DOCTORAL THESIS

Immunoregulation of porcine dendritic cells by influenza viruses and *Haemophilus parasuis*

Lessons from *in vitro* studies

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Universitat Autònoma de Barcelona

IMMUNOREGULATION OF PORCINE DENDRITIC CELLS BY INFLUENZA VIRUSES AND *HAEMOPHILUS PARASUIS*

LESSONS FROM *IN VITRO* STUDIES

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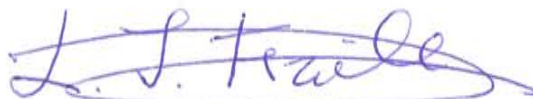
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De y Para Mis Amores

Yannich y Thay

What doesn't kill me makes me stronger.
Friedrich Nietzche

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.........termino uma etapa e começo outra. Cheguei a meta desta estapa graças a muitas pessoas de boa fé que me mostraram o caminho e me incentivaram a seguir em frente. São demasiados os sentimentos que se assomam quando depois de um percurso cheio de dificuldades, se chega a meta. Estou feliz e orgulhosa por terminar esta etapa. Primeiro agradeço a Allah subhanawataãla por pôr no meu caminho a todos vocês que contribuiram direta ou indirectamente para que chegasse a meta.

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> "*Não chores, meu filho;*
>
> *Não chores, que a vida É luta renhida;*
>
> *Viver é lutar.*
>
> *A vida é combate, Que os fracos abate,*
>
> *Que os fortes, os bravos Só pode exaltar*".

Muito obrigada a todos.

ABSTRACT/RESUMEN

ABSTRACT

Swine influenza virus (SwIV) is considered a zoonosis and the fact that swine may act as "mixing vessels" of influenza viruses potentially infectious for humans illustrates its relevance and the need to understand the interaction of different influenza viruses and other respiratory pathogens with the porcine immune system. Moreover, the clinical status of influenza infected animals may deteriorate due to secondary bacterial infection. Some of these bacteria such as *Haemophilus parasuis* are commensal of the respiratory tract of pigs. *H. parasuis* is a gram negative bacteria in the family *Pasteurellaceae* and the causal agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis and meningitis. Dendritic cells (DCs) mediate the induction of immunity against pathogens and the availability of DCs beneath the epithelium of respiratory organs makes them suitable targets for pathogens. However, their interaction with different influenza viruses or the role of DCs in co-infections has not been fully characterized.

In this thesis, three studies were undertaken in order to provide knowledge about the interaction of dendritic cells with porcine respiratory pathogens such as influenza viruses and *H. parasuis*.

In the first study, the goal was to study *in vitro* the interaction of porcine bone marrow derived DCs (poBMDCs) with SwIV. Thus, poBMDCs were exposed to a circulating strain of H3N2 SwIV. Infection of poBMDCs resulted in structures resembling influenza virus inside poBMDCs in vesicles and also free in cytoplasm. Viral progeny was undetectable in supernatant but limited replication was detected in the first 8h after infection. Moreover, viral particles from infected-poBMDCs were able to induce cytopathic effect in susceptible cells only when cell-to-cell interaction was favoured.

I

In the second study, poBMDCs were infected with swine, human and avian influenza viruses for 4, 8, 16 and 24h. Our main goal was to characterize *in vitro* the interaction of poBMDCs with different influenza viruses that may be found by the porcine immune system, such as: SwIV *A/Swine/Spain/SF32071/2007(H3N2)* (here referred as H3N2 SwIV), the 2009 pandemic influenza virus *A/Catalonia/63/2009(H1N1)* (here referred as hH1N1), the low pathogenic avian influenza virus (LPAIV) *A/Anas plathyrhynchos/Spain/1877/2009(H7N2)* (here referred as aH7N2) and the high pathogenic avian influenza virus (HPAIV) *A/Chicken/Italy/5093/1999(H7N1)* (here referred as aH7N1). Swine influenza virus H3N2 infection induced an increase of SLA-I and CD80/86 at 16 and 24h after infection (hpi) whereas the other viruses did not. All viruses induced expression of *NF-ϰB, TGF-β* and *IL-10* in poBMDCs to different extent and in a time dependent manner suggesting a specific function in cytokine production. The secretion of IL-12 was virus dependent mostly detected at 24hpi whereas IL-18 was secreted in response to all viruses. The IFN-α was detected only in H3N2 SwIV-infected cells while TNF-α was secreted in response to H3N2 SwIV, aH7N2 and aH7N1 infection. Inhibition of NF-ϰB resulted in a decrease of IFN-α and IL-12 secretion in H3N2 SwIV infected-poBMDCs at 24hpi, suggesting a role of this transcription factor on the synthesis of these cytokines.

In the third study, the immune response of poBMDCs in an *in vitro* co-infection with the H3N2 SwIV and non-virulent *H. parasuis* serovar 3 (SW114) or virulent serovar 5 (Nagasaki) was performed. At 1hpi, SW114 interaction with poBMDCs was higher than that of Nagasaki, while at 8h both strains showed similar levels of interaction. However, at ultrastructural level, Nagasaki induced more drastic cell damage when compared to SW114 infection. At 1hpi, no significant changes in SLA-I, SLA-II and CD80/86

expression were observed in DCs infected with either *H. parasuis* strain. Similar levels of IL-1β, IL-6, TNF-α, IL-8, IL-18, IL-10 and IFN-α were secreted by poBMDCs infected with both *H. parasuis* strains, being IL-12 differentially secreted by SW114-infected poBMDCs compared to Nagasaki-infected poBMDCs. However, poBMDCs co-infected with H3N2 SwIV and SW114 or Nagasaki showed a significant decrease in SLA-II expression compared to mock cells. Cells co-infected with either SW114 or Nagasaki secreted higher levels of IL-1β, TNF-α, IL-6, IL-12 and IL-10 compared to mock or H3N2 SwIV infection alone. Moreover, IL-12 and IFN-α secretion differentially increased in cells co-infected with H3N2 SwIV and Nagasaki.

Three main general conclusions can be drawn from this work: *(i)* poBMDCs are infected by H3N2 SwIV and they transmit the infection to permissive cells only when cell-to-cell contact was favoured; *(ii)* the cytokine profile and the mRNA expression of transcription factors after H3N2 SwIV, hH1N1 and aH7N2 or aH7N1 may indicate a specific immune response activation against these viruses; *(iii)* previous infection of poBMDCs with H3N2 SwIV followed by *H. parasuis* infection resulted in a profile of cytokines biased mainly towards inflammatory response.

Overall, these data pave the way for understanding the relation between respiratory pathogens such as influenza viruses and/or *H. parasuis* and porcine dendritic cells for triggering possible mechanisms driving to protective immune response.

RESUMEN

El virus de la gripe porcina (SwIV) es una zoonosis y el hecho de que los cerdos pueden actuar como "cocteleras virales" del virus de la gripe, potencialmente infecciosos para el ser humano, pone de manifiesto su relevancia y la necesidad de comprender la interacción de los diferentes virus de la influenza y otros patógenos respiratorios con el sistema inmune porcino. Además, el estado clínico de los animales infectados con la gripe puede agravarse debido a una infección bacteriana secundaria. Algunas de estas bacterias como el *Haemophilus parasuis* son comensales del tracto respiratorio de los cerdos. El *H. parasuis* es una bacteria gram-negativa de la familia *Pasteurellaceae* y es el agente causal de la enfermedad de Glässer, que se caracteriza por poliserositis fibrinosa, poliartrites y meningitis. Por otro lado, las células dendríticas (DCs) son importantes en la inducción de una respuesta inmune efectiva contra los patógenos. Su localización por debajo del epitelio de las vías respiratorias como células centinelas, les convierte en dianas de los patógenos. Sin embargo, su interacción con los virus de la gripe o su papel en las co-infecciones no ha sido totalmente caracterizado.

En esta tesis, se llevaron a cabo tres estudios con el fin de obtener conocimientos acerca de la interacción de las células dendríticas con los patógenos respiratorios porcinos, tales como los virus de la gripe y/o con *H. parasuis*.

En el primer estudio, el objetivo era estudiar la interacción *in vitro* de las células dendríticas derivadas de la médula ósea de cerdos (poBMDCs) con el virus de la gripe porcina. Para ello, las poBMDCs fueron expuestas a una cepa circulante de la gripe porcina (H3N2 SwIV). La infección de las poBMDCs resultó en la formación de estructuras similares a virus de la gripe dentro de

vesículas (de las poBMDCs) pero también libres en el citoplasma. En los sobrenadantes de las células infectadas no se detectó progenie viral pero se ha detectado una ligera replicación en las primeras 8h después de la infección. Además, las partículas virales de las poBMDCs infectadas fueron capaces de inducir efecto citopático en células permisivas sólo cuando se favorecía la interacción célula-célula.

En el segundo estudio, las poBMDCs fueron infectadas por los virus de la gripe porcina, humana y aviar durante 4, 8, 16 y 24 horas. Nuestro principal objetivo era caracterizar *in vitro* la interacción de las poBMDCs con los diferentes virus de gripe que pueden entrar en contacto con el sistema inmune del cerdo en la naturaleza como son: el virus porcino *A/Swine/Spain/SF32071/2007(H3N2)* (referenciado como H3N2 SwIV), el virus humano de la pandemia del 2009 *A/Cataluña/63/2009(H1N1)* (referenciado como hH1N1), el virus de la gripe aviar de baja patogenicidad (LPAIV) *A/Anas/plathyrhynchos/Spain/1877/2009(H7N2)* (referenciado como aH7N2) y el virus de la gripe aviar de alta patogenicidad (HPAIV) *A/Chicken/Italy/5093/1999(H7N1)* (referenciado como aH7N1). El virus de la gripe porcina H3N2 indujo el aumento de la expresión de SLA-I y CD80/86 a las 16 y 24h después de la infección (hpi), mientras los otros virus no. Todos los virus indujeron diferentes niveles de expresión de *NF-κB, TGF-β e IL-10* en las poBMDCs a largo del tiempo sugiriendo una función específica de estos factores/genes en la producción de citoquinas. La secreción de IL-12 fue dependiente del virus y detectada en su mayoría a las 24hpi mientras la IL-18 fue secretada en las células infectadas por todos los virus. El IFN-α se ha detectado solamente en la infección con el H3N2 SwIV mientras el TNF-α se ha detectado en respuesta a los virus H3N2 SwIV, aH7N2 y aH7N1. La inhibición de NF-κB resultó en una disminución de las cantidades de IFN-α e IL-12 a las 24hpi en las poBMDCs con el H3N2 SwIV,

sugiriendo un papel importante de este factor de transcripción en la síntesis de estas citoquinas.

