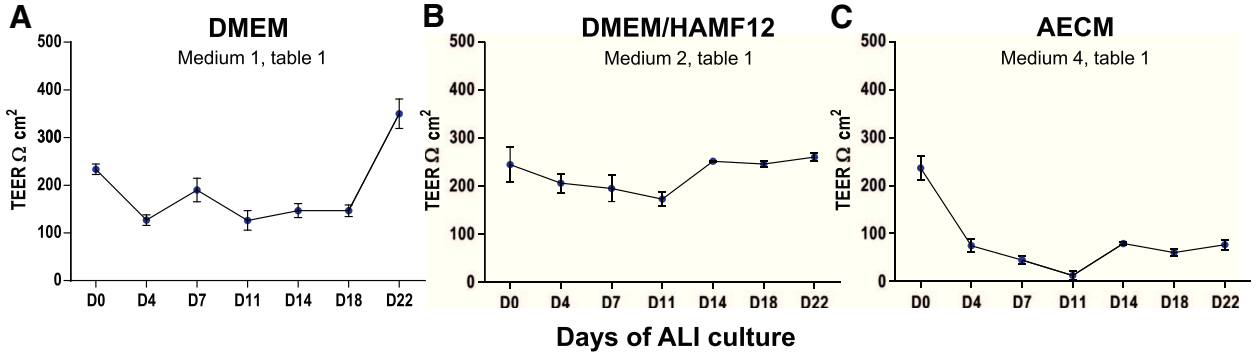

Figure 2 Measurement of transepithelial electrical resistance (TEER) in NPTr cells cultured in ALI conditions. Cells were cultured for a maximum of twenty-two days using different media **(A**, **B**, and **C)**. Data are representative of two independent experiments and are presented as means +/− SEM, (n = 6).

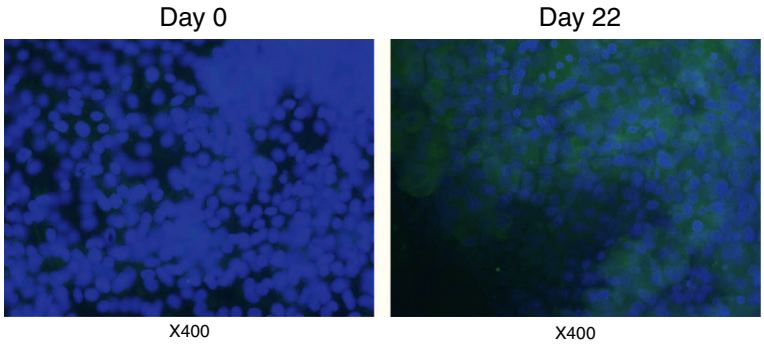

Figure 3 Immunofluorescence staining of the tight junction specific protein ZO-1 in NPTr cells. The staining was carried out at day 0 and day 22 of ALI culture.

was similar except that the expression of OCLN mRNA was not increased after 3 weeks of culture (Figure 5B). Moreover, the expression of the transcripts after three weeks of culture was even more significantly modified ($p < 0.01$) (Figure 5B).

Discussion

In vitro models using cell lines are indispensable for understanding the response of epithelium to infectious agents. In the current report we assessed the capacity of NPTr cells to polarize and differentiate when cultured under ALI conditions. Using immunofluorescence and electronic microscopy we evaluated the presence of goblet and ciliated cells, the epithelial junction organization, and the transepithelial electrical resistance. We have shown that it is possible to identify both mucin-producing cells and non-mucin-producing β-tubulin-positive cells in the NPTr population. However β-tubulin staining was quite diffuse and cilia were not observed at the apical side of the cells. Moreover, although the heterogeneity in cell population increased when

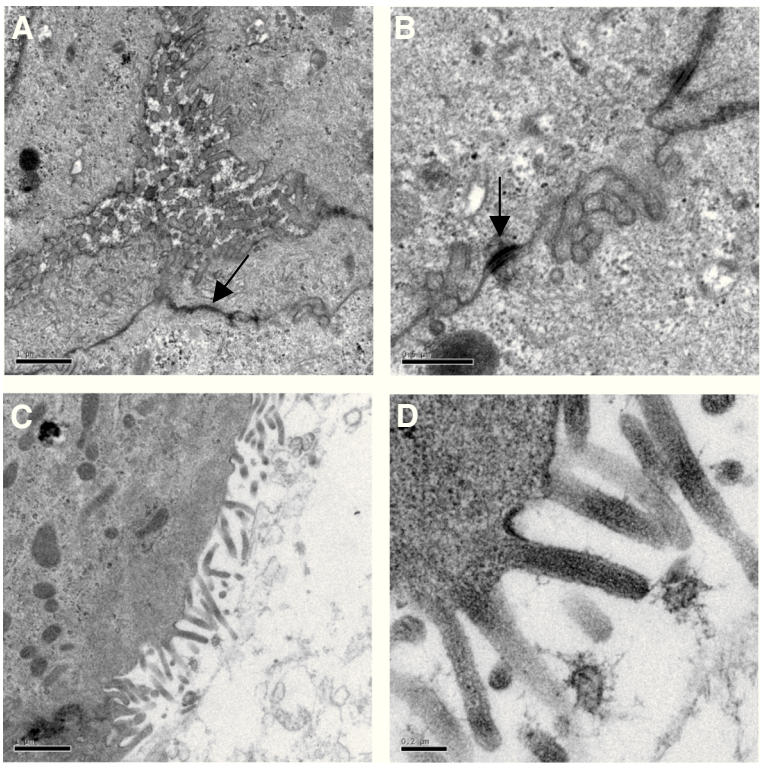

Figure 4 Ultrastructural views of NPTr cells cultured in ALI conditions. Views of the tight junction **(A**, black arrow**)** and the desmosome **(B**, black arrow**)** between adjacent cells and microvilli **(C, D)** after three weeks of culture in ALI conditions are presented. Scale bar 1 μm **(A)**, 0.5 μm **(B)**, 1 μm **(C)**, and 0.2 μm **(D)**.

Table 3 Primer sequences, annealing temperatures of primer sets (°C), expected PCR fragment sizes (bp) and accession numbers or references

Primer name	Primers sequence	Annealing temperature (°C)	PCR product (bp)	Accession number or reference
ACTB	CACGCCATCCTGCGTCTGGA AGCACCGTGTTGGCGTAGAG	63	100	[24]
B2MI	CAAGATAGTTAAGTGGGATCGAGAC TGGTAACATCAATACGATTTCTGA	58	161	[24]
GAPDH	CTTCACGACCATGGAGAAGG CCAAGCAGTTGGTGGTACAG	63	170	AF017079
HMBS-2	AGGATGGGCAACTCTACCTG GATGGTGGCCTGCATAGTCT	58	83	[24]
HPRT-1	GGACTTGAATCATGTTTGTG CAGATGTTTCCAAACTCAAC	60	91	[24]
KRT 8	TGACCGACGAGATCAACTTC TGATGTTCCGGTTCATCTCC	60	294	NM_001159615
MUC1	TAAAGAAGACGGGCTTCTGG CCGCTTTAAGCCGATCAAAC	60	134	XM_001926883
MUC2	ACCCGCACTACGTCACCTTC GGCAGGACACCTGGTCATTG	62	150	BX671371
MUC4	CTGCTCTTGGGCACTATATG CCTGTGACTGCAGAATCAAC	60	133	DQ848681
OCLN	CTACATAATGGGCGTCAACC GGGCTGCTCGTCATAAATAC	60	298	NM_001163647
RPL-19	AACTCCCGTCAGCAGATCC AGTACCCTTCCGCTTACCG	60	147	[25]
SDHA	CTACAAGGGGCAGGTTCTGA AAGACAACGAGGTCCAGGAG	58	141	[24]
TBP-1	AACAGTTCAGTAGTTATGAGCCAGA AGATGTTCTCAAACGCTTCG	60	153	[24]
VIL1	AGAAGTGGACGGTGCCCAAC TCTCGCCGATGAGGTAGGTG	64	273	XM_001925167
ZO-1/TJP1	GAGGGCATTTCCCACGTTTC GCTTTAGAGCCGAGTCCTTG	62	256	XM_003353439

Reference genes are underlined.

the cells were cultured under ALI conditions, this condition was not absolutely necessary to generate this heterogeneity. Indeed, the two kinds of cells were detected at the beginning of the culture even when the conventional impermeable plastic support was used. NPTr cells would be, independent of the culture conditions, a heterogeneous population of cells including both cells specialized in the production of mucins, and non-mucin-producing β-tubulin-positive cells. Nevertheless, we cannot exclude the possibility of just one kind of cell only producing mucus under specific stimuli. The presence of two cell types would be interesting in the context of the study of host/pathogen interactions as viruses or bacteria sometimes discriminate between goblet and ciliated cells [20-22,25,26]. We did not culture primary trachea epithelial cells in parallel to our cultures of NPTr cells. Recently (unpublished data), some experiments involving primary bronchial epithelial cells have been initiated in the lab. Preliminary results showed significant differences between primary and NPTr cells in terms of TEER and immunostaining, strongly suggesting that the protocol and conditions used in our study could only partially account for the limited differentiation of NPTr cells.

Regarding TEER, it was observed that DMEM and serum free DMEM/HAMF12 supplemented with dexamethasone media were the only ones enabling the development of a higher resistance, with values close to the 300 Ω cm^2 after three weeks of culture under ALI conditions. The development of high TEER values coupled with the observations we made with transmission electron microscopy and staining of ZO-1 demonstrated the development of strong intercellular junctions. The presence of multiple layers of cells also likely contributed to the increase in TEER. Curiously the mRNA expression of ZO-1 was significantly lower after three weeks of ALI culture than at the beginning, which is the opposite of what was expected. A similar result was observed also when the cells were cultured on the impermeable support. Discrepancies between the expression of ZO-1 mRNA and protein, and the TEER have also been observed by others [22]. One explanation for this could be variations in mRNA stability or protein synthesis and turn-over. The difference in the expression of OCLN mRNA observed between NPTr cells cultured on impermeable support and cells cultured under ALI conditions is probably due to a higher development of intercellular junctions when the cells were cultured on the transwells under ALI conditions. Villin -a protein associated with the actin core bundle of the brush border- transcripts (VIL1) were not expressed more after three weeks of culture on either the impermeable support or the transwell. Nossol and collaborators demonstrated variability between different cell lines, using intestinal porcine epithelial cells 1 (IPEC-1) and IPEC-J2 (isolated from the jejunum). With IPEC-1 cell culture they detected a significant increase of villin mRNA levels in conventional membrane and ALI cultures compared to impermeable dish cultures [21]. However with IPEC-J2 villin

