Co-infection of porcine respiratory epithelial cells
by influenza viruses and *Streptococcus suis*

THESIS

Submitted in partial fulfilment of the requirements for the degree

**DOCTOR OF PHILOSOPHY**

(PhD)

awarded by the University of Veterinary Medicine Hannover

by

Nai-Huei Wu

(Taichung, Taiwan)

Hannover, Germany 2016
Supervisor: Prof. Dr. Georg Herrler

Supervision Group:
Prof. Dr. Georg Herrler
Prof. Dr. Peter Valentin-Weigand
Prof. Dr. Wolfgang Garten

1st Evaluation: Prof. Dr. Georg Herrler
Institute of Virology, University of Veterinary Medicine
Hannover

Prof. Dr. Peter Valentin-Weigand
Institute of Microbiology, University of Veterinary Medicine
Hannover

Prof. Dr. Wolfgang Garten
Institute of Virology, Philipps-Universität Marburg
Marburg

2nd Evaluation: Prof. Dr. Dr. Thomas W. Vahlenkamp
Institute of Virology, University of Leipzig
Leipzig

Date of final exam: 05. April, 2016

This work was supported by grants from the Bundesministerium für Bildung und Forschung (BMBF) to G.H. (FluResearchNet; project code 01KI1006D), the Deutsche Forschungsgemeinschaft (DFG) to G.H. (He1168/15-1), PVW (Va23917-1).
Parts of this thesis have been communicated or published previously in:

**Publications:**


**Wu, N.H., Yang W., Meng, F., Dijkman R., Thiel, V., Valentin-Weigand, P., Herrler, G.** The differentiated airway epithelium infected by influenza viruses maintains the barrier function despite a dramatic loss of ciliated cells. Submitted.

**Oral presentations:**

20/05/2015 Seminars in Virology and Biochemistry, University of Veterinary Medicine Hannover, Hannover, Germany

Sialic acid-dependent interactions between influenza viruses and *Streptococcus suis* affect the infection of porcine tracheal cells.

N.-H. Wu


Poster presentations (selected)

Characterization of the growth of swine H1N1 influenza A viruses with mutations in the NS1 protein in differentiated respiratory epithelial cells. N.-H. Wu, W. Yang, F. Meng, J. Wang, Z. Wu, C. Lu, G. Herrler

CONTENTS

LIST OF ABBREVIATIONS ........................................................................................................... I

LIST OF FIGURES ........................................................................................................................ III

ABSTRACT ..................................................................................................................................... V

ZUSAMMENFASSUNG ....................................................................................................................... VII

1 INTRODUCTION .......................................................................................................................... - 1 -

1.1 Influenza A viruses .................................................................................................................... - 1 -

1.1.1 Background .......................................................................................................................... - 1 -

1.1.2 Taxonomy ............................................................................................................................ - 2 -

1.1.3 Virus structure and replication ............................................................................................ - 2 -

1.1.3.1 Hemagglutinin (HA) ....................................................................................................... - 6 -

1.1.3.2 Neuraminidase (NA) .................................................................................................... - 7 -

1.1.4 Antigenic variation .............................................................................................................. - 7 -

1.1.5 Influenza A viruses in swine ................................................................................................ - 8 -

1.1.5.1 North American swine influenza viruses ........................................................................ - 8 -

1.1.5.2 European swine influenza viruses .................................................................................. - 9 -

1.1.6 Swine-original influenza virus, H1N1pdm09 ....................................................................... - 12 -

1.2 Streptococcus suis .................................................................................................................... - 13 -

1.2.1 Etiology ............................................................................................................................... - 13 -

1.2.2 Streptococcus suis infection ............................................................................................... - 13 -

1.2.3 Pathogenesis ....................................................................................................................... - 15 -

1.2.4 Virulence factors ............................................................................................................... - 16 -

1.2.4.1 Capsule polysaccharide (CPS) ...................................................................................... - 16 -

1.2.4.2 Suilysin .......................................................................................................................... - 17 -

1.3 Sialic acid ............................................................................................................................... - 18 -

1.3.1 Receptor for influenza viruses ........................................................................................... - 18 -

1.3.2 Sialic acid on the bacterial capsular polysaccharide .......................................................... - 19 -

1.4 Bacterial co-infection with influenza virus ............................................................................. - 20 -

1.5 Swine models for study influenza viruses ............................................................................... - 22 -
1.5.1 Precision-cut lung slices (PCLS) .......................................................... - 23 -
1.5.2 Air-liquid interface (ALI) cultures .......................................................... - 23 -

1.6 Aim of the study .................................................................................................. - 25 -

2. MANUSCRIPT I ..................................................................................................... - 27 -

Sialic acid-dependent interactions between influenza viruses and
*Streptococcus suis* affect the infection of porcine tracheal cells ................ - 27 -

3. MANUSCRIPT II .................................................................................................. - 29 -

Dynamic virus-bacterium Interactions in a porcine precision-cut lung slice
coinfection model: swine influenza virus paves the way for *Streptococcus
suis* infection in a two-step process ............................................................... - 29 -

4. MANUSCRIPT III ............................................................................................... - 31 -

The differentiated airway epithelium infected by influenza viruses maintains
the barrier function despite a dramatic loss of ciliated cells ................. - 31 -

5. MANUSCRIPT IV ............................................................................................... - 69 -

Efficient suilysin-mediated invasion and apoptosis in porcine respiratory
epithelial cells after streptococcal infection under air-liquid interface
conditions ............................................................................................................. - 69 -

6. DISCUSSION ....................................................................................................... - 71 -

6.1 Sialic acid-dependent interactions between influenza viruses and
*Streptococcus suis* .......................................................................................... - 71 -

6.2 Host-pathogen interaction in an air-liquid interface culture system for
differentiated porcine airway epithelial cells .............................................. - 79 -
   6.2.1 An adequate model reflects porcine airway epithelium functions .... - 79 -
   6.2.2 Interplay of PBEC and influenza viruses, the story of host-pathogen
interaction .......................................................................................................... - 83 -

6.3 Infection strategies of *Streptococcus suis* in well-differentiated porcine
respiratory epithelial cells .............................................................................. - 90 -

6.4 Conclusions and outlooks ............................................................................... - 94 -
7. REFERENCES .......................................................................................................................... - 97 -

8. APPENDIX .............................................................................................................................. - 113 -

8.1 Acknowledgments ............................................................................................................... - 113 -
LIST OF ABBREVIATIONS

A549  human lung adenocarcinoma epithelial cells
AIV  avian influenza viruses
ALI  air-liquid interface cultures
BSA  bovine serum albumin
BBB  blood-brain barrier
CDC  cholesterol-dependent cytolysin
cFU  colony forming units
CO₂  carbon dioxide
CPS  capsular polysaccharide
cRNA  complementary ribonucleic acid
CNS  central nervous system
Cy3  indocarbocyanine
DuoR  double reassortant
dpi  days post infection
e.g.  exempli gratia (for example)
et al.  et alii (and others)
FFU  focus-forming units
Fig.  figure
FITC  fluorescein isothiocyanate
GBS  group B streptococcus
H1N1pdm09  swine-origin pandemic H1N1 virus
HA  hemagglutinin
HAE  human airway epithelial
HAT  human airway trypsin-like protease
HEp-2  human epithelial type 2 cells
HPAIV  highly pathogenic avian influenza
hpi  hours post infection
IAV  Influenza A virus
i.e.  id est (that is; in other words)
LPAI  low-pathogenic avian influenza
M  matrix proteins
MDCK  Madin-Darby canine kidney cells
mL  milliliter
m.o.i.  multiplicity of infection
mRNA  messenger ribonucleic acid
NA  neuraminidase
NEP  nuclear export protein
NP  nucleoprotein
NPTr  newborn pig trachea cells
NS  non-structural proteins
PA  polymerase acidic protein
PB  polymerase basic protein
PBEC  porcine bronchial epithelial cells
PBS  phosphate buffered saline
PCLS  precision-cut lung slices
PFA  paraformaldehyde
pH  negative logarithm of the hydrogen ion concentration
PK 15  pig kidney 15 cells
PTEC  porcine tracheal epithelial cells
RNA  ribonucleic acid
PRDC  porcine respiratory disease complex
RBS  receptor binding site
S. suis  *Streptococcus suis*
SIV  swine influenza virus
SOIV  swine-origin influenza virus
TEER  transepithelial electrical resistance
TMPRSS2  transmembrane protease serine 2
TRIG  triple reassortant internal genes
vRNP  viral ribonucleoprotein complexes
LIST OF FIGURES

FIGURE 1. THE STRUCTURE OF INFLUENZA A VIRUS. ........................................ - 3 -
FIGURE 2. VIRAL REPLICATION. ........................................................................... - 4 -
FIGURE 3. ENTITY RELATIONSHIP DIAGRAM OF SWINE-INFLUENZA VIRUSES. .... - 11 -
FIGURE 4. AIR-LIQUID INTERFACE (ALI) CULTURE................................................. - 24 -
ABSTRACT

Co-infection of porcine respiratory epithelial cells by influenza viruses and *Streptococcus suis*

Nai-Huei Wu

The severity of influenza virus-induced disease may be enhanced by secondary bacterial infections in both humans and animals. The pathogenesis of viral-bacterial co-infections is much more complex than that of viral mono-infections. Furthermore, the action and counteractions between the two pathogens and the host still remain poorly understood. Aim of this study was to investigate the interaction between a viral and a bacterial pathogen in a specific host during mono-infection or co-infection. *Streptococcus suis* (*S. suis*) was chosen as the secondary bacterial pathogen to analyze the co-infection with swine influenza virus (SIV). Both infectious agents have a zoonotic potential and represent important respiratory pathogens associated with the porcine respiratory disease complex (PRDC). I applied three different infection models: (i) immortalized newborn pig trachea (NPTr) cells, (ii) porcine precision-cut lung slices (PCLS), and (iii) air-liquid interface (ALI) cultures of primary porcine airway epithelial cells to analyze the infection by SIV and/or *S. suis* in more detail.

In the first part of this study, I investigated the co-infection process with the NPTr cell line as well as with PCLS, to analyze the interaction between SIV, *S. suis* and the host in an *in vitro* and an *ex vivo* model, respectively. Two SIV field strains of different subtypes (H1N1 and H3N2), designated SIV-H1N1 and SIV-H3N2, were used to figure out whether there are differences between these influenza virus subtypes. The importance of the bacterial capsular polysaccharide during SIV-*S. suis* co-infection was studied by comparing the *S. suis* strain 10 and its noncapsulated isogenic mutant. I could show a bilateral interaction mediated by the SIV haemagglutinin (HA) protein which recognized the α2,6-linked sialic acid present on the *S. suis* capsular polysaccharide. This direct viral-bacterial interaction delayed the replication of SIV-H1N1 and SIV-H3N2. On the other hand, the HA protein expressed on the surface of SIV-infected cells promoted *S. suis* adherence to and colonization of NPTr cells. This effect was also observed in PCLS at the early stage of co-infection. Afterwards, at the late stage of infection, SIV damaged the mucociliary clearance function of PCLS and promoted *S. suis* adherence and invasion. My results indicate that SIV-infected cells facilitate the bacterial adherence and invasion, first via a sialic acid-dependent interaction and later by a damage of the epithelium. In addition,
bacterial co-infection had a negative effect on influenza virus replication mediated by the sialic acid-dependent interaction.

In the second part of my thesis, I established two primary well-differentiated ALI cultures, porcine tracheal epithelial cells (PTEC) and porcine bronchial epithelial cells (PBEC) cultures, to study the action and counteraction between airway epithelial cells and influenza viruses. The infection characteristics of SIV were determined with SIV-H1N1 and SIV-H3N2 whereas two recombinant human viruses, R1 and R2, were used to analyze the phenotype of viruses with a different sialic acid-binding preference. I discovered that both SIV strains targeted ciliated cells and non-ciliated cells but not mucus-producing cells. Furthermore, they induced apoptosis in infected cells. SIV infection resulted in a dramatic loss of cilia, reduction of the epithelial thickness and exposure of basal cells on the apical surface of the epithelial cell layer. Lectin staining indicated that these cells have a different expression pattern of surface markers compared to well-differentiated epithelial cells. This may explain why influenza virus infection may predispose the host to viral/bacterial co-infections. On the other hand, despite the detrimental effect of SIV infection, the epithelium still maintained its barrier function and the regeneration of differentiated cells from basal cells was initiated. My results provide a deeper insight into the action and counteraction between influenza viruses and airway epithelial cells in long term infections. Additionally, ALI cultures supply a model to study not only SIV-induced effects but also the regeneration process of the epithelium after influenza virus infections.

Furthermore, in collaboration with Fandan Meng, we used the ALI culture system to analyze the mono-infection of well-differentiated respiratory epithelial cells by S. suis. Suilysin, a soluble cytolsyn of S. suis, was found to facilitate adherence to and invasion of porcine respiratory epithelial cells. During infection, S. suis induced suilysin-mediated apoptosis resulting in an impairment of the epithelium. These findings demonstrate that the secretion of suilysin by S. suis contributes to adherence to and invasion of porcine epithelial cells as well as to apoptosis.

Having analyzed the mono-infection of ALI cultures by SIV and S. suis, this culture system can now be used to investigate the co-infection.
ZUSAMMENFASSUNG

Co-Infektionen porziner respiratorischer Epithelzellen durch Influenzaviren und *Streptococcus suis*

Nai-Huei Wu


Weiterhin wurde in Kollaboration mit Fandan Meng das ALI-Kultursystem genutzt um die Mono-Infektion enddifferenzierter respiratorischer Epithelzellen durch S. suis zu untersuchen. Es wurde gezeigt, dass Suilysin, ein lösliches Cytolysin von S. suis, die Adhärenz an und die Invasion von porzinen respiratorischen Epithelzellen erleichtert. Im Verlaufe der Infektion induzierte S. suis eine suilysin-vermittelte Apoptose, die zu einer Schädigung des Epithels führte. Diese Befunde zeigen, dass die Sekretion von Suilysin durch S. suis sowohl zur Adhärenz und Invasion als auch zur Apoptose beiträgt.

Nachdem mit den ALI-Kulturen die Mono-Infektionen durch SIV und S. suis charakterisiert wurden, kann dieses Kultursystem in Zukunft auch für die Untersuchung der Co-Infektion genutzt werden.
1 INTRODUCTION

1.1 Influenza A viruses

1.1.1 Background

Influenza A virus (IAV) infection is one of the most common respiratory diseases in mammals. The clinical symptoms include high fever, coughing, coryza, and inflammation of the upper respiratory tract. Symptoms persist for 7 to 10 days, but patients may experience weakness and fatigue lasting for weeks (Cate, 1987; Taubenberger and Morens, 2008). During an influenza pandemic, severe cases are primarily due to high morbidity and increased mortality in infants and elderly, in some cases patients are also suffering from chronical illness (Nicoll et al., 2012; Taubenberger and Morens, 2008). Until now, three predominant HA subtypes of IAV (H1N1, H2N2 and H3N2) have caused epidemics in the human population referred to as seasonal influenza viruses. Whereas several severe pandemics have occurred in the past century, the 1918 “Spanish flu” caused by the H1N1 subtype resulted in approximately more than 500,000 deaths in the United States and taken up to 50 million human lives worldwide (Johnson and Mueller, 2002; Taubenberger and Morens, 2006). Another pandemic was caused by the H2N2 subtype in 1957, known as the “Asian flu” in China, and was replaced by the “Hong Kong flu” with the H3N2 subtype in 1968 (Taubenberger and Kash, 2010). In 2009, a new pandemic H1N1 virus (H1N1pdm09) emerged which differed from the previous pH1N1 viruses (Smith et al., 2009a) and started to replace the circulating seasonal H1N1 virus (Neumann and Kawaoka, 2011). H1N1 strains are co-circulating in the human population together with seasonal H3N2 strains.
1.1.2 Taxonomy

Three genera of influenza viruses including *Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus C* together with the *Thogotovirus* and *Isavirus* genera form the family of *Orthomyxoviridae* (Greek: "orthos" = true "myxo" = mucus) (Horimoto and Kawaoka, 2005). In the last few years, a novel influenza D virus has been separated from the *Influenzavirus C* genus (Hause et al., 2014). The classification of influenza viruses in type A, B, or C is based on antigenic differences in the nucleoprotein (NP) and matrix protein (M) of the virus, as well as other different molecular characteristics (Horimoto and Kawaoka, 2005). Influenza B viruses were found only in humans and seals, influenza C viruses were found in humans and pigs (Guo et al., 1983; Osterhaus et al., 2000). In contrast, a wide range of infectious IAV has been shown to cover a variety of mammalian and avian species (Baigent and McCauley, 2003). Influenza A viruses are further divided into subtypes with respect to the antigenicity of their surface proteins, hemagglutinin (HA or H) and neuraminidase (NA or N). Up to now, 18 HA and 11 NA subtypes have been discovered, included the bat derived H17N10 and H18N11 strains (Neumann et al., 2009; Wu et al., 2014). The official designation of an influenza A virus strain (e.g. A/sw/Bad Griesbach/IDT5604/2006 (H1N1)) represents the viral isolate in the form of "A (virus type) / host / origin of geographic / number of the isolates / year of isolated (H- and N-subtype) " (CDC, 2010; WHO, 1980).

1.1.3 Virus structure and replication

The virus particles of influenza A viruses show a pleomorphic shape with a diameter of about 100 nm; filamentous particles are 300 nm long (Fujiyoshi et al., 1994). The genome of IAV contains eight single-stranded RNA segments of negative polarity which encode 13 proteins. The virion is surrounded by a lipid bilayer envelope derived from the host membrane; and virus-encoded glycoproteins are incorporated in the
viral envelope. These glycoproteins, are visible under the electron microscope as 10-14 nm long "spikes" protruding from the viral membrane (Nayak et al., 2009). The HA protein forms trimers, while the NA protein occurs as tetramers. Additionally, matrix protein 2 (M2) can also be found on the lipid envelope surface forming a proton channel. Whereas the matrix protein 1 (M1) is underneath the lipid envelope, the viral ribonucleoprotein complexes (vRNP) are bound to it for stabilization. The vRNPs contain an RNA segment associated with the nucleoprotein and polymerase proteins (Brown, 2000a).