En el tercer estudio, se evaluó *in vitro* la respuesta inmune de las poBMDCs en una co-infección con el H3N2 SwIV y con *H. parasuis* cepa no virulenta del serotipo 3 (SW114) o con la cepa virulenta del serotipo 5 (Nagasaki). Una hora después de la infección, la cepa SW114 interaccionó mas con las poBMDCs que la Nagasaki, mientras que a las 8h ambas cepas mostraron niveles similares de interacción. Sin embargo, Nagasaki indujo un daño ultraestructural más drástico en comparación con SW114. Después de 1h de infección, no se observaron cambios significativos en la expresión de SLA-I, SLA-II y CD80/86 en las células infectadas por ambas cepas de *H. parasuis*. Mientras, niveles similares de IL-1β, IL-6, TNF-α, IL-8, IL-18, IL-10 e IFN-α fueron secretados por las poBMDCs infectadas con ambas cepas de *H. parasuis*, siendo la IL-12 secretada diferencialmente por las poBMDCs infectadas con el SW114 en comparación con las infectadas con Nagasaki. Sin embargo, las poBMDCs co-infectadas con H3N2 SwIV y SW114 o Nagasaki mostraron una disminución significativa en la expresión de SLA-II en comparación con las células control. La células co-infectadas sea con SW114 o Nagasaki secretaron niveles más altos de IL-1β, TNF-α, IL-6, IL-12 e IL-10 en comparación con las células control o infectadas con H3N2 SwIV. Por otra parte, la secreción de IL-12 y de IFN-α fue diferencialmente mayor en las células co-infectadas con H3N2 SwIV y Nagasaki.

Tres conclusiones generales se pueden obtener de estos estudios: *(i)* las poBMDCs se infectan con el virus de la gripe porcina H3N2 y transmiten la infección a las células permisivas sólo cuando se favorece la interacción célula-célula; *(ii)* el perfil de secreción de citoquinas, la expresión de los mRNA de citoquinas y factores de transcripción después de la infección con los virus

H3N2 SwIV, hH1N1 y aH7N2 o aH7N1 pueden indicar una activación de la respuesta inmune específica contra estos virus; *(iii)* la previa infección de las poBMDCs con el virus de la gripe porcina (H3N2) seguida por la infección con *H. parasuis* resultó en un perfil de citoquinas sesgada mayoritariamente hacia una respuesta inflamatoria.

En conjunto, estos datos nos ayudan a comprender la interacción entre los patógenos respiratorios (como los virus de la gripe y/o *H. parasuis*) con las células dendríticas así como conocer posibles mecanismos involucrados en el desarrollo de la respuesta inmune protectora en el cerdo.

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ABBREVIATIONS

LIST OF ABBREVIATIONS

A

Abs- Antibodies

ADCC- Antibody-Dependent Cell-Mediated Cytotoxicity

AFC- Antibody Forming Cell

ANOVA- Analysis of Variance

APCs- Antigen Presenting Cells

B

BCR- B Cell Receptor

BMDCs- Bone Marrow Dendritic Cells

BMHCs- Bone Marrow Hematopoetic Cells

BSA- Bovine Serum Albumin

C

CAPE- Caffeic Acid Phenethyl ester

CCL- Chemokine Ligand

CCR- Chemokine Receptor

CD- Cluster of Diferentiation

cDCs- conventional Dendritic Cells

CFU- Colony Forming Unit

CPE- Cytopathic Effect

CPS- Capsular Polysaccharide

cRNA- complementary RNA

Ct- Threshold cycle

CTLs- Cytotoxic T Lymphocytes

CTLA4- Cytotoxic T Lymphocyte Antigen 4

D

DAPI- 4',6-diamidino-2-phenylindole

DCs- Dendritic Cells

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Dimethil Sulphoxide

DNA- Deoxyribonucleic Acid

Dp- Dendritic process

E

ELISA- Enzyme-Linked Immunosorbent Assay

EM- Electron Microscopy

F

FASl- FAS Ligand

Fc- Fragment crystallizable

FS- Foward Scatter

FITC- Fluorescein Isothiocyanate

FLT3- Fms-like tyrosine kinase 3

G

GM-CSF- Granulocyte-Macrophage Colony Stimulating Factor

GTPase- Guanosine Triphosphate hydrolyze

H

H_2SO_4 - Sulphuric Acid

HA- Hemagglutinin

HIV- Human Immunodeficiency Virus

HPAIV- High Patogenic Avian Influenza Virus

I

iBALT- inducible Bronchus Associated Lymphoid Tissue

ICA- Infectious Center Assay

ICAM1- Intercellular Adhesion Molecule 1

IFN- Interferon

Ig- Immunoglobulin

IL- Interleukin

iNKT- invariant NK T cells

iNOS- inducible Nitric Oxide Synthase

IRF- Interferon Regulatory Factor

ISGs- Interferon Stimulating Genes

J

JAK/STAT- Janus kinase/ Signal Transducer and Activator of Transcription

L

LCs- Langerhan Cells

LOS- Lipooligosaccharide

LPAIV- Low Pathogenic Avian Influenza Virus

LPS- Lipopolysaccharide

M

M1 or M2- Matrix protein 1 or 2

MAA-II- *Maackia amurensis* type II

MDCK- Madin-Darby Canine Kidney cells

MFI- Mean Fluorescence Intensity

MHC- Major Histocompatibility Complex

MLST- Multilocus Sequence Typing

MoDCs- Monocyte derived DCs

MOI- Multiplicity of Infection

mRNA- messenger RNA

Mx- Mixovirus gene

N

NA- Neuraminidase

NALP 3- NOD Pyrin domain containing protein 3

NEP- Nuclear Export Protein

NF-𝑥B- Nuclear Factor kappa B

NIPCs- Natural Interferon Producing Cells

NK- Natural Killer

NKG2D- NK activating receptor

NKp46- NK cytotoxic receptor

NLSs- Nuclear Localization Signals

NOD- Nucleotide Oligomerization Domain

NOS- Nitric Oxide Synthase

NP- Nucleoprotein

NS1 or NS2- Non-Structural protein 1 or 2

O

OIE- International Office of Epizooties (World Organization for Animal Health)

ORF- Open Reading Frame

P

PA- Polymerase Acidic

PACE- Paired basic Amino Acid Cleaning Enzyme

PAMs - Porcine Alveolar Macrophages

PAMPs- Pathogens Associated Molecular Patterns

PB1 or PB2- Polymerase Basic protein 1 or 2

PB- Phosphate Buffer

PBMC- Peripheral Blood Mononuclear Cells

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PCV2- Porcine *Circovirus* type 2

pDCs- plasmacytoid Dendritic Cells

PE- Phycoerythrin

PFA- Paraformaldehyde

PI3- Phosphatidylinositol 3-kinase

PK-15- Porcine Kidney cells

poBMDCs- porcine BMDCs

Poly:IC- Polyinosine-Polycytidylic acid salt

PRRs- Pattern Recognition Receptors

PRRSV- Porcine Reproductive and Respiratory Syndrome Virus

R

RIG- I- Retinoic Inducible Gene I

RLR - RIG-like Receptos

RNA- Ribonucleic Acid

RNP- Ribonucleoprotein

rpGM-CSF- recombinant porcine GM-CSF

RPMI- Roswell Park Memorial Institute medium

RT- Room Temperature

RT-qPCR- Real Time quantitative PCR

S

SLA- Swine Leukocyte Antigen

SNA- *Sambucus nigra*

SPF- Specific Pathogen Free

SS- Side Scatter

SWC- Swine Workshop Cluster

SwIV- Swine Influenza Virus

T

TA- Transwell Assay

$TCID_{50}$- 50% Tissue Culture Infective Dose

TCR- T Cell Receptor

TGF-β- Tumour Growth Factor beta (β)

Th- T helper

TLR- Toll-like Receptor

TNF-α- Tumour Necrosis Factor alpha (α)

TRAIL- TNF-Related Apoptosis-Inducing Ligand

TRITC- Tetramethylrhodamine B isothiocyanate

V

VLPs- Virus Like Particles

vRNPs- viral Ribonucleocapsids

W

WHO- World Heath Organization

1. GENERAL INTRODUCTION

The most beautiful experience we can have is the mysterious. It is the fundamental emotion which stands at the cradle of true art and true science.

Albert Einstein

1.1. Influenza viruses.

1.1.1. Nomenclature.

Influenza viruses are enveloped, single stranded RNA viruses in the family *Orthomyxoviridae*. This family comprises five genera: influenza virus A, B, C, Thogotovirus (which includes Thogoto and Dhori viruses) and Isavirus (which includes infectious salmon anemia virus) [1]. Influenza A viruses are further classified into subtypes based on the antigenicity of their HA and NA molecules. Currently, 17 HA (H1-H17) being the H17 recently described in bats [2] and 9 NA subtypes (N1-N9) are known [3]. The present nomenclature system includes the type of virus, the host of origin (except for humans), the geographic site of isolation, the strain number, and the year of isolation, followed by the antigenic description of the HA and NA subtypes in parenthesis [1,4]. For example, *A/swine/Spain/SF32071/2007(H3N2)* describes an influenza A virus isolated from pig in Spain in 2007 with an identification number SF32071 and it is a H3N2 subtype. Although, many types of HA and NA has been identified among influenza A genera, reassortment between members of different genera has never been reported. This absence of genetic exchange between viruses of different genera is one manifestation of speciation as a result of evolutionary divergence [1].

1.1.2. Molecular biology of influenza A viruses.

Influenza A and B viruses posses segmented genome of eight single-stranded negative-sense RNA molecules that typically encode 11 or 12 viral proteins [5] (**Fig. 1**). The influenza virion is pleiomorphic, forming spherical virions that are ~100 nm in diameter [6], as well as filamentous virions that are ~100 nm in diameter but reaching over 20 µm in length [7] or filamentous forms that exceed 300 nm in length [8].
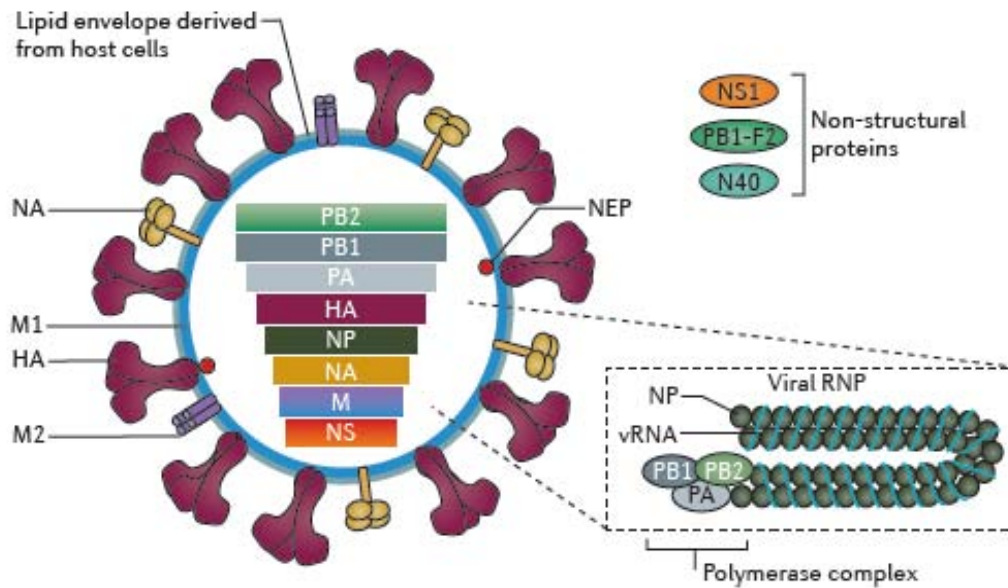
Figure 1. **Molecular composition of influenza virus**. The influenza A virus genome consists of eight single-stranded RNAs that encode 11 or 12 proteins. These are nuclear export protein (NEP; also known as NS2) and the host antiviral response antagonist non-structural protein 1 (NS1), which are encoded by the NS segment; the matrix protein M1 and the ion channel M2, which are encoded by the M segment; the receptor-binding protein hemagglutinin (HA), the sialic acid-destroying enzyme neuraminidase (NA), nucleoprotein (NP), and the components of the RNA-dependent RNA polymerase complex (PB1, PB2 and PA), all expressed from their respective genome segments; and the newly identified N40 protein, which is expressed from the PB1 segment. Within the virion, each of the eight viral segments forms a viral ribonucleoprotein (RNP) complex: viral RNA is wrapped around NP, and this structure is then bound to the viral polymerase complex. Image from reference [5].