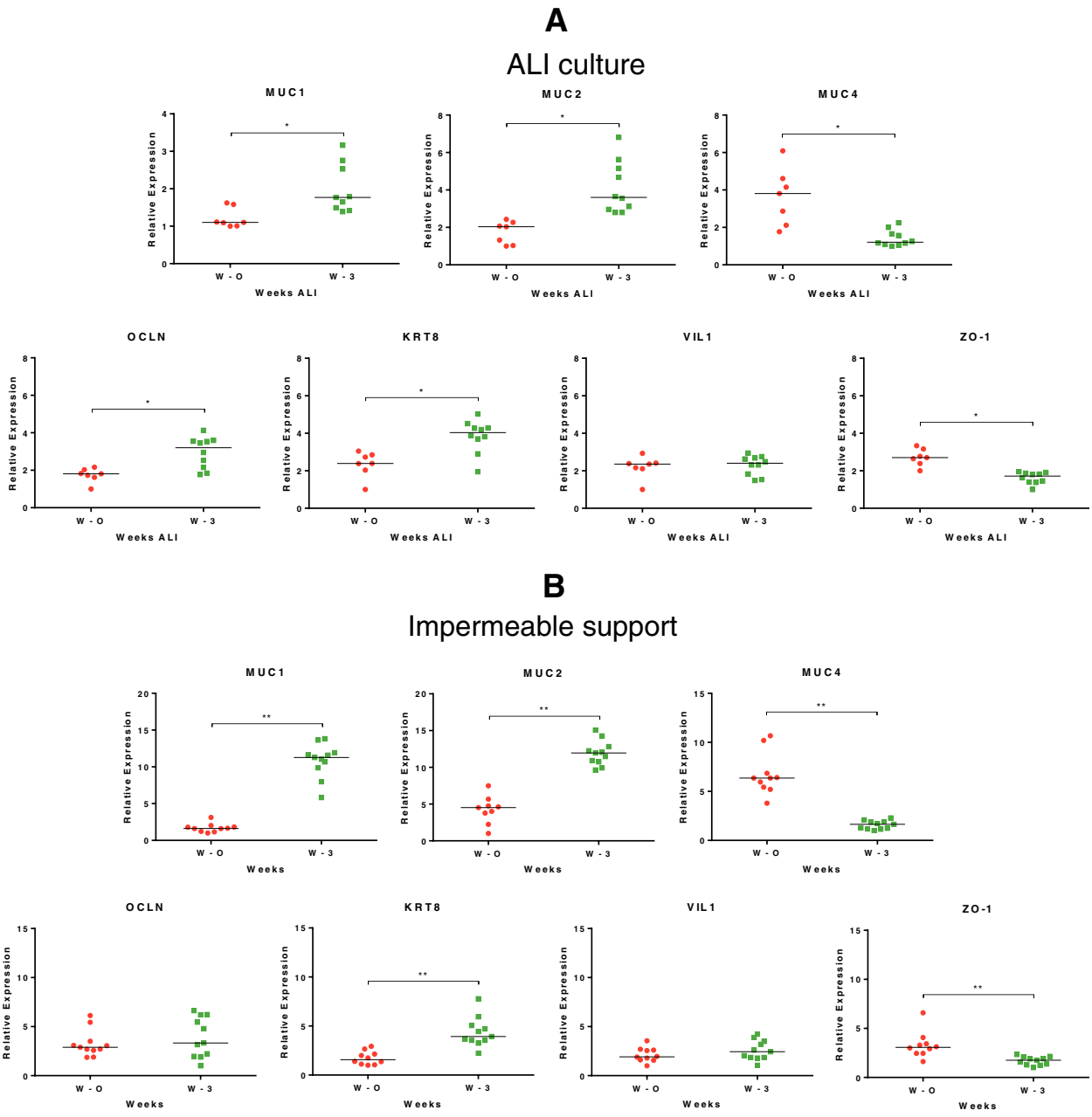

Figure 5 Assessment of the mRNA expression of various cellular genes after ALI culture. (A) Relative mRNA expressions of various cellular genes at the beginning of ALI culture (W – 0) and after three weeks of culture (W – 3). **(B)** Relative mRNA expressions of various cellular genes at the beginning of the culture on impermeable support (W – 0) and after three weeks of culture (W – 3). The median of the data is presented for a maximum of 10 independent W – 0 and W – 3 transwells. *, $p < 0.05$, **, $p < 0.01$, ns: not significant. Comparisons were carried out using non-parametric Mann-Whitney test.

mRNA was significantly increased in cells cultured conventionally on membranes but it was not increased in cells cultured under ALI conditions, in comparison to dish culture [21]. Regarding mucins, we assessed the mRNA expression of one secreted gel-forming mucin (MUC2) and two cell surface mucins (MUC1 and MUC4) [27]. These three mucins are produced in the respiratory tract as well as in other systems [27]. The mRNA expression of both MUC1 and MUC2 was significantly increased after three weeks of culture on impermeable support and under ALI conditions while we observed a decrease in the mRNA expression of MUC4. The significant increase of MUC1 and MUC2 mRNA expression was consistent with the higher percentage of mucus-producing cells under

ALI conditions after three weeks of culture. The decrease of MUC4 mRNA expression is more difficult to explain and could also be related to mRNA stability or protein synthesis and turn-over.

The cells cultured under ALI conditions with AECM and DMEM/HAMF12 without serum did not growth well nor differentiate as well as the cells cultured with DMEM supplemented with serum. Together these results show the importance of fetal calf serum in obtaining the most favorable development of NPTr cells in our ALI conditions. After three attempts using AECM medium with similar results, this medium seems more adapted for the culture of primary epithelial cells than for NPTr cell line. In previous studies focusing on the culture of respiratory tissue explants or primary respiratory epithelial cells in various species the absence of serum and retinoic acid did not prevent the harmonious development of the ciliated cells [19,20,22,28-31]. However, in two of these studies, bovine serum albumin was added to the medium [19,29]. In other studies [12,21,22], serum at various concentrations was added to the cell line or the primary cells. In our study, we were not able to fully differentiate NPTr cells under the conditions we selected. With ALI conditions using DMEM medium supplemented with serum the NPTr cells did develop intercellular junctions and cellular polarity, however "real" goblet cells and cilia did not develop. This lack of full differentiation of NPTr cells could be due to several possible factors: 1) the need to supplement the culture medium with serum, retinoic acid, or other additives despite other studies demonstrating mucociliary differentiation without this supplementation [32-34]; 2) the potential irreversible loss of the ability to develop cilia; 3) the timing we selected for our different attempts; and 4) the potential need to supplement the culture medium with still undetermined factors that would allow a full differentiation of the cells. Regarding the effect of retinoic acid, our attempts using DMEM/HAMF12 supplemented with serum, dexamethasone and retinoic acid were not convincing, as they resulted in a degraded cellular monolayer. The origin of the serum could also be particularly critical as recently demonstrated with porcine cell line IPEC-J2 [35]. Authors showed that porcine serum was allowing a better differentiation of the cell line than previously used bovine serum [35].

Conclusions

Briefly our data showed that both mucus-producing cells and non-mucus-producing β-tubulin-positive epithelial cells were already detectable at the beginning of the ALI culture with an increase in the number of mucus-producing cells after a few weeks under ALI conditions. Transepithelial electrical resistance increased slowly over time and strong intercellular junctions were observed at the end of the culture period. Nevertheless, even when well-developed microvilli were identified on the cells, no cilia were detected. Moreover, the generated epithelium was globally more similar to a stratified squamous than a pseudo-stratified epithelium. In our study, the culture of NPTr cells in ALI conditions enabled the development of a system intermediate between the conventional cell line culture and the culture of primary tracheal epithelial cells in ALI conditions. However, it was not possible to mimic the pseudo-stratified epithelium seen with primary epithelial cells. Improvement of the cell culture conditions may allow the full differentiation of NPTr cells to both ciliated and goblet cells even if we cannot exclude the possibility that NPTr cells somehow have lost definitely the capacity to develop cilia.

Methods

Culture support

Culture support was prepared according to Bals and collaborators [36] except that 50 μl of 0.01% collagen solution (Sigma–Aldrich, Saint-Quentin, France) were used to coat a 6.54 mm ThinCert™ - TC Inserts (Greiner bio-one, Courtaboeuf, France).

Newborn pig trachea cell culture

The NPTr cells [5] (between 30 and 50 passages) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich), 20 IU/ml of penicillin and 20 mg/ml of streptomycin (Invitrogen). Cells were plated onto 24-well plastic plates (Greiner bio-one, Courtaboeuf, France) and incubated at 37°C in 5% CO_2 in a humidified atmosphere. Sub-passages were made when cells reached 100% confluence. After trypsinization, collected cells were seeded onto coated ThinCert™ - TC Inserts (Greiner bio-one). A total of 0.8 ml of fresh medium was added to the lower reservoir and 0.25 ml of a 10^5 cells/ml suspension was added to the upper reservoir. As a control, cells were also plated onto conventional 24-well plastic plates for twenty-two days.

Culture after seeding cells on the insert

After seven days of culture at 37°C in 5% CO_2 in a humidified atmosphere, when cells were completely confluent, medium was removed from the upper reservoir. The cells were gently washed with Ca/Mg-free phosphate buffered saline (PBS) every two days at the apical side. Half of the basolateral culture medium was replaced every other day. The culture was kept in ALI conditions for twenty-two days.

Parallel experiments were carried out in order to evaluate the cells' capacity to fully differentiate using other culture media. NPTr cells were cultured with different types of media (Table 1). Again, after seven days of culture, when cells were completely confluent, the

apical medium was removed. The culture medium in the lower reservoir was replaced by serum-free 50% Dulbecco's Modified Eagle's Medium (DMEM)–50% DMEM/Ham's F-12 (HAMF12) medium (Sigma-Aldrich) supplemented with 10^{-7} M dexamethasone (Sigma-Aldrich), 20 IU/ml of penicillin and 20 mg/ml of streptomycin (Invitrogen) or DMEM/HAMF12 supplemented with insulin (5 mg/ml), transferrin (5 mg/ml), selenium (5 ng/ml), epidermal growth factor (5 mg/ml) (all supplied by Sigma-Aldrich), and 20 IU/ml of penicillin and 20 mg/ml of streptomycin (Table 1). Every two days, the basal medium was changed and the apical surface washed with Ca/Mg-free PBS. The cultures were kept for twenty-two days in ALI conditions to induce cell differentiation. Finally, another experiment was performed to evaluate the impact of retinoic acid on NPTr cell differentiation. The procedure was identical to the one described above except that the culture medium in the lower reservoir was replaced either by serum free airway epithelial cell medium (AECM) (Promocell, Heidelberg, Germany) supplemented as recommended by the supplier with bovine pituitary extract (0.004 ml/ml), epidermal growth factor (10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), triiodo-L-thyronine (6.7 ng/ml), transferrin holo (human) (10 μg/ml), and retinoic acid (0.1 ng/ml) (all supplied by Promocell) or DMEM/HAMF12 supplemented with 5% FCS, 20 IU/ml of penicillin and 20 mg/ml of streptomycin, dexamethasone 10^{-7} M, and retinoic acid (0.1 ng/ml) (Table 1).

Transepithelial electrical resistance measurements

Transepithelial electrical resistance (TEER) measurement provides an indirect measure of the formation of tight junctions [37]. Among the cell-cell junctions (tight junctions, adherens junctions, gap junctions, and desmosomes), tight junctions are the most important for maintaining epithelial integrity. TEER is also used as a marker of disruption of epithelial cells. TEER was measured using a MILLICELL® ERS volt-ohm meter (Millipore, Molsheim, France). On day 0 and every fourth day up to day 22 in ALI conditions, 150 μl of medium was added apically into the insert and the measurement taken. Apical medium was then aspirated to restore ALI conditions. Prior to testing the culture's TEER an empty culture insert was used as a blank and subtracted from each subsequent sample reading. Data are presented as resistance values (Ω cm^2).

Immunofluorescence staining

Immunofluorescence staining was performed directly on cells cultured onto the ThinCert™ - TC Inserts (Greiner bio-one), on ThinCert™ - TC Insert frozen sections and on lung tissue frozen sections as described below.

Inserts

Cell cultures were washed three times with PBS prior to fixation for 15 min with 3% paraformaldehyde (Sigma-Aldrich). After one wash with PBS containing 0.1 M glycin (Fisher Scientific, Illkirch, France) cells were treated for permeabilization with 0.2% Triton X-100 (Sigma-Aldrich) over 15 min. Finally, inserts were washed three times with Ca/Mg-free PBS before staining.

Insert frozen sections

Insert membranes were removed from the ThinCert™ membrane supports, then immersed in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Flemingweg, The Netherlands), snap-frozen, and stored at −80°C. Serial transverse sections (7 μm thick) of the membrane were cut at −20°C using a LEICA CM3050 microtome (Leica, Nanterre, France), collected onto treated glass slides (SuperFrost Plus, Menzel-Glaser, Braunschweig, Germany), air-dried, fixed in acetone (Sigma-Aldrich) for 10 min at 4°C, and then stored at −80°C until use. Insert frozen sections were washed three times with Ca/Mg-free PBS before staining.

Lung tissue frozen sections

Small pieces of lung tissue (6 mm × 6 mm) were collected from a two-month-old healthy pig provided by INRA experimental unit (Nouzilly, France). The pig was cared for in accordance with the guidelines of the Institutional Animal Care and Use committee at INRA. The pieces were then immersed in Tissue-Tek® O.C.T. Compound (Sakura Finetek), snap-frozen, and stored at −80°C. Serial transverse sections (7 μm thick) of the membrane were cut at −20°C using a LEICA CM3050 microtome and treated as described above for the insert frozen sections.