Figure 1. The structure of influenza A virus.

The infectious virion contains eight genome segments within the vRNP complexes and the following structural proteins: hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), non-structural proteins 1 and 2 (NS1 / 2), nucleoprotein (NP), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). The latter four proteins together with the RNA segments form the RNP complex. Figure adapted from (Horimoto and Kawaoka, 2005)
The viral infection initiated by the binding between viral HA and sialic acid on the cell surface. After the endocytosis, uncoating process, the viral genomes replicated and synthesized its viral protein and viral RNAs in the nucleus. After vRNP formation and post-translational modification, the newly generated viruses will bud into extracellular fluid for next infection. Figure modified from (Garten et al., 2015).

The infection and replication of influenza A viruses starts with the attachment of virus to target cells. The viral HA proteins first bind to the host cell receptors via sialylated macromolecules (glycoproteins or glycolipids) on the cell surface (Weis et al., 1988). The virion is then taken up by receptor-mediated endocytosis (Sieczkarski and Whittaker, 2002). Acidification inside the endosomal vesicles results in a conformational change of HA proteins (Bui et al., 1996) that is required for the fusion of the viral membrane with the endosomal membrane. Via the channel function of the M2 protein, protons get from the endosomal lumen into the virus particles and result in
the dissociation of the RNP complexes from the M1 protein. After the fusion step has been induced by the exposed fusogenic region of HA and the help of M1 protein. The viral ribonucleoproteins get into the cytoplasm in a process referred to as “uncoating” (Harrison, 2008). The vRNPs are transported through the pores of the cell nucleus. The viral RNAs can serve as a template for the synthesis of messenger RNA (mRNA) to be used for protein translation, and it can also act as a template for full-length complementary RNA (cRNA) required for the synthesis of new vRNA. The 5' and 3' ends of viral RNA form promoter structures which bind to the viral RNA-dependent RNA polymerase and initiate the subsequent viral RNA replication (Elton et al., 2006). A unique feature of influenza A virus replication is the “Cap-snatching”, a process in which 5'-terminal nucleotides of newly synthesized cellular mRNA are taken by the viral polymerase and used as a primer for the synthesis of viral mRNA (Shapiro and Krug, 1988).

After the translation of viral proteins, viral glycoproteins (HA, NA) are translocated into the ER and transported to the Golgi apparatus where oligomerization and glycosylation occur. After the post-translational modification, the HA and NA proteins are transported to the plasma membrane together with the M2 proteins. On the cells surface, these three viral proteins cooperate to initiate the budding process. On the other hand, the translated NS1 protein is synthesized in large quantities and functions as an interferon suppressor (Garcia-Sastre et al., 1998; Kochs et al., 2009). PA, PB1, PB2, and NP proteins are transported back into the nucleus for viral RNA replication (Resa-Infante et al., 2011). A function of the M1 protein is to bind the vRNP complex and terminate the transcription together with NS2 protein (also referred as nuclear export protein, NEP) (O'Neill et al., 1998). This vRNP-M1-NEP complex will be further transported into the cytoplasm and interact with the cytoplasmic surface of the plasma
membrane to be incorporated into new virions. After the budding process, newly formed viral particles are released from the host cell with the help of neuraminidase (Nayak et al., 2004).

1.1.3.1 Hemagglutinin (HA)

The viral HA protein is synthesized as a HA precursor, referred to as HA0. Subsequently, this single polypeptide chain will be glycosylated and cleaved into two chains, HA1 (globular head) and HA2 (rod-like stalk) held together by disulfide bonds (Sriwilaijaroen and Suzuki, 2012). Most HA subtypes contain a single basic amino acid residue in the proteolytic cleavage site (Laver, 1971); Serine proteases are known to be the host proteases involved in the proteolytic activation of HA (Garten et al., 2015). In avian hosts, the virulence is determined by the number of basic amino acids in the cleavage site of the hemagglutinin. Viruses of the H5 and H7 subtypes contain a multi-basic amino acid sequence and are cleaved by furin or furin-like proteases that are ubiquitously present in the organism. Such viruses are designated highly pathogenic avian influenza (HPAI) viruses, which induce systemic infection and show a zoonotic potential (Alexander, 2000). A functional HA is a homo-trimeric integral membrane protein which mediates attachment to target cells by binding to the sialic acids of host cell surface components. The globular head region (HA1) has a sialic acid binding pocket which is referred to as the receptor binding site (RBS); it is surrounded by antigenic sites (Gerhard et al., 1981; Webster and Laver, 1980; Wiley et al., 1981). Amino acid differences in the RBS determine which linkage type of sialic acid is recognized, α2,6-linked sialic acid (human-like receptors) or α2,3-linked sialic acid (avian-like influenza virus receptors) (Matrosovich et al., 2000).
1.1.3.2 Neuraminidase (NA)

The viral NA protein is an integral membrane protein which cleaves sialic acid. During the virus budding, the NA protein desialylates the cell surface receptor and releases the viral hemagglutinin and allows viral release (Palese and Compans, 1976). The functional NA protein consists of four identical monomers, which include four compartments: the cytoplasmic tail, transmembrane domain, stalk domain, and the globular ectodomain with the enzyme activity (Air, 2012). The NA projects slightly further from the viral envelope surface than do HA proteins (Blok and Air, 1982); the variable stalk length is associated with host specificity (Castrucci and Kawaoka, 1993). The number of NA proteins on the virion membrane is approximately 40-50 NA spikes, while 300-400 HA spikes are present on the same virion (Harris et al., 2006). Although the enzyme active site is surrounded by the antigenic residues (Tulip et al., 1992; Venkatramani et al., 2006), the active site still contains 15 consensus amino acids in all influenza viruses (Colman et al., 1983; Varghese et al., 1983). Therefore, the bladed propeller-like structure of active site becomes the target for anti-influenza treatment, for example, oseltamivir and zanamivir (Air, 2012).

1.1.4 Antigenic variation

Influenza A viruses are divided into different serological subtypes with respect to the antigenic differences on the hemagglutinin and neuraminidase proteins. The antigenic variation is driven by two phenomenons which are termed as antigenic drift and antigenic shift. Antigenic drift takes place during the viral replication where the virus-encoded RNA-dependent RNA polymerase generates errors which lead to nucleotide exchanges, i.e. mutations, in viral genomes. The mutations may result in antigenic diversity which may a benefit for the newly synthesized virus to become
more competitive against host defense challenges. Antigenic drift primarily occurs in the viral surface HA and NA proteins due to pressure from host innate and adaptive immune system (Carrat and Flahault, 2007). Antigenic shift occurs during the co-infection of host cells; within a cell infected by two or more viruses, the new viruses may be formed that contain a hybrid mixture of genome segments derived from the genomic segments of the different progenitors. This event is termed reassortment and may produce emerging viruses which cross the defensive barriers (Essere et al., 2013).

1.1.5 Influenza A viruses in swine

In swine, most influenza A virus infections result in a sudden onset of acute respiratory disease, which includes fever, inactivity, nasal or conjunctivitis discharge, respiratory distress, and inappetence (Zell et al., 2013). Viruses are shed by the nasal discharges which allow the transmission between pigs via direct contact or aerosols (Brown, 2000b). The course of disease is usually mild or asymptomatic (Loeffen et al., 1999); typically the infected pigs recover shortly after the onset of symptoms (Ma et al., 2009). While morbidity is high, mortality is usually low in the field (Dee, 2014; Vincent et al., 2014). However, SIV infection is often accompanied by secondary bacterial infections. Such co-infections are part of the porcine respiratory disease complex (PRDC), a multifactorial syndrome caused by a combination of pathogens and/or environmental factors (Bochev, 2007).

1.1.5.1 North American swine influenza viruses

SIV were first described in the literature during the 1918 pandemic (Koen, 1919), but it was not until 1930, that the first SIV H1N1 strain was isolated from North American
pigs which are referred to as “Classical swine H1N1 virus” (Shope, 1931). The classical SIV was considered to be a result of adaptation from its progenitor, “the Spanish flu 1918” virus. It predominated until the late 90s in the US pig population (Memoli et al., 2009). In 1998, several outbreaks of SIV with H3N2 subtype were found; one was a double reassortant H3N2 (DuoR H3N2) strain with the backbone of classical SIV H1N1 and the HA and NA from human seasonal H3N2 virus. Another reassorted SIV-H3N2 strain was a result based on the DuoR H3N2 reassortment but included additional PB2 and PA segments from an avian influenza virus (Webby et al., 2000; Zhou et al., 1999). This triple reassortant H3N2 virus later reassorted with other SIVs or evolved through antigenic drift by introductions of human IAV genes. The main part of the internal segments: avian-like PB2 and PA; human H3N2-like PB1; and classical swine H1N1-like NP, M, and NS were consequence and have been termed the triple reassortant internal genes (TRIG) (Gramer et al., 2007; Vincent et al., 2008). Base on TRIG cassette, further reassortments have been identified among the North America pigs, including reassorted SIV H1N1 and an SIV H1N2 strain. Accordingly, these two new lineages became endemic in US pigs (Lorusso et al., 2013). Additionally, the SIV H1N2 contains the TRIG cassette, the H1 from the classical swine H1N1, and also the N2 gene from the former triple reassortant H3N2 (Karasin et al., 2002; Webby et al., 2004). This SIV H1N2 virus was later considered as the progenitor of the swine-original pandemic H1N1 virus (SOIV or H1N1pdm09) which appeared in 2009.

1.1.5.2 European swine influenza viruses

Unlike the situation in US, the classical SIV-H1N1 lineage in European pigs has not been well documented. It is believed that the classical SIV-H1N1 was circulating
throughout the years while the first importation happened during World War II (Lange et al., 2014) and the re-importation from North America to Europe took place in 1976 (Nardelli et al., 1978). Different from the classical SIV H1N1, in 1979 outbreaks of influenza among European pigs were caused by a new H1N1 subtype (Brown, 2000b). This H1N1 virus resulted from a bird-pig transmission event which is referred to as “avian-like H1N1” afterwards (Pensaert et al., 1981; Scholtissek et al., 1983). In addition, this avian-like H1N1 gradually replaced the classical SIV in European pig population.

Beside the H1N1 subtype, a H3N2 virus which was spread from Asia was circulating throughout Europe (Ottis et al., 1982; Turnova et al., 1980). This H3N2 was found to result from an adaptation event during the “Hong Kong flu” pandemic in 1968 (Miwa et al., 1987) and spread widely all over Eurasian pigs. During the 80s, a reassortment event took place between the circulating viruses. The adapted H3N2 virus from HK68 kept its HA and NA segments but acquired other gene segments from the avian-like H1N1, which created a new “human-like SIV H3N2 virus”. Compared to the original adapted H3N2 virus, this human-like H3N2 showed a higher pathogenicity for infected pigs with intermediate symptoms (Campitelli et al., 1997).

In a similar way, a “human-like SIV H1N2” occurred in 1994 and contained both HA and NA of human-like origin (Brown et al., 1998); an “avian-like SIV H1N2” contained HA of avian-like origin (Gourreau et al., 1994). Additionally, other reassortment events also took place between 1987 and 2000s (Kyriakis et al., 2013; Marozin et al., 2002), but none of them showed a triple reassortment as in North America.
Figure 3. Entity relationship diagram of swine-influenza viruses.

The swine influenza viruses in North America and Europe were closely related to the reassortment events based on the different lineages. The classical SIV H1N1 and the TRIG cassette were the main course of the reassortment in US. The avian-like H1N1 and human-like H3N2 circulating in Europe became the backbone of emerging SIV reassortants.
1.1.6 Swine-original influenza virus, H1N1pdm09

In 2009, an emerging virus was isolated from patients in the United States which later caused an influenza pandemic in the human population. This virus was found to be a mix from four different progenitors: North American swine influenza (NP, NS), North American avian influenza (HA, PB2, PA), human influenza (PB1), and Eurasian swine influenza (M, NA). Based on the genetic characteristics, six of its segments were mainly from American swine TRIG-H1N2 virus, while the rest from Eurasian avian-like SIV-H1N1 virus. Therefore, the pandemic virus was often called “swine-origin influenza virus, SOIV” or “H1N1pdm09” due to the WHO pandemic declaration. This H1N1pdm09 can cause pulmonary embolism with respiratory complications, which has been surmised as a leading cause of human death (Smith et al., 2009b).

In pig population, the H1N1pdm09 was detected worldwide, (Howden et al., 2009; Simon et al., 2014; Welsh et al., 2010; Yang et al., 2016). For example, in European pigs the H1N1pdm09 started to co-circulate with the enzootic SIV, and showed the ability of reassortment. With different subtypes the H1N1pdm therefore persists in the field and threats the livestock and mankind (Starick et al., 2012). Although the pig-to-human transmission cases are barely found, the H1N1pdm09 virus underlined the potential of pigs to reassort novel or pandemic influenza strains (Itoh et al., 2009; Patriarca et al., 1984; Robinson et al., 2007).
1.2 *Streptococcus suis*

1.2.1 Etiology

*Streptococcus suis* (*S. suis*) is an encapsulated, Gram-positive, facultative anaerobic coccus. According to the antigenicity of the capsular polysaccharides, 35 serotype of *S. suis* are defined (Higgins et al., 1995; Perch et al., 1983). *S. suis* has a world-wide distribution and the serotype 2 is most prevalent in animals all over the world. In Europe and Asia, serotype 2 is most frequently isolated from clinical cases in pigs and humans, but in some European countries serotype 9 has been predominant after the year 2000 (Gottschalk and Segura, 2000; Goyette-Desjardins et al., 2014). In contrast, in North America, serotypes 2 and 3 are the two most prevalent serotypes isolated from diseased pigs (Goyette-Desjardins et al., 2014).

1.2.2 *Streptococcus suis* infection

*S. suis* is one of the important porcine bacterial pathogens having a huge economic impact in porcine industry. In the United States alone, losses of over 300 million dollars were estimated to be caused by *S. suis* infection (Staats et al., 1997). This bacterium can induce meningitis, septicemia, arthritis, peritonitis, endocarditis and pneumonia in pigs (Gottschalk et al., 2007; Staats et al., 1997). Apart from pig populations, *S. suis* infection has been reported also in a wide range of other animal species such as cats, dogs, deer, horses, ruminants and birds (Devriese and Haesebrouck, 1992; Devriese et al., 1994; Staats et al., 1997).

Pigs harbor *S. suis* in their upper respiratory tract, especially on the tonsils and nasal cavities and become asymptomatic carriers. The carrier rates of *S. suis* serotype 2 in herds are variable but high (up to 100%), and the carrier status in tonsils may persist even after penicillin treatment (Clifton-Hadley et al., 1984; Staats et al., 1997). Some
of the carriers may develop diseases. In spite of the fact that S. suis causes diseases in pigs of all age, the weaned pigs and growing pigs show the highest susceptibility (Gottschalk, 2014; Staats et al., 1997). Both vertical and horizontal transmission can be a possible route for spreading of S. suis between pigs herds. Although S. suis serotype 2 is considered to be mainly transmitted through the nasal and oral route of infection (Berthelot-Herault et al., 2001; Staats et al., 1997), the infection may also be acquired from contaminated piglets during farrowing since the genital tract is an additional natural habitat of S. suis (Gottschalk and Segura, 2000). Moreover, the environmental factors such as contaminated faeces, dust, water and feed may also be sources of infection (Staats et al., 1997).

There are several reports that S. suis is a zoonotic agent and responsible for meningitis and septicemia in humans; most human cases are caused by serotype 2 (Goyette-Desjardins et al., 2014). To date, more than 1600 cases from 34 countries have been reported as S. suis infection after the first case had been diagnosed in Denmark in 1968 (Goyette-Desjardins et al., 2014; Perch et al., 1968; Wertheim et al., 2009). Despite most human cases are sporadic, in 1998 and 2005, two outbreaks of S. suis infections occurred in China resulting in high mortality rates. The enhanced severity is reflected in the designation streptococcal toxic shock-like syndrome (Yu et al., 2006). Different from pigs, S. suis is transmitted through direct contact to open wounds on skin or by the food uptake via the oral route in humans. The ingestion of and contact with contaminated meat or pork products are considered as risk factors for human infection (Segura et al., 2014; Wertheim et al., 2009).
1.2.3 Pathogenesis

The pathogenesis of S. suis infection is yet poorly known due to the complex involvement of factors and mechanisms. In general, S. suis first has to adhere, to colonize and invade the respiratory epithelium to initiate disease. Studies revealed that the adherence of S. suis to epithelial cells is affected by the capsule polysaccharide (CPS). Additionally, the adhesion of S. suis serotype 2 to epithelial cells such as porcine kidney (PK15), madin-darby canine kidney (MDCK), A549, HeLa or Human epithelial type 2 (HEp-2) cells is enhanced when the bacterium is lacking CPS (Benga et al., 2004; Lalonde et al., 2000). Hence, it is hypothesized that in the early stage of infection, S. suis down-regulates expression of CPS resulting in better adhesion of bacteria to the host cells. After initial adherence, the suilysin secreted from S. suis may impair the epithelial barrier together with other virulence factors via the cytolytic activity, followed by breaching mucosal epithelia in the upper respiratory tract (Gottschalk and Segura, 2000; Lalonde et al., 2000; Seitz et al., 2013). As a result, S. suis can reach the bloodstream and disseminate systemically. However, the mechanism of how S. suis gets access to deeper tissues and induces dissemination is still unclear.