The **viral envelope** consists of a lipid bilayer containing transmembrane proteins on the outside and matrix protein (M1) on the inside. The lipids that compose the envelope are derived from the host plasma membrane and are selectively enriched in cholesterol and glycosphingolipids. Three transmembrane envelope proteins hemagglutinin (HA), neuraminidase (NA), and M2 (ion channel) are anchored in the lipid bilayer of the viral envelope.

HA, a type I transmembrane protein[1], is a homotrimer and is the major envelope protein (~80%) forming the spikes [3]. The HA provides the receptor-binding site and elicits neutralizing antibodies. Cleavage of HA is essential for fusion and virus infectivity. The *NA, a type II transmembrane protein*, is present as a homotetramer on the viral envelope. The NA removes the cell surface receptor (sialic acid) and is critical for release of virus particles from the cell surface and spread of virus. Finally, the *M2 a type III transmembrane protein*, is a minor protein of the viral envelope. The M2 is a homotetramer, functions as an ion channel and is crucial during uncoating for dissociating the virus ribonucleocapsids (vRNP) from M1 in the early phase of the infectious cycle [3].

The **viral core** consists of helical vRNP containing vRNA (negative stranded) and nucleoprotein (NP) along with minor amounts of the nuclear export protein (NEP) (also called non-structural protein NS2) and three polymerase (3P) proteins (PB1, PB2, PA) which form the vRNA polymerase complex (3P complex) [1,9,10]. Recently a new protein, N40 expressed by the PB2 segment was discovered which similarly to PB1-F2[2] is non-essential for virus viability but its loss is detrimental to virus replication [11]

1.1.3. Infection cycle of influenza A viruses.

The replication cycle of influenza virus is summarized on **Figure 2**. Overall, six complex steps compose the replication cycle of influenza virus namely: attachment, the entry and uncoating of vRNP, synthesis of vRNA, synthesis of viral proteins, packaging of viral RNA and assembly, virus budding and release. *In vitro,* for different purposes including vaccine development, studies

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[1] They are single-pass trans-membrane proteins which have their N-terminus exposed to the extracellular or luminal space, while type II membrane proteins have their C-terminus exposed to the extracellular or luminal space. Type III membrane proteins have multiple transmembrane domains in a single polypeptide chain.

[2] It is a pro-apoptotic protein, which is encoded by a second ORF in the PB1 segment.

on influenza virus are mainly carried out in polarized cells [12]. Thus, MDCK were used for many years as a model to study the replication cycle of influenza virus *in vitro*. A body of accumulating evidences indicate that complete influenza viral particles are not found inside the infected cell and the process of assembly, morphogenesis, budding and release of progeny virus particles takes place at the plasma membrane of the infected cells [7]. These events are crucial for the production of infectious virions and pathogenesis of influenza virus. Although defective particles[3] occurs in RNA viruses, they do not replicate independently, but they may alter the course of an infection by recombination with the genome of a replication-competent virus [13]. Defective particles can profoundly influence the course of virus infection. In some cases they appear to moderate the pathogenesis, whereas in other cases they potentiate it making the symptoms of disease much more severe. These particles cause restricted gene expression and result in a persistent infection by a virus that normally causes an acute infection and is rapidly cleared from the body [13].

a) Virus attachment.

Influenza virus particles bind to cell surface sialic acid, ubiquitously present on glycoproteins or glycolipids of host cells through HA [14,15]. The specificity of the sialic acid (α-2,3-linked or α-2,6-linked sialic acid) and the selective binding of a particular strain of influenza virus to a specific sialic acid receptor are important determinants for species-specific restriction of influenza viruses [16-18]. Once the virus is attached, cleavage of HA occurs and this is an absolute requirement for infectivity [10,19]. The nature of the HA cleavage site is an important virulence determinant for influenza viruses. Cleavage efficiency of HA varies depending on the presence of single or multiple basic

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[3] They are virus particles containing partial deletion of the genome and they are replication defective.

residues at the cleavage site and plasminogen[4] binding ability of NA [19]. After cleavage, the HA2 portion mediates the fusion of virus envelope with the cell membrane, while the HA1 contains the receptor binding and antigenic sites [20,21]. When the HA is cleaved, the virus enter the cell through clathrin mediated endocytosis or macropinocytosis [8,22-26]. The mechanism by which the virus enters to cells will be described further in the **section 1.1.4.**

b) Virus entry, vRNA uncoating and transport into the nucleus.

During the infectious cycle, virus particles, that bound to cell surface sialic acid, are internalized by receptor-mediated endocytosis and those with cleaved HA undergo fusion with the endosomal membrane at low pH (pH 5.0). The low pH of the endosome has two main important functions: *firstly* it triggers a conformational change in the HA, exposing a fusion peptide that mediates the merging of the viral envelope with the endosomal membrane, thus opening a pore through which the vRNP are released into the host cell cytoplasm. *Secondly*, the hydrogen ions from the endosome are pumped into the virus particle via the M2 ion channel [26-28] allowing acidification of the virion. The acidification disrupts internal protein-protein interactions, allowing the release of vRNP from the viral matrix into the cytoplasm [29].

c) Synthesis of viral mRNA.

When vRNP are release into the cell cytoplasm, they are trafficked to the host nucleus, by viral proteins nuclear localization signals (NLSs), where replication occurs [30,31]. In the nucleus, vRNA is synthesized. Here, the vRNA is transcribed by the viral RNA-dependent RNA polymerase into two positive sense RNA species: *(i)* the messenger RNA (mRNA) which serve as template for viral protein synthesis and *(ii)* a complementary RNA (cRNA) from which

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[4] It is a precursor enzyme (zymogen) which, following partial cleavage by a plasminogen activator is converted to its active and proteolytic form, plasmin. Its primary target is fibrin, but it is also able to degrade several constituents of the extracellular matrix and to convert a number of pro-hormones and cytokine precursors to their active form.

the RNA polymerase will transcribe more copies of negative sense, genomic vRNA, that will form the virion [8]. Differently from the host cell mRNA, which is polyadenilated by a specific poly(A) polymerase, the poly(A) tail of influenza virus mRNA is encoded in a negative sense vRNA as a stretch of 5-7 uracil residues, which the viral polymerase transcribes into the positive sense as adenosines [8,32-34]. This capped process occur as a "cap-snatching" phenomena in which the virus PB1 and PB2 proteins "steal" 5´capped primers from host pre-mRNA transcripts to initiate viral mRNA synthesis [35,36]. Then, the mRNA is exported from the nucleus to the cytoplasm where it is translated. The nuclear export of vRNA segments is mediated by the M1 and NEP/NS2 complex [30].

d) Synthesis of viral proteins.

In this phase the proteins PB1, PB2, PA, NP, NS1, NS2 and M1 are produced in the cytoplasm. While the envelope proteins, HA, NA and M2 are synthesized from viral origin mRNA on ribosome into the endoplasmic reticulum, where they are folded and trafficked to the Golgi apparatus for post-translational modification, little is known about the synthesis of non-envelope proteins (PB1/2, PA and NS1/2). All the three envelope proteins have apical sorting signals that will direct them to the cell membrane for virion assembly. M1 interacts with both vRNA, NP and with NEP mediating the M1-RNP export via nucleoporins into the nucleus [8] or bringing the complex RNP-NEP into contact with the envelope-bound HA, NA and M2 proteins for packaging at the host cell membrane [8,31].

e) Packaging of RNA and virion assembly.

Formation of vRNP complexes takes place in the nucleus. It results from binding of newly synthesized PB1, PB2, PA, NP and NS2 proteins to vRNA. To be infectious, a single virus particle must contain each of the eight unique

RNA segments [3,8]. Since virus assembly is a highly inefficient process, where more than 90% of the virus particles are non infectious as they have packed very few or excessive viral gene segments; packaging signals on all vRNA segments are important at the moment that full genome is incorporated into the new formed virus particle [37].

f) Virus budding and release.

Finally, when all the new viral proteins were formed, the M1 protein bring the complex RNP-NEP into contact with the envelope-bounded HA, NA and M2 proteins for packaging at the host cell membrane [8,31]. Then, NA releases progeny from host cells by using its destroying activity to cleave the terminal sialic acid residues of the cell surface glycotoproteins and gangliosides. Also, viral NA by removing the sialic acids from its virus envelop prevents the virus aggregation and enhances virus infectivity [38-40].

It is important to note that, influenza viruses are genetically unstable due to drift[5] and shift[6] antigenic mechanisms [41,42]. Apart from the drift and shift mechanisms, that occurs in ssRNA viruses, the presence on pig's trachea of both receptors for human (2,3 α-sialoadesins) and avian (2,6 α-sialoadesins) viruses [5] makes the possibility of reassortment and outbreaks of new and more pathogenic virus reliable [9,43]. Examples are the 1918 "Spanish flu" pandemic influenza virus which had a common ancestor with the porcine H1N1 virus that caused influenza outbreaks in pigs in the same period [44,45]; and more recently the 2009 pandemic influenza virus originated from pigs which had six genomic RNA segments from triple reassortant swine viruses.

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[5] It refers to random mutations in the genes of a virus or to accumulation of mutations within the HA or NA antibody-binding sites and as consequence, the resulting viruses cannot be inhibited well by antibodies against previous strains.