Staining

In the case of filter cultures, the reagents were added to the apical filter chamber. Each incubation period with the selected antibodies was performed at room temperature for 20 min in the dark. The goblet cells were stained indirectly by using monoclonal anti-human gastric mucin 5 AC clone 45 M1 antibodies (dilution 1/200) (Sigma-Aldrich) followed by AF488-labeled secondary antibodies (dilution 1/600) (Invitrogen) (see Table 2). Tight junctions were stained with purified monoclonal mouse anti-human ZO-1 antibodies (dilution 1/100) (BD Biosciences, Rungis, France). For cilium staining, cells were treated with Cy3-labeled monoclonal antibodies recognizing β-tubulin (dilution 1/500) (clone TUB 2.1, Sigma-Aldrich). β-tubulin is often expressed as a cytoskeletal protein, however, its apical expression is a marker of ciliated cells [38]. 4', 6'-diamidino-2-phenylindole (DAPI) (Life Technologies Inc., Carlsbad, CA, USA) at 0.5 μg/ml was used as counterstaining before the cells were washed three times with Ca/

Mg-free PBS. Controls were incubated with primary isotype control antibodies followed by secondary antibodies (Table 2). All samples were observed with a Nikon Eclipse 80i microscope connected to Nikon intensilight C-HGF and the imaging software NIS Elements D (Nikon Instruments Europe BV, Amsterdam, The Netherlands).

Transmission electron microscopy

The filter membranes with NPTr cells were fixed by incubation for 24 h in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) (Sigma-Aldrich) and post-fixed by incubation for 1 h with 2% osmium tetroxide (Electron Microscopy Science, Hatfield, PA, USA). They were then dehydrated in a graded series of ethanol solutions, cleared in propylene oxide, and embedded in Epon resin (Sigma-Aldrich) which was allowed to polymerize for 48 h at 60°C. Ultrathin sections were cut and placed on 300 mesh copper grids and then stained with 5% uranyl acetate and 5% lead citrate (Sigma-Aldrich). The grids were then observed with Jeol 1230 TEM (Tokyo, Japan) connected to a Gatan slow scan digital camera and digital micrograph software (Gatan, Pleasanton, CA, US) for image acquisition.

Scanning electron microscopy

The filter membranes with NPTr cells were washed in PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich) and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) (Sigma-Aldrich) and post-fixed by incubation for 1 h with 2% osmium tetroxide. Then, specimens were dehydrated in a graded series of acetone and dried in hexamethyl-disilazan solution (HMDS) (Sigma-Aldrich). Dried specimens were coated with a thin layer of platinum with ion beam coater PECS (Gatan France, Evry, France) and observed with Zeiss Ultra + Field Emission Gun Scanning electron microscope (FEGSEM) (Carl Zeiss S.A.S, Le Pecq, France).

Real time polymerase chain reaction assays and validation of reference genes

NPTr cells were lysed and total RNA was isolated using RNeasy Mini kit (Quiagen, Courtaboeuf, France). Quantitative real-time PCR (qPCR) was performed using cDNA synthesized as previously described [39]. Primers were designed using Clone Manager 9 (Scientific & Educational Software, Cary, NC, USA) and were purchased from Eurogentec (Liège, Belgium) (Table 3). Diluted cDNA (10X) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. The qPCR conditions were 98°C for 30 seconds, followed by 37 cycles with denaturation at 95°C for 15 seconds and annealing/elongation for 30 seconds (annealing temperature, Table 3). Real time assays were run on a Bio-Rad Chromo 4 (Bio-Rad, Hercules, CA, USA). The specificity of the qPCR reactions was assessed by analyzing the melting curves of the products and size verification of the amplicons. To minimize sample variations, we used an identical amount of cells and high quality RNA. The quality of RNA was assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Massy, France) and RNA integrity numbers (RIN) were calculated. RIN were always ≥8.7 demonstrating the high quality of the RNA. Samples were normalized internally using simultaneously the average *cycle quantification* (*Cq*) of the three most suitable reference genes in each sample to avoid any artifact of variation in the target gene. These three most suitable reference genes were selected among eight commonly used reference genes which were investigated in each tissue using qPCR with SYBR green. The genes included beta-actin (*ACTB*), beta-2-microglobulin (*B2MI*), glycera ldehyde-3-phosphate dehydrogenase (*GAPDH*), hydroxymethylbilane synthase (*HMBS*), hypoxanthine phosphoribo syltransferase-1 (*HPRT-1*), ribosomal protein L-19 (*RPL-19*), succinate dehydrogenase complex subunit A (*SDHA*) and TATA box binding protein 1 (*TPB-1*). The stability of these reference genes in all the selected tissues was investigated using the geNorm application [40]. The threshold for eliminating a gene was M ≥0.5 as recommended [41]. The correlation coefficients of the standard curves were >0.995 and the concentration of the test samples was calculated from the standard curves, according to the formula $y = -M*Cq + B$, where M is the slope of the curve, *Cq* the first positive second derivative maximum of amplification curve calculated using PCR Miner [42] and *B* the y-axis intercept. All qPCRs displayed efficiency between 90% and 110%. Expression data are expressed as relative values after Genex macro analysis (Bio-Rad, Hercules, CA, USA) [40].

Statistical analysis

Data for the comparison of differences in relative mRNA expression between NPTr cells (W – 0 and W – 3) were expressed as relative values. Because data were independent and non-normally distributed, the Mann–Whitney test was selected for statistical analysis (GraphPad Prism software version 3.00, GraphPad Software Inc., San Diego, CA, USA). Differences between groups were considered significant when $p < 0.05$.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MDO and MO carried out most of the experiments, participated in the analysis of the data and drafted the manuscript. PYS performed the electronic microscopy analysis. GS provided the NPTr cells, participated in the design of some experiments, and helped to draft the manuscript. FM conceived the study, actively participated in its design and coordination, analyzed the data, drafted and revised the manuscript. All authors read and approved the final manuscript.

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Discussion et perspectives

Les infections respiratoires sont associées à des taux de morbidité et de mortalité élevés partout dans le monde. Parmi celles-ci, la grippe constitue depuis plus d'un siècle, une préoccupation sanitaire majeure chez l'Homme et chez de nombreuses espèces animales (Alexander 1982, Brown 2000, de Wit and Fouchier 2008). Actuellement dans les élevages porcins, la grippe est le plus souvent une maladie endémique aux conséquences économiques importantes (Kuntz-Simon and Madec 2009, Brown 2000). De nos jours, les conditions rencontrées dans les élevages intensifs permettent aux souches du virus influenza, lorsqu'elles s'adaptent à l'espèce porcine, de se transmettre très efficacement horizontalement, de porc à porc. Étant donné son rôle de « *mixing vessel* » lors des infections grippales, le porc constitue un hôte fondamental dans l'écologie des virus influenza (Crisci et al. 2013). Même s'ils se montrent plus stables que les virus humains, les SIV subissent régulièrement des modifications génomiques et antigéniques (Rose et al. 2013). Il est donc important de mieux connaître l'ensemble des éléments contribuant au développement de la maladie dans l'espèce porcine. En outre, grâce aux similarités existant entre cet animal et l'Homme, l'espèce porcine constitue un modèle d'étude particulièrement pertinent de l'infection grippale humaine (Meurens et al. 2012).

Dans ce contexte, l'ensemble des études présentées ici avait pour objectif une meilleure compréhension de la réponse immunitaire innée antivirale contre un SIV de sous-type H3N2, en utilisant différents systèmes *in vitro* et *ex vivo*, et plus particulièrement sa régulation potentielle par les protéines SOCS.

À ce jour, de nombreuses études concernant l'expression et l'implication des protéines SOCS chez l'humain et la souris ont été réalisées (Alexander 2002, Kubo et al. 2003, Yoshimura et al. 2005). Toutefois, dans l'espèce porcine, ce type d'études reste fort rare (Bruel et al. 2010, Nelli et al. 2012). Jusqu'à récemment, il n'existait aucune d'information sur l'expression constitutive des transcrits de SOCS chez le porc. Afin d'obtenir ces informations préliminaires, l'expression des ARNm des SOCS a été caractérisée dans un contexte non infectieux. Pour ce faire différents tissus ont été prélevés : thymus, poumon, trachée, estomac, intestin grêle, colon, ganglion lymphatique mésentérique, foie, rate et rein. Pour cette analyse, la validation des gènes de référence a été effectuée sur la base de leur stabilité dans chacun des tissus, afin de déterminer de manière fiable par la suite les niveaux d'expression des transcrits d'intérêt. Parmi les gènes de référence testés, B2MI, RPL19 et

HPRT-1 ont été sélectionnés comme étant les plus stables dans nos conditions d'expérimentation. Cette sélection est en concordance avec des études précédentes (Facci et al. 2011, Nygard et al. 2007) dans lesquelles RPL19 et HPRT-1 avaient déjà été sélectionnés comme gènes de référence en raison de la stabilité de leur expression.

En ce qui concerne l'expression des ARNm de SOCS et CISH, une expression constitutive des transcrits a été retrouvée dans l'ensemble des tissus sélectionnés. L'expression des transcrits SOCS dans l'espèce porcine confirme ce qui a été décrit précédemment dans des études réalisées notamment chez la souris (Hilton et al. 1998, Krebs and Hilton 2000, Yoshimura et al. 2007). A l'homéostasie, l'expression des SOCS et CISH peut être induite par différentes cytokines comme l'INFγ, le *granulocyte colony-stimulating factor* (G-CSF), le *granulocyte-macrophage colony-stimulating factor* (GM-CSF), l'IL2, l'IL3, l'IL4, l'IL6, des hormones telles que la *growth hormone* (GH) et la prolactine, impliquées dans différents processus physiologiques (Krebs and Hilton 2000).

Parmi les observations réalisées au cours de cette étude, des niveaux significatifs d'expression des transcrits de SOCS1 ont été retrouvés notamment dans le thymus et le colon. Ces données sont conformes à celles obtenues lors d'études réalisées chez la souris (Chong et al. 2003, Catlett and Hedrick 2005, Marine et al. 1999). Ces études ont démontré une expression constitutive de SOCS1 dans le thymus et ont mis en évidence le rôle de SOCS1 dans la différenciation des cellules T CD4+ (Catlett and Hedrick 2005, Marine et al. 1999). Chez des souris SOCS1 -/-, les thymocytes ne se différencient pas en cellules T CD4+ et présentent un phénotype CD8 immature, caractérisé par une interaction anormale avec le CMH de type I au lieu du CMH de type II. Ces cellules T immatures stimulent une importante production d'INFγ. Cette observation est associée à un taux de mortalité élevé des animaux en corrélation avec la surproduction d'INFγ (Catlett and Hedrick 2005). Les souris SOCS1 -/- développent un syndrome néonatale de nécrose hépatique, accompagné de troubles hématopoïétiques et d'une lymphopénie sévères, responsables du taux de mortalité de 100% (Alexander et al. 1999, Starr et al. 1998, Metcalf et al. 1999).

Au niveau respiratoire, des niveaux variables d'expression des transcrits de SOCS et CISH ont été mesurés. Pothlichet et collaborateurs (Pothlichet, Chignard and Si-Tahar 2008), ont évalué l'expression des ARNm de SOCS et CISH dans des cellules épithéliales bronchiques humaines *human bronchial epithelial cells-2B* (BEAS-2B) non infectées ou en présence du virus influenza. Ils ont observé qu'à l'exception de CISH, les transcrits des 7

protéines SOCS étaient constitutivement exprimés dans les cellules épithéliales. Après stimulation par le virus influenza A/Scotland/20/74 (H3N2), seules les protéines SOCS1 et SOCS3 étaient surexprimées, suggérant un rôle probable de ces 2 protéines dans la régulation de la réponse antivirale. Cette réponse passe par le récepteur des interférons de type I (IFNAR), impliqué dans la voie d'activation RIG-I/MAVS/IFNAR1 (Pothlichet et al. 2008). Plus récemment, Nelli et collaborateurs (Nelli et al. 2012) ont évalué la réponse de cellules épithéliales primaires et de macrophages, d'origine humaine et porcine, afin de comparer l'action d'une souche aviaire hautement pathogène et d'une souche humaine moyennement pathogène. L'ensemble des résultats ont montré une surexpression de SOCS3 dans les cellules d'origine porcine associée à une régulation plus efficace de la réponse inflammatoire (Nelli et al. 2012). En effet, l'expression plus importante de SOCS3 chez le porc contribuerait au développement d'une réponse inflammatoire modérée.