A “modified Trojan horse theory” may explain how S. suis survives in the bloodstream and how it disseminates (Gottschalk and Segura, 2000; Williams and Blakemore, 1990). The presence of CPS protects S. suis from phagocytosis of monocyte/macrophage or against the killing mechanism by neutrophils. Additionally, S. suis may adhere to monocytes via the sialic acid which is presented on its CPS. By these functions, bacteria may survive, “travel” and disseminate in the blood by being bound to but not ingested by macrophages (Fittipaldi et al., 2012; Gottschalk and Segura, 2000). As a result, S. suis may cross the blood-brain barrier (BBB) or the
blood-cerebrospinal fluid (CSF) barrier and reach the central nervous system (CNS) followed by meningitis induction (Fittipaldi et al., 2012; Vanier et al., 2004).

1.2.4 Virulence factors

Several cell-associated or secreted factors are important for the pathogenesis of *S. suis* infection, including CPS, suilysin, muramidase-released protein, extracellular factor and fibronectin-fibrinogen binding protein (Fittipaldi et al., 2012; Vanier et al., 2004). Until now, *S. suis* serotype 2 is considered as the most virulent serotype and most of studies have been carried out on this serotype, while the overall picture of the virulence factors is still limited. Thus, to reach a better understanding the difference between the serotypes needs to be investigated.

1.2.4.1 Capsule polysaccharide (CPS)

Based on the serotyping result of CPS, *S. suis* is now divided into 35 different serotypes (Higgins et al., 1995; Perch et al., 1983; Wisselink et al., 2000). So far, CPS is considered the most critical virulence factor. By using isogenic nonencapsulated mutant strains in *in vivo* or *in vitro* models, previous reports showed that the absence of CPS results in increased phagocytosis by monocytes or macrophages and accelerated clearance in the bloodstream (Charland et al., 1998; Segura et al., 2004; Smith et al., 1999). Moreover, it has been hypothesized that when *S. suis* colonizes the respiratory epithelium, the CPS expression is down-regulated. After invasion, up-regulation of encapsulation can protect the bacterium from phagocytosis (Gottschalk and Segura, 2000).
The CPS of *S. suis* serotype 2 is consists of the sugars rhamnose, galactose, glucose, N-acetylglucosamine, and N-acetyl-neuraminic acid (sialic acid) with the repeating unit: \([4]^{\text{Neu5Ac(a2–6)Gal(b1–4)GlcNAc(b1–3)Gal(b1–4)[Gal(a1–3)]Rha(b1–4)Glc(b1–2)]n}\) (Gottschalk and Segura, 2000; Van Calsteren et al., 2010). Apart from serotype 2, the CPS of serotype 1, 14, 16, 27 and 1/2 also contain sialic acid (Smith et al., 2000; Van Calsteren et al., 2013; Wang et al., 2011). The sialic acid is considered to be related to adherence of *S. suis* to monocytes in “modified Trojan horse theory” (described in Pathogenesis) (Gottschalk and Segura, 2000). However, although CPS is regarded as the most important virulence factor in *S. suis*, some avirulent strains are well encapsulated (Fittipaldi et al., 2012; Gottschalk and Segura, 2000).

1.2.4.2 Suilysin

Suilysin is the hemolysin of *S. suis*, which belongs to the cholesterol-dependent cytolysins (CDC) family (Jacobs et al., 1994; Segers et al., 1998). Suilysin shows high similarity to the toxic pneumonolysin produced by *Streptococcus pneumoniae* (Segers et al., 1998), but yet its role in virulence and pathogenicity has not been confirmed. Although most of the European *S. suis* strains are suilysin-positive, the North American strains contain variants of this secreted protein (Segers et al., 1998). Recent studies have shown that suilysin acts on the mucosa of the epithelium and contributes to the invasive injury in *S. suis* infection (King et al., 2001; Takeuchi et al., 2014). Others showed that not only the epithelial but also the endothelial cells, and even immunocytes, can suffer from its cytotoxic effects (Norton et al., 1999; Segura and Gottschalk, 2002), which are dependent on the presence of cholesterol (Charland et al., 2000; Lv et al., 2014).
1.3 Sialic acid

1.3.1 Receptor for influenza viruses

The first step of a viral infection is the virus attachment to host cells via the binding of HA proteins to receptors on the cell surface. In mammalian cells, a variety of cellular glycans may be involved in viral attachment (de Graaf and Fouchier, 2014; Varki and Varki, 2007). Sialic acids in a terminal position of glycans serve as receptor determinants. The sialic acids are nine-carbon monosaccharides with N-acetyleneuraminic acid (Neu5Ac) and N-glycolyneuraminic acid (Neu5Gc) being the most frequent forms. Most influenza viruses prefer to bind to Neu5Ac rather than to Neu5Gc (Suzuki et al., 1986). Sialic acids are connected to the neighboring sugar via an α-linkage extending from the 2-carbon (Nicholls et al., 2008). Among different linkage types, α2,3 linkages and α2,6 linkages are preferentially recognized by viral HA proteins. Human and human-like influenza viruses have a preference for α2,6-linked sialic acids, while avian and avian-like influenza viruses bind to α2,3-linked sialic acids (Rogers and Paulson, 1983).

Generally speaking, α2,6-linked sialic acid is the most abundant influenza virus receptor determinant distributed over the respiratory tract of humans (Shinya et al., 2006); an increasing amount of α2,3-linked sialic acid is present in the lower airways (Nicholls et al., 2008). Additionally, children express a higher level of α2,3-linked sialic acid compared to the expression pattern in adults. For avian species, α2,3-linked sialic acid was found in the respiratory and intestinal tract (Franca et al., 2013). Pigs express both sialic acid forms (Sriwilaijaroen et al., 2011), which suggested to fit to their role as “mixing vessel” for emerging influenza viruses by reassortment of gene segments (Ma et al., 2008). However, recent findings demonstrate the abundance
and distribution of sialic acids in the porcine airways are very similar to those of humans (Van Poucke et al., 2013).

**1.3.2 Sialic acid on the bacterial capsular polysaccharide**

Many pathogenic bacteria contain a terminal sialic acid residue on their capsular polysaccharide. For instance, *Escherichia coli* K1 (α-2,8-linked sialic acid), *Neisseria meningitides* (group B and C contain α2,8- or α2,9-linked sialic acid, respectively), Group B *Streptococcus* (GBS) (α2,3-linked sialic acid) and *S. suis* (α2,6-linked sialic acid) (Lewis et al., 2004; Swartley et al., 1997; Troy, 1992; Van Calsteren et al., 2013; Van Calsteren et al., 2010). The sialic acid on the bacterial surface can function as a resistance factor against the innate immune response of the host (Severi et al., 2007). It is known that the sialic acid on the capsular polysaccharide of GBS can protect the bacteria from phagocytosis by inhibition of alternative-pathway activation (Marques et al., 1992). However, different from GBS, *S. suis* may adhere to monocytes via the sialylated capsular polysaccharide (Gottschalk and Segura, 2000). Accordingly, *S. suis* may survive and “travel” in the bloodstream. It has to be noted that not all *S. suis* contain sialic acid residues. The α2,6-linked sialic acid was found on serotype 2 and 14 but not serotype 3 (Van Calsteren et al., 2013; Van Calsteren et al., 2010; Wang et al., 2013).
1.4 Bacterial co-infection with influenza virus

During an influenza pandemic, a highly virulent virus may directly cause pneumonia and lead to severe symptoms. Most of the individuals that have died from influenza infections are associated with extremely high frequency of bacterial colonization in the nasopharynx (Chertow and Memoli, 2013). During the 1918 H1N1 pandemic (Spanish flu), people had no knowledge of virus infections and antibiotic therapy was not available. The only documentary records related to epidemiological features of the infection are the medical histories in military. The major outbreaks of purulent bronchitis resulting from influenza virus infections are associated with streptococci, staphylococci, Haemophilus influenzae, and pneumococci (Brundage, 2006). In the 1957 and 1968 pandemic, the influenza infections showed a high co-infection rate with Streptococcus pneumoniae, H. influenzae, and Staphylococcus aureus. A notable high mortality rate has been reported in Staphylococcus aureus co-infected patients (Petersdorf et al., 1959; Schwarzmann et al., 1971). In the last pandemic by H1N1pdm09, co-infection with H. influenza, Streptococcus pneumoniae, Staphylococcus aureus (both methicillin-resistant and susceptible strains) were common (Joseph et al., 2013). Thus, it is necessary to further investigate the impact on morbidity and mortality caused by severe secondary infection. Nevertheless, the mechanisms of co-infection and/or secondary infection remain complex. In brief, the course of viral-bacterial co-infection may result in the disruption of physical defense barrier, immune responses dysregulation, and invasive sepsis (Dawood et al., 2009).

Previous reports suggested some mechanisms during influenza virus-bacteria co-infection in the respiratory epithelium. The first interaction between viral and bacterial pathogens is associated with the receptor on the host cell surface (Plotkowski et al., 1993; Plotkowski et al., 1986). For influenza viruses, sialic acid is
the major target for viral entry (Suzuki et al., 1986). After budding of influenza viruses from the apical side of the epithelial cell, the sialic acid will be cleaved with the help of the NA protein (Air, 2012). The viral neuraminidase cleaves the sialic acid not only from epithelial cells but also from mucin and thus promotes bacterial adherence (McCullers, 2014). The influenza virus neuraminidase has been shown to release sialic acid from the surface of the airway epithelium and thus to expose receptors that facilitate pneumococcal adherence (Chertow and Memoli, 2013; McCullers and Bartmess, 2003).

On the other hand, the influenza virus induces a cytolytic effect on epithelial cells and damages the epithelium. This impairment of the mucociliary escalator function decelerates clearance of bacteria (Pittet et al., 2010). Moreover, a reduction of epithelial cell repair and regeneration was observed during H1N1pdm09 influenza virus and Streptococcus pneumoniae co-infection in a mouse model (Kash et al., 2011).

Furthermore, an increase of the cytokine release and the innate immune response will overcome the dysregulation of alveolar macrophages for bacterial clearance (Braciale et al., 2012). Beside the specific virulence factors expressed from the bacterial agent, depletion of macrophages may be beneficial for the invasion and dissemination of pathogens (Lee et al., 2010). Pneumococcal surface protein A, staphylococcal adhesive molecules or other fibrinogen modulator proteins are more active during the onset of bacterial infection after prior influenza virus infection (Foster and Hook, 1998; McCullers, 2014).
1.5 Swine models for study influenza viruses

Influenza viruses remain one of the major threats to human health. Pigs are known to be a source of emerging novel influenza viruses with zoonotic potential. After the swine-origin influenza virus pandemic, various models were established to further study the infection of the pig respiratory tract, where the “mixing vessel” is located (Ma et al., 2008). Diverse research approaches have been applied (Crisci et al., 2013). The *in vivo* pig infection primarily focuses on the immune response and the pathogenicity after influenza virus infection (Meng et al., 2013). Although most IAV infections are asymptomatic in the pig population, the cytokine expression pattern and the innate/adapt immunocyte involvement still provide valuable insight. For instance, the acute phase proteins such as C-reactive proteins give a hint for the possible interspecies transmission cycles in pigs (Brookes et al., 2010). Most of the *in vitro* studies focus on the infectivity of IAV rather than the host response. “NPTr cells” (newborn pig trachea epithelial cells) (Ferrari et al., 2003), “NSK” cells (newborn swine kidney cells) (Lombardo et al., 2012) and “SD-PJEC” cells (intestinal epithelial cells) have been used to analyze infection by IAV from different origins (Sun et al., 2012).

The most widely used models in swine are the primary epithelial explants or primary cell cultures. For a closer look into how different influenza viruses target the epithelium, several explants from the nasal respiratory tract, trachea, bronchi or lung sections were applied to investigate the host-pathogen interactions (Nunes et al., 2010). The *ex vivo* explants from pigs include the first host defence barrier and can be used to analyze virus infection (Londt et al., 2013). Two primary porcine culture systems, precision-cut lung slices (PCLS) and air-liquid interface (ALI) cultures, which were used in my study, will be described in the following chapters.
1.5.1 Precision-cut lung slices (PCLS)

Similar to explants, the precision-cut lung slices (PCLS) are another porcine ex vivo culture model which was set up in our lab for infection studies. This culture system contains the original setting of airway cells as found in the organ including bronchi, bronchioles and alveoli. So far, PCLS have been prepared from different species such as human, porcine, bovine, caprine, murine and avian (Abd El Rahman et al., 2010; Ebsen et al., 2002; Kirchhoff et al., 2014a; Kirchhoff et al., 2014b; Meng et al., 2013; Neuhaus et al., 2013). The porcine PCLS consist of well-differentiated epithelial cells, including ciliated cells, mucus-producing cells and basal cells, and the cells remain viable for around one week (Punyadarsaniya et al., 2011). In addition, by using PCLS as a model we cannot only qualify but also quantify the ciliary activity to determine the epithelial response during IAV infection (Meng et al., 2013; Punyadarsaniya et al., 2011). Hence, porcine PCLS is an interesting model to analyze the infection of the porcine epithelium by IAVs as well as by other respiratory pathogens.

1.5.2 Air-liquid interface (ALI) cultures

The air-liquid interface (ALI) culture is another model of well-differentiated epithelial cells which is obtained by culturing primary respiratory epithelial cells under ALI conditions. The ALI culture consists of a pseudostratified epithelial layer and contains ciliated cell, mucus-producing cells and basal cells. Since maintained under ALI conditions, this in vitro culture has a polarized organization and represents a situation similar to the in vivo situation. Until now, various ALI cultures from different species have been established, such as human, porcine, ferret and mouse ALI cultures (Bateman et al., 2013; Ibricevic et al., 2006; Zeng et al., 2013). The human ALI culture is widely used to study respiratory pathogens, including influenza viruses, coronaviruses, adenovirus, mycoplasma and fungi (Dijkman et al., 2013; Homma et
al., 2016; Kogure et al., 2006; Krunkosky et al., 2007; Lam et al., 2011). Furthermore, it is also a suitable model to study the effect of cigarette smoking on the airway epithelium (Kuehn et al., 2015). Besides, different from immortalized cell lines, IAVs can infect and replicate in human and porcine ALI cultures in the absence of exogenous trypsin (Bateman et al., 2013; Chan et al., 2010). In short, since porcine ALI cultures can mimic the *in vivo* situation, it is a valuable model to study entry and infection strategies of IAVs and other respiratory pathogens.

![Figure 4. Air-liquid interface (ALI) culture.](image)
The ALI culture consists of a pseudostratified epithelial layer and contains ciliated cell, mucus-producing cells and basal cells.
1.6 Aim of the study
The severity of influenza virus induced disease may be enhanced by secondary bacterial infections in both human and animals. Although there are several studies that have investigated the mechanisms of how influenza viruses interact with bacteria during co-infections, the pathogenesis is much more complex than mono-infection and still remains poorly understood. In order to understand the interaction between viral and bacterial pathogens on specific host, *Streptococcus suis* (*S. suis*) was chosen as the bacterial pathogen to perform co-infection with swine influenza virus (*SIV*). Both infectious agents have a zoonotic potential and represent important respiratory pathogens associated with the porcine respiratory disease complex (*PRDC*). In my study, three different infection models: NPTr cells, PCLS and primary porcine airway ALI cultures are applied to analyze the co-infection in more detail.

In the first part of my thesis work, I want to investigate the co-infection process with an *in vitro* model, the NPTr cell line, as well as an *ex vivo* model, PCLS, to analyze the interaction between SIV and *S. suis*. The NPTr cells are immortalized porcine tracheal cell line whereas PCLS represent the differentiated airway epithelium. The viral subtype differences of SIV will be studied via comparing the outcomes of infection by two field isolates of SIV, A/sw/Bad Griesbach/IDT5604/2006 (SIV H1N1) and A/sw/Herford/IDT5932/2007 (SIV H3N2). As bacterial agents, *S. suis* serotype 2 and a noncapsulated isogenic mutant will be used to investigate the influence of the bacterial capsular polysaccharide in the SIV co-infection. To simulate the secondary bacterial infection in the field, NPTr cells and PCLS will be first inoculated with SIV, followed by *S. suis* infection. Immunofluorescence microscopy will be used to monitor the course of infection, and the virus titers at different time points will be determined. Furthermore, a co-sedimentation and hemagglutination inhibition assay will be
performed to provide more insight into the direct relationship between the influenza virus hemagglutinin protein and the sialic acid on the *S. suis* capsular polysaccharide. (Manuscript I & II)

In the second part of my thesis, I want to establish an ALI culture system of well-differentiated airway epithelial cells from the swine trachea and bronchus. Compared to the NPT*r* cells or the PCLS model, the ALI cultures show similar features as the pseudostratified epithelium of the porcine respiratory tract, i.e. it contains the mucociliary clearance function and is suitable to investigate virus entry and release. SIV strains of H1N1 and H3N2 subtype isolated from clinical cases and representing two lineages circulating in European pigs will be selected for determining the infection characteristics of SIV; two recombinant viruses, R1 and R2, will be used to analyze the phenotype of viruses with different sialic acid binding preference. The viruses will be inoculated from the apical and basal side of ALI cultures to study the differences between these infection routes. Moreover, the influences of long term infection in the porcine respiratory tract will be studied by using different influenza virus strains to infect ALI cultures. Additionally, the action and counteraction between airway epithelial cells and influenza viruses, the changes of cell morphology and the distribution of sialic acids will be analyzed. (Manuscript III)

Furthermore, the infection process of *S. suis* in the porcine respiratory epithelium will be analyzed by using ALI cultures. The interaction between cells and *S. suis* serotype 2 or its suilysin-deficient mutant will be investigated. The contribution of suilysin, a secreted cholesterol-dependent cytolsin, during the infection process will be described. (Manuscript IV)
2. MANUSCRIPT I

Sialic acid-dependent interactions between influenza viruses and *Streptococcus suis* affect the infection of porcine tracheal cells

Nai-Huei Wu¹, Fandan Meng¹, Maren Seitz², Peter Valentin-Weigand², Georg Herrler¹#

¹ Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany
² Institute of Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany

M.S., P.V.-W., and G.H. contributed equally to this work.