[6] Refers to the process by which at least two different strains of a virus (or different viruses), specially influenza A, combine to form a new subtype having a mixture of the surface antigens of the two original strains.

During the reassortment in pigs this virus acquired the HA and M segments from the Eurasian avian-like swine virus [46,47].
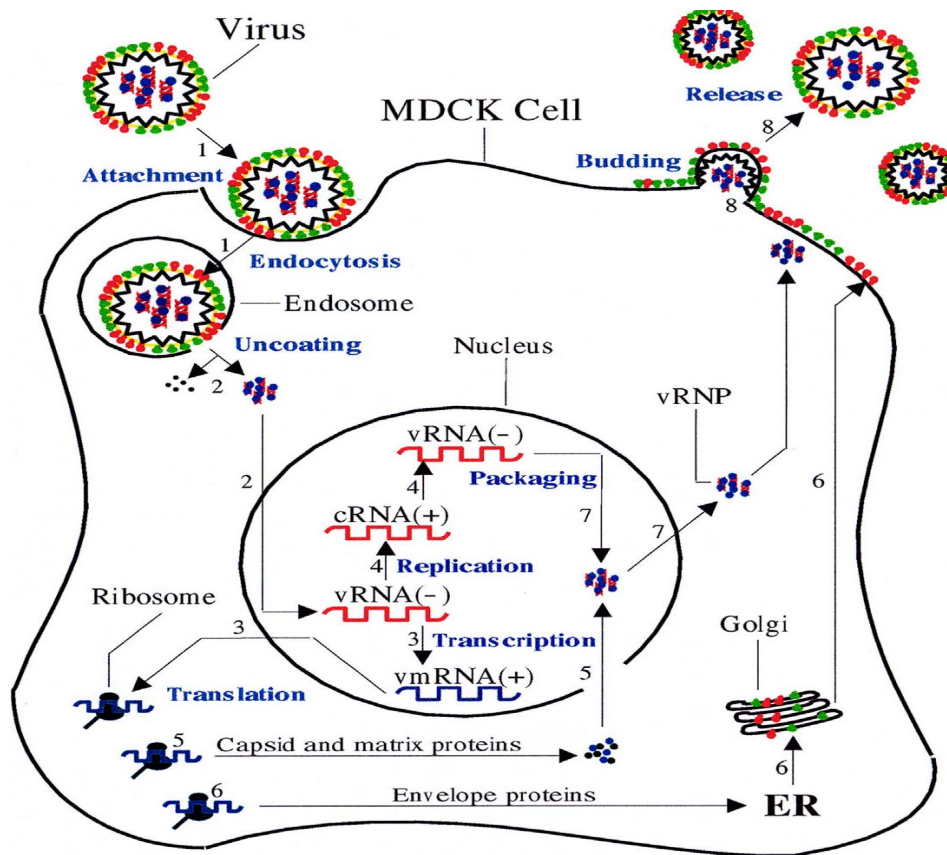
Figure 2. **Schematic illustration of influenza A virus replication cycle**. The different steps are assigned by numbers. *1:* Virus attachment and entry into the cell which is a receptor mediated process. *2:* Virus uncoating and import of vRNA into the nucleus through nuclear localization signals (NLS). *3:* Synthesis of viral mRNA and proteins. *4:* Synthesis of viral genome. *5 and 6:* Synthesis of viral proteins (genome, non structural, matrix and envelop proteins). *7:* Packaging of viral components. *8:* Budding and release of progeny. Image modified from reference [48].

1.1.4. Portal of entry into the cell.

The plasma membrane is a dynamic structure that functions to separate the intracellular milieu from the extracellular environment by regulating and coordinating the entry and exit of small and large molecules. In this context,

macromolecules and pathogens must be carried into the cell in membrane bounded vesicles derived by the invagination and pinching-off of pieces of the plasma membrane in a process called endocytosis **(Fig. 3)** [23].

Once influenza virus attaches to the target cells, it is internalized through clathrin mediated endocytosis or macropinocytosis [8,22-26]. The endocytosis is a complex process that occurs by multiple mechanisms which fall into two broad categories, phagocytosis and pinocytosis.

Phagocytosis[7] is typically restricted to specialized mammalian cells, whereas pinocytosis occurs in all cells by at least four basic mechanisms: macropinocytosis[8], clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin-and-caveolae independent endocytosis [23,24]. These mechanistically diverse and highly regulated endocytic pathways function to control complex physiological processes such as hormone-mediated signal transduction, immune surveillance, antigen presentation, and cellular and organism homeostasis [23].

Apart from mediated endocytosis and phagocytosis, DCs can ingest antigens by micropinocytosis and macropinocytosis processes that do not involve specific recognition receptors but capture whatever might be in the fluid phase in the surrounding area of DCs [49]. For example, recently was shown that Vaccinia virus, an enveloped virus, enters to DCs via macropinocytosis [50].

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[7] It is an active and highly regulated process involving specific cell-surface receptors and signalling cascades mediated by Rho-family GTPases (small proteins that regulate many aspects of intracellular actin dynamics, and are found in all eukaryotic organisms including yeasts and some plants).

[8] It is also regulated by Rho-family GTPases, which trigger the actin-driven formation of membrane protrusions. However, unlike phagocytosis, these protrusions do not 'zipper up' along a ligand coated particle, but instead they collapse onto and fuse with the plasma membrane.
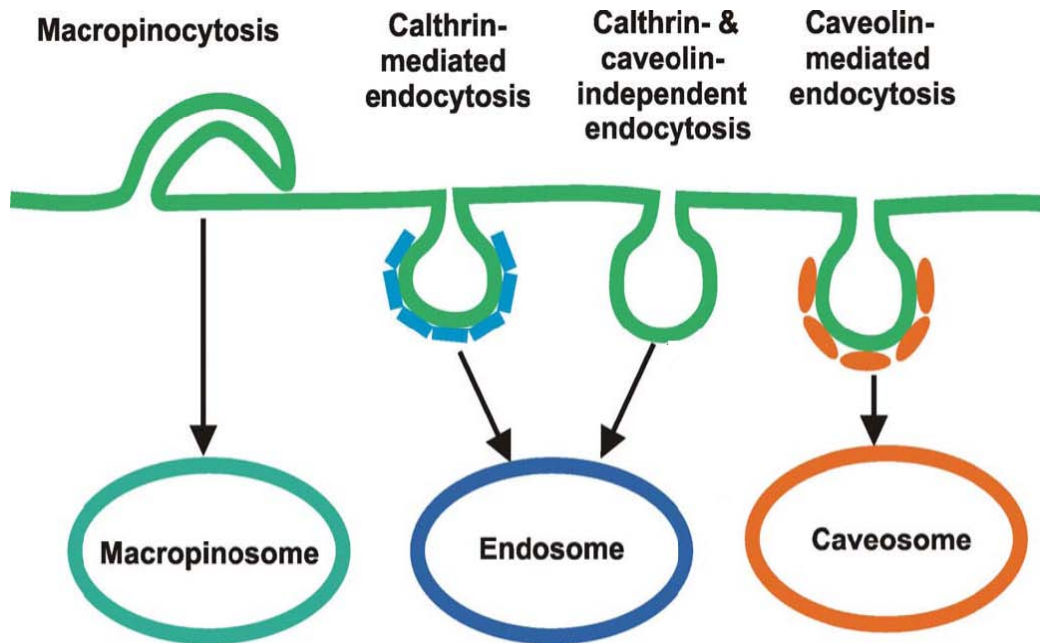
Figure 3. Possible pathways exploited by influenza viruses to infect cells. Figure modified from reference [24].

Another mechanism of cell uptake is trogocytosis; which involves the transfer of plasma membrane fragments from the presenting cells to T lymphocytes through the immunological synapse. This mechanism is specifically triggered by TCR and BCR on T and B cells respectively [51,52], by NKG2D and NKp46 receptors in NK cells [53] and by various receptors on the other cells including Fc receptors [54]. Moreover, it is thought that trogocytosis may involve components of both the endocytosis and phagocytosis machinery such as ubiquitinylation, dynamin[9], clathrin recruitment, PI3 kinase, Src family kinase and actin rearrangement [54].

1.1.5. Pigs: an interesting model for human research.

Pigs are well accepted as "mixing vessels" of influenza viruses and their role on the emergence and epidemiology of influenza pandemic is well documented. However, the molecular viral characteristics of influenza A viruses that determine their ability to be transmitted within and between species are poorly understood. Therefore, a big interest of studying the role of pigs on the molecular biology and virus life cycle is emerging. Also, the

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[9] It is a GTPase responsible for the endocytosis in eukaryotic cells, by scission of new formed vesicles.

immunological factors associated to the antigenic shift and drift are of great interest.

For these purposes one good approach is using pigs or porcine immune cells to understand these mechanisms. The selected approach (animals or cells) should be easy to handle and available for multiple researchers [55]. Actualy, it is believed that by using pigs or swine immune cells, the conditions under investigation will better achieve and replicate the response of humans to a certain infection [55,56]. Indeed, recently was demonstrated that porcine bone marrow derived macrophages resemble human macrophages in response to LPS; sharing with humans the genes involved in tryptophan metabolism, lymphoattractant, chemokines and vitamin D3-converting enzyme [57]. Additionally, humanized piglets have a "great potential" for use in microbiome research [58].

When pigs are selected as model, the possibility of using surgical and non-surgical procedures used in human medicine such as catheterization, heart surgery, valve manipulation, endoscopy and broncho-alveolar lavages, canulation of lymph vessels, allows the study of DCs migration from periphery during several days, which is not possible with human or mice. Moreover, the pig is more closely related to the human, anatomically, genetically and physiologically, when compared to mice [55,56,59,60]. Highlighting these similarities, pig-to-primate organ xenotransplation are being used successfully [61].

Because, in this thesis different influenza A viruses were employed in *in vitro* studies, through the next pages, a brief description of SwIV, the 2009 pandemic H1N1 and avian influenza viruses is presented. The intention is showing some particularities of the pathogenesis of these viruses.

1.2. Swine influenza viruses.

The disease caused by influenza viruses in pigs is essentially similar to that recorded in human, with low mortality but high morbidity [62,63]. Infection with SwIV is generally limited to the respiratory tract, with virus replication demonstrated in epithelial cells of the nasal mucosa, tonsils, trachea, lungs, and tracheobronchial lymph nodes [64]. Natural or experimental infection with influenza in swine is characterized by a rapid onset of high fever, dullness, loss of appetite, laboured abdominal breathing and coughing. Weight loss can be considerable, but mortality is low and recovery occurs within 7–10 days [65,66].

Actual evidences demonstrate the co-circulation of swine H1N1, H1N2 and H3N2 subtypes in Europe [67-69]. The maintenance of H3N2 and H1N1 viruses in pigs and the frequent introduction of new viruses from other species may be a key factor in the emergence of pandemic strains of human influenza as reported in the last years [42,46,47,65]. Subclinical infections are also very common and many, if not most pigs become infected with one or more influenza virus subtypes without ever showing clinical signs [68].