Cette première étude nous a incités à approfondir l'analyse de la réponse immunitaire innée porcine induite par le virus influenza et à mieux caractériser l'expression des SOCS et CISH au cours de cette réponse. La réponse immune innée contre les SIV a déjà été décrite précédemment (Crisci et al. 2013, Kreijtz et al. 2011). Néanmoins, la plupart de ces études se focalisaient uniquement sur une seule population cellulaire ou étaient directement réalisées chez l'animal, ce qui limitait ou compliquait l'analyse des résultats. Dans notre étude, afin d'avoir une représentation globale de la réponse immune innée et afin de mieux comprendre les contributions respectives des diverses populations cellulaires, plusieurs systèmes *in vitro* et *ex vivo* ont été utilisés. Nous avons comparé une lignée de cellules épithéliales porcines (*newborn porcine trachea cells*, NPTr), des macrophages alvéolaires porcins (*porcine alveolar macrophages*, PAMs) et des explants pulmonaires (*precision-cut lung slices*, PCLS). Actuellement, trois sous-types du virus influenza sont en circulation en Europe : les sous-types H1N1, H3N2 et H1N2. Les sous-types européens ont des caractéristiques différentes de celles retrouvées chez les virus originaires d'Amérique du nord et d'Asie (Kuntz-Simon and Madec 2009, Rose et al. 2013). Ces différences sont déterminées par des points de mutation, substitutions ou délétions-insertions au niveau de HA et NA, ou par l'échange des gènes entre virus (Webster et al. 1992). L'ensemble des expériences *in vitro* et *ex vivo* a été réalisé avec un sous-type H3N2 de SIV (A/Swine/ Bissendorf/IDT1864/2003), isolé en Allemagne.

Pour estimer la réplication virale et plus particulièrement la transcription virale, l'expression des ARNm du gène viral codant pour la protéine M a été évaluée. Des niveaux

d'expression plus importants ont été observés dans les cellules NPTr en comparaison aux niveaux d'expression observés dans les PAMs et les PCLS. Ces observations sont conformes avec celles obtenues dans des études précédentes dans lesquelles les cellules épithéliales ont été reconnues comme cibles privilégiées des virus influenza (Taubenberger and Morens 2008, Vareille et al. 2011). Ces résultats ont encore été confirmés par l'identification du virus, grâce à l'expression de la NP virale, au niveau des cellules épithéliales dans les PCLS. Des observations similaires ont été obtenues dans une autre étude réalisée également avec des PCLS infectées par différents sous-types de SIV (Punyadarsaniya et al. 2011). L'utilisation d'une approche *ex vivo*, telle que celle des PCLS, offre une vision plus réaliste par rapport aux systèmes unicellulaires grâce à la présence *in situ* des différentes populations cellulaires, telles que les macrophages et les cellules dendritiques, dans leur contexte tissulaire. Concernant les transcrits impliqués dans la reconnaissance des agents pathogènes, RIG-I est clairement induit dans les cellules NPTr, les PAMs et les PCLS. Toutefois pour d'autres transcrits impliqués dans la reconnaissance des agents pathogènes, comme TLR3 et TLR8, une augmentation de leur expression a été exclusivement observée dans les PAMs. Ce résultat est étonnant car Ioannidis et collaborateurs (Ioannidis et al. 2013) ont récemment rapporté que les cellules épithéliales respiratoires reconnaissaient les virus Influenza essentiellement par les TLR3 exprimés à leur surface et au niveau endosomal. Cette reconnaissance aboutit à la production d'INF (Ioannidis et al. 2013, Noppert et al. 2007). Comparant les trois systèmes, nous avons principalement observé l'expression des transcrits d'INFβ après stimulation de RIG-I. Dans les cellules NPTr, des expressions significatives d'INFβ et d'INFλ1 ont été observées, contrairement aux PAMs dans lesquels les INF de type III n'ont pas montré d'augmentation significative d'expression en présence du virus. Ce résultat est conforme à une étude précédente, qui a montré que les INF de type III étaient essentiellement produits par les cellules épithéliales (Ioannidis et al. 2013). Suite à la reconnaissance des ARN viraux, RIG-I induit l'activation des IRF3/7 et de NFκB, stimulant la transcription d'INFβ dans les cellules épithéliales (Dai, Zhang and Hong 2011, Yoneyama and Fujita 2007). Globalement, en réponse aux interférons, les transcrits de Mx1, Mx2, PKR et OAS1 ont été retrouvés dans les trois systèmes. Particulièrement dans les cellules NPTr, l'expression de ces ISG était plus précoce et plus élevée comparativement aux expressions retrouvées dans les autres systèmes étudiés. Cela pourrait être dû à la nature épithéliale et à une plus grande homogénéité de la population cellulaire NPTr, qui est une lignée cellulaire, par rapport aux cellules primaires (PAMs) et aux explants de poumon (PCLS). Les résultats obtenus ont indiqué une réplication

virale efficace et l'établissement d'une réponse antivirale contre celle-ci dans les trois systèmes *in vitro* et *ex vivo*.

Afin de progresser dans la caractérisation de la régulation de la réponse immunitaire précoce, nous nous sommes ensuite intéressés à l'expression des SOCS lors de l'infection par le virus Influenza. Actuellement, il n'y a que quelques études chez les mammifères suggérant ou montrant l'implication des protéines SOCS dans la régulation de la réponse immunitaire vis-à-vis du virus influenza (Pauli et al. 2008, Pothlichet et al. 2008, Jia et al. 2010, Nelli et al. 2012, Ramirez-Martinez et al. 2013). Lors de l'analyse de l'expression des transcrits des huit membres de la famille SOCS – CISH en réponse à l'infection, nous avons uniquement observé une surexpression des ARNm de SOCS1 à 24h post-infection dans les trois systèmes de culture. Ce résultat est conforme avec celui obtenu lors de notre première étude qui montrait de faibles niveaux d'expression constitutive pour SOCS1 au niveau des poumons et de la trachée, suggérant une surexpression en réponse à l'agression virale. D'un autre côté, ces résultats sont différents de ceux obtenus dans d'autres études faites chez le porc, dans lesquelles une surexpression de SOCS3 dans les cellules épithéliales respiratoires était observée en réponse à un virus humain H1N1 et à un virus aviaire hautement pathogène H5N1 (Nelli et al. 2012). Une autre étude a montré également la surexpression des ARNm et protéine SOCS3 dans des cellules épithéliales humaines afin de réguler la réponse des INF de type I via la voie de signalisation JAK/STAT (Pauli et al. 2008). Dans nos conditions d'expérimentation, les niveaux d'expression des ARNm de SOCS3 étaient variables et ne montraient pas de différences significatives entre les différents groupes expérimentaux. Ces différences entre études pourraient être liées au type de virus HPAI H5N1 (A/turkey/Turkey/1/05), H1N1 (A/USSR/77) et H1N1 (A/Puerto Rico/8/34 (PR8)) et/ou au modèle expérimental utilisés.

Les virus influenza peuvent activer différentes voies de signalisation impliquées notamment dans la réponse immunitaire et la régulation de l'apoptose (Ludwig et al. 2006, Ehrhardt et al. 2010). Des études précédentes ont montré l'implication des voies MAPK (JNK, p38 et ERK1/2), PI3K/Akt et JAK/STAT dans la pathogenèse du virus influenza (Dai et al. 2011, Ehrhardt et al. 2010). Afin de déterminer quelles voies sont activées en présence du SIV dans les cellules NPTr, nous avons évalué l'activation des voies MAPK (p38, ERK 1/2), PI3K/Akt et JAK/STAT dans les cellules épithéliales NPTr lors de l'infection par le virus influenza. Parmi les voies de signalisation évaluées, seule PI3K/Akt n'était pas activée

en présence du SIV dans les cellules NPTr. Ce résultat était quelque peu surprenant car la voie PI3K/Akt a été décrite comme activée pendant le processus de réplication virale des virus influenza A. (Ehrhardt et al. 2010, Shin et al. 2007). Le moment d'observation qui a été choisi (4 premières h) pourrait expliquer cette différence. En effet, dans l'étude de Shin et collaborateurs, l'activation de PI3/Akt était plus tardive à partir de 6h post-infection (Shin et al. 2007). En revanche, l'activation de la voie JAK/STAT en réponse au virus a été clairement observée. Cette voie est impliquée notamment dans la réponse INF de type I. Les INF de type I vont se fixer sur le récepteur IFNAR pour activer la voie JAK/STAT (Ehrhardt et al. 2010). Cette activation est en concordance avec les résultats observés précédemment qui montraient une forte expression des transcrits d'INFβ dans les cellules NPTr en réponse au virus. Concernant les MAPK, nous avons observé l'activation de p38 qui est connue pour être activée en réponse aux situations de stress et en réponse aux cytokines pro-inflammatoires, tandis que ERK1/2, également activée, participe plutôt aux processus de prolifération cellulaire et d'apoptose (Ehrhardt et al. 2010, Ludwig et al. 2006). Ces résultats peuvent être corrélés avec les travaux réalisés par Gao et ses collaborateurs dans des macrophages porcins où une activation de la voie ERK1/2 en présence du SIV a été observée (Gao et al. 2012). Parallèlement et de manière similaire à nos observations, ils n'ont pas non plus observé de surexpression significative des transcrits d'IL1β en réponse au virus tandis que les niveaux d'expression de TNFα étaient quant à eux significativement augmentés à partir de 3h post-infection.

Par la suite, nous avons procédé à des inhibitions spécifiques des différentes voies activées par le SIV afin de déterminer l'impact de ces inhibitions sur la transcription de SOCS1. Le choix de SOCS1 reposait sur l'observation de l'activation de cet unique membre de la famille des SOCS en réponse à l'infection virale. Pour ce faire, des pré-traitements avec des inhibiteurs chimiques ont été réalisés sur les cellules NPTr et l'efficacité du blocage a été vérifiée par Western blot à 15 et 30 minutes, temps correspondant au pic d'activation pour chacune des voies. Parmi les voies bloquées, seule l'inhibition de la voie JAK/STAT a montré un impact significatif sur l'expression des transcrits de SOCS1, leur expression étant diminuée. Ce résultat confirme les observations faites dans des études précédentes qui mettaient en évidence un lien privilégié entre les protéines SOCS et la voie JAK/STAT, et plus particulièrement entre CISH, SOCS1 et SOCS3 et JAK/STAT (Endo et al. 1997, Naka et al. 1997, Starr et al. 1997, Yoshimura et al. 2007, Dalpke et al. 2008). Une réduction d'expression en réponse au traitement a aussi été observée pour les transcrits des protéines de

la réponse antivirale. Ainsi 24h post-infection, les ARNm de RIG-I, INFβ, INFλ1, PKR et MX1 étaient significativement moins exprimés lors du blocage de JAK/STAT.

Ces résultats encouragent à aller plus loin dans l'étude du lien entre SOCS1 et la réponse antivirale. D'ailleurs, des expériences dans lesquelles je suis également impliqué, utilisant un peptide inhibiteur de SOCS1 et SOCS3 (Ahmed et al. 2010), sont actuellement en cours. L'inhibiteur appelé *pJAK2* (1001-1013) *inhibitor* (Ahmed et al. 2010, Waiboci et al. 2007, Flowers et al. 2004) correspond à la séquence d'activation de JAK2 (LPQDKEYYKVKEP) et offre une inhibition efficace de SOCS1 et SOCS3 par le ciblage de leur région KIR. Ce peptide permettra d'analyser plus finement la réponse immune dirigée contre SIV H3N2 après inhibition de SOCS1 et SOCS3. Cependant, ce blocage n'est pas spécifique de SOCS1 et des techniques alternatives de siRNA voire de shRNA devront être développées pour préciser le rôle exact de SOCS1 dans la pathogenèse du SIV. Additionnellement des expériences de surexpression des différentes SOCS sont envisagées. Pour ce faire, le clonage des séquences codant pour les protéines SOCS et CISH ainsi que d'autres acteurs de la signalisation des cytokines comme des STATs et JAKs est nécessaire afin de construire des plasmides d'expression (pcDNA3.1, Invitrogen) et des adénovirus recombinants (pAdenoX vector, Clontech). Récemment SOCS1, SOCS2, SOCS3 et CISH ont été clonés dans le plasmide pCR Blunt Vector (Invitrogen) et les inserts ont été vérifiés par séquençage. Ces constructions permettront *in fine* de surexprimer les SOCS dans les cellules NPTr, et aussi dans des cellules primaires, comme les PAMs ou celles présentes dans les PCLS. L'ensemble de ces manipulations permettront de compléter et de clarifier plusieurs aspects concernant la régulation de la réponse antivirale contre le SIV.