# Correspondence to: Georg Herrler email: georg.herrler@tiho-hannover.de

State of publication: published

**J Gen Virol. 2015 Sep;96(9):2557-68. doi:10.1099/jgv.0.000223.**

Available at:

[http://jgv.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.000223](http://jgv.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.000223)

**Authors contributions:**

NW, FM, SM, PVW and GH conceived and designed the experiments; NW, FM, and SM performed the experiments; NW and FM analyzed the data; NW, FM, SM, PVW and GH wrote or helped to draft the paper. All authors read and approved the final manuscript.

**The extent of contribution from Nai-Huei Wu to this article:**

Scientific design: 70%

Performance of experiments: 80%

Analysis of experiments: 80%

Writing of the paper: 50%
Abstract

Bacterial co-infections are a major complication in influenza-virus-induced disease both in humans and animals. Either of the pathogens may induce a host response that affects the infection by the other pathogen. A unique feature in the co-infection by swine influenza viruses (SIV) and *Streptococcus suis* serotype 2 is the direct interaction between the two pathogens. It is mediated by the haemagglutinin of SIV that recognizes the α2,6-linked sialic acid present in the capsular polysaccharide of *Streptococcus suis*. In the present study, this interaction was demonstrated for SIV of both H1N1 and H3N2 subtypes as well as for human influenza viruses that recognize α2,6-linked sialic acid. Binding of SIV to *Streptococcus suis* resulted in cosedimentation of virus with bacteria during low-speed centrifugation. Viruses bound to bacteria retained infectivity but induced only tiny plaques compared with control virus. Infection of porcine tracheal cells by SIV facilitated adherence of *Streptococcus suis*, which was evident by co-staining of bacterial and viral antigen. Sialic-acid-dependent binding of *Streptococcus suis* was already detectable after incubation for 30 min. By contrast, bacterial co-infection had a negative effect on the replication of SIV as indicated by lower virus titres in the supernatant and a delay in the kinetics of virus release.
3. Manuscript II

Dynamic virus-bacterium Interactions in a porcine precision-cut lung slice coinfection model: swine influenza virus paves the way for *Streptococcus suis* infection in a two-step process

Fandan Meng¹, Nai-Huei Wu¹, Andreas Nerlich³, Georg Herrler¹, Peter Valentin-Weigand²#, Maren Seitz²

¹ Institute for Virology, University of Veterinary Medicine Hannover, Hannover, Germany
² Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany
³ Charité Universitätsmedizin Berlin, Department of Internal Medicine/Infectious Diseases and Respiratory Medicine, Berlin, Germany

G.H., P.V.-W., and M.S. contributed equally to this work.

#Correspondence to: Peter Valentin-Weigand, email: Peter.valentin@tiho-hannover.de

State of publication: published


Available at: [http://iai.asm.org/content/83/7/2806.long](http://iai.asm.org/content/83/7/2806.long).

**Authors contributions:**

NW, FM, GH, PVW and SM conceived and designed the experiments; NW, FM, and SM performed the experiments; FM and SM analyzed the data; NW, FM, AN, SM performed the confocal microscopy; NW, FM, GH, PVW and SM wrote or helped to draft the paper. All authors read and approved the final manuscript.

**The extent of contribution from Nai-Huei Wu to this article:**

Scientific design: 30%

Performance of experiments: 20%

Analysis of experiments: 10%

Writing of the paper: 10%
Abstract

Swine influenza virus (SIV) and Streptococcus suis are common pathogens of the respiratory tract in pigs, with both being associated with pneumonia. The interactions of both pathogens and their contribution to copathogenesis are only poorly understood. In the present study, we established a porcine precision-cut lung slice (PCLS) coinfection model and analyzed the effects of a primary SIV infection on secondary infection by S. suis at different time points. We found that SIV promoted adherence, colonization, and invasion of S. suis in a two-step process. First, in the initial stages, these effects were dependent on bacterial encapsulation, as shown by selective adherence of encapsulated, but not unencapsulated, S. suis to SIV-infected cells. Second, at a later stage of infection, SIV promoted S. suis adherence and invasion of deeper tissues by damaging ciliated epithelial cells. This effect was seen with a highly virulent SIV subtype H3N2 strain but not with a low-virulence subtype H1N1 strain, and it was independent of the bacterial capsule, since an unencapsulated S. suis mutant behaved in a way similar to that of the encapsulated wildtype strain. In conclusion, the PCLS coinfection model established here revealed novel insights into the dynamic interactions between SIV and S. suis during infection of the respiratory tract. It showed that at least two different mechanisms contribute to the beneficial effects of SIV for S. suis, including capsule-mediated bacterial attachment to SIV-infected cells and capsule-independent effects involving virus-mediated damage of ciliated epithelial cells.
4. Manuscript III

The differentiated airway epithelium infected by influenza viruses maintains the barrier function despite a dramatic loss of ciliated cells

Nai-Huei Wu¹, Wei Yang¹, Fandan Meng¹, Ronald Dijkman², Volker Thiel², Peter Valentin-Weigand³, Georg Herrler¹#

¹Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany
²Institute of Virology and Immunology, University of Bern, Bern, Switzerland
³Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany

#Correspondence to Georg Herrler, email: Georg.Herrler@tiho-hannover.de

State of publication: submitted

Authors contributions:

NW, WY, FM, RD, VT, PVW and GH conceived and designed the experiments; NW, WY and FM performed the experiments; NW analyzed the data; NW, WY, FM, RD, VT, PVW and GH wrote or helped to draft the paper. All authors read and approved the final manuscript.

The extent of contribution from Nai-Huei Wu to this article:

Scientific design: 80%
Performance of experiments: 80%
Analysis of experiments: 95%
Writing of the paper: 50%
Abstract
The airway epithelium is a primary barrier to infections by respiratory pathogens. We developed an air-liquid interface culture system for differentiated porcine respiratory epithelial cells to study the long term infection by influenza viruses. Release of infectious virus from infected cells was maintained at a high titer for more than seven days. During this infection period, infected cells were subject to apoptosis resulting in the loss of ciliated cells and a reduction of the thickness of the epithelial cell layer. Despite the detrimental effect of the influenza virus infection, the airway epithelium retained its barrier function as indicated by the transepithelial resistance that remained unaffected during the whole infection period. The loss of ciliated cells was compensated by the generation of polarized epithelial cells that appeared to have originated from basal cells but not yet differentiated into ciliated cells. These cells still expressed KRT5, a marker for basal cells and showed an expression pattern of sialic acids that was intermediate between basal cells and well-differentiated airway epithelial cells. Our results provide insight into the action and counteraction between influenza viruses and airway epithelial cells resulting in a localized respiratory infection. They enable experimental approaches to questions concerning the regeneration process of the epithelium during recovery from virus infection.
Introduction

The respiratory tract is the portal of entry used most often by microorganism to get access to their host. The airway epithelium is the primary barrier to infection. Viruses have found different ways to get across the epithelial barrier, e.g. by transcytosis as in the case of Epstein-Barr-virus (Tugizov et al., 2013) or by using immune cells as a ferry as in the case of measles virus (Lemon et al., 2011; Ludlow et al., 2009). The most straightforward strategy, however, is the infection of the epithelial cells. In order to successfully infect the respiratory epithelium, the pathogens have to overcome the mucociliary clearance system. This effective defence mechanism consists of a layer of mucus made up from mucins released by mucus-producing cells. Foreign material entrapped by the mucus is transported out of the respiratory tract by the movement of the cilia present on a specialized type of airway epithelial cells, the ciliated cells (Ganesan et al., 2013; Munkholm and Mortensen, 2014).

Influenza viruses (IV) are rather efficient in overcoming the defence mechanisms of the host. Having entered the airway system, they are entrapped in the mucus layer because mucins are rich in sialic acids, which act as a receptor determinant for influenza viruses (Cohen et al., 2013). The viral sialic acid binding activity is a function of the hemagglutinin (HA), one of the surface glycoproteins of influenza viruses (Weis et al., 1988). Another viral surface glycoprotein is the neuraminidase (NA) which serves as a receptor-destroying enzyme (Gamblin and Skehel, 2010). The NA protein not only facilitates the release of virus particles from infected cells and thus the spread of infection, it also cleaves sialic acid residues from mucins and thus enables the virus to get through the mucus layer (Cohen et al., 2013). Infection of the airway epithelial cells is initiated by the binding of the hemagglutinin to cell surface glycoconjugates. Human and porcine influenza viruses preferentially bind to α2,6-linked sialic acid,
whereas most avian influenza viruses have a preference for α2,3-linked sialic acid (Matrosovich et al., 2000).

Among the 16 HA serotypes (H1-16) of influenza A viruses, several strains of the H5 and H7 subtypes - present in avian viruses - are designated highly pathogenic avian influenza viruses (HPAIV), because their infection is characterized by a viremic phase that usually results in the death of the animals (Bottcher-Friebertshauser et al., 2013). A hallmark of these avian viruses is a multibasic cleavage site in the HA protein that allows proteolytic activation by furin or furin-like enzymes (Alexander, 2000). As these proteases are ubiquitously distributed in the organism, the fusion function and consequently the infectivity of HPAIV is activated in all tissues and organs. In contrast to HPAIV, other avian viruses are designated low-pathogenic avian influenza viruses (LPAIV). LPAIV and mammalian IV have hemagglutinins that lack a multibasic cleavage site and thus are not susceptible to the action of furin-like proteases. Some of the proteases that may be responsible for proteolytic activation of influenza viruses in the respiratory tract are TMPRSS2 and HAT (Garten et al., 2015). These proteases are not restricted to the airways but their distribution is not as ubiquitous as that of furin-like enzymes.

Human and swine influenza viruses may cause disease but in most cases, infections remain restricted to the respiratory tract and do not result in death. The distribution of activating proteases may in part explain the localized infection induced by these viruses (Peitsch et al., 2014). However, the interactions between influenza viruses and airway epithelial cells that result in cellular damage and disease on the one side and in the prevention of virus spread to other tissues and organs and in the recovery of the respiratory epithelium on the other side are not well characterized.

The primary target cells of mammalian influenza viruses are the differentiated airway epithelial cells. We have established an air-liquid interface (ALI) culture system from
porcine airway cells to analyze the infection of differentiated respiratory epithelial cells by human and swine influenza viruses. This culture system comprises mucus-producing cells and ciliated cells, i.e. cells of the mucociliary clearance system as well as basal cells, the progenitor cells of the differentiated epithelial cells. The ALI cells were susceptible to infection by human and swine influenza viruses resulting in release of infectious virus at a constant level for more than seven days. The detrimental effect of the virus infection was apparent in the appearance of apoptotic cells and in a dramatic loss of ciliated cells. On the other hand, the counteraction of the epithelial cells was documented by the finding that the transepithelial electrical resistance (TEER) of the epithelial cell layer was not decreased during the whole infection period analyzed. The maintenance of the TEER is explained by the appearance of polarized epithelial cells that – by lectin staining – showed a phenotype intermediate between basal cells and well-differentiated epithelial cells. Our results help to understand the localized infection by influenza viruses and provide an approach to study questions related to the recovery of epithelial cells from influenza virus infection.

Results

An air-liquid interface culture system for differentiated porcine airway epithelial cells

To study the influenza virus infection in the actual target cells, the differentiated airway epithelial cells, we established an air-liquid interface (ALI) culture system derived from the porcine airway. Primary porcine tracheal epithelial cells (PTEC) and porcine bronchial epithelial cells (PBEC) were isolated from the trachea and bronchi, respectively, of swine that were shown by multiplex PCR to be negative for porcine respiratory tract pathogens (porcine circovirus-2, porcine reproductive and respiratory
syndrome virus, porcine cytomegalovirus, porcine influenza A virus, porcine respiratory coronavirus, *Mycoplasma hyorhinis* and *Mycoplasma hyopneumoniae*). After 5 days maintained under ALI conditions, the cilia movement of PTEC and PBEC cultures was observed under a light microscope (data not shown). The trans-epithelial electrical resistance (TEER) values of PTEC and PBEC cultures became stable after four weeks of incubation under ALI conditions (not shown). Therefore, PTEC and PBEC were cultured under ALI conditions at least four weeks prior to experiments. In general, there were no major differences in the results between PTEC or PBEC. Therefore, only results obtained with bronchial cells (PBEC) will be shown here. PBEC cultured under ALI conditions for four weeks were analyzed for the presence of cilia by fluorescent staining with an anti-β-tubulin antibody (Fig. 1A, red). More than 65% of the total surface area of PBEC was covered by cilia. The positive staining of mucus on the apical surface of the cultures (Fig. 1B, green) indicated the presence of mucus-producing cells. Cryosections were stained for the presence of cytokeratin 5 (KRT5), a marker protein of basal cells. As shown in Figure 1C, the basal cells (KRT5, green) were located just above the filter support. In brief, we established cultures of well-differentiated airway epithelial cells which contained ciliated, mucus-producing cells, and basal cells.

**Sialic acid distribution on well-differentiated PBEC cultures**

Sialic acid is the crucial receptor determinant for infection by influenza viruses with avian viruses having a preference for α2,3- and human and swine viruses preferring α2,6-linked sialic acids. Therefore, the sialic acid distribution on well-differentiated PBEC cultures was determined by staining with the lectins MAA II and SNA which recognize α2,3- or α2,6-linked sialic acids, respectively. As shown in Fig. 2A, both α2,3- (MAA II staining, red) and α2,6-linked sialic acids (SNA staining, green) were
expressed on the apical surface of PBEC cultures but the α2,6-linkage type was predominant. Colocalization of MAA II and SNA signals (Fig 2A, arrows) indicates that some cells expressed sialic acids in both linkage types. In contrast to the apical staining, when lectins were applied from the basal side, PBEC were primarily stained by MAA II and hardly by SNA (Fig. 2B). Strong MAA II staining is found in the same area that is stained by the basal cell marker (compare 1C and 2B) indicating that basal cells mainly express α2,3-linked sialic. The distribution of sialic acids in well-differentiated airway epithelial cells was analyzed also by combining the lectin staining with cilia staining (anti-β-tubulin) or mucus staining (mucin5AC antibody). As shown in Fig 2C, both α2,3 and α2,6-linked sialic acids (green) were detected on ciliated cells (red). By contrast, non-ciliated cells predominantly expressed α2,6-linked sialic acid (Fig. 2C). Mucin/mucus-producing cells (MAA II staining in green, SNA staining in red) expressed α2,6-linked but not α2,3-linked sialic acids (Fig 2D).

**Kinetics of virus release from well-differentiated airway epithelial cells infected by human influenza viruses with different sialic acid binding preference**

We used two recombinant human influenza viruses, R1 and R2, to analyze the course of infection in our ALI culture system. R1 is derived from the pandemic strain A/Hong Kong/1/68 (H3N2) and has a sialic acid binding activity with a preference for the α2,6-linkage type. R2 differs from R1 by two mutations in the hemagglutinin which uses α2,3-linked sialic acid as the preferred receptor determinant (Matrosovich et al., 2007). Well-differentiated PBEC were inoculated by R1 and R2 from the apical surface at an MOI of 0.25. The inoculum was incubated for 2 h, and then removed by rinsing the cells with PBS to remove unbound viral particles. PBEC were maintained under ALI conditions during the infection. At different time points, 100μL harvests were collected from the apical surface of PBEC, and the course of infection was
monitored. As shown in Fig 3A1, the titer of R1 virus released from the apical side of PBEC was about 13-fold higher than that of R2 at 8 hpi and 6-fold on 2 dpi. The difference in the amount of released virus was significant (P<0.01). Taken together, influenza virus with a preference for α2,6-linked sialic acid replicated more efficiently in PBEC when inoculated from the apical side, which is consistent with the abundant expression of this linkage type on the apical surface. When influenza virus was applied from the basal side of PBEC, there was no significant difference in the replication kinetics irrespective of the binding preference for α2,3- or α2,6-linked sialic acids (Fig 3A2).

Replication kinetics of swine influenza viruses in ALI cultures of porcine airway epithelial cells

In order to analyze the replication of swine influenza viruses (SIV) in porcine well-differentiated airway epithelial cells, PBEC cultures were infected by either of two SIV strains, sw/Bad Griesbach/06 (H1N1 subtype) and sw/Herford/07 (H3N2 subtype). These viruses had been isolated from clinical cases and represent two virus lineages circulating in European pigs. To investigate the route of infection, PBEC were inoculated from the apical or basal side as described above for the R1 and R2 viruses. The replication kinetics of the two SIV strains is shown in Fig 3B (3B1, apical inoculation; 3B2 basal inoculation). Starting from 2 dpi, the titer of released SIV-H1N1 was significantly higher than that of SIV-H3N2 (P<0.05). By contrast, the observed differences were not significant when the two SIV strains were inoculated from the basal side (Fig 3B2). The infectivity of the virus released from PBEC was determined by a focus-forming assay on MDCK cells. Surprisingly, a difference was noted in the foci morphology of SIV-H1N1 released from well-differentiated airway cells at early and late times of infection. As shown in Fig 3C1, the foci morphology of infectious SIV
H1N1 at 3 dpi was similar to that of the original virus, whereas the foci size of virus collected at 6 dpi was significantly increased (from 20 μm to 200 μm in diameter). This increase in size of the foci was observed only when SIV-H1N1 was inoculated from the apical side of PBEC (Fig 3C1). By contrast, when PBEC were infected by SIV-H1N1 from the basal side, the morphology of the foci remained the same as that from the original virus (Fig 3C1). SIV-H3N2 induced larger foci than did the original SIV-H1N1 and this size of the foci did not change significantly during the whole infection period (Fig 3C2).