Furthermore, it is well known that SwIV can contribute to more chronic, multifactor respiratory disease problems in combination with other viruses or bacteria [65,66,70].

1.3. The 2009 human pandemic influenza virus.

Human pandemics[10] are caused by viruses that people have little or no immunity [71]. Recently, in spring of 2009, a novel influenza A virus of H1N1 subtype with human-to-human transmission emerged, and by june 2009, reached pandemic levels [72]. Molecular epidemiology studies revealed that the outbreak was caused by the new swine-origin influenza A (H1N1) virus

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[10] It refers to outbreaks on a global scale with sustained human-to-human transmission.

emerged in Mexico and United States. This new virus had the capacity of human-to-human transmission and was a result of several viruses circulating in swine. The initial transmission to humans occurred several months before recognition of the outbreak [47]. This novel virus was also shown to be different from the seasonal virus as it posses six genomic RNA segments from triple reassortant swine viruses and acquired their HA and M segments from the Eurasian avian-like swine virus [46,47].

In humans, this virus mostly caused a mild self-limiting upper respiratory tract illness with fever, cough and sore throat, myalgia, malaise, chills, rhinorrhea, conjunctivitis, headache and shortness of breath. The spectrum of clinical presentation varies from asymptomatic cases to primary viral pneumonia resulting in respiratory failure, acute respiratory distress, multi-organ failure and death [73,74]. These findings indicated that 2009 H1N1 virus causes lower respiratory tract infections, in contrast to seasonal influenza viruses, whose typically affect the upper respiratory tract [75]. Secondary bacterial infections were reported in a substantial number of cases and its presence exacerbated the course of the disease [71].

Also, during the pandemic, it was observed that children had been more susceptible to the virus while the elderly had some pre-existing immunity [71]. Moreover, certain factors such as asthma, autoimmune diseases, cardiovascular disease, diabetes, obesity and pregnancy (mostly in the 3rd trimester) were shown to be involved in an increased risk of developing severe disease [76,77].

In contrast to efficient replication in humans and pigs, avian species such as chickens, ducks, and turkeys were shown to be resistant to experimental infection with the 2009 H1N1 virus [78,79].

1.4. Avian influenza viruses.

In the nature wild birds, predominantly ducks, geese and gulls are the reservoir of influenza A viruses. Till now, from the 17 known antigenic HA subtypes, 16 subtypes and all 9 NA subtypes have been described in birds [3,80].

Besides the classification based on HA and NA, avian influenza A viruses are also classified by their pathogenic properties in chickens. Influenza A viruses of subtypes H5 and H7, but not other subtypes, may become highly pathogenic after introduction into poultry and can cause outbreaks of *highly pathogenic avian influenza (HPAI)*. The transition from the *low pathogenic avian influenza (LPAI)* phenotype to the HPAI phenotype is achieved by the introduction of basic amino acids into the hemagglutinin cleavage site, which facilitates systemic virus replication, causing an acute generalized disease in poultry in which mortality may be as high as 100% [81,82]. As a result of those mutations, most LPAI viruses show only a basic amino acid in its HA cleavage site, whereas the HPAI viruses acquire an increased number of basic amino acids in their proteolytic cleavage site [83,84]. As a consequence, the LPAI viruses can only be cleaved by trypsin-like proteases produced by the epithelial cell of the respiratory and gastrointestinal tract. Therefore, these viruses cause only mild symptoms restricted to these organs. In contrast, the presence of multibasic cleavage sites in the HA of HPAI viruses make them more accessible to ubiquitous proteases of the furin[11] enzyme family [71,85]. This differential cleavage of the HA in HPAI viruses allow their replication in a larger number of tissues, including brain, heart, pancreas, adrenal gland and kidney, among others, causing an extremely severe systemic disease with high fatality rates [83]. However, this characteristic is only present in viruses of H5 and H7 subtypes, although most of H5 and H7 subtype viruses are of low

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[11] Also named PACE (Paired basic Amino acid Cleaving Enzyme) they are pro-protein convertase that process latent precursor proteins into their biologically active products.

virulence [86]. It is also worth mentioned that viruses of H5 and H7 subtypes have been able to infect humans, causing severe and fatal infections such as the ones reported with the Asian HPAI virus H5N1, and flu-like symptoms and conjunctivitis with H7 virus [87].

Pigs are also susceptible to natural or experimental infection with LPAI and HPAI avian influenza viruses. And, most of the LPAI virus subtypes found in pigs have an H1 or H3 usually restricted to birds [65]. However, as for LPAI viruses, it is doubtful whether HPAI viruses replicate efficiently in pigs and whether they are readily transmitted among pigs [65]. Indeed, recently surveillance studies found that H5N1 avian influenza virus was circulating in pigs in Indonesia [88] without causing disease. Moreover, in this study authors reported that one isolate recognized human-type receptor, meaning that the H5N1 can replicate for longer periods undetected and facilitate the adaption of avian virus to mammalian hosts [88].

1.5. Transmission of influenza A viruses.

In humans, avian and pigs, the general routs of influenza virus transmission include aerosol, large droplet and direct contact with secretions of infected individuals or contaminated fomites[12] [89]. Transmission via direct contact with infected individuals is thought to be the major transmission route [90-92].

1.6. Prevention/control of influenza A viruses.

Vaccination is the primary strategy for the prevention of influenza in humans [93,94] or to control virus spread among herds and prevent possible

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[12] It is any inanimate object or substance capable of carrying infectious organisms (such as bacteria, viruses or parasites) and hence transferring them from one individual to another.

transmission of swine viruses to humans [95,96]. In humans, although both inactivated and live attenuated vaccines are effective in preventing influenza and its associated complications, the protection they confer varies widely depending on the antigenic match between the viruses in the vaccine and those circulating during a given influenza season and on the recipients age and health status [97]. It is why the emergence of novel pandemic (avian or swine origin) influenza virus illustrate the need of developing novel vaccines as well as improved methods of immunizations to control the spread of these novel virus [98]. To counteract these difficulties in humans, recombinant DNA techniques are being used allowing the production of vaccine candidates as soon as the HA genetic sequence of the virus causing the outbreak is known [99].

In swine even being the H3N2, H1N1 and H1N2 the only influenza A virus infecting predominantly pigs worldwide, the current commercially available inactivated vaccines are not efficacious due to the diverse multitude of genetically different viruses co-circulating in swine herds [96]. The lack of efficay may be due to the fact that SwIV vaccines manufacturers are not obliged to regularly replace their vaccine strains in order to antigenically match the currently circulating viruses. Most commercial SwIV vaccines contain different H1N1 and/ or H1N2 virus strains, including older isolate from the 1970s or the 1980s or more recent viruses isolated after the 2000 [95,96].

Considering the efforts in improving the influenza vaccine efficacy, the goal in human and veterinary fields is to achieve an "universal vaccine" that is safe, elicits humoral and cellular responses identical to those triggered by a natural infection, produce long-lasting and cross-strain protection and that can be manufactured rapidly in large amounts under well-controlled conditions [96,98,99].

1.7. Diagnosis of influenza A viruses.

For a definitive diagnosis of influenza, laboratory confirmation is required. The success of virus diagnosis mostly depends on the quality, handling, transport and storage conditions of the sample before it is processed in the laboratory. The samples for virus isolation (in cell cultures or embryonated chicken eggs) or for the direct detection of viral antigen or nucleic acids should be taken during the first 3 days after onset of clinical symptoms of influenza [93].

In humans and pigs, influenza is primarily a respiratory tract infection while in avian species influenza can be an infection of both respiratory and large intestinal tract [100].

Influenza viruses can be isolated in cell lines, primary cells susceptible to influenza infection or embryonated chicken eggs. Madin-Darby canine kidney cells (MDCK) is the preferred cell line, however, for SwIV, primary swine kidney, swine testicle, swine lung or swine tracheal cells can be used [100,101]. For virus typing, hemagglutination inhibition test, neuraminidase inhibition test, fluorescent antibody test, immunohistochemistry, antigen-capture ELISA and PCR are used. The standard procedures for these techniques are described in the OIE Terrestrial Manual and WHO Manual on Animal Influenza Diagnosis and Surveillance [100,101].

1.8. Respiratory disease complex.

The respiratory disease in pigs is common in modern swine production worldwide and is often referred as porcine respiratory disease complex. This disease complex results from infection with various combinations of primary and secondary respiratory pathogens such as *Mycoplasma hyopneumoniae, Pasteurella multocida, Actinobacillus pleuropneumoniae, Haemophilus parasuis* and *Streptococcus suis* [102]. *S. suis, H. parasuis, Escherichia coli* and *P. multocida* were

isolated frequently in association with the porcine reproductive and respiratory syndrome virus (PRRSV) [103]. Among these pathogens, PRRSV, SwIV and porcine circovirus type 2 (PCV2) are common virus involved in the respiratory disease complex [104].

1.8.1. *Haemophilus parasuis.*

a) Taxonomy and pathology.

Haemophilus parasuis is a non-motile, gram negative, small pleomorphic rod, varying from single cocobacillus to long, thin, filamentous chains, in the family *Pasteurellaceae* [105-107]. Fifteen serovars of *H. parasuis* have been defined, and the virulence of each reference strain was determined. Thus, serovars 1, 5, 10, 12, 13 and 14 have been classified as highly virulent; serovars 2, 4 and 15 moderately virulent; and serovars 3, 6, 7, 8, 9 and 11 have been considered non-virulent [108]. *H. parasuis* is the causal agent of Glässer's disease, which is characterized by fibrinous polyserositis and polyarthritis [106,109].

b) Pathogenesis.

The pathogenesis of *H. parasuis* remains unclear, but disease production is influenced by stress, early colonization of pigs by virulent strains and immune status of the animals [110]. However, the ability to isolate *H. parasuis* from the nasal cavity and trachea but not from tonsils or lung specimens from slaughter houses indicates that the bacteria preferentially colonize the upper respiratory tract [106]. Thus, virulent strains derived from systemic lesions, were found to be conformed into the systemic clade on the multilocus sequence typing[13] (MLST) and were classified as being phagocytosis and serum-resistant, while

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[13] It is a technique used in molecular biology for typing of multiple loci. This procedure characterizes isolates of bacterial species using the DNA sequences of internal fragments of multiple housekeeping genes.

the non virulent strains derived from the nostril of healthy piglets belonged to the nasal clade on MLST and were classified as being phagocytosis and serum-sensitive [111-113].

The mechanisms of virulence are not fully elucidated; however, recent studies have identified proteins from *H. parasuis* membrane such as the trimeric autotransporter proteins that could be involved in virulence [114,115].