En plus de ces travaux *in vitro*, des expériences *in vivo* en collaboration avec l'agence nationale de sécurité sanitaire de l'alimentation (ANSES) de Ploufragan sont en cours. Ces travaux visent à mieux caractériser la réponse immune innée et le rôle régulateur potentiel des SOCS dans un contexte de co-infection avec le virus influenza H1N1 A/Sw/Cotes d'Armor/0231/06 (SIV H1N1) et *Mycoplasma hyopneumoniae* (Mhp). Les premier résultats obtenus ont montré que parmi les transcrits SOCS, seulement ceux de SOCS1 sont surexprimés significativement 24h post-infection dans le groupe SIV et le groupe co-infecté SIV/Mhp par rapport aux groupes *mock* infectés avec les milieux de culture ou les groupes non infectés. Cette surexpression à 24h post-infection a également été observée pour les transcrits de RIG-I, INFβ, Mx1, OAS1 et PKR, suivie par une diminution jusqu'au niveau

basal 48h post-infection. Ces observations sont en concordance avec les analyses réalisées dans les cellules NPTr, les macrophages alvéolaires et les explants pulmonaires. Ces résultats ouvrent donc des perspectives dans la poursuite de l'étude du rôle potentiel de SOCS1 lors de la réponse immune précoce à l'infection par le SIV.

Dans ce travail de thèse, nous avons travaillé en parallèle au développement d'un modèle alternatif de culture en interface air-liquide (ALI) d'une lignée cellulaire obtenue à partir des cellules épithéliales de la trachée du porc, la lignée NPTr (Ferrari et al. 2003). En effet le but était de développer un système de culture plus proche de la réalité tout en restant relativement simple. Il est connu que les cellules différenciées ne répondent pas toujours de la même façon que les cellules non différenciées aux agents pathogènes notamment suite à une localisation cellulaire et une expression différentes des PRRs (Ioannidis et al. 2013, Ciencewicki, Brighton and Jaspers 2009). La lignée NPTr provenant d'un élevage porcin indemnes de pathogènes spécifiques, offre un large spectre de sensibilités pour l'analyse des interactions hôte/pathogènes (Ferrari et al. 2003). La culture en condition ALI des cellules NPTr pourrait nous permettre de mieux caractériser la réponse immune innée des cellules épithéliales porcines aux différents agents pathogènes. Ce système plus réaliste, permet d'obtenir une organisation spatiale des récepteurs de l'immunité innée proche de celle observée *in vivo* (Ioannidis et al. 2013, Lopez-Souza et al. 2004, Gruenert, Finkbeiner and Widdicombe 1995). Ensuite l'utilisation d'une lignée cellulaire offre plusieurs avantages par rapport à l'utilisation de cellules primaires. La durée de vie des cellules est prolongée ; la variabilité entre passages est faible ; et la manipulation des cellules est plus simple avec moins de risques de contaminations (Prytherch et al. 2011). Habituellement, les cellules épithéliales primaires ou issues de lignées cellulaires sont mises en culture sur des supports en plastique conventionnels et sont maintenues submergées. Cette méthode de culture présente des inconvénients. Parmi ceux-ci il y a notamment la perte de la capacité des cellules à développer des cils (de Jong et al. 1994, Jorissen et al. 1991, Whitcutt, Adler and Wu 1988, Wu, Nolan and Turner 1985). Des variations entre espèces animales sont néanmoins rapportées. Ainsi chez le hamster, des cellules primaires cultivées submergées sur des supports en plastique conventionnels peuvent garder la capacité à développer des cils et peuvent se différencier en cellules caliciformes (Kim et al. 1985, Lee et al. 1984). En revanche, plusieurs études ont démontré que la culture de cellules épithéliales primaires en condition ALI maintenait et/ou stimulait la différenciation des cellules jusqu'à l'obtention de

structures épithéliales similaires à celles retrouvées dans l'arbre respiratoire (Bateman et al. 2013, Clark et al. 1995, de Jong et al. 1993, Goris et al. 2009).

Nous avons émis l'hypothèse que les cellules épithéliales NPTr pourraient bénéficier de la culture en condition ALI et potentiellement se redifférencier pour *in fine* montrer des caractéristiques similaires à celles observées avec les cellules épithéliales primaires, comme par exemple la présence de cils cellulaires.

Dans un premier temps, des marquages par immunofluorescence ont été réalisés afin d'identifier les différents types de populations présents avant et après la mise en culture en conditions ALI. Dès le début de la culture, la présence de cellules productrices du mucus et de cellules β-tubuline positives a été observée. Cependant, le marquage β-tubuline était un peu diffus et les cellules positives localisées à la périphérie ne montraient pas de cils à leur surface. Afin d'évaluer la capacité de différenciation des cellules NPTr en conditions conventionnelles non ALI, des cultures sur support plastique ont été réalisées. Nous avons observé la présence de deux mêmes types de populations cellulaires dans les conditions non ALI. Ces observations suggèrent que les cellules NPTr se développent similairement quelle que soit la technique de culture utilisée. Toutefois la culture sur support plastique ne permet pas la polarisation des cellules. La présence de deux types de cellules peut être utile notamment pour l'étude des interactions hôte/pathogène avec des agents pathogènes qui visent une population cellulaire particulière comme les cellules caliciformes ou les cellules ciliées. C'est ce qui a été effectué par Goris et collaborateurs lors de l'infection de cellules bovines par le virus parainfluenza 3 bovin (BPIV3) et le virus respiratoire syncytial bovin (BRSV). Ils ont observé que le BPIV3 avait une plus grande affinité pour les cellules ciliées que pour les cellules caliciforme même à une multiplicité d'infection faible (0,1) (Goris et al. 2009).

Afin d'évaluer la qualité de « l'épithélium » formé, un suivi de l'évolution de la résistance électrique transépithéliale (TEER) dans les différentes conditions de culture a été réalisé. Parmi les différents milieux de culture testés pendant trois semaines en condition ALI, seulement les cultures avec le DMEM et le DMEM/HAMF12 ont permis d'obtenir des valeurs de TEER proches des 300 Ωcm^2. Ces valeurs sont à rapprocher des observations faites par microcopie électronique à transmission et des marquages dirigés contre la protéine associée aux jonctions serrées *zonula occludens-1* (ZO-1), démontrant le développement de jonctions serrées entre les cellules. La présence de multiples couches cellulaires pourrait aussi

contribuer à l'augmentation des valeurs de TEER. Ensuite l'expression des ARNm de ZO-1 a été mesurée après trois semaines de culture ALI. L'expression des transcrits de ZO-1 était diminuée. Des résultats similaires ont été déjà observés dans une autre étude qui compare les cellules épithéliales primaires humaines HBEC, et les lignées Calu-3 et BEAS-2B en condition ALI (Stewart et al. 2012). Dans cette étude les cellules Calu-3 montrait des valeurs de TEER élevées et des expressions d'ARNm et de protéine ZO-1 faibles par rapport aux cellules primaires (Stewart et al. 2012). En revanche, les ARNm associés à l'occludine, une autre protéine impliquée dans la formation des jonctions serrées, étaient significativement plus exprimés après trois semaines de culture ALI. En ce qui concerne la villine, les niveaux d'expression des transcrits sont restés faibles tout au long de la culture. Ces résultats diffèrent partiellement de ceux obtenus dans une étude réalisée par Nossol et collaborateurs (Nossol et al. 2011) où l'expression des ARNm de villine avait aussi été évaluée mais cette fois dans des cellules épithéliales intestinales porcines (IPEC-1 et IPEC-J2). En effet, les cellules IPEC-1 cultivées en conditions ALI ont montré des niveaux d'expression des ARN de villine significativement plus élevés que ceux observés lors de la culture conventionnelle. Cependant, les cellules IPEC-J2 cultivées en ALI ont montré une expression faible des ARNm de villine en comparaison à la culture sur support plastique (Nossol et al. 2011).

Afin d'identifier le type de mucines produites suite à la culture ALI, l'expression des transcrits de MUC1, MUC2 et MUC4 a été quantifiée. MUC2 est une mucine formatrice du gel MUC1 et MUC4 sont des mucines de surface (Linden et al. 2008, Ross et al. 2007). Une augmentation significative de l'expression des ARNm de MUC1 et MUC2 a été observée après culture conventionnelle et après culture en condition ALI. Ces résultats sont en concordance avec l'apparition des cellules productrices de mucus après trois semaines de culture en conditions conventionnelles et en condition ALI. Cependant, l'expression des ARNm de MUC4 ne montre pas des différences significatives. Cette diminution d'expression pourrait être causée par une perte de stabilité de l'ARNm.

Par la suite, nous avons privé les cellules NPTr du sérum de fœtus de veau pour évaluer la capacité de différenciation des cellules avec les différents types de milieux de culture sans sérum. En revanche, les milieux ont bénéficié de l'ajout de dexaméthasone et d'acide rétinoïque afin de mimer les conditions de culture testées précédemment dans d'autres études (Wu, Zhao and Chang 1997). Nous avons observé que les cellules NPTr sont finalement très dépendantes du sérum et que malgré la présence de dexamethasone et d'acide

rétinoïque ainsi que d'autres facteurs de croissance, les cellules n'arrivent plus à former des cils, contrairement aux observations faites dans d'études précédents en absence de ces additifs (de Jong et al. 1993, Finkbeiner, Carrier and Teresi 1993, Kondo, Finkbeiner and Widdicombe 1993). Ces résultats suggèrent que la présence du sérum est fondamentale pour la culture de la lignée NPTr. Au contraire, des études précédents effectuées avec des explants de tissu et des cellules primaires ont montré que l'absence de sérum et d'acide rétinoïque permettait un développement optimal des cellules ciliées (Chopra 1982, Clark et al. 1995, Goris et al. 2009, Gray et al. 1996, Jetten et al. 1987, Marchok, Cone and Nettesheim 1975, Stewart et al. 2012).

Dans notre modèle, nous avons réussi à différencier partiellement les cellules NPTr en conditions de culture ALI. Les cellules NPTr se polarisent et développent des jonctions serrées. Cependant, de vraies cellules caliciformes et des cellules ciliées restent absentes dans nos conditions. L'optimisation du modèle en est toujours en ce moment insuffisante quant à la différenciation totale des cellules et des questions restent encore à élucider. Le manque de différenciation pourrait être expliqué par l'absence des facteurs qui puissent améliorer la composition du milieu de culture, la possible perte irréversible de la capacité de former des cils par les cellules NPTr ou l'utilisation des temps de culture inadéquats. Malgré ses limitations, le modèle *in vitro* ALI pour la culture des cellules NPTr offre une alternative par rapport à l'utilisation des cellules primaires.

Conclusion

Le travail de thèse présenté ici s'inscrit dans le cadre de l'étude de la réponse immune antivirale et de son contrôle dans l'espèce porcine. Dans un premier temps, l'expression des SOCS à l'homéostasie a été évaluée afin d'obtenir des informations préliminaires concernant cette famille de régulateurs des cytokines. Les transcrits de SOCS1 sont surexprimés dans le thymus à l'homéostasie.

Dans la deuxième étude et afin d'aller plus loin dans les observations précédentes et de confirmer les observations réalisées dans d'autres études, l'implication des SOCS dans l'infection par un SIV de sous-type H3N2 a été évaluée. Pour cela, deux systèmes *in vitro* avec des cellules épithéliales NPTr et des macrophages alvéolaires ainsi qu'un système *ex vivo* avec des explants pulmonaires ont été utilisés afin d'obtenir une vision globale de la réponse immune innée contre SIV. La comparaison des trois systèmes, nous a permit d'observer l'expression des transcrits d'INFβ après stimulation de RIG-I. Plus particulièrement, dans les cellules NPTr, des expressions significatives d'INFβ et d'INFλ1 ont été observées, contrairement aux PAMs dans lesquels les INF de type III n'ont pas montré d'expression significative en présence du virus. Globalement, en réponse aux interférons, les transcrits de Mx1, Mx2, PKR et OAS1 ont été retrouvés dans les trois systèmes. Parmi les SOCS en réponse à l'infection, une surexpression des ARNm de SOCS1 à 24h post-infection dans les trois systèmes de culture a été observée.