**Cell tropism of SIV among differentiated airway epithelial cells**

We further analyzed which cell type among well-differentiated airway epithelial cells is preferentially infected by SIV. PBEC were inoculated with either of the two SIV strains from the apical side as described above. The infected cultures were fixed at 1 dpi, followed by co-immunostaining of viral nucleoprotein, cilia, and mucus using anti-NP, anti-β-tubulin, and mucin5AC antibodies, respectively. As shown in Fig 4, SIV-antigen (green) was associated with β-tubulin-positive (red, Fig 4A) and also with β-tubulin negative cells, but not with mucin5AC-positive cells (red, Fig 4B). These results demonstrate that both SIV strains preferentially infect both ciliated cell and some non-ciliated cells different from mucus-producing cells.

**SIV-induced apoptosis**

To analyze airway epithelial cells for a detrimental effect of infection, we performed an immunofluorescent staining of SIV-infected PBEC to detect apoptotic cells. As shown in Fig 5, cells positive for activated caspase-3 (green) were present in SIV-infected PBEC at 2 dpi, indicating that SIV infection induces apoptosis in well-differentiated PBEC. Moreover, co-localization of cleaved caspase-3 (green), β-tubulin (red) and
viral NP (cyan) was observed when SIV-infected PBEC were analyzed by confocal microscopy (Fig 5A, arrow), showing that SIV infection induces apoptosis in ciliated cells.

**Effect of infection on the mucociliary apparatus**

To get more information about the cell damage caused by influenza virus infection, PBEC cultures were inoculated by either of the influenza viruses from the apical side as described above. The cultures were fixed at 8 dpi and subjected to analysis by immunofluorescence microscopy. The presence of cilia on virus-infected PBEC was detected by staining with an anti-β-tubulin antibody (red). As shown in Fig 6A, a loss of cilia was observed in all virus-infected cultures compared to the mock-infected cultures. By applying a software to determine the area lacking cilia, the loss of cilia was quantified (Fig. 6B). SIV-infected cultures lost more cilia compared to R1- and R2-infected samples. The effect of influenza virus infection was also evident when the epithelial thickness was analyzed. The thickness of SIV-infected PBEC was reduced at 8 dpi (Fig. 7). By applying a software to quantify the effect on the immunofluorescence microscopy pictures, we measured the thickness of infected PBEC cultures. As shown in Fig 7C (or combined with 7B), the thickness of SIV-infected cultures was reduced by about 50%. In R1- or R2-infected PBEC, the epithelial thickness was not significantly reduced. This may be explained by the finding that the loss of cilia was less pronounced in R1- and R2-infected cultures than it was in SIV-infected samples. The reduction in height started to become evident after 2 dpi as shown in Fig 7A for a SIV-H1N1-infected culture stained by an antibody directed against the junctional protein β-catenin (green at 2 dpi; red at 8 dpi). Additionally, in spite of the failure to detect infection of mucus-producing cells at 1 dpi (Fig. 4), colocalization of mucin and NP was observed at 8 dpi (Fig 6C, arrow).
**Maintenance of the barrier function of virus-infected PBEC**

To analyze whether the detrimental effect of the influenza virus infection affects the barrier function of the epithelial cell layer, we determined the trans-epithelial electrical resistance (TEER). The TEER values of SIV-infected PBEC (Fig 8B) were not decreased compared to mock-infected cultures during the whole infection period analyzed, up to 8 dpi. This finding is consistent with the staining for the tight junction protein ZO-1. As shown in Fig. 8A (or combined with 7A) the pattern of tight junctions between individual cells was observed both at 2 and 8 dpi (ZO-1, red in 2 dpi; green in 8 dpi). As mentioned above (Fig. 7A) another cell adhesion protein, β-catenin, also had a similar distribution, both at 2 and 8 dpi.

The cells maintaining the barrier function at 8 dpi were analyzed in more detail by staining the cultures for the basal cell marker KRT5 and for the presence of sialic acids. As shown in Fig. 9A, in PBEC infected by SIV-H1N1, cells that had survived infection were positive for KRT5. Some of the cells were infected by SIV as indicated by staining for NP. Furthermore, these KRT5-positive cells were stained by both lectins, MAA II and SNA (Fig. 9B). By contrast, in mock-infected samples, MAA II staining was mainly detected in the basal portion of the cell layer whereas SNA preferentially stained the apical portion. These results are consistent with the conclusion that the cells maintained at 8 dpi are derived from basal cells that are in the process of differentiating into specialized cells.

**Discussion**

The airway epithelium is equipped with the mucociliary clearance system to prevent the detrimental effect of foreign substances including infection by microorganisms. This defence mechanism is based on the mucins produced by mucus-producing cells and the ciliary activity of specialized epithelial cells that transport the mucus out of the
respiratory tract (Ganesan et al., 2013; Munkholm and Mortensen, 2014). To understand the infection of airway epithelial cells, it is necessary to use culture systems that comprise mucus-producing cells and ciliated cells. Therefore, we established the ALI system as these cultures are made up not only from cells of the mucociliary clearance system but also from non-ciliated, non-mucus-producing epithelial cells as well as basal cells, the progenitors of the differentiated epithelial cells. We chose porcine ALI cultures as they can be used to analyze the infection by both human and swine influenza viruses ((Bateman et al., 2013; Powell et al., 2014); this study). Compared to the human counterpart, swine ALI cells have the advantage that the source of cells is well-defined and reproducible as far as the age and genetic background of the animals are concerned. The growth conditions of swALI cultures were different from those described for human ALI cultures (see Materials and Methods). This difficulty in establishing this system may explain why so far only few reports about differentiated respiratory epithelial cells of swine are available (Bateman et al., 2013; Lam et al., 2011; Powell et al., 2014).

As we were interested to use the ALI culture for infection studies with influenza viruses, it was important to analyze the distribution of sialic acids which are the receptor determinant for influenza viruses. Consistent with another report about swALI, the apical surface of differentiated epithelial cells was found to predominantly express α2,6-linked sialic acid (Bateman et al., 2013). Our data agree with a report about ex vivo-samples of the respiratory tract of swine where the α2,6-linked sialic acid was predominant in the upper airway (Van Poucke et al., 2010). Only in the lower parts of the respiratory tract, in the bronchioli and alveoli, a substantial amount of α2,3-linked sialic acid was detected on the apical surface of the respiratory epithelium (Nelli et al., 2010; Van Poucke et al., 2010). Strong expression of α2,3-linked sialic
acid has been reported on subepithelial cells of swALI cultures (Bateman et al., 2013). Here, we have shown that the basal cells are predominantly labeled by MAA II and thus expressing α2,3-linked sialic acid. Moreover, we found non-ciliated cells showed abundant expression of α2,6-linked sialic acids while ciliated cells contained both sialic acid linkage types in PBEC cultures. When the sialic acid distribution of different species is compared, only the porcine airway epithelium shows an expression pattern similar to that of human cells indicating that the PBEC cultures are more suitable to investigate human influenza virus infection than MAE and ferret airway epithelial cells (Ibricevic et al., 2006; Thompson et al., 2006; Zeng et al., 2013).

The ALI culture system revealed characteristics of the influenza virus infection that were different from those obtained with immortalized cells or that could not be addressed by conventional cell cultures. The amount of infectious virus released into the supernatant remained high during the whole infection period analyzed, i.e. up to eight dpi. This long period of virus release may be related to the ALI culture conditions. As influenza virions are released from the apical side of the polarized epithelial cells, spread of infection is expected not to be as efficient as in the case of immortalized cells which are covered by medium. In differentiated epithelial cells, virus spread has to occur via the periciliary fluid surrounding the cilia or in the mucus blanket on top of the periciliary layer that is transported by the ciliary activity (Tilley et al., 2015). Interaction with mucins may further hamper the spread of infection, because the viral binding to sialic acids has to be counteracted by the viral neuraminidase (Matrosovich et al., 2004). The primary target cells among swALI cultures were ciliated cells and, among non-ciliated cells, non-mucus-producing cells. Infection of mucus-producing cells was observed only at later stages of infection. The infection by influenza viruses had a detrimental effect on the airway epithelial cells as indicated by the detection of
apoptotic cells. Another effect of the infection was a significant reduction in the amount of cilia detected on infected airway epithelial cells. The reduction was more pronounced in SIV-infected cultures compared to cells infected by either of the two human influenza viruses. The difference is consistent with a more efficient infection by SIV as indicated by the 10-fold increased amount of virus released into the supernatant. The reduced staining of tubulin is not due to a loss of cilia by dedifferentiation of ciliated cells; it is rather explained by the loss of ciliated cells, probably due to the apoptotic effect of the infection mentioned above. The loss of cilia and the reduction in epithelial thickness are also observed in SIV-infected porcine trachea tissue explants (Patrono et al., 2015). This conclusion is based on the reduced thickness of the epithelial cell layer at late times of infection that was observed in parallel to the loss of cilia staining. The loss of ciliated cells was clearly detected with cultures infected by SIV. In the case of human influenza viruses, where the foci of infected cells were not as large as those of SIV, the thickness of the epithelial layer was not significantly reduced.

Despite the detrimental effect of the infection by influenza virus, the transepithelial electrical resistance of the epithelial layer was retained during the whole infection period. The finding of epithelial cells with tight junctions indicates that the lost ciliated cells were replaced by polarized cells that were able to maintain the barrier function of the epithelium. It appears that basal cells had started to differentiate into specialized cells. This process had not yet proceeded to the appearance of ciliated cells and mucus-proceeding cells, but the differentiation had resulted in polarized epithelial cells that were sufficient to maintain the barrier function as indicated by the transepithelial electrical resistance. The reorganization of the epithelial cells has also been observed in another report, where mice were infected by IV in vivo, and a loss of suprabasal cells was observed in the early stage of infection, but the epithelium was
regenerated and cells re-differentiated at later time points after IV clearance (Tata et al., 2013). The intermediate position of these polarized cells is shown by our results of the lectin staining, which may indicate that the cells are in a re-differentiation process. Undifferentiated human airway epithelial cells have been shown to express α2,3-linked sialic acids and the expression of α2,6-linked sialic acids is increasing overtime during the differentiation phase (Chan et al., 2010). Whereas well-differentiated cells predominantly expressed α2,6-linked sialic acid, and basal cells mainly contained α2,3-linked sialic acid, sialic acids in both linkage types were detected in polarized cells at late times of infection. Our results are consistent with data from influenza virus infections in swine. Airways infected by infection have been reported to be lined by flattened epithelial layers (Janke, 2014).

The results obtained from analyzing the infection of ALI cultures by influenza viruses provide an explanation of the influenza virus infection. On the one side, there is the detrimental effect that can result in disease. On the other side, the loss of well-differentiated cells is compensated by the generation of polarized cells that maintain the barrier function of the epithelial cell layer which contributes to the restriction of the infection to the respiratory tract. Thus, the localized infection by influenza virus is not only a matter of the availability of proteases required for proteolytic activation of virus infectivity but is also the result of action and counteraction in the interplay between virus and airway epithelial cells. As the cells that replace the well-differentiated cells are not yet ciliated the infected areas of the epithelium may for a certain time not contribute to the mucociliary clearance system. Furthermore, the lectin staining of the infected ALI cultures at late times of infection indicates that the surface expression of these cells is different from the pattern of surface proteins of well-differentiated epithelial cells. This provides an explanation
why prior virus infection renders the host more susceptible to the co-infection by another viral or bacterial pathogen.

Our results not only provide new insights into the infection of the airway epithelium by influenza viruses, they also show an experimental access to questions related to the recovery of infection, e.g. the re-differentiation of the epithelium and the susceptibility to co-infections.

**Material and Methods**

**Influenza viruses**

Two swine influenza viruses (SIV), A/sw/Bad Griesbach/IDT5604/2006 (SIV H1N1 subtype, SIV H1N1) and A/sw/Herford/IDT5932/2007 (SIV H3N2 subtype, SIV H3N2) and two recombinant human influenza viruses, R1 and R2 were used in this study. The information of the virus strains and preparation of virus stocks have been described in detail in previous study (Matrosovich et al., 2007; Meng et al., 2013; Wu et al., 2015).

**Differentiated porcine airway epithelial cells cultures**

Primary porcine airway epithelial cells were isolated from pigs in the local slaughterhouse in Hannover. Primary porcine tracheal epithelial cells (PTEC) and porcine bronchial epithelial cells (PBEC) were obtained from swine trachea and bronchus, respectively. Tracheal and bronchial segments were washed by phosphate-buffered saline (PBS) and incubated for 1 day (bronchus) or 2 days (trachea) in Eagle’s minimal essential medium (EMEM, Gibco) supplemented with penicillin (100 units/ml, Sigma), streptomycin (100 μg/ml, Sigma), amphotericin B (2.5 μg/ml, Sigma), gentamicin (50 μg/ml, Gibco), DNase (10 μg/ml, Roche) and Protease
(1 mg/ml, Sigma). Primary cells were harvested as previous described (Lam et al., 2011) and were seeded on type I collagen (Sigma)-coated flask with bronchial epithelial cell serum-free growth medium (BEGM). The BEGM contained the BEBM basal medium (Lonza) and supplemented with the required additives (Sigma and BD Biosciences) modified from previous studies (Dijkman et al., 2013; Fulcher et al., 2005). BEGM was refreshed at 2 days intervals until cells reached 70 to 80% confluence. PTEC and PBEC were transplanted to type IV collagen-coated Transwell® polycarbonate membrane (24 well, 0.4 μm pore size, Corning Costar) at a density of 2.5 × 10^5 cells per filter support, the cells were then grew with air-liquid interface (ALI) medium in both apical and basal compartments. The ALI medium consists a mixture of DMEM (Gibco) and BEBM basal medium (1:1) with additives (Sigma and BD Biosciences) which is adapted from previous studies (Fulcher et al., 2005). After PTEC and PBEC reached confluence, the medium on the apical side was removed and cells were maintained under ALI conditions at least 4 weeks at 37°C in a humidified 5% CO₂ atmosphere. The ALI medium in basal compartments was refreshed at 2 days intervals and the apical surface of cultures was washed with Hanks' balanced salt solution (HBSS, Gibco) weekly.

Additionally, PTEC and PBEC were validated for porcine specific respiratory tract pathogens including porcine circovirus-2, porcine reproductive and respiratory syndrome virus, porcine cytomegalovirus, porcine influenza A virus, porcine respiratory coronavirus, *Mycoplasma hyorhinis* and *Mycoplasma hyopneumoniae* by multiplex Polymerase Chain Reaction (PCR) (Harder and Huebert, 2004) which was done in the Institute of Virology, University of Veterinary Medicine, Hannover. All the PTEC and PBEC used in this study were free from above pathogens.
Measurement of trans-epithelial electrical resistance (TEER)

The TEER developed by PTEC and PBEC cultures was measured by using the Millicell® ERS-2 Voltohmmeter (Millipore) according to the manufacturer’s instructions.

Virus infection of differentiated epithelial cells

Well-differentiated PTEC and PBEC were washed 5 times with PBS and were inoculated influenza viruses from apical or basal side at an MOI (multiplicity of infection) of 0.25. The filter supports with PTEC and PBEC were inverted for basal inoculation. After 2 h of incubation at 37 °C, PTEC and PBEC were rinsed with PBS twice to remove unbound viral particles and fresh ALI medium were added. The infected PTEC and PBEC were further maintained under ALI conditions at 37 °C in a 5% CO₂. At different time points, 100μL of DMEM were added to the apical surface of infected-cells and followed by 30 min incubation at 37 °C in a 5% CO₂. The harvests were collected at 2h, 8h, 1d, 2d, 3d, 4d, 6d and 8d post virus-infection (hpi or dpi) and the viruses were titrated by focus forming assay on MDCK cells. The entire infection period was absence of exogenous trypsin should be noted. On the other hand, the infected PTEC and PBEC were fixed with 3% paraformaldehyde (PFA) directly or performed cryosections for immunofluorescence analysis at 1, 2 and 8 dpi. The cryosections of PTEC and PBEC were carried out with a cutting thickness of 20 μm as previously description (Punyadarsaniya et al., 2011).

Lectin staining

The Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin II (MAAII) were used to detect sialic acid. After fixation with 3% PFA, the cells or the cryosections were blocked with Streptavidin/Biotin Blocking Kit (Vector laboratories). FITC-labeled SNA (Vector laboratories) and biotinylated MAAII (Vector laboratories)
were used to detect α2,6- and α2,3-linked sialic acids, respectively. The binding of biotinylated lectins was visualized by incubation of the samples with streptavidin-Cy3 (Sigma) or streptavidin-DyLight 488 (Vector laboratories) and observed by fluorescence microscopy.

**Immunofluorescence analysis (IFA)**

Whole-mount and cryosections of PTEC and PBEC were fixed with 3% PFA at RT for 1h and 20 min, respectively, and permeabilized with 0.5% Triton X-100. Followed by PBST (PBS+0.1% tween 20) washing step, cells and sections were further blocked with 5% goat serum for 30 min and incubated with primary antibody overnight at 4°C (anti-NP) or 1h at RT (others). Cells and sections were then washed 3 times with PBST followed by Alexa Fluor® conjugated secondary antibody incubation for 1 h at RT. The nuclei were stained by DAPI (4’,6-diamidino-2-phenylindole) and the samples were embedded with ProLong® Gold Mountant (Life Technologies).