Regarding the virulence of some strains of *H. parasuis,* it is suggested that invasion of endothelial cells may be related to the ability of some strains to cause meningitis [116]; additionally the adherence and invasion of virulent strains to porcine kidney cells PK-15 compared to no-virulent strains, could be involved in the lesions caused by *H. parasuis* [117]. It is also suggested that *H. parasuis* uses cellular adhesion, induction of apoptosis (in a caspase-3 dependent manner) and up-regulation of inflammatory mediators such as IL-8 and IL-6 to invade the newborn pig tracheal cells and the central nervous system via the blood brain barrier [118,119].

The majority of studies mentioned before were done in non-immune cells (PK-15, brain endothelial cells, pig epithelial cells….etc); however no studies were conducted in porcine DCs.

In relation to virus and bacteria co-infection *in vivo*, it was reported that combined infection of pigs with typically low pathogenic organisms like PCV2 and *M. hyopneumoniae* can result in severe respiratory disease [104]. Also, an initial infection with *M. hyopneumoniae* before SwIV (H1N1) inoculation increased influenza clinical signs and pathogenesis which was not observed with H1N2 infection [120]. Authors suggest that *M. hyopneumoniae* and H1N1 SwIV appeared to act synergistically, whereas with H1N2 would compete, since H1N2 infection led to the elimination of *M. hyopneumoniae* in lung

diaphragmatic lobes [121]. In another study it was shown that previous infection of caesarean and colostrum deprived pigs with *M. hyopneumoniae* followed by SwIV induced severe lung lesions [122].

1.9. Dendritic cells (DCs).

1.9.1. Discovery and main functions of DCs.

In 1973, Ralph Steinman and Zanvil Cohn, identified cells with a distinctive dendritic morphology **(Fig. 4)** as a rare component of mouse spleen cell suspension [123]. From then, many studies were done, and actually DCs are considered to be the critical antigen presenting cells (APCs) that mediate between the innate and adaptive immune systems. DCs collect and process antigens for presentation on major histocompatibility complex (MHC) molecules to T lymphocytes. Also they sense the environment via innate receptors for inflammatory mediators, for damaged cells or for microbial products, and then direct an appropriate adaptive immune response from cells reactive with the presented antigen. In the quiescent state, the presentation of self-antigens by the DCs serves to maintain self-tolerance. Following pathogen invasion, the DCs are activated by the signals that receive via their innate receptors. Then, T cells reactive with the foreign antigens presented by DCs are driven to an immune response directed to infection [124-128].

Several properties of DCs make them the most efficient APCs for initiation of primary T cell response: *(i)* DCs are strategically located at the common sites of entry of microbes and foreign antigens and in tissues that may be colonized by microbes, *(ii)* DCs express receptors that enable them to capture microbes and to respond to microbes, *(iii)* these cell migrate from epithelia and tissues preferentially to the T cell zones of lymph nodes, through which naïve T lymphocytes circulate, searching for foreign antigens and finally *(iv)* mature

DCs express high level of peptide-MHC complexes, costimulators, and cytokines, all of which are needed to activate T cells [49,129-132].

1.9.2. Origin and differentiation of DCs.

Dendritic cell progenitors are in bone marrow, and they give rise to circulating precursors that home to tissues where they reside as immature cells with high phagocytic capacity. Following tissue damage, immature DCs capture antigens and subsequently migrate to the lymphoid organs, where they select rare Ag-specific T cells thereby initiating immune responses [129].
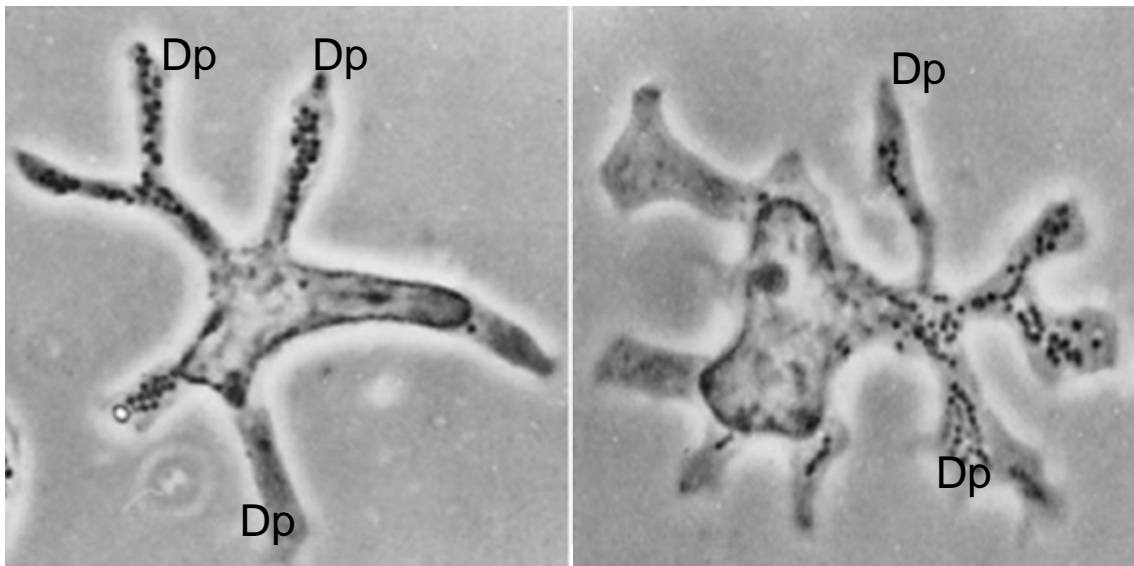
Figure 4. Phase-contrast micrographs of spleen dendritic cells fixed in glutaraldehyde. The dendritic processes (Dp) described by Steinman, at that time, may be observed. Images from reference [123].

a) Heterogeneity of DC subsets in humans and mice.

DCs are a heterogeneous group of cells that have been divided into different subsets. This segregation is based on their distinct patterns of cell surface molecule expression. Thus, DCs can be divided into two major categories: **conventional DCs (cDCs)** and **plasmacytoid DCs (pDCs)** [133].

Conventional DCs constitute the major groups of DCs important in viral infections. Among the cDCs three subpopulations are described *(i)* the migratory DCs, which differentiate in the peripheral tissues, such as skin and mucosal tissues, *(ii)* the lymphoid tissues-resident DCs that do not migrate, but develop *in situ* and live their entire life within the lymphoid tissues and *(iii)* the monocyte-derived or inflammatory DCs which appears to differentiate into DCs in response to inflammation [124].

The migratory DCs are derived from precursors that develop in the tissues or traffic from bone marrow. The classical example are the Langerhans cells (LCs) which are found in the squamous epithelium of the skin, while DCs migrating from the subcutaneous tissue (dermis, sub mucosa, lamina propria and intestinal layers underlying the epithelium) are collectively known as interstitial DCs [134,135]. Migratory DCs constitutively migrate to lymph nodes via the afferent lymphatic vessels, potentially providing a mechanism by which the immune system can continuously receive information about the tissue environment [124].

On other hand, the *Lymphoid-resident DCs* are the only DCs type found in mice spleen and thymus, differentiated by CD4 and CD8 co-receptor expression [136-139]. These cells develop *in situ* as result of precursors seeded from the blood [140]. These cells also play important roles in the initial activation of protective T cells in viral infections [124,133].

Finally within the cDCs population are the *Monocyte derived DCs (MoDCs)* which differentiate from blood circulating monocytes in an inflammatory environment [124,140,141].

The second groups of DCs are the **(pDCs) or naturally interferon producing cells (NIPC).** Albeit their few amounts in the peripheral blood cells (0.2%–0.8% of peripheral blood mononuclear cells (PBMCs)) [142], they are the major sources of IFN for their ability to rapidly release high levels of IFN-α during pathogen infections [142,143]. This feature of pDCs is due in part to the fact that these cells, more than other cell types, abundantly express the endosomal TLR 3, 7, 8 and 9, which recognize nucleic acids of viruses that have been internalized into the cell [144]. Phenotypically, pDCs appear to be relatively immature, expressing no co-stimulatory molecules and only low levels of MHC II molecules [145].

In general, pDCs are considered to be poor stimulators of T cells compared with cDCs during viral infection as their main role is migrate from blood, enter lymphoid tissues and produce large amounts of type I IFN to suppress viral invasion [146,147].

Although research in DCs were mostly carried out in mice and human, immune cells with the same phenotype and functions as DCs were identified in horses, cat, cattle, sheep, rat, non-human primates, dog, chicken, guinea pigs, rabbits, and other species such as reptiles, amphibians and pigs (reviewed in [148]).

Considering that pigs are the target specie in this thesis, through the next pages we will describe some knowledge on porcine immune system with special interest in DCs.

1.9.3. Porcine DCs.

The percursors of pig DCs in bone marrow or in blood are SWC3 or CD172a positive cells an indicative of their myeloid origin [149]. Bone marrow derived

DCs (BMDCs) have been used as a successfully tool to study the biology [150-154] and the immune response against influenza virus [155-158] or porcine respiratory bacteria [159] *in vitro*. The data generated by using this approach, may support the knowledge of using pigs or swine immune cells as suitable approach to study human diseases and also animal health.

The use of BMDCs has advantages compared to the use of MoDCs since the lasts require large amounts of blood, more than one cytokine to induce differentiation and time to isolate monocytes and then derive them into DCs.

1.9.4. Porcine DC subsets and its differentiation *in vitro*.

In vitro, porcine DCs can be differentiated from blood monocytes and from bone marrow hematopoietic cells (BMHCs).

Porcine DCs can be obatined by using **blood monocytes** with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [150,153], GM-CSF/IL-4 or GM-CSF/IL-4/IFN-α "cocktails" [152]. For this generation, IL-4 can be replaced by IL-13 [160]. After 3-7 days of culture, non-adherent or semi-adherent cells with dendritic morphology can be obtained.

Additionally, porcine DCs can be obtained by using **BMHCs** with GM-CSF/TNF-α "cocktail" or GM-CSF alone for 7-10 days [150]. In contrast to GM-CSF, the use of Flt3 ligand (Flt3L) in BMHCs induces the differentiation of both cDCs and pDCs as in human and mice [142]. Furthermore, cDCs derived from Flt3L, are phenotypically and functionally different from that generated with GM-CSF, either from monocytes or from BMHCs [150].

The MoDCs differentiated with GM-CSF and IL-4 resemble human MoDCs since they are in an immature state, with up-regulated CD80/86, MHC-I and II, and T-cell stimulatory activity upon maturation, while inflammatory chemokine receptors such as CCR1 and macropinocytic activity are down

regulated [150,152,153,161]. The transforming growth factor beta (TGF-β) allows the generation of cells with Langerhans cells characteristics [153]; meaning that cytokine modulation is important for manipulating the type of DCs generated.