Les voies MAPK (p38, ERK 1/2), PI3K/Akt et JAK/STAT sont activées dans les cellules épithéliales NPTr suite à l'infection par SIV. Suite au blocage spécifique de chaque voie évaluée, JAK/STAT à un impact négatif sur la transcription de SOCS1 à 24h. Une réduction d'expression a aussi été observée pour les transcrits des protéines de la réponse antivirale. Les ARNm de RIG-I, INFβ, INFλ1, PKR et MX1 étaient significativement moins exprimés lors du blocage de JAK/STAT. L'ensemble de résultats contribuent à affiner la connaissance de la réponse immune innée chez le porc et la potentielle implication des protéines SOCS dans la régulation de la réponse antivirale. L'utilisation d'un peptide inhibiteur correspondant à la région KIR de SOCS1 et SOCS3 permettra d'analyser plus finement dans un premier temps la réponse immune dirigée contre SIV H3N2 après inhibition de SOCS1 et SOCS3. Des techniques alternatives de siRNA voire de shRNA devront être développées pour la réalisation d'une inhibition spécifique qui permettra préciser le rôle exact de SOCS1 dans la pathogenèse du SIV. Additionnellement, des expériences de surexpression

de SOCS dans les cellules NPTr, et aussi dans des cellules primaires, comme les PAMs ou les PCLS pourront donner plus d'informations concernant la fonctionnalité des SOCS. L'ensemble de ces manipulations permettront de compléter et de clarifier plusieurs aspects concernant la régulation de la réponse antivirale contre le SIV.

Dans la troisième étude, nous avons travaillé en parallèle au développement d'un modèle alternatif de culture en interface air-liquide (ALI) d'une lignée cellulaire obtenue à partir des cellules épithéliales de la trachée du porc, la lignée NPTr. Plusieurs aspects de la différenciation cellulaire ont été évalués dans différentes conditions de culture. La présence de cellules productrices du mucus et de cellules β-tubuline positives a été observée ainsi que le développement de jonctions serrées entre les cellules et une augmentation de la TEER. Cependant, de vraies cellules caliciformes et des cellules ciliées son absentes. Malgré ses limitations, le modèle *in vitro* ALI pour la culture des cellules NPTr offre une alternative par rapport à l'utilisation des cellules primaires. Cette approche *in vitro* pourrait apporter des nouvelles informations pour mieux comprendre la réponse immune innée dans l'étude des infections respiratoires chez le porc.

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Annexes

Annexe 1 :

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

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

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ABSTRACT

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

1. Introduction

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

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

Respiratory infectious agents can be divided into primary and secondary pathogens. Primary pathogens include bacteria such as *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, and *Mycoplasma hyopneumoniae*, and viruses including porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus (SIV), pseudorabies virus, and porcine circovirus type 2 (Opriessnig et al., 2011). Other primary pathogens are described but they are rarely encountered or have less impact on porcine health. Among the secondary pathogens, common bacteria such as *Actinobacillus suis*, *Haemophilus parasuis*, *Pasteurella multocida*, *Salmonella choleraesuis*, and *Streptococcus suis* are frequently reported (Choi et al., 2003; Fablet et al., 2012a,b, 2011; Opriessnig et al., 2011). Together, primary and secondary pathogens are involved in the well-described porcine respiratory disease complex (PRDC) (Hallbur, 1998). PRRSV and SIV, together and individually, are frequently encountered in the fields (Choi et al., 2003; Fablet et al., 2012a, 2011). In a study by Choi and collaborators, 109 samples (17%) of 636 SIV-positive cases were also positive for co-infections with PRRSV, behind *P. multocida* (148 samples, 23.2%) and *M. hyopneumoniae* (122 samples, 19.2%) (Choi et al., 2003). Previous studies dealing with PRRSV/SIV dual infections (Pol et al., 1997; Van Reeth et al., 1996, 2001) showed various outcomes with respect to dual infection. In a study where feeder pigs were infected first with PRRSV, then with porcine respiratory coronavirus or SIV, more severe disease and growth retardation were observed with dual infection than with PRRSV infection alone (Van Reeth et al., 1996). In another study where 3-week-old specific-pathogen-free piglets were intra-nasally infected with PRRSV, followed one week later with a H3N2 SIV strain, observations indicated that the previous PRRSV infection did not influence clinical signs during influenza infection (Pol et al., 1997). Then, in a study with PRRSV and a European H1N1 SIV strain, authors observed variable clinical outcomes of dual PRRSV-SIV infection, depending on both the time interval between infections and the health status of pigs used in the study (Van Reeth et al., 2001). Aside from these various following co-infections, our understanding of the impact of one virus on the other at the molecular level is still extremely limited. Most of the studies on PRRSV/SIV co-infections were performed more than ten years ago at a time where the porcine toolbox was much less developed. Thus, the aim of the current study was to determine at the molecular level how dual infections, compared to single infections, influence the response of porcine alveolar macrophages (PAMs) and precision cut lung slices (PCLS) to PRRSV and SIV. PAMs and PCLS were used because of their relevance in the context of infections with PRRSV and SIV. PAMs, pulmonary intravascular macrophages (PIMs), and interstitial macrophages (ISMs) are the main targets of PRRSV (Meulenberg, 2000; Sang et al., 2011). PAMs can also be infected by SIV (Crisci et al., 2013; Taubenberger and Morens, 2008). PCLS have previously been used for infection studies in birds (Abd El Rahman et al., 2010), cattle (Goris et al., 2009), and pigs (Punyadarsaniya et al., 2011). The PCLS culture system has several advantages over other systems: (1) slices can be obtained in large numbers; (2) the general architecture of the tissue is preserved so differentiated epithelial cells, which are the main target cells of SIV, and various other cellular types are maintained *in situ*; and (3) the slice viability extends past 7 days (Punyadarsaniya et al., 2011). Moreover, PAMs and PCLS systems together allow us to study host/pathogen interactions in a single cell type population *versus* a multi-cellular tissue, granting more accurate analysis of the contribution of PAMs to the global disease response.

2. Materials and methods

2.1. Ethics statement

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

2.2. Precision-cut lung slices

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

2.3. Cells and viruses

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

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

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

2.4. Virus infection

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

For superinfections, six wells of PAMs (10^5 cells/well, one well corresponding to one pig) were first infected with SIV (MOI of 10), then superinfected with PRRSV (MOI of 10) 3 h later. In parallel, six wells of PAMs were infected with PRRSV (MOI of 10) and superinfected with SIV (MOI of 10) 3 h after infection with PRRSV. The 3 h delay between infections was selected based on previous studies where interference between related viruses of another family was intensively assessed *in vitro* and *in vivo* (Banfield et al., 2003; Glazenburg et al., 1994; Meurens et al., 2004a,b; Schynts et al., 2003). Many interference mechanisms take place early in the viral cycle (Meurens et al., 2003). After the first infection, virus attachment was allowed for 1 h at 4 °C. Cells were then further incubated at 37 °C and superinfections were performed 3 h after the temperature shift. One hour after the temperature shift and 1 h after each superinfection cells were washed once with PBS and further incubated at 37 °C in 1 ml of RPMI 1640 (GIBCO®-BRL) supplemented with 10% FBS and 1% antibiotic/antimycotic (Anti-Anti 100×, GIBCO®-BRL). Additionally 6 non-infected wells were used as controls. Fifteen hours post-superinfection, the culture medium was removed, clarified twice by centrifugation (1000 × *g*), divided into aliquots, and stored at −80 °C. For PCLS the procedure was identical except that 10^6 pfu of SIV and 10^6 $TCID_{50}$ of PRRSV were administered. Six slices, prepared from the same left lung lobe, were used for each condition. The experiment was repeated four times using 4 different animals.

2.5. Immunofluorescence analysis of precision-cut lung slices

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

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

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

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

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

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

Table 1 (*Continued*)

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

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

2.7. Interferon alpha and beta enzyme-linked immunosorbent assays

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

2.8. Statistical analysis

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

3. Results

3.1. Viability of porcine precision-cut lung slices

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

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

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

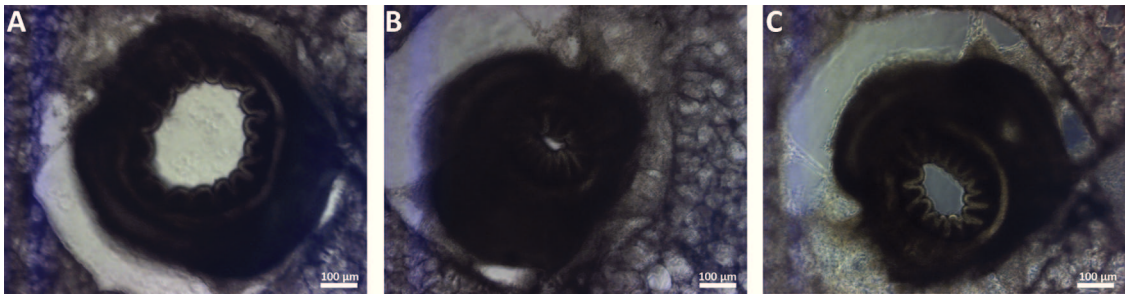

Fig. 1. Viability of PCLS evaluated by bronchoconstriction 96 h after the PCLS preparation. Untreated slice (a) was incubated with 10^{-4} M methacholine (b) to induce bronchoconstriction. Removal of the drug resulted in a reverse effect (c). Viability was tested at 24 h and 96 h after the PCLS preparation.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

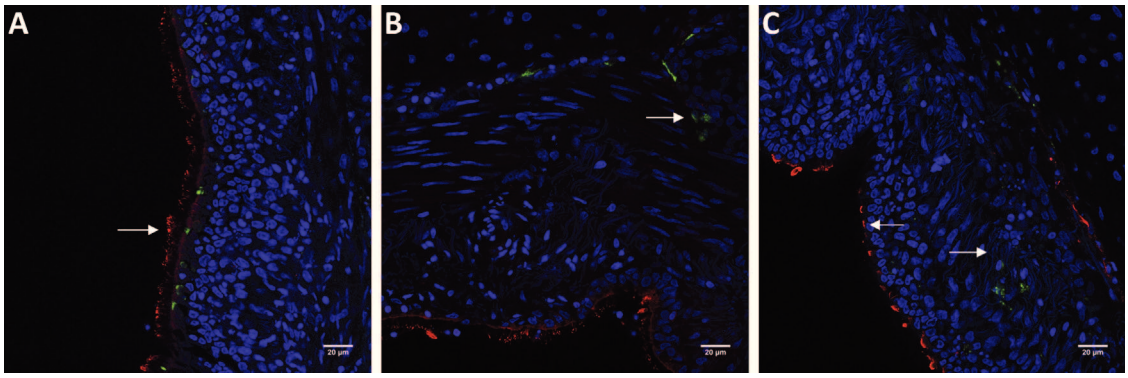

Fig. 2. Infection of PCLS by SIV and PRRSV characterized by immunostaining. PCLS were infected by SIV (A), PRRSV (B) or both viruses (C). Cryosections were prepared after 18 h of infection and image data was collected using a laser-scanning confocal microscope. Infected cells were stained with an anti-nucleoprotein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

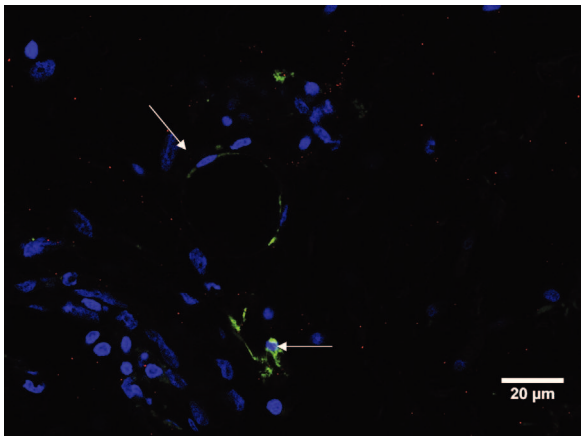

Fig. 3. PRRSV cellular targets in alveolar tissue. PCLS was infected by PRRSV and SIV. Cryosections were prepared after 18 h of infection and used for detection of infected cells in the alveoli. Infected cells were stained with an anti-nucleocapsid monoclonal antibody for the detection of PRRSV (green). Ciliated cells were stained using an anti-beta-tubulin monoclonal antibody (red). Arrows indicate infected cells (presumably type 1 cell and macrophage, horizontal arrow), scale bar = 20 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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

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

Regarding PRRSV VR-2385 nucleoprotein transcript expression in the PCLS, no expression was observed in the control slices or the slices only infected with SIV H1N1 (Fig. 4). In most of the conditions where the PRRSV viruses were used, PRRSV transcript expression was detected at similar levels (detection around *Cq* 22) with no statistically significant differences between conditions (Fig. 4). PRRSV transcript expression was only significantly lower ($P < 0.05$) than in the "PRRSV" condition when slices were superinfected with PRRSV 3 h after SIV infection (SIV-3h-PRRSV) (Fig. 4). Similar observations were made for SIV (M protein transcripts) in PCLS (detection around *Cq* 20), except for "SIV-3h-PRRSV", where the transcript expression was not significantly lower than in the other conditions where SIV had been used (Fig. 4).