The primary antibodies used in this study were as follows: anti-influenza A virus nucleoprotein (NP) antibody (mouse, 1:750, AbDSeroTec), anti-mucin-5AC antibody (mouse, 1:250, Acris), anti-cytokeratin 5 (KRT5) antibody (rabbit, 1:500, Abcam), anti-ZO-1 antibody (rabbit, 1:100, Life Technologies), anti-β-catenin antibody (mouse, 1:500, Sigma), cleaved caspase-3 antibody (rabbit, 1:300, Cell Signaling) and Cy3-labeled antibody against β-tubulin (1:300, Sigma). Secondary antibodies were Alexa Fluor® 488, 568 or 633 conjugated antibodies (1:1000, Life Technologies). All antibodies were diluted in 1% bovine serum albumin. IFA was performed by using a Leica TCS SP5 AOBS confocal laser scanning microscope. For confocal Images analysis and processing, LAS AF Lite software (Leica) and ImageJ/Fuji (NIH) software were used.
Viral titration by focus forming assay

The infectivity of the viruses was evaluated by focus forming assay on MDCK cells as previously described (Shin et al., 2015) with modifications. In brief, serial 10-fold dilutions were performed for each sample and added on MDCK cells in 96-well plate. After 20 h incubation, cells were fixed with 3% PFA and permeabilized, followed by incubation with antibodies against influenza A virus NP (mouse, 1:1000, AbDSeroTec). The secondary antibody with horseradish peroxidase (HRP) conjugated anti-mouse IgG (H+L) antibody (1:2000, KPL) was used. Subsequently, the immunological staining was performed by TrueBlue substrate (KPL) and the foci number was measured. The calculated viral titer is indicated in foci-forming units per mL (FFU/mL).

Statistical analysis

Data in the figures are shown as the means ± SEM. All statistical analyses were done by using Prism 5 software (GraphPad Software).

Acknowledgements

This work was performed by N. W., W. Y. and F. M. in partial fulfillment of the requirements for the PhD degree from the University of Veterinary Medicine Hannover. N.W. was a recipient of a Georg-Christoph-Lichtenberg scholarship from the Hannover Graduate School for Veterinary Pathobiology, Neuroinfectiology, and Translational Medicine (HGNI). W.Y. and F.M. was a recipient of a fellowship from China Scholarship Council. This work was supported by grants from Deutsche Forschungsgemeinschaft to GH (He1168/15-1) and PVW (Va23917-1). We are grateful to Michaela Schmidtke (University of Jena, Germany), Ralph Dürrwald (IDT Biologika GmbH, Dessau-Roßlau, Germany) and Mikhail Matrosovich (Philipps-University Marburg, Germany) for kindly providing viral strains. We gratefully thank
Christine Bächlein (University of Veterinary Medicine Hannover, Germany) for performing multiplex-PCR of porcine specific respiratory tract pathogens.
References


Bateman, A.C., Karasin, A.I., and Olsen, C.W. (2013). Differentiated swine airway epithelial cell cultures for the investigation of influenza A virus infection and replication. Influenza and other respiratory viruses 7, 139-150.


Depends on Genetic Background. Journal of virology 89, 9998-10009.


**Figure**

**Fig. 1:** Characterization of porcine well-differentiated airway epithelial cell cultures.
Fig. 1: Characterization of porcine well-differentiated airway epithelial cell cultures.

Porcine tracheal epithelial cells (PTEC) and porcine bronchial epithelial cells (PBEC) were cultured under air-liquid interface (ALI) condition at least 4 weeks and detected cilia (A, B and C), mucus (B) and basal cells (C) by immunofluorescence staining. (A) immunofluorescence staining of PTEC and PBEC ALI cultures for cilia by using anti-β-tubulin antibody (red, in horizontal planes). (B) immunofluorescence staining of PTEC and PBEC ALI cultures for cilia (red) and mucus (green, mucin 5AC monoclonal antibody, in vertical planes). (C) immunofluorescence staining of cryosections for cilia (red) and basal cells (green, antibody against cytokeratin 5). Scale bars, 50 μm (A), 25 μm (B and C).
Fig. 2: Expression of sialic acid on porcine well-differentiated airway epithelial cell cultures.
**Fig. 2: Expression of sialic acid on porcine well-differentiated airway epithelial cell cultures.**

(A) PBEC apical surface was stained by SNA (green) and MAAII (red) and showed in horizontal (A1) or vertical (A2) planes. (B) Cryosection of PBEC cultures was stained by SNA (green) and MAAII (red). (C) PBEC was co-staining SNA or MAAII (green) with cilia (red) and showed in horizontal (C1) or vertical (C2) planes. (D) PBEC was stained by SNA (green) or MAAII (red), followed by mucus staining (red, when co-staining with SNA; green, when co-staining with MAAII). α2,3- and α2,6-linked sialic acid were detected by MAAII and SNA lectin staining, respectively. Cilia was stained using anti-β-tubulin antibody and mucus was stained by antibodies against mucin 5AC. The arrows show colocalization. Scale bars, 25 μm.
Fig. 3: Replication of influenza A viruses in porcine well-differentiated airway epithelial cell cultures.
Fig. 3: Replication of influenza A viruses in porcine well-differentiated airway epithelial cell cultures.

(A) Replication kinetics of R1 and R2 viruses in PBEC ALI culture. (B) Replication kinetics of SIV in PBEC ALI culture. PBEC ALI culture was inoculated influenza viruses from apical (A1, B1 and C middle panels) or basolateral (A2, B2 and C lower panels) side at an MOI 0.25. Viruses released from apical side were harvested at different time point and titrated by focus forming assay in MDCK cells. The results are shown as means ± SEM from 3 (SIV) or 2 (R1 and R2) independent experiments with triplicates samples. Statistical analysis with unpaired Student’s t-test (***:P<0.001, **:P<0.01, *:P<0.05). (C) Foci morphology of SIV in MDCK cells. Original SIV and samples harvested at 3 and 6 days post SIV infection in PBEC ALI cultures were perform focus forming assay in MDCK cells. The foci was stained with monoclonal antibody against influenza nucleoprotein. Scale bars: 200 μm.
Fig. 4: Cell tropisms of swine influenza viruses in porcine well-differentiated airway epithelial cells.

PBEC ALI cultures were infected with SIV H1N1 or H3N2 form apical surface at an MOI of 0.25 and fixed at 1 dpi, followed by immunofluorescence staining to detect viral nucleoprotein (green), cilia (A, red) and mucus (B, red). Scale bars, 25 μm.
Fig. 5: Immunofluorescence staining of apoptotic cells in PBEC at 2 days post infection.

PBEC ALI culture was infected with SIV from apical surface at an MOI of 0.25 and fixed at 2 dpi. (A) immunofluorescence staining for viral nucleoprotein (cray), cilia (red) and cleaved caspase-3 (green). (B) immunofluorescence staining for viral nucleoprotein (cyan), mucin (red) and cleaved caspase-3 (green). Confocal images were showed in horizontal (top panels) or vertical (lower panels) planes. The arrows show colocalization and the dashed lines show the location of membrane filter. Scale bars: 25 μm.
Fig. 6: Immunofluorescence staining of porcine well-differentiated airway epithelial cells at 8 days post infection.
Fig. 6: Immunofluorescence staining of porcine well-differentiated airway epithelial cells at 8 days post infection.

PBEC ALI culture was inoculated influenza viruses from apical side at an MOI 0.25 and fixed at 8 dpi. (A) PBEC culture was stained by viral nucleoprotein (green) and cilia (red). (B) Quantification of ALI cultures cilia area at 8 dpi. Results are shown as percentages (means ± SEM) compare to mock-infected ALI cultures from 3 independent experiments with duplicates samples. (C) immunofluorescence staining for viral nucleoprotein (green) and mucin (red). The arrows show colocalization. Scale bars, 25 μm (C) or 50 μm (A).
Fig. 7: Porcine well-differentiated airway epithelial cell cultures decreased thickness after influenza viruses infection.

PBEC ALI culture was inoculated influenza viruses from apical (A, middle panels, B and C) or basolateral side (A, right panels) at an MOI 0.25. (A) PBEC ALI culture was inoculated SIV H1N1 and fixed at 2 or 8 dpi, followed by immunofluorescence staining with anti-β-catenin antibody (A1, 2 dpi, green; A2, 8 dpi, red). Confocal images were showed in vertical planes. (B) PBEC was fixed at 8 dpi and stained by viral nucleoprotein (green) and cilia (red) and showed in vertical planes. (C) Quantification of PBEC ALI cultures thickness at 8 dpi. Results are shown as percentages (means ± SEM) compare to mock-infected ALI cultures from 2 independent experiments with duplicates samples. Scale bars, 25 μm
Fig. 8: Porcine well-differentiated airway epithelial cell cultures remain tight junctions after SIV infection.

(A) PBEC ALI culture was inoculated SIV H1N1 from apical (middle panels) or basolateral (right panels) side at an MOI 0.25. ALI cultures were fixed at 2 (top panels) and 8 dpi (lower panels), followed by staining with anti-ZO-1 antibody (top panels, red; lower panels, green) to detect tight junction protein. Scale bars, 10μm. (B) PBEC ALI culture was inoculated SIV from apical or basolateral side. The transepithelial electrical resistance (TEER) value of mock-infected and SIV-infected PBEC was determined at the indicated time points. The results are shown as means ± SEM (n=3).
Fig. 9: Sialic acid expression on PBEC after SIV infection.

PBEC ALI cultures were inoculated SIV H1N1 from apical (middle panels) or basolateral (right panels) side at an MOI 0.25 and performed cryosections at 8 dpi. (A) immunofluorescence staining for cytokeratin 5 (red, basal cells) and viral nucleoprotein (green). (B) immunofluorescence staining to detect α2,3- and α2,6-linked sialic acid by MAAII and SNA lectin staining, respectively. Scale bars: 25 μm
5. MANUSCRIPT IV

Efficient suilysin-mediated invasion and apoptosis in porcine respiratory epithelial cells after streptococcal infection under air-liquid interface conditions

Fandan Meng¹, Nai-Huei Wu¹, Maren Seitz², Georg Herrler¹, Peter Valentin-Weigand²#

¹Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany
²Institute of Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany

G.H. and P.V.-W. contributed equally to this work.

#Correspondence to: Peter Valentin-Weigand, email: Peter.valentin@tiho-hannover.de

State of publication: accepted

Sci Rep, DOI: 10.1038/srep26748

Authors contributions:

NW, FM, SM PVW and GH conceived and designed the experiments; NW and FM performed the experiments; FM analyzed the data; NW, FM, SM, PVW and GH wrote or helped to draft the paper. All authors read and approved the final manuscript.

The extent of contribution from Nai-Huei Wu to this article:

Scientific design: 20%
Performance of experiments: 20%
Analysis of experiments: 10%
Writing of the paper: 10%
Abstract

Streptococci may colonize the epithelium in the airways and other entry sites. While local infection often remains asymptomatic, severe or even fatal diseases occur when streptococci become invasive and spread to different sites in the infected host. We have established porcine respiratory air-liquid interface cultures (ALI) from the porcine lung to analyze the interaction of streptococci with their primary target cells. As representative of the streptococcal family we chose *Streptococcus suis* (*S. suis*) that is not only a major swine respiratory pathogen but – occasionally also infects humans. Suilysin, a cholesterol-dependent cytolysin (CDC), is an important virulence factor. By comparing an *S. suis* wt strain with a suilysin-deficient mutant (10Δsly), we demonstrate that suilysin contributes to (i) adherence to airway cells (ii) loss of ciliated cells (iii) apoptosis, and (iv) invasion. A striking result of our analysis was the high efficiency of *S. suis*-induced apoptosis and invasion upon infection under ALI conditions. These properties are rather inefficient when analyzed with immortalized cells. We hypothesize that soluble effectors such as suilysin are present at higher concentrations in cells kept at ALI conditions and thus more effective. These results should be relevant also for infection of the respiratory tract by other respiratory pathogens.
6. DISCUSSION

6.1 Sialic acid-dependent interactions between influenza viruses and *Streptococcus suis*

Bacterial co-infection is a major factor of promotion in the morbidity and mortality associated with influenza epidemics in human (McCullers, 2014). In spite of the few studies on the mechanisms of how influenza viruses interact with bacteria (Rynda-Apple et al., 2015; Short et al., 2012), the modulation of the pathogenicity by co-infections is an important issue that needs to be investigated. Compared to mono-influenza virus infections, the action and counteractions between both pathogens and the host are still poorly understood.

To further investigate the interaction between viral and bacterial pathogens in a specific host, in my studies, *S. suis* was chosen as the bacterial pathogen to perform co-infections with SIV. Both infectious agents have a zoonotic potential, and represent important pathogens associated with the porcine respiratory disease complex (PRDC) in the porcine respiratory tract (Brockmeier SL, 2002). To characterize the mechanisms of interaction between SIV and *S. suis*, my colleague and I performed the co-infection with two different approaches, NPTr and PCLS. The immortalized porcine tracheal cells, NPTr cells are easy to handle and can provide a deeper insight to the molecular mechanism. On the other hand, PCLS consist of differentiated epithelial cells and maintain the original setting of the cells within the organs and thus reflect the *in vivo* conditions.

In my study, NPTr cells were first infected by either of the two different SIV strains. After having removed unbound viral particles, *S. suis* was applied as a secondary
infectious agent. My results show that in vitro infection of SIV enhanced S. suis wt adhesion to respiratory epithelial cells. The adhesion of S. suis wt to NPT r cells was significantly increased when cells had been pre-infected with either SIV-H1N1 or SIV-H3N2 virus. Moreover, most S. suis wt were associated with SIV-infected cells. A previous study shows that pre-infection of NPT r cells with SIV-H1N1, increased not only the adhesion but also the invasion rate of S. suis (Wang et al., 2013). By contrast, whether the NPT r cells were pre-infected with SIV or not, the adhesion number of S. suis Δcps to NPT r cells showed no difference. Similar results were also obtained at the initial infection stage in PCLS (24 and 48 hpi). These results indicate that the capsular polysaccharide of S. suis plays a critical role for the enhancement of S. suis adhesion to and invasion of SIV-infected cells.

On the other hand, although SIV had a positive effect on S. suis infection, our results showed that the bacterial co-infection rather has a negative effect on the SIV replication. Compared to the virus titer of released infectious particles at 24 hpi by mono-infected cells, the virus titer was 20 to 30-folds lower when cells were co-infected with SIV-H1N1 and S. suis wt. For the H3N2 SIV, the difference between the co-infected and mono-infected groups was less pronounced. This difference between SIV-H3N2 and SIV-H1N1 can be explained by the faster replication of SIV-H3N2. When this virus was applied at a lower m.o.i., the difference between the co-infected and mono-infected group became more significant. These results demonstrate that the capsular polysaccharide of S. suis is crucial for the interactions between these two pathogens. Possible explanations of the reduced viral infectivity are (i) that the binding of bacteria to infected cells inhibits the budding process and thus the release of virions or (ii) that the direct binding of SIV to bacteria in the supernatant hampers the spread of infection.
It is widely known that the influenza virus hemagglutinin (HA) protein recognizes sialic acids and has a binding preference for either α2,3- or α2,6-linked sialic acid (Rogers and Paulson, 1983; Yamada et al., 2006). Previous reports have shown that the capsular polysaccharide of *S. suis* serotype 2 contains a terminal α2,6-linked sialic acid residue (Smith et al., 2000; Van Calsteren et al., 2010). Some of the pathogenic bacteria decorate their cell surfaces with sialic acids. For instance, the capsules of Group B *Streptococcus* (GBS) contain terminal α2,3-linked sialic acid residues (Lewis et al., 2004). *S. suis* serotype 14 contains α2,6-linked sialic acid (Van Calsteren et al., 2013). The terminal sugars of *Neisseria meningitides* serogroups B and C consist of α2,8- or α2,9-linked sialic acids, respectively (Swartley et al., 1997). The sialic acid on the bacterial surface can render the bacteria resistant against the innate immune response of the host (Severi et al., 2007). In my study, by using lectin staining, I confirmed that *S. suis* wt strain 10 only expressed α2,6-linked sialic acid on its capsular polysaccharide. In contrast, neither α2,6- nor α2,3-linked sialic acid was presented on non-capsulated mutant *S. suis* Δcps. To further investigate how sialic acid is involved in the co-infection process, an exogenous neuraminidase from *Clostridium perfringens* was used to cleave the terminal sialic acid residues (Newstead et al., 2008). After neuraminidase treatment, α2,6-linked sialic acid was undetectable on the capsular polysaccharide of *S. suis* wt.

I investigated the direct interaction of virus and streptococci by applying a co-sedimentation assay to validate whether or not the bacterial effects were related to sialic acid. After the centrifugation step, I detected the lack of measurable HA activity and a decrease of viral infectivity in the supernatants of the samples. Additionally, the infectious viral particles could be detected in the pellet fraction indicating that both SIV
strains were co-sedimented with *S. suis* wt. Interestingly, SIV which bound to wt bacteria showed a different plaque morphology in MDCK cells compared to virus incubated in the absence of bacteria. Many of the plaques were distinctly smaller in size compared to the original control virus. The change of plaque morphology suggested a delayed virus replication and may reflect the co-infection situation in NPT*Tr* cells. When *S. suis* wt was pretreated with neuraminidase, the amount of SIV which co-sedimented with bacteria was limited. Furthermore, when I used *S. suis* Δ*cps* instead of *S. suis* wt in the co-sedimentation assay, the HA activity and viral infectivity were only marginally affected. These results demonstrate that there exists a direct binding between SIV and *S. suis*, and the sialic acids on the capsular polysaccharide were the key factor in this viral-bacterial interaction. Although by using scanning electron microscopy, a previous report obtained evidence that influenza viruses and paramyxoviruses binds to GBS (Hosaka et al., 1998), the binding efficiency and mechanism have not yet been well studied. According to my results, the direct binding between SIV and *S. suis* may act as a modulator in the viral-bacterial interplay which may affect the outcome of co-infection. Interestingly, the direct binding between SIV and *S. suis* could be reversed neither by viral neuraminidase nor by exogenous neuraminidase from *Clostridium perfringens*, suggesting that the linkages between SIV and *S. suis* were very tight and irreversible.