Phenotypically porcine MoDCs and BMDCs (generated with GM-CSF alone) are characterized as being $CD172a^+$ $CD1^+$ $CD4^-$ $CD11R1^-$ $CD14^+$ $CD16^+$ $CD80/86^+$ and $SLA-II^+$ [56,150,153,160,162].

In the small intestine of pigs, **mucosal DCs** can be found in the Peyer´s patches, as $MHC-II^+$ cells without T and B-cell markers and in the lamina propria of the intestine [163].

In the upper **respiratory tract DCs** are $CD16^+$ and $MHC-II^+$ cells. In the tracheal mucosa many DCs are located above the basal membrane and inside the epithelial layers where they form a dense network with many cytoplasmatic processes probably related to their important role as immunological sentinels in this organ [164].

As in humans and mice, **porcine pDCs** are specialized in secreting large amounts of type I IFN [142,151]. These cells represent less than 0.5% of the porcine peripheral blood mononuclear cells (PBMCs) [151,154].

Regarding pDCs in pigs, their phenotype remains to be fully characterized. While Summerfield *et al.,* (2003, 2009) describes porcine pDCs as $CD172a^{low}$ $CD4^{high}$ $CD14^-$ $CD163^-$ within the PBMCs population expressing low levels of CD3, CD5 and CD6 as well as no CD21 nor CD2 [56,154], Calzada-Nova *et al.,* (2009) describes that pDCs are within the $CD172a^+$ $CD4^{high}$ subset but also

express CD1a$^+$ CD11a$^+$ CD16$^+$ CD11c$^-$ CD11b$^-$ CD18^{low} CD44$^+$ CD29$^+$ CD45RC$^-$ CD56$^-$ CD163$^-$ and MHC-II^{low} phenotype [165].

1.10. Innate and adaptive immune responses.

An immune response is the culmination of a sequencial interactions/signalling between the presenting cells and the effector cells. This response starts with the interaction of T cells with APCs through the TCR and MHC molecules. During this interaction three signals are delivered. The signal one requires the recognition of the peptide loaded on MHC by TCR. Therefore a second singal is delivered by co-stimulatory molecules which together with signal one induce immunity. Finally, signal three results from the APCs to T cells and determine the differentiation of the lasts in effector cells [131]. Signal three is mainly composed by soluble mediators such as cytokines that will induce the differentiation of T cells in effector cells [166-170]. The cytokines involved in immune responses are summarized in **table 1.**

Cytokine Profile	Th1	Th2	Th17	Treg
Secreted cytokines	IFN-γ; TNF-α	IL-4; IL-2; IL-5; IL-10; IL-13	IL-17; IL-21; IL-22	TGF-β IL-10
Inductor cytokine	IL-12; IL-18; IL-27; IFN-γ	IL-4	IL-6/TGF-β; IL-21/ TGF-β; IL-23	IL-10 TGF-β IL-2
Pathogens cleared	Intracellular bacteria; protozoal parasites; fungi; viruses.	Extracellular pathogens; including helminths and nematods.	Similar to Th1 but also in autoimmune diseases.	Maintain self-tolerance.

Table 1. **Types, differentiation and functions of effector T cells**. Table modified from reference [170].

1.10.1. Immune response against influenza A viruses.

Infections with influenza evoke host immune responses which result ultimately in abortion of virus replication.

a) Innate immunity against influenza virus.

The innate immune system forms the first line of defence against influenza virus infection. It consists of components, such as mucus and collectins, which aim to prevent infection of respiratory epithelial cells. Rapid innate cellular components like **Macrophages, DCs and Natural killer cells** are recruited with the objective of controlling virus replication and dissemination. These cells secrete different types of chemical mediators such as cytokines that will activate the T cells and induce their differentiation in memory cells or elicit adaptive responses (**Fig. 5**).

Influenza A virus infection, is sensed by infected cells via PRRs that recognize viral RNA, the main PAMP of influenza. The PRRs are Toll-like recepors (TLRs), retinoic inducible gene I (RIG-I)-like receptors (RLR) and the nucleotide-binding oligomerization domain [171]-like receptor family pyrin domain containing protein 3 (NALP3) [172].

The TLR7 binds single-stranded viral RNA especially in pDCs; while TLR3 and RIG-I bind double-stranded viral RNA in most other infected cells. Signalling by these receptors leads to production of pro-inflammatory cytokines and type I interferons (IFN-α, β) [173,174]. In this way, firstly, IFN-β is produced and via positive feedback, regulated by interferon regulatory factor 7 (IRF-7), the expression of both IFN-α and IFN-β is stimulated [175-177]. These interferons have strong antiviral activity by inhibiting protein synthesis in host cells and limiting virus replication. Type I IFNs also induce interferon stimulating genes (ISGs) via the JAK/STAT signalling pathway [178] (and reviewed in [179]) and one of ISGs is the

orthomyxovirus resistance gene (Mx) gene that encodes the MxA protein, a GTPase, with strong antiviral activity that can inhibit influenza virus replication [180]. Besides viral inhibition, the type I IFNs secreted by infected cells also induces an "antiviral state" in neighbouring un-infected cells [176], blocking in this way the infection.

While the immune system has developed mechanisms to inhibit virus replication by IFN system, the virus also evolved strategies to attenuate the IFN response to lower levels that allow replication and transmission within their host [179]. These mechanisms of virus evasion include the NS1 protein which is highly expressed in the cytoplasm and nuclei of infected cells where it is able to interact with different components involved in the IFN response and inhibiting it [175]. Moreover, other proteins from the virus such as PB1-F2, PA and the M2 (reviewed in [179]) or PB2 [157,181] have been implicated in the inhibition of IFN system.

The activation of innate immune cells by PAMPs may activate the nuclear factor ϰB (NF-ϰB) a central orchestrator of inflammation and immune responses. NF-ϰB has been shown to have a critical role in homeostasis of cells of the immune system by maintaining the expression of pro-survival genes [182,183] and up-regulating a variety of antiviral genes [184]. NF-ϰB has been described as the major host signalling pathway implicated in the replication of influenza virus [185,186]. Moreover, in influenza virus infection, the activation of NF-ϰB was shown to be a result of an over expression of viral proteins such as HA, NP and M1 during virus infection [187]. Thus, NF-ϰB is commonly activated upon virus infection resulting in the activation of an array of cytokines and chemokine genes [188].

Recently it was reported that porcine DCs infected with porcine H1N1 virus expressing the PB2 of avian H5N1 influenza virus, induced more nuclear translocation of NF-ϰB compared to native porcine H1N1 virus [157].

After influenza virus infection, innate immune cells are recalled to block virus propagation, either by phagocytosis of infected cells or by producing cytokines that will activate NK or T cytotoxic cell to kill infected cells. In mice, upon infection, **macrophages** in one hand become activated and phagocytose influenza virus infected cells limiting viral spread [189], and on the other hand, they produce nitric oxide syntase 2 (NOS2) and TNF-α, contributing to influenza virus induced pathology [190]. In pigs, alveolar macrophages were shown to be indispensable for controlling influenza virus in lung [189] and TNF-α was associated with fever and pulmonary lesions in influenza infected animals [191].

The **natural killer cells (NK)** are important effectors cells of the innate immune response. In mice, NK cells recognized antibody-bounded influenza virus infected cells through the receptors NKp44 and NKp46, and lysed them in a process called antibody dependent cell cytotoxicity (ADCC) [192,193]. Also in mice, invariant NKT (iNKT) cells stimulate the induction of cellular immunity and regulated infection induced pathology [194]. In pigs, only recently a mAb specific for NK was identified [195]. And also recently, iNKT cells were identified in pig lungs [196]. With the identification of NK cell marker in pigs, probably many studies on the role of these cells in influenza infected pigs will be addressed.

The **DCs** are important innate components on influenza virus infection. Their location underneath the airway epithelium barrier and above the basal membrane allows them to monitor the airway lumen via their dendrites which

are extended through the tight junctions between the airway epithelial cells [197], performing in this way their APC functions.

In mice, cDCs detect and opsonize virions and apoptotic bodies from infected cells, then migrate dependent on CCR7 via the afferent lymphatic system to the draining lymph node. Here they present the influenza virus derived antigen to T cells and activate them [198]. Next, cDCs degradate the viral protein and subsequently the immuno-peptides are presented by MHC-I or II molecules to T CD8^{+} or T CD4^{+} cell respectively [199]. Moreover, some specialized DCs have the ability to ingest virus-infected cells or cellular fragments and present antigens from these cells to CD8^{+} T cells in a process called cross-presentation or cross-priming [49]. Among various other activities of DCs in influenza virus infection, in mice, they also exert cytolytic activity (interferon killer DCs) and contribute to the formation of bronchus associated lymphoid tissue (iBALT) (CD11^{+} DCs) [197,199,200].

Porcine DCs also recognize PAMPs through PRRs. This interaction results in a robust cytokine and chemokine response, along with DC activation and maturation, all important for adaptive immune response occurs [56] (**Fig. 6**).