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

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

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

Table 2
Viral and host transcript expression summary.

	PCLS	PAMs
Conditions:		
PRRSV	-*Viral*: Moderate expression (*Cq*: 22) -*Host*: Low response	-*Viral*: High expression (*Cq*: 13) -*Host*: Low to moderate response
SIV	-*Viral*: Moderate expression (*Cq*: 20) -*Host*: Moderate response	-*Viral*: Moderate expression (*Cq*: 18) -*Host*: Moderate response
PRRSV + SIV	-*Viral*: Similar to single infections -*Host*: Additive effects and synergy	-*Viral*: SIV replication reduced -*Host*: Similar to single infection
PRRSV-3h-SIV	-*Viral*: Similar to single infections -*Host*: Decreased response	-*Viral*: Similar to single infections *Host*: Similar to single infections or slightly increased (IFNα)
SIV-3h-PRRSV	-*Viral*: PRRSV replication reduced -*Host*: Similar to single infections	-*Viral*: PRRSV replication reduced -*Host*: Similar to single infections

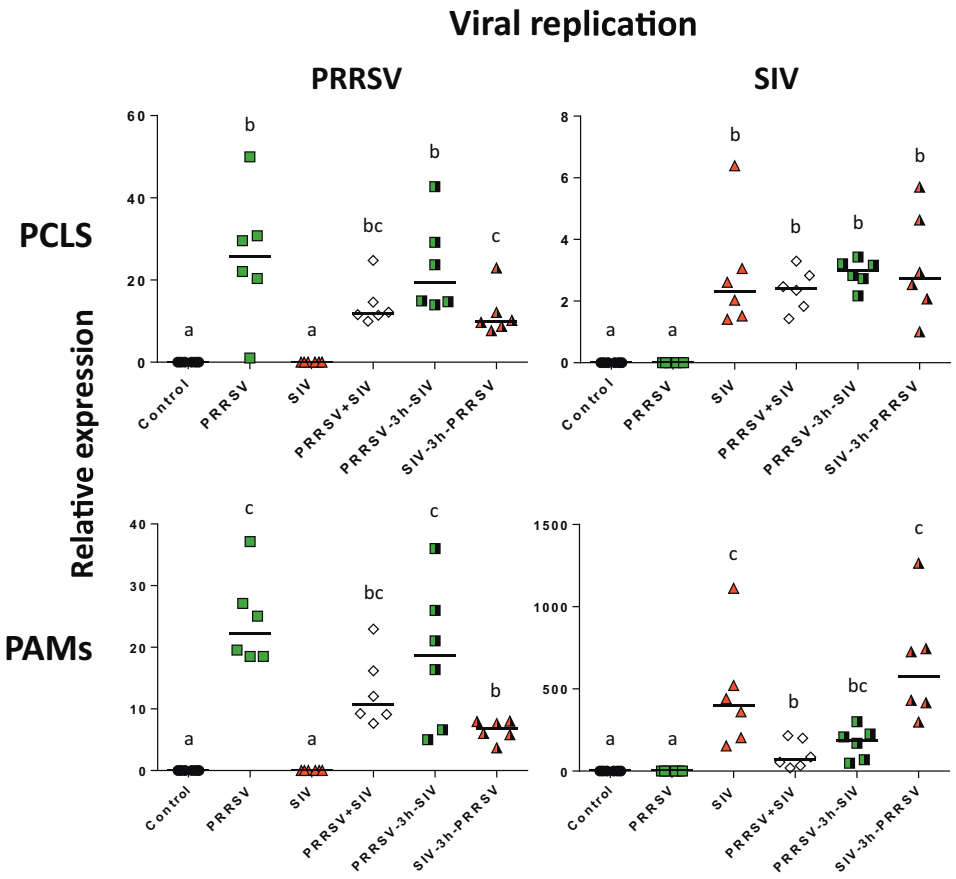

Fig. 4. Viral replication – relative expression of viral transcripts (nucleoprotein-PRRSV and M protein-SIV) after 18 h of infection of PCLS and PAMs. For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common superscripts are significantly different ($P < 0.05$).

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

3.4.1. Transcripts involved in viral recognition

Regarding transcripts involved in viral recognition (Tables 1 and 2), several observations were made in experiments where PCLS (Fig. 5A) and PAMs (Fig. 5B) were used. None of the pathogen recognition receptor (PRR) transcripts was significantly over-expressed in response to PRRSV in the PCLS although one of them (TLR9) had a small, but not statistically significant, increase (Fig. 5A). On the contrary, SIV significantly ($P < 0.05$) induced the expression of DAI, LGP2, MDA5, RIG-I, and TLR3 genes (Fig. 5A). These transcripts were generally expressed at a similar level to when PRRSV was superinfecting the PCLS 3 h after SIV (SIV-3h-PRRSV) (Fig. 5A). However, when PRRSV was administered to the slices 3 h prior to SIV (PRRSV-3h-SIV), a lower expression than in the co-infection condition "PRRSV + SIV" was observed for most of the transcripts, with the exception of TLR8 and TLR9. This lower induction was statistically significant for MDA5, RIG-I, and TLR3 transcripts in some conditions (see Fig. 5A). Except for TLR3 and RIG-I, the induction of transcript expression was not statistically higher in the situation of co-infection compared to single infections (Fig. 5A). In PAMs, PRRSV did not significantly induce the expression of viral recognition transcripts, similarly to PCLS (Fig. 5B). However, for DAI, LGP2, MDA5, RIG-I, and TLR7 there was a marked increase in the expression of the transcripts in response to PRRSV when compared to controls (Fig. 5B). Similarly to what was observed in PCLS, the expression of transcripts for DAI, LGP2, MDA5, and RIG-I was significantly higher in the SIV infected cells ($P < 0.05$) than in the controls (Fig. 5B). Regarding TLR3, TLR8, and TLR9 transcripts, no significant difference was observed between control and SIV conditions (Fig. 5B), although expression of TLR3 transcripts seemed higher with SIV than PRRSV. However, this observation was not confirmed by statistical analysis. Furthermore, no statistically significant differences were identified between the various conditions for TLR transcripts, with the exception of TLR7 transcripts (Fig. 5B). Moreover, "SIV" condition was not significantly different amongst the co-infection and superinfection conditions for all the transcripts (Fig. 5B).

3.4.2. Interferon transcripts

Next, the expressions of interferon transcripts were analyzed using the same viral infection combinations

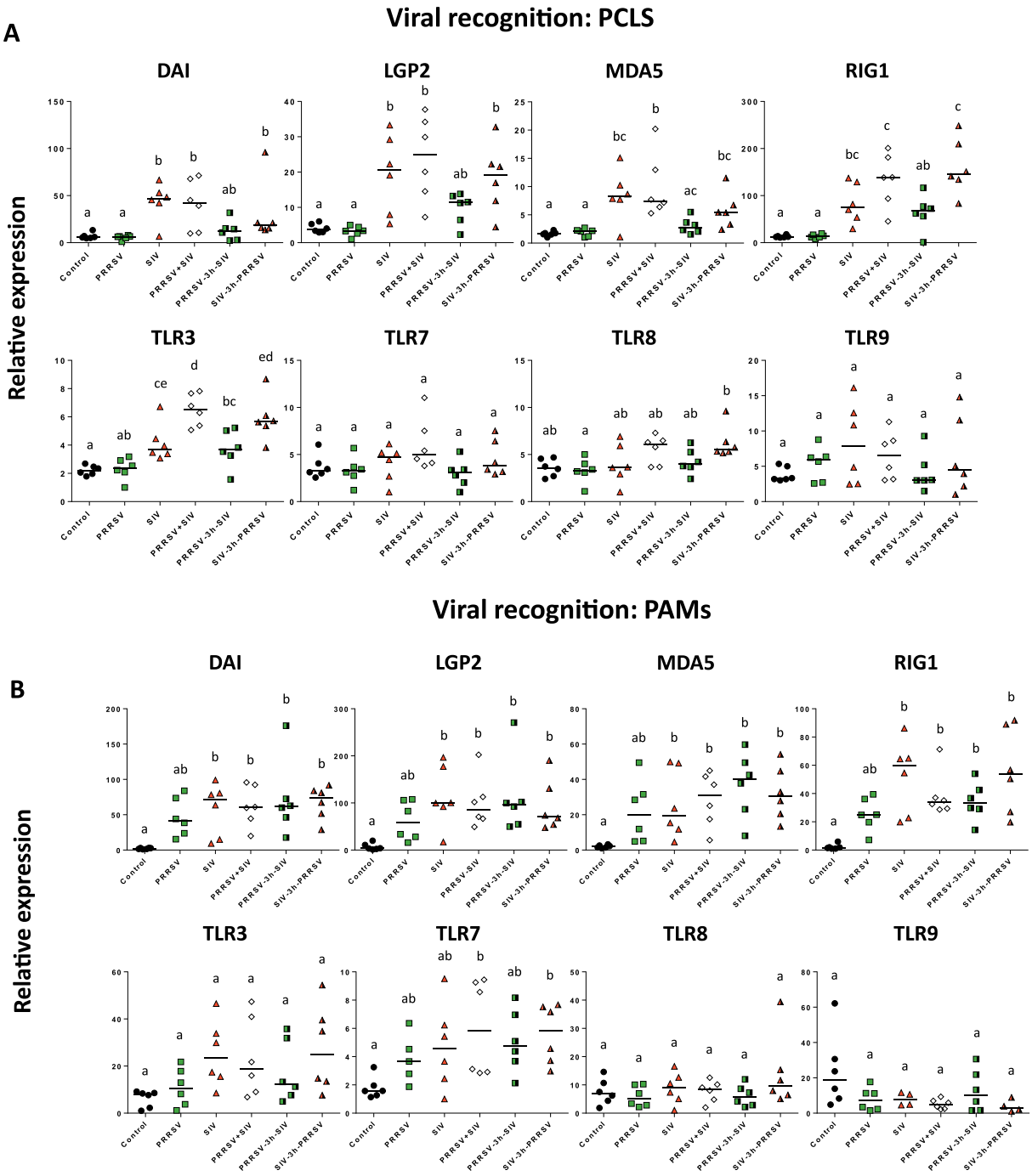

Fig. 5. Viral recognition – relative expression of viral recognition transcripts after 18 h of infection of PCLS (A) and PAMs (B). For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common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

discussed above in PCLS and PAMs (Fig. 6 and Table 2). In PCLS, no statistically significant differences between conditions were reported for IFNα mRNA (Fig. 6). IFNβ transcripts were expressed at higher levels in response to SIV alone or in association with PRRSV (Fig. 6) ($P < 0.05$). When SIV was co-administered with PRRSV, the expression of the transcript was significantly higher than when SIV and PRRSV were administered alone (Fig. 6) ($P < 0.05$). The median of relative expression in the "PRRSV + SIV" condition is higher than the sum of the median of relative expression of "PRRSV" and "SIV" conditions. For IFNγ, only the conditions "PRRSV + SIV" and "SIV-3h-PRRSV" showed