To further investigate the influence of the direct binding between SIV and *S. suis*, I performed a comparative hemagglutination inhibition (HI) assay. Both *S. suis* wt and Δ*cps* were used to analyze their ability to prevent agglutination of chicken erythrocytes by different influenza virus strains. In my results, encapsulated *S. suis* (wt) competitively inhibited the hemagglutination activity by all human and porcine viruses which have a binding preference for α2,6-linked sialic acid. Other viruses
which recognize α2,3-linked sialic acid, were not inhibited in its hemagglutination activity by *S. suis* wt. Consistent with the result of the co-sedimentation experiment, the HI activity of the bacteria was lost after pretreatment of *S. suis* with neuraminidase. Furthermore, I observed differences in the HI titer between different virus strains as already shown for SIV-H1N1 and SIV-H3N2 in the co-sedimentation analysis. Thus, these data indicate that the HA proteins of the respective viruses recognize the *S. suis* capsular polysaccharide with different efficiencies, and they also demonstrate that the direct interaction of SIV with *S. suis* may be regulated by the binding efficiency of the viral HA protein.

Whereas the HI and co-sedimentation assays indicated a direct interaction between the HA protein of SIV and the sialic acid on the capsular polysaccharide of streptococci, the increasing number of adherent *S. suis* still needs to be explained. To determine whether the adhesion ability of *S. suis* is affected by prior SIV infection or not, I performed another co-infection experiment under conditions which restricted the bacterial multiplication. It is known that mutants of *S. suis* lacking a capsule bind more efficiently to cultured cells than encapsulated wt bacteria (Benga et al., 2004). I obtained the same result when NPTr cells were only infected by *S. suis* under conditions that allowed bacteria multiplication. An increased number of adherent bacteria was observed after incubation for 2 h at 37 °C. But when the incubation time was shortened to 30 min at 4 °C, to obtain conditions that restricted multiplication, no binding of *S. suis Δcps* was detected on the NPTr cells, regardless whether the cells were pre-infected by SIV or not. On the other hand, *S. suis* wt bound to SIV-infected cells efficiently under these restricted adhesion conditions. My results indicate that the adherence of *S. suis* wt to SIV-infected cells is more efficient than that of streptococci lacking a sialylated capsular polysaccharide. Moreover, the two recombinant viruses
R1 and R2 differ only by two mutations in the sialic acid binding site that result in different binding preferences for sialic acid (Matrosovich et al., 2007). The preferential binding of R1 to *S. suis* wt was the direct evidence that the adherence of influenza virus to *S. suis* was mediated by the viral HA. The enhancement of adherence only occurs when cells were co-infected with *S. suis* and influenza virus which recognizes α2,6-linked sialic acid. This explains the mechanism of the increased bacterial adherence during the early stages of SIV and *S. suis* co-infection in the PCLS model. From the results, I hypothesize that the infection of pigs by SIV may facilitate the colonization by *S. suis*. Streptococci lacking sialic acid such as *S. suis* serotype 3 (Wang et al., 2013) cannot adopt this strategy to enhance bacterial infection. They can apply alternative strategies to enhance the infection efficiency during co-infections with influenza viruses. For instance, the host sialic acids released by the viral neuraminidase can be used as metabolites to support bacterial colonization (Siegel et al., 2014). Moreover, the removal of sialic acids from the cell surface may expose cellular ligands that can be used for bacterial adhesion (McCullers and Bartmess, 2003; Peltola and McCullers, 2004).

Interestingly, although SIV infection had a positive effect on the colonization by *S. suis*, the bacterial co-infection had a negative effect on SIV replication. The direct binding of influenza HA protein to sialic acid on the capsular polysaccharide may delay the SIV replication by the following reasons: (i) The viral-bacterial interaction may affect the attachment and internalization of SIV and result in a delayed replication. (ii) Prior to infection, the viruses have to be released from the bacterial surface by the viral neuraminidase and this enzymic action may be responsible for the delay in the replication. (iii) *S. suis* bound on the surface of SIV-infected cells has a detrimental effect on virus budding resulting in a reduced number of virus particles released into
the supernatant. However, it should be noted that the effect on SIV-infection may vary in co-infection with different streptococci. For example, when mice were infected by influenza virus (PR8) and *Streptococcus pneumoniae*, which lack sialic acid on the capsular polysaccharide, the infectivity of influenza virus was even enhanced under co-infection conditions compared to mono-infection (Smith et al., 2013).

During a single pathogen infection cycle, I found that a cytopathic effect of NPTr cells was induced only by influenza virus infection but not by *S. suis* inoculation (not shown). Similar results were observed in our PCLS *ex vivo* experiments including ciliostatic effects combined with loss of ciliated cells after SIV infection. In co-infections, the ciliary activity of PCLS was affected by SIV-H3N2 but remained unaffected by *S. suis*. This may impair the mucociliary clearance function, followed by a disruption of the primary defense barrier of epithelium, and increases the risk of secondary bacterial infection. Furthermore, my colleague and I observed that at a later stage (72 hpi) of infection, the ciliated epithelial cells which were damaged by SIV could promote *S. suis* adherence and invasion to the deeper tissue layers in PCLS.

Taken together, in this part of my thesis work, I discovered that in NPTr cells, the mutual interaction between SIV and *S. suis* has a benefit for the bacterial partner but an inhibitory effect on virus infection. The binding of SIV to *S. suis* was mediated by viral HA protein and the sialic acid on the bacterial capsular polysaccharide. A similar result was also observed at the early infection stage in the co-infection of PCLS, whereas at the later infection stage in PCLS the bacterial adherence was not sialic acid-dependent. The impairment of the mucociliary clearance function by SIV may play a crucial role in the promotion of bacterial adherence and invasion. These
findings provide an explanation of how secondary infection enhances the detrimental effect of influenza virus infection. However, viral-bacterial co-infection has to be considered as a multifactorial interaction between the two partners. Sialic-acid-dependent interaction is only one factor among these complex events. A previous study has shown that *S. suis* releases a protease which is able to activate influenza viruses and thus enhances infectivity in MDCK cells (Wang and Lu, 2008). This may be also a possible factor which enhances virus replication under bacterial co-infection conditions. Last but not least, it has to be noted that not all bacteria which contain capsular sialic acid interact with influenza virus via the HA protein. A report shows that influenza virus enhances *Neisseria meningitides* adhesion to the cells which is mediated by direct interaction between viral neuraminidase and capsular sialic acid on bacteria (Rameix-Welti et al., 2009). It would be interesting to investigate the interaction between influenza virus and other bacteria with sialic acid, for instance, influenza viruses which recognize α2,3-linked sialic acid and group B streptococci (GBS) which contain α2,3-linked sialic acid.
6.2 Host-pathogen interaction in an air-liquid interface culture system for differentiated porcine airway epithelial cells

6.2.1 An adequate model reflects porcine airway epithelium functions

Cultures of human differentiated airway epithelial cells (HAE) are widely used to analyze the infection by many respiratory pathogens, including influenza viruses, coronaviruses, adenovirus, mycoplasma and fungi (Dijkman et al., 2013; Homma et al., 2016; Kogure et al., 2006; Krunkosky et al., 2007; Lam et al., 2011), They are also applied as a model to study the effect of cigarette smoking on the airways (Kuehn et al., 2015). Differentiated porcine airway epithelial cells cultures promised to be also a suitable model to investigate the interaction between swine respiratory pathogens and pigs. The major challenge lies in the model establishment since the primary porcine epithelial cells turned out to be more sensitive than cells from other species and the suitable culture conditions had to be optimized. Although a previous study used NPTr cells under air-liquid interface (ALI) conditions to mimic the porcine airway epithelium, the differentiation process was restricted, e.g. no ciliated cells were generated (Delgado-Ortega et al., 2014). Therefore, I established cultures of primary differentiated porcine tracheal epithelial cells (PTEC) and porcine bronchial epithelial cells (PBEC) for the infection studies. PTEC and PBEC were isolated and maintained under ALI conditions to represent the tracheal and bronchial epithelium of pigs, respectively. The PTEC and PBEC cultures comprised ciliated cells, mucus-producing cells and also basal cells, which have been already observed in HAE cultures (Chan et al., 2010; Ibricevic et al., 2006; Kikuchi et al., 2004), and the cilia beating was detected under the light microscope. The presence of mucus and cilia indicated that both PTEC and PBEC cultures have a mucociliary clearance function, the primary defense mechanism against respiratory pathogens (Munkholm and Mortensen, 2014). Moreover, by using immunofluorescence microscopy, I confirmed that the PTEC and
PBEC cultures contained tight junctions and adhesion junctions, which are crucial for intercellular contacts and for the barrier function of the epithelium (Niessen, 2007). In brief, the differentiated PTEC and PBEC showed characteristics of the porcine airway epithelium and the cultures were polarized and pseudostratified similar to the in vivo situation.

A suitable well-differentiated epithelial cells culture may reduce the number of experiment animals, following the 3R principle of reduction, refinement, and replacement (Aufderheide et al., 2015). However, the major restriction in the establishment of a successful culture is the donor. Unlike other animal species, one of the advantages of PTEC and PBEC cultures is that the donors are available more easily. For example, in the ferret and mouse airway, the total amounts of collectable cells are limited due to the tiny airway, and the donors for human cells are rare and their health status is uncertain. To stabilize the source of donors, the Rho-associated protein kinases inhibitor Y27632 is widely used to enhance proliferation of human and mouse airway basal cells (Horani et al., 2013). In my study, I made a preliminary trial with Y27632 treatment of porcine cells. In spite of the human and mouse airway basal cells, the primary porcine airway basal cells could only be sub-cultured for 4 passages, and the cell differentiation was limited after reaching confluence on the filter (not shown). These results suggested that PTEC and PBEC were not suitable for Rho kinases inhibitor Y27632 treatment. The initiator of differentiation of swine airway cells appears to be different from HAE or mouse differentiated airway epithelial cells (MAE).

The ALI culture system of well-differentiated epithelial cells is a suitable model for influenza A virus (IAV) infection studies. It shows characteristics of the airway
epithelium: an environment made up from polarized cells with separate access to the apical and basal compartments, different expression pattern of sialic acids, and the mucociliary clearance function (Bateman et al., 2013). Compared to the in vivo pig infection model, both PTEC and PBEC may provide a deeper insight into the molecular changes in the respiratory epithelium, which represent the major target for IAV infection (Ibricevic et al., 2006; Matrosovich et al., 2004). The SIV strains used in this study only induce mild symptoms in infected pigs (Meng et al., 2013). This might be due to the effect of the innate and adapted immune response which shows the difficulty in studying the pathogenicity of viruses. Exogenous addition of immune cells may provide a closer look into the cell-cell interactions of the respiratory tract. For instance, the effect of adding macrophage to the infected HAE from the apical or basal surface may modulate the pathogen clearance as has been shown in recent studies (Lehmann et al., 2011; Ugonna et al., 2014). As far as the cell tropism study is concerned, influenza A viruses are not only targeting the airway epithelium, but also infecting alveolar type II pneumocytes (Weinheimer et al., 2012). Since the porcine PCLS comprise both of the cell types, it is impossible to distinguish whether the viruses were released from bronchioles or alveoli when we investigated the influenza virus replication in PCLS. Furthermore, unlike PCLS or immortalized cells which are maintained in medium, our PTEC and PBEC cultures were more suitable to study the virus entry and release due to the polarized cell morphology. Additionally, PCLS can only be maintain in medium for one week (Punyadarsaniya et al., 2011) which may not be adequate for the long term influenza infection to study the regeneration of the epithelium while the ALI cultures could be maintained over 8 weeks. Accordingly, by using the PTEC and PBEC cultures, I investigated the action and counteraction between influenza viruses and airway epithelium.
The sialic acids are widely known as receptor determinant on the host cell surface for the attachment of influenza A viruses (Londrigan et al., 2011). I have shown that both α2,3- and α2,6-linked sialic acids were expressed on the apical surface of PTEC and PBEC cultures with the α2,6-linkage type being predominant. The distribution of sialic acids on PTEC and PBEC was similar to that reported for porcine tissue sections (Punyadarsaniya et al., 2011; Sauer et al., 2014; Van Poucke et al., 2010) and showed the same pattern that is found in the porcine airway. On the other hand, although a previous study showed that the ciliated cells in porcine bronchioles epithelium contain both sialic acid linkage types (Punyadarsaniya et al., 2011), there is no report differentiating between sialic acids of the porcine trachea and bronchus, respectively. In this study, non-ciliated cells showed abundant expression of α2,6-linked sialic acids while ciliated cells contained both sialic acid linkage types in PTEC and PBEC cultures. Mucins and mucus-producing cells possessed α2,6-linked but not α2,3-linked sialic acids. My results obtained for the sialic acid expression on different cell types of the porcine airway epithelium were similar to that reported for the human airway epithelium (Ibricevic et al., 2006; Thompson et al., 2006). In contrast, mouse airway epithelium expresses only α2,3-linked sialic acid which is only present on ciliated cells (Ibricevic et al., 2006). The ferret airway epithelium mainly expresses α2,6-linked sialic acids whereas α2,3-linked sialic acids are detected in low amounts and present only on non-ciliated cells (Zeng et al., 2013). When the sialic acid distribution of different species is compared, only the porcine airway epithelium shows an expression pattern similar to that of human cells indicating that the PTEC and PBEC cultures were more suitable to investigate human influenza virus infection than MAE and ferret airway epithelial cells.
6.2.2 Interplay of PBEC and influenza viruses, the story of host-pathogen interaction

Throughout my studies, SIV strains sw/Bad Griesbach/06 (H1N1 subtype) and sw/Herford/07 (H3N2 subtype) were selected to determine the infection characteristics of SIV. Both viruses were isolated from clinical cases and represented two SIV lineages circulating in European pigs. The SIV strains were first inoculated from the apical surface of PBEC to simulate the classical infection route via the airway. SIV showed the same cell tropism for ciliated cells in both the PBEC and PCLS infection models. However, unlike the results from the PCLS study (Meng et al., 2013), both SIVs could infect non-ciliated but not mucus-producing cell in PBEC at 1 dpi. This might due to the differences between the culture conditions: the PCLS were maintained in medium while PBEC were maintained under ALI conditions. In other words, these results indicated that most SIV have a cell tropism toward ciliated and non-ciliated cells when infected from the apical side.

Human influenza viruses preferentially bind to α2,6-linked sialic acids whereas avian influenza viruses have a binding preference for α2,3-linked sialic acids (Rogers and Paulson, 1983). Most of the human influenza viruses target ciliated and non-ciliated cells which express α2,6-linked sialic acids in the human epithelium (Ibricevic et al., 2006; Thompson et al., 2006). By contrast, avian-like influenza viruses infect exclusively ciliated cells as α2,3-linked sialic acids are found in the human epithelium only in ciliated cells (Ibricevic et al., 2006; Matrosovich et al., 2004; Thompson et al., 2006). Combined with the results from previous studies, our findings show that porcine and human influenza viruses have the same cell tropism and porcine and human airway epithelial cells show a similar sialic acid expression pattern.
To further investigate the effect of the sialic acid binding preference, I used two human recombinant viruses, R1 and R2. R1 virus is derived from a human pandemic strain A/Hong Kong/1/68 (H3N2) and has a binding preference for human-like receptors ($\alpha$2,6-linked sialic acids). R2 differs from R1 by two mutations in the hemagglutinin which recognizes avian-like receptors ($\alpha$2,3-linked sialic acids) (Matrosovich et al., 2007). My results showed that both human-like (R1) and avian-like (R2) viruses could infect ciliated and non-ciliated cells in the PTEC and PBEC culture (not shown). These findings may partially support the mixing vessel theory that via aerosol infection, both human and avian influenza viruses could reassort in the pig respiratory epithelium. On the other hand, although a previous report suggested that the cellular entry of influenza virus does not only rely on sialic acids (Thompson et al., 2006), I could show that R1 virus replicated more efficiently than R2 in PBEC after apical inoculation. My results indicate that R1 virus might benefit from the predominant $\alpha$2,6-linked sialic acids presentation on the apical surface of the porcine epithelium.

In contrast to the airway infection route, I also inoculated the influenza viruses from the basal side of PBEC cultures to study the infection process related to systemic infection or opportunistic infections. The basal cells on the filter in PTEC and PBEC cultures present $\alpha$2,3-linked sialic acids as also reported by other studies (Bateman et al., 2013; Sauer et al., 2014) and may provide a site for influenza virus attachment. In my study, the replication rate of viruses showed no significant differences between R1 and R2 viruses when inoculated from basal side. These results indicated that the replication kinetics are not dependent from the binding preference for $\alpha$2,3- or $\alpha$2,6-linked sialic acids when virus is inoculated from the basal side. Moreover, both SIV strains could release large amount ($10^6$-$10^7$ FFU/mL) of infectious virus from the
apical surface of the epithelium for more than 8 days even when the viruses were inoculated from the basal side. This observation demonstrates that the infection via the airway is not the only possible route for influenza viruses.

Interestingly, SIV-H1N1 replicated significantly faster than H3N2 (P<0.05) when PBEC were infected from the apical surface, whereas in PCLS, it was the other way round (Meng et al., 2013). The difference in these results may be explained in that way that both SIV strains (SIV-H1N1 and SIV-H3N2) prefer to infect different airway locations. We could detect infectious virus released from lungs and bronchioles in our in vivo and PCLS experiments (Meng et al., 2013), which represent the lower part of porcine airway; by contrast, PBEC are derived from the primary bronchi.

Surprisingly, infectious SIV-H1N1 released from PBEC cultures at the later time points showed foci morphology different from that of virus harvested during early infection. The foci morphology of infectious viruses harvested at 3 dpi was similar to that of the original virus, whereas the foci size of virus collected at 6 dpi was increased from 20 μm to 200 μm in diameter when SIV H1N1 were infected from the apical surface. The change in the morphology could only be observed when SIV-H1N1 was inoculated from the apical side. No change in the foci morphology was observed when virus was inoculated from the basal side. A possible explanation of this difference in the foci morphology may be related to the proteolytic cleavage of the hemagglutinin. Previous studies have shown that the trypsin-like serine proteases, human airway trypsin-like protease (HAT) and transmembrane protease serine 2 (TMPRSS2) are presented at the apical surface of epithelial cells (Garten et al., 2015; Miki et al., 2003; Orikawa et al., 2012; Takahashi et al., 2001). Since I have confirmed by RT-PCR that PBEC expressed HAT and TMPRSS2 (not shown), change in the foci morphology after
Discussion

apical inoculation may be explained by the apical expression of HAT and TMPRSS2. But further studies are required to clarify this point. Nevertheless, SIV-H3N2 induced larger foci compared to the original SIV-H1N1, and this foci size of H3N2 was subject to no significant change during the whole infection period.