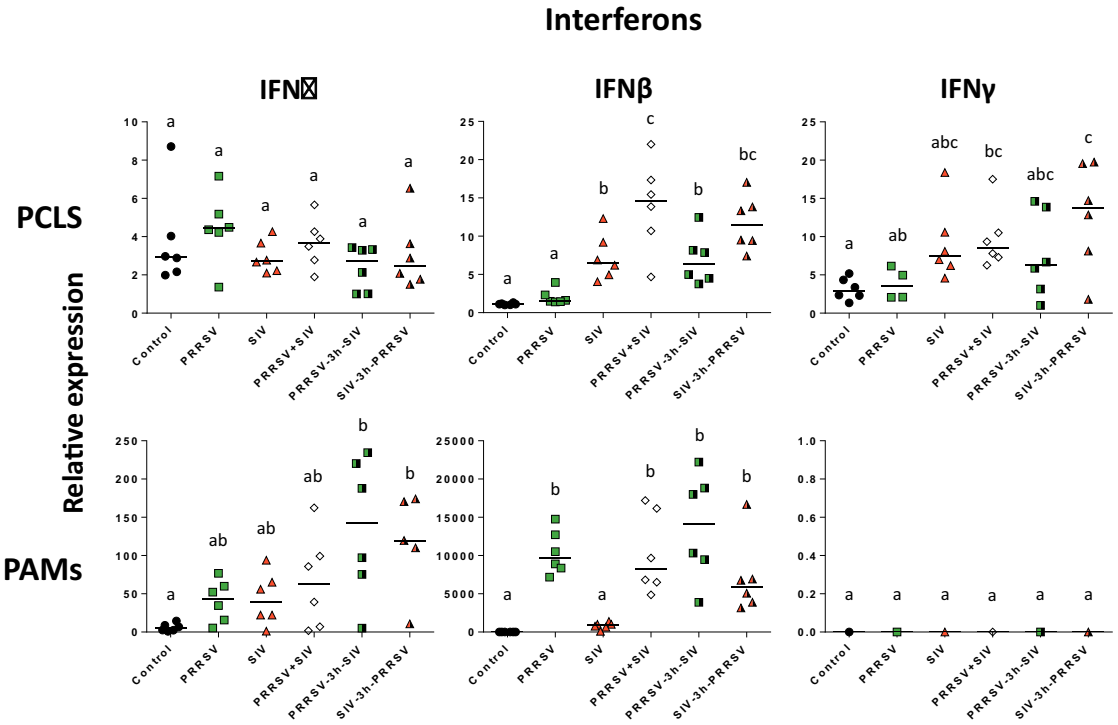

Fig. 6. Interferons – relative expression of interferon transcripts after 18 h of infection of PCLS and PAMs. For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common superscripts are significantly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

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

3.4.3. Antiviral transcripts in response to interferons

In response to the IFNs, various antiviral transcripts may be expressed (Tables 1 and 2). In PCLS, PRRSV did not induce any significant increase in the expression of the transcripts under study, while SIV was able to significantly induce ($P < 0.05$) their expression (Fig. 7). Similarly, when co-infected (PRRSV + SIV), the expressions of antiviral transcripts were significantly higher ($P < 0.05$) than in the "control" conditions (Fig. 7). In the superinfection condition, "PRRSV-3h-SIV", the expression of the transcripts was slightly higher for some (Mx2 and PKR) but not all (Mx1 and OAS1), relative to the control (Fig. 7). When PRRSV was administered 3 h after SIV (SIV-3h-PRRSV), the expressions of transcripts were similar to when both viruses were administered together or when the SIV was administered alone (Fig. 7). In the PAMs, PRRSV was unable to induce a statistically significant stimulation in the expression of the various transcripts, despite the subtle increase observed (Fig. 7). In nearly all the conditions where SIV was administered, a higher expression of the transcripts ($P < 0.05$) was observed (Fig. 7). However, as previously observed (Figs. 5A and 6), the increase was less important and sometimes not statistically significant comparatively to the control when the PRRSV was administered 3 h before SIV (Fig. 7).

3.4.4. Cytokines and related transcripts

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

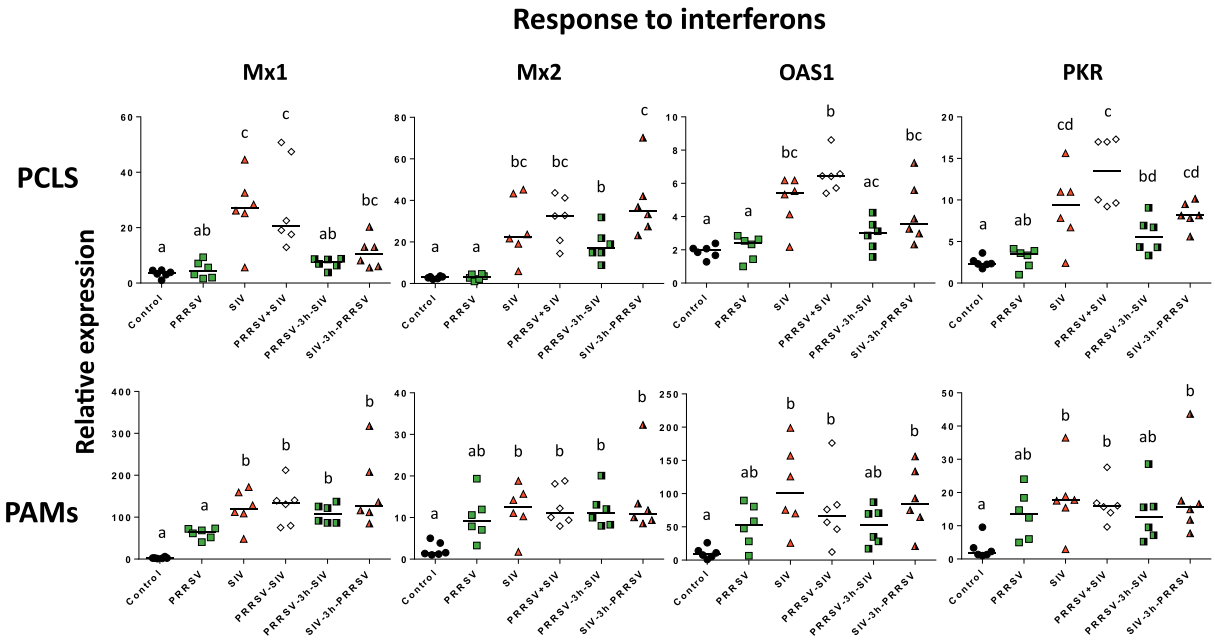

Fig. 7. Response to interferons – relative expression of interferon induced gene transcripts after 18 h of infection of PCLS and PAMs. For the PCLS, $n = 6$ slices and median value in one representative pig out of four and for PAMs, $n = 6$ pigs and median value. Dot plots within each graph with no common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

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

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

3.5. IFNα and IFNβ quantification

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

4. Discussion

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

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

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

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

Regarding the PRR transcripts (DAI, LGP2, MDA5, RIG-I, and TLR7) in PAMs, PRRSV showed a positive trend toward an increase in their expression; however it was not statistically significant, possibly due to the small number of samples examined. One hypothesis to explain the complete absence of transcript over-expression in response to the PRRSV in the PCLS is the relatively small percentage of macrophages in the slices and their cellular heterogeneity in comparison to a pure population such as PAMs. In the literature, PRRSV was associated with an increase of most, if not all, viral-sensing TLRs in the lungs, including TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 (Liu et al., 2009; Xiao et al., 2010a,b). In our study, after 18 h of infection, an increase in expression was demonstrated for TLR7. Notably, the relative expression of TLR3 transcripts was particularly high in "PRRSV/SIV" condition in PCLS. For TLR8 and TLR9, there were no statistically significant alterations in transcriptional expression in all infection conditions. In agreement with previous reports (Xiao et al., 2010a,b), our data suggest a significant stimulation of RIG-I and MDA5 after infection of lungs with another type 2 PRRSV. Additionally, after PRRSV infection, we observed an increase in the mRNA expression of two known detectors of virally derived RNA and DNA, DAI and LGP2 transcripts (Pichlmair and Reis e Sousa, 2007; Sang et al., 2011). Contrary to PRRSV, SIV significantly induced the up-regulation of the expression of all the selected transcripts (*i.e.* DAI, LGP2, MDA5, and RIG-I) and TLR3 transcripts in the PCLS ($P < 0.05$). In the PAMs, increases in the expression of viral recognition transcripts were statistically significant (DAI, LGP2, MDA5, and RIG-I) or showed a slight but not significant increase (TLR3 and TLR7). These results are in agreement with previous studies in pigs (Husser et al., 2011; Li et al., 2011) and other species (Wu et al., 2011). Conversely, NLRP3, a detector of SIV replication (Wu et al., 2011), was not induced but rather slightly repressed in our conditions. This is possibly due to the chosen time point after viral infection. In co-infection and superinfection conditions, relative expression of the viral recognition transcripts was not generally statistically different from relative expression of the same transcripts in response to SIV infection alone. However, in the case of TLR3 and RIG-I transcripts in the PCLS, they were clearly additive and even displayed synergistic effects of the two viruses with possible contribution of TLR7 transcripts to the additive effects (Fig. 5A). When a 3 h delay was introduced between the administration of the two viruses, observations were similar, with the exception of "PRRSV-3h-SIV" in PCLS, where expression of the transcripts was generally lower. Again, the 15 h incubation time *versus* 18 h could account for the observed difference even if we cannot exclude a PRRSV-specific effect.

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

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

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

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

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

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Mario DELGADO-ORTEGA

Étude comparative de la réponse immune innée à une souche porcine d'influenza de sous-type H3N2 et implication potentielle des protéines SOCS

Résumé

L'objectif de ce travail de thèse s'inscrit dans le cadre de l'étude de la réponse immune innée contre le virus influenza porcin (SIV) et de son contrôle dans l'espèce porcine par les protéines *suppressors of cytokine signaling* (SOCS) et la *cytokine-inducible SH2 domain containing protein* (CISH). L'analyse de l'expression des ARNm de SOCS à l'homéostasie a montré une expression significative dans le thymus suggérant un rôle dans la différenciation des cellules T.

La réponse immune innée contre une souche de SIV de sous-type H3N2 a été analysée *in vitro* et *ex vivo*. L'expression de transcrits impliqués dans la réponse antivirale et de SOCS a été évaluée. Une surexpression des ARNm des gènes antiviraux et de SOCS1 a été observée notamment à 24h post-infection. L'infection expérimentale des cellules NPTr par le virus H3N2 induit une activation des voies de signalisation impliquant MAPK et JAK/STAT. L'utilisation d'inhibiteurs spécifiques de la voie JAK/STAT a conduit à une diminution de l'expression des transcrits antiviraux et ceux de SOCS1 ainsi que l'expression des interférons de type I et III.

Afin de développer un outil alternatif *in vitro* d'étude de la réponse immune innée, la culture en interface air-liquide (ALI) des cellules NPTr a été réalisée. Des cellules à mucus, des jonctions serrées et une résistance transépithéliale élevée ont été observées. Cependant, ces cellules n'ont pas développé de cils. La culture des cellules NPTr dans des conditions ALI, a permis une représentation partielle de l'épithélium respiratoire porcin et constitue ainsi une alternative d'étude *in vitro*.

Mots clés : Influenza, porc, homéostasie, réponse immune innée, SOCS, signalisation, interface air-liquide

Abstract

The aim of this work was to investigate the innate immune response to swine influenza virus (SIV) and its regulation in swine by the suppressors of cytokine signaling SOCS and the cytokine-inducible SH2 domain containing protein (CISH). The assessment of SOCS constitutive mRNA expression showed significant mRNA expression of SOCS1 in thymus suggesting a key role of this protein in T cell differentiation.

The innate immune response against an SIV H3N2 subtype was then assessed *in vitro* and *ex vivo* by measuring antiviral and SOCS transcripts expression. The induction of several antiviral genes along with SOCS1 gene was observed. Experimental infection of NPTr cells with H3N2 virus induced MAPK and JAK/STAT signaling pathways activation. The inhibition of JAK/STAT pathway clearly reduced antiviral transcript expression, SOCS1 and both interferon types I and III mRNA expression as well.

In order to develop an alternative *in vitro* tool to study the innate immune response, NPTr epithelial cell line were cultured at the air-liquid interface. This system promotes the differentiation of mucus producing cells, tight junctions development and enables high trans-epithelial electronic resistance values. Nonetheless, the NPTr cells do not develop cilia. The culture of NPTr cells in ALI conditions allows a partial *in vitro* representation to investigate some aspects of host/respiratory pathogen interaction in pigs.

Keywords: Influenza, pig, homeostasis, innate immune response, SOCS, signaling, air-liquid interface