As far as the long term infection process is concerned, I studied the action and counteraction between airway epithelial cells and influenza viruses by using PBEC culture as a model. Previous reports have shown that influenza viruses target ciliated cells and induce apoptosis of infected cells (Ibricicvic et al., 2006; Zeng et al., 2013), as we found with SIV-infected PBEC. In addition, I observed not only apoptosis but also a dramatic loss of cilia at 8 dpi. This may be related to our results with SIV infected PCLS where we found a reduction of the ciliary activity and an induction of a ciliostatic effect (Meng et al., 2013). Furthermore, SIV-infected cells, including ciliated and non-ciliated cells, were found to be subject to apoptosis and detached from infected PBEC. The removal of dead cells resulted in a decrease of the epithelium thickness. Both the loss of cilia and the reduction in epithelial thickness are also observed in SIV-infected porcine trachea tissue explants and avian influenza virus (H1 and H7 subtypes)-infected human epithelium (Patrono et al., 2015; Qi et al., 2014). Likewise, although the mucus-producing cells were not targeted by SIV infection at the early time point, I observed virus-infected mucus-producing cells at 8 dpi. My results show that SIV damaged the mucociliary system and thus the primary defense mechanism of the epithelium. As a consequence of the impaired mucociliary clearance function of the porcine epithelium, the risk of secondary infection by inhaled microbes may be increased after SIV infection. In brief, IAV infection results in the loss of cilia, reduction in epithelial thickness and decreased mucociliary clearance of the porcine epithelium. However, only SIV infection had a detrimental effect on
porcine epithelium whereas human influenza virus infection only showed a mild effect in my study.

On the other hand, since the dead cells were detached from SIV-infected PBEC, the basal cells were exposed to the epithelial apical surface. I detected changes in the sialic acid distribution on the apical surface of PBEC, while the basal cells mainly presented α2,3-linked sialic acids. Therefore, IAV which recognize the avian-like receptor (α2,3-linked sialic acids) might gain benefit and attach to these modified basal cells of the porcine epithelium. With the expression of α2,3-linked sialic acids on the apical surface of SIV-infected PBEC at 8 dpi, the risk for secondary infection by avian influenza virus may be increased. When these cells are infected by two different influenza viruses, it may result in reassortment between SIV and an avian influenza virus as previously described (Castrucci et al., 1993; Scholtissek et al., 1985).

Interestingly, in my results, although SIV infection induced cell damage and showed a detrimental effect on the porcine epithelium, the physical barrier function of the epithelium, the tight junctions and the adherens junctions, were still maintained. Despite part of the infected cells were detached from the apical surface of the epithelium, the trans-epithelial electric resistance of PBEC showed no difference when compared to mock-infected control cells. Therefore, I hypothesized that the damaged ciliated cells were replaced by “specialized cells” by initiating the differentiation of basal cells, to compensate the gap and to form tight junctions. The basal cells in infected PBEC began the regeneration process but had not yet differentiated to ciliated cells or mucus-producing cells, respectively. Such a reorganization has also been observed in another report, where mice were infected by IAV in vivo, and a loss of suprabasal cells was observed in the early stage of infection,
but the epithelium was regenerated and cells re-differentiated at later time points after IAV clearance (Tata et al., 2013).

In parallel to regeneration of the epithelium, a change in the sialic acid expression pattern was detected. The majority of sialic acids exposed on basal cells was α2,3-linked sialic acids, whereas the reorganized basal cells presented both α2,3- and α2,6-linked sialic acids. This may indicate that the cells are in a differentiation process. Undifferentiated HAE cells have been shown to express α2,3-linked sialic acids and the expression of α2,6-linked sialic acids is increasing overtime during the differentiation phase (Chan et al., 2010). I hypothesize that the SIV-infected PBEC were recovering from infection, but still release large amounts of infectious virus. These results further explain why SIV showed a high mobility but low mortality in the field (Dee, 2014; Simon et al., 2014).

Taken together, the differentiated PTEC and PBEC are suitable models to study the interaction between porcine airway epithelial cells and respiratory pathogens. The polarized and pseudostratified epithelial cells show similarity to the in vivo situation and can be used to study the entry and infection strategies of respiratory pathogens. In my study, I used PTEC and PBEC to study the long term infection of influenza viruses. Dramatic loss of cilia, desquamation of infected cells and subsequent reduction of the epithelial thickness show the destruction of the epithelium induced by the influenza virus infection. Despite the detrimental effect of SIV infection, the epithelium still remains its barrier function and the basal cell regeneration is initiated. Moreover, in the intermediate of infection, the epithelial cells have lost their mucociliary clearance function and the basal cells are exposed on the apical surface. Therefore, the risk of secondary infection by inhaled pathogens, including avian
influenza viruses, may be increased. In short, my results showed the action and counteraction between SIV and the porcine epithelium and provide a promising approach to study the process of epithelial cell recovery after SIV infection. Last but not least, SIV-infected PTEC and PBEC supply a suitable platform to investigate secondary respiratory pathogens infection of the porcine airway epithelium.
6.3 Infection strategies of *Streptococcus suis* in well-differentiated porcine respiratory epithelial cells

*Streptococcus suis* is an important respiratory pathogen in piglets after weaning which can cause economic loss in the porcine industry worldwide (Goyette-Desjardins et al., 2014). While the capsular polysaccharide of *S. suis* serotype 2 is a critical virulence factor, other virulence (associated) factors might play a role (Gottschalk, 2014; Vanier et al., 2004). Among others, secreted proteins, such as suilysin are also considered as virulence factors for *S. suis* serotype 2 strains (Fittipaldi et al., 2012), but the related function and contribution toward *S. suis* pathogenesis are still under discussion. In order to study the mechanisms of bacterial infection and how the clinical symptoms are initiated, we further focused on the *S. suis* infection process on porcine respiratory epithelial cells. Although there are studies using differentiated human bronchial epithelial cells to investigate the infection of streptococci, such as *Streptococcus pneumoniae* (Nguyen et al., 2015), until now, there is no report related to *S. suis* infection. Therefore, my colleague and I designed and performed the bacterial infection on the porcine well-differentiated respiratory epithelial cells, including PCLS and porcine ALI cultures (PTEC and PBEC).

In this study, encapsulated *S. suis* serotype 2 strain 10 (wt), its noncapsulated mutant (Δcps; refer as 10cpsΔEF in Manuscript II) and its suilysin-deficient mutant (Δsly; refer as 10Δsly in Manuscript II & IV) were used to inoculate PCLS to study the adhesion ability of the bacteria. Compared to the results from *S. suis* wt and Δcps, Δsly showed a weak affinity towards the luminal surface of bronchioli. In contrast, *S. suis* wt and Δcps efficiently adhered to both ciliated cells and mucus/ mucus-producing cells of the bronchiolar epithelium, and colonized certain regions of the epithelium. These results demonstrate that the adherence of *S. suis* to PCLS may be facilitated by suilysin, in
agreement with the previous studies using immortalized human epithelial cell line (HEp-2) (Norton et al., 1999; Seitz et al., 2013).

The mechanisms of how suilysin promotes \textit{S. suis} adherence to the porcine epithelium were also studied by using porcine ALI cultures. In this part, the differentiated PTEC and PBEC cultures were inoculated by \textit{S. suis} wt, \textit{Δ}sly or suilysin-deficient unencapsulated mutant of wt strain 10 (\textit{Δ}cps\textit{Δ}sly; referred to as \textit{10Δ}cps\textit{Δ}sly in Manuscript IV). The results were similar to the PCLS infection studies: Adherent bacteria were mainly detected on ciliated cells, and the number of \textit{S. suis} wt adhering to PTEC and PBEC was significantly higher than for \textit{Δ}sly. Therefore, we concluded that suilysin can promote the adherence of \textit{S. suis} to well-differentiated porcine epithelial cells, which is in agreement with a previous study with the \textit{in vitro} HEp-2 model and the \textit{ex vivo} PCLS model (Meng et al., 2015; Seitz et al., 2013). Moreover, the adhesion number of \textit{S. suis} \textit{Δ}cps\textit{Δ}sly to PTEC and PBEC was significantly more efficient compared to \textit{Δ}sly, which considered a negative correlation between encapsulation and the adhesion ability. Depending on my previous work with the immortalized NPT\textit{r} cells and the published results from our collaborator with HEp-2 cells (Benga et al., 2004; Wu et al., 2015), the capsular polysaccharide of \textit{S. suis} modulated the adhesion capacity of \textit{S. suis}, despite the observation in our \textit{ex vivo} models, in which both \textit{S. suis} wt and \textit{Δ}cps showed similar numbers of adhering bacteria on PCLS (Meng et al., 2015). In contrast, for \textit{S. suis} \textit{Δ}cps\textit{Δ}sly only a meagerly invasive bacterial number could be observed, unless this strain showed the highest capacity of adherence to both PTEC and PBEC. Therefore, these data also point out that a lack of the capsule does not facilitate \textit{S. suis} invasion.
Interestingly, the invasive rate of *S. suis* wt into PBEC and PTEC were significantly higher than the two suilysin-deficient mutant strains, ∆sly and ∆cps∆sly. The same phenotype of the suilysin-deficient strain was also found *in vitro* using HEp-2 cells (Benga et al., 2004). These data demonstrate that suilysin promotes both the adherence and also the invasion of *S. suis*. Furthermore, although not directly comparable, suilysin-mediated invasion of *S. suis* into ALI cultures seems to be more efficient than in the immortalized cells system. This might be related to the different culture conditions. Since immortalized cell maintained in medium, the concentration of secreting suilysin from *S. suis* may be lower, due to dilution by the culture medium. As PBEC is maintained under ALI condition, the suilysin released from *S. suis* at the apical side of PBEC is not diluted and more efficacious than in the case of cells cultured with medium.

The cytotoxic effect on PTEC and PBEC was analyzed in this study since the effect has been reported to be correlated with the suilysin production (Segura and Gottschalk, 2002). The results showed that *S. suis* wt induced cytotoxic effects in both PTEC and PBEC whereas ∆sly and ∆cps∆sly did not damage the epithelial cells. Cytotoxic effects of suilysin can also explain the observed cell lesions at 48 hpi in PBEC which partially expanded toward the whole epithelium. Similar lesions were found in our previous co-infection studies in PCLS (Meng et al., 2015). These lesions and the impairment of the mucociliary clearance function may lead to the exposure of basal cells and promoted streptococcal infection. Furthermore, most of the apoptotic cells were found at the edge of area where the epithelial cell layer was damaged. These apoptotic cells co-localized with suilysin, indicating an involvement of suilysin in cytopathic effects, which may further be responsible for the damage and death in porcine epithelium.
Taken together, we demonstrated that suilysin contributes to (i) the adherence to airway epithelial cells, (ii) the loss of ciliated cells, (iii) apoptosis, and (iv) invasion by using two porcine well-differentiated respiratory epithelial cell models, PCLS (i) and PBEC (i to iv). Culture conditions may affect the result of invasion studies. For example, the enhanced efficiency of suilysin-mediated cytotoxic effects is only observed under ALI conditions but not in cells covered by medium. Accordingly, it will be interesting to analyze the co-infection of PBEC and PTEC by SIV and S. suis, since these pathogens show different characteristics when they are analyzed under ALI conditions or with PCLS/ NPTr cells.
6.4 Conclusions and outlooks

In my thesis, the interactions between porcine respiratory pathogens and the host have been analyzed under mono-infection and co-infection conditions. My colleague and I applied three different infection models: immortalized newborn pig trachea (NPT) cells, porcine precision-cut lung slices (PCLS) and air-liquid interface (ALI) cultures of primary porcine well-differentiated airway cells to analyze infection by swine influenza virus (SIV) and/or *Streptococcus suis* (*S. suis*) in more detail. I discovered several mechanisms of interaction between SIV and/or *S. suis* during the infection process:

(i) SIV has a beneficial effect on the bacteria whereas *S. suis* has an inhibitory effect on virus replication during co-infection in NPT cells. This effect could be explained by the interaction mediated by the SIV haemagglutinin (HA) protein that recognizes α2,6-linked sialic acid present on the *S. suis* capsular polysaccharide. This virus-bacteria binding delays the replication of SIV-H1N1 and SIV-H3N2. By contrast, the HA protein expressed on the SIV-infected cell surface promotes *S. suis* adherence and colonization to NPT cells. The sialic acid-dependent effect is also observed at the early stage in the co-infection of PCLS. At the late stage of infection, SIV damages the mucociliary clearance function of PCLS and promotes *S. suis* invasion.

(ii) SIV H1N1 and H3N2 target ciliated cells and non-ciliated cells but not mucus-producing cells in porcine ALI cultures and induce apoptosis in infected cells. SIV infection results in a dramatic loss of cilia, reduction of the epithelial thickness and exposure of basal cells on the apical surface of the epithelium. This increases the risk of secondary infection by inhaled pathogens including avian influenza viruses.
(iii) Despite the detrimental effect of SIV infection, the epithelium still maintains its barrier function and the regeneration of the epithelium is initiated by differentiation of basal cells into specialized cells.

(iv) Soluble suilysin facilitates \textit{S. suis} adherence and invasion to porcine respiratory epithelial cells. During the infection process, \textit{S. suis} induces suilysin-mediated apoptosis resulting in porcine epithelium impairment.

Apart from this, both culture systems, PCLS and ALI cultures, are valuable models to study the interactions between porcine respiratory pathogens and the host. Different from PCLS, porcine ALI cultures are maintained under ALI conditions and provide a similar environment as in the \textit{in vivo} situation. Compared to the irreversible ciliostatic effect on infected PCLS, it is striking that the recovery of the infected epithelium was observed in porcine ALI cultures. This finding suggests that porcine ALI cultures are a valuable model to study (i) virus entry and release, (ii) the SIV-induced effect and regeneration of the epithelium, (iii) the molecular changes during long term influenza virus infection. Accordingly, it is also worth to further study the action and counteraction between SIV, \textit{S. suis} and epithelial cells in co-infection by using the porcine ALI model.

In sum, these findings provide deeper insights into the pathogenesis of SIV and \textit{S. suis} co-infection in porcine epithelial cells. However, since co-infection is much more complex than mono-infection, the direct viral-bacterial interactions may not be the only mechanism during infection. Other factors such as immune response have to be analyzed to get a more comprehensive picture. Last but not least, since both SIV and \textit{S. suis} have a zoonotic potential, these findings may relevant also for viral-bacterial co-infection of human cells.
7. REFERENCES


Bateman, A.C., Karasin, A.I., and Olsen, C.W. (2013). Differentiated swine airway epithelial cell cultures for the investigation of influenza A virus infection and replication. Influenza and other respiratory viruses 7, 139-150.


Ebsen, M., Mogilevski, G., Anhenn, O., Maiworm, V., Theegarten, D., Schwarze, J., and Morgenroth, K. (2002). Infection of murine precision cut lung slices (PCLS) with respiratory syncytial virus (RSV) and chlamydophila pneumoniae using the Krumdieck technique. Pathology, research and practice 198, 747-753.


observed by electron cryo-microscopy. The EMBO journal 13, 318-326.


and genetic diversity of suilysin in Streptococcus suis isolated from different diseases of pigs and characterization of the genetic basis of suilysin absence. Infection and immunity 69, 7572-7582.


508-515.


of general virology 33, 159-163.


meningococcal capsules. Infection and immunity 77, 3588-3595.


present. Veterinary research communications 21, 381-407.


Wang, Y., Gagnon, C.A., Savard, C., Music, N., Srednik, M., Segura, M., Lachance, C., Bellehumeur, C.,


Yamada, S., Suzuki, Y., Suzuki, T., Le, M.Q., Nidom, C.A., Sakai-Tagawa, Y., Muramoto, Y., Ito, M., Kiso,


8. Appendix

8.1 Acknowledgments

First, I am particularly grateful to Prof. Dr. Georg Herrler for giving me the opportunity to join your group and to work on this very interesting topic. I truly appreciate your advice, encouragement and support throughout my research and Ph.D. study. Thank you and Bi-Ju for the care and support for my life in Germany.

I would like to express my thanks to Prof. Dr. Valentin-Weigand and Prof. Dr. Wolfgang Garten for the interesting discussions and suggestions during our meetings, as well as for evaluating this thesis.

In addition, I am very grateful to Dr. Fandan Meng for the perfect collaboration and the assistance in every field, as well as all the good time we had. Thanks to Dr. Maren Seitz for the collaboration and the recommendations during the co-infection projects.

I would like to thank Dr. Nadine Krüger, Dr. Markus Hoffmann, Dr. Jana Kirchhoff and Sabine Uhlenbruck for the technical support and for sharing your knowledge. That helped me a lot. Besides, I really enjoy spending time with you outside the lab!

Furthermore, I would like to thank all the colleagues and former members of our institute, in particular, Christel, Christine, Anna, Ann-Kathrin, Anne, Frau Kaps, Martina, Sandra B., Sandra G., Tanja, Tim and Wei. I am so glad to work with all of you.

I am grateful to the financial support of Georg-Christoph-Lichtenberg-Stipendium during my PhD study.

Last but not least, many thanks to my family, especially my husband, Dai-Lun Shin. This thesis could not have been finished without your support and encouragement! I also like to thanks my Taiwanese friends in Hannover. Our beer time is one of my best memories in Germany.