Many studies related to the role of DCs in influenza infection were done *in vivo* using a mice model and *in vitro* using mice or human DCs. However, is now known that the extrapolation of the data generated in mice not always reproduce the physio-pathological events that occur in humans. Therefore, the use of an animal/cell approach that is closer to humans may complement the knowledge related to the immune response against influenza virus.
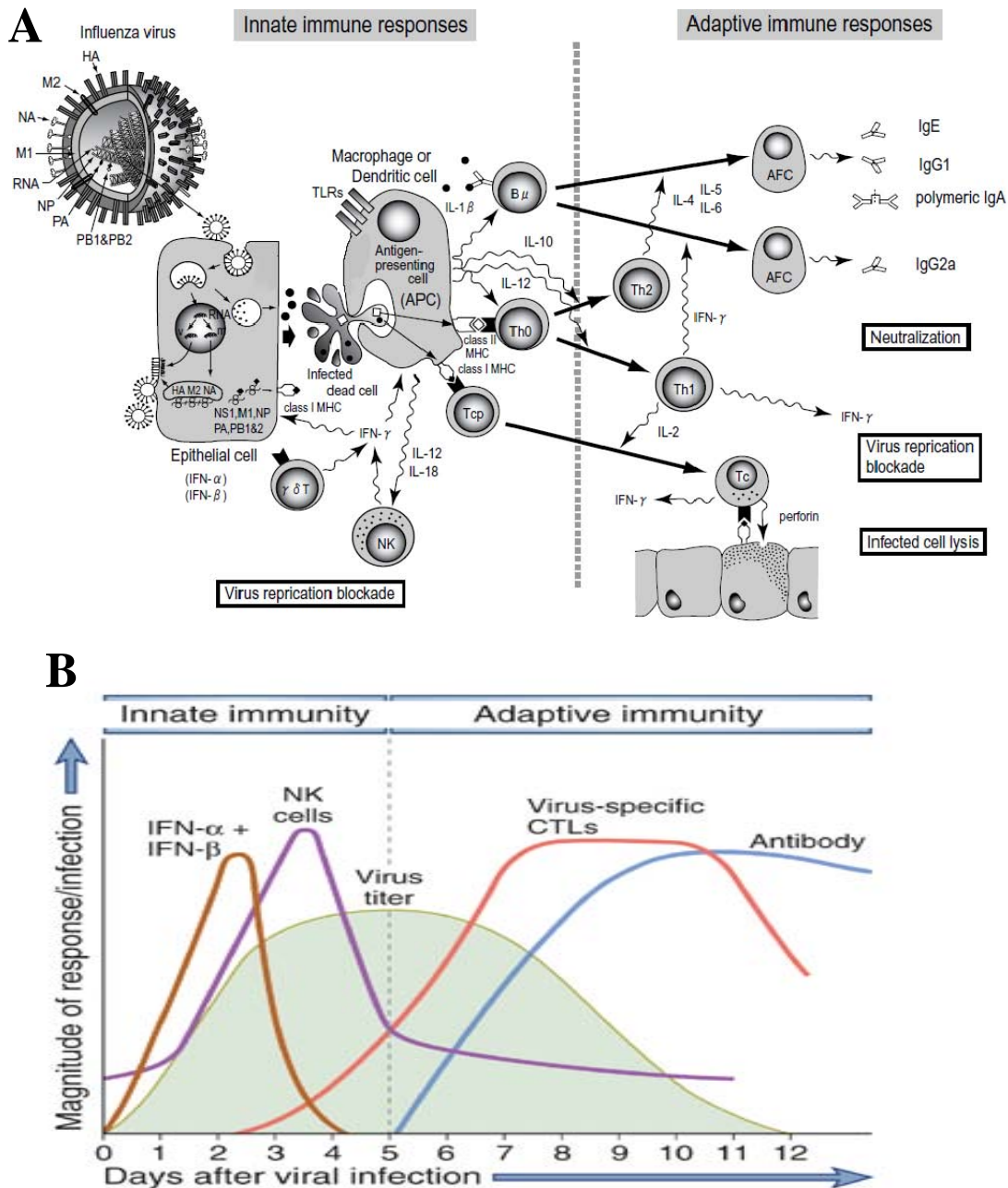
Figure 5. (A) Illustration of some defence mechanisms induced by influenza virus infection. Influenza virus infects and replicates in epithelial cells. Infected cells secrete type I IFN as an antiviral mechanism. APCs phagocyte infected cells and produce IL-12 and IL-18 wich activate NK cells to kill infected cells. Upon processing, the APCs present the MHC-loaded peptides to naïve T lymphocytes which undergo differentiation to initiate adaptive immune responses. The adaptive responses include the secretion of Abs by antibody forming cells (AFC) or cytokines by Th cells that will block virus replication or the release of granzimes by CTLs to lyse infected cells. Figure modified from reference [201]. **(B)** Kinetic of innate and adaptive immune response to virus infection Figure from reference [202].

b) Adaptive immune response to influenza A viruses.

The adaptive immune response forms the second line of defence against influenza virus infection. It consists of humoral virus specific antibodies (Abs) and cellular immunity (T cells).

The **humoral response** is orchestrated by influenza-specific Abs such as IgA, IgM and IgG. *In vivo*, locally produced mucosal or secretory IgA afford protection to the airway epithelial cells [197]. Serum IgA are produced rapidly after influenza infection and the presence of these Abs is an indicative of recent influenza virus infection [203], while IgM Abs initiate complement mediated neutralization of influenza virus and are hallmark of primary infection [204]. Finally, IgG Abs provides long-lived protection [205]. The virus-specific Abs induced by influenza infection, are against the major proteins of the virus, especially specific for the two surface glycolproteins HA and NA, since Abs recognizing these proteins correlate with protective immunity [206].

The **HA-specific Abs** bind predominantly to the trimetric globular head of the HA and inhibit virus attachment and entry in the host cell [207]. The HA Abs facilitate phagocytosis of virus particles by Fc receptor expressing cells and by binding of the Abs to the HA expressed on infected cells that mediates ADCC. Moreover, HA-specific antibodies are a solid correlate of protection providing an indication that they match the virus causing the infection [208].

In contrast to the relatively variable HA globular head the HA stem region is highly conserved mainly because this part of the glycoprotein is physically masked for the immune system. During influenza infection, Abs are formed against the stem region, although at lower titers. Interestingly, some of these Abs are able to recognize and bind HA molecules from different subtypes and have broad neutralizing capacity [209]. Recently a human Ab that is directed

against the HA receptor-binding pocket and able to neutralize antigenically diverse influenza viruses of the same subtype was identified [210].

Antibodies against NA have protective potential. By binding NA antibodies does not directly neutralise the virus but inhibits enzymatic activity and consequently limit virus spread. NA-specific Abs facilitate ADCC and also may contribute to the clearance of virus-infected cells [4,211].

The M2 protein is highly conserved among influenza viruses of different subtypes. As the protein is present at low concentration in infected cells, **M2-specific Abs** are raised after natural infection to a limited extends [197,207].

The NP is an important target for protective T cells, but also **NP-specific Abs** may contribute to protection against influenza virus [212]. Although the exact mechanism of protection remains unclear Abs against NP can induce ADCC of infected cells [213].

The major **cells of the adaptive immune** response to influenza virus are the T CD4$^+$, CD8$^+$ and T regs cells. CD4$^+$ T cells are activated after recognition of virus-derived MHC class II- associated peptides on APCs that express co-stimulatory molecules. The most important phenotype of these CD4$^+$ T cells is that of T helper cells. Different subsets of Th cells are distinguished based on their cytokine expression profiles. Th1 cells produce IFN-γ and IL-12 and are involved mainly in cellular immune response, while Th2 cells produce IL-4 and IL-13 and are considered to promote B cell response predominantly [4,201]. T regs and Th 17 cells have been identified regulating the immune response to influenza virus infection. The first controls both Th and the CD8$^+$ T cell response after infection [214] and the lasts cells improve Th responses by producing IL-6 which inhibits T reg function [215]. The main function of virus-specific CD8$^+$ T cells is that of cytotoxic T lymphocytes

(CTLs) [207]. After infection these cells are activated in the lymphoid tissue and recruited to the site of infection. There they recognize and eliminate influenza virus infected cells thus preventing the production/release of progeny virus. Their lytic activity is mediated by the release of perforin and granzymes [4,201]. However, even in the absence of granzyme influenza virus-specific CTLs are able to lyse target cells *in vivo* [216,217]. In humans, CTLs induced by influenza virus, are mainly directed against NP, M1 and PA proteins [218], however, in the most severe cases, following infection with highly pathogenic avian influenza virus, there are indicators that, CTL-mediated immunopathology can be fatal [219,220].

In experimental infection of pigs with the pandemic H1N1 was observed that CD4$^+$ T cells became activated immediately after infection and both CD4 and CD8 T cells expanded from day 3-7 post infection coinciding with clinical signs [91]. Also in lungs of swine H1N1-infected pigs, innate, proinflammatory, Th1 and Th2 cytokines were detected. Moreover, higher frequency of cytotoxic T cells, γσ T cells, DCs, activated T cells, CD4$^+$ and CD8$^+$ cells were found in infected animals [221].

An important phenomenon that occurs in influenza virus infection is the original antigenic sin firstly described by Davenport *et al.,* [222] in 1953. Later this phenomenon was observed in humans [223], rabbits [224] and ferrets [225]. In the context of infection with two influenza viruses (difted viruses), two thing need to happen to ensure a successful protection, *(i)* activation of memory B cells that recognize shared epitopes and *(ii)* activation of naïve B cells that recognize novel epitopes [226,227]. In the case of repeated infection with variant influenza viruses, the latter response is not induced and this phenomenon is called **antigenic sin**.

In humans and mice, sequential infections with influenza virus variants lead to a predominat Ab response against cross-reactive epitopes on the HA, which are shared with the first infecting virus, whereas, the response to strain-specific epitopes may be lower [226].

Consecutive experimental infection of pigs with the antigenically distinct SwIV of subtype H1N1 and H1N2 causes strong boost of already existing hemagglutation inhibition Ab titers to the first infecting virus as shown in longitudinal studies [228,229]. This theoretical explanation could take place in SwIV infection in pigs according to the results oberved by Busquets *et al.*, [227] and Kyriakis *et al.,* 2010 [228].
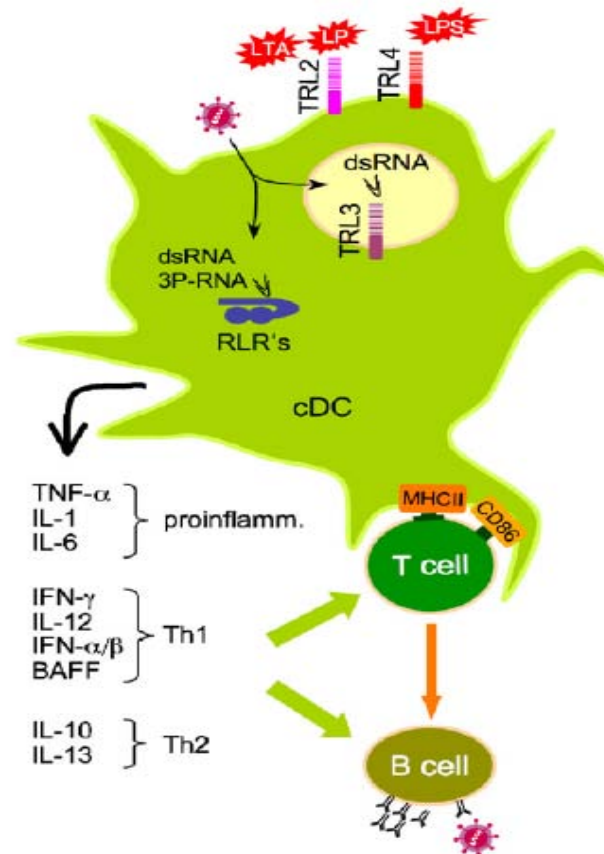
Figure 6. Porcine cDCs, are involved in antigen presentation and regulate lymphocyte responses. This response is partially controlled by the cytokine profile wich depends on stimulation of TLR ligands, RLR ligands, viruses and bacterias as well as other factors such as the tissue environment. The cytokine profiles resulted from those

interactions can be classified as proinflammatoy, Th1 and Th2 like. Image modified from reference [56].

1.10.2. Immune response against *H. parasuis.*

Extracellular bacteria can induce inflammation which results in tissue destruction or they can produce toxins with diverse pathologic effects [202]. The principal mechanisms of innate immunity to extracellular bacteria are *(i)* complement activation by the bacterial LPS, *(ii)* phagocytosis of opsonised or extracellular bacteria and *(iii)* inflammatory response mediated by the cytokines secreted by infected cells (**Fig. 7).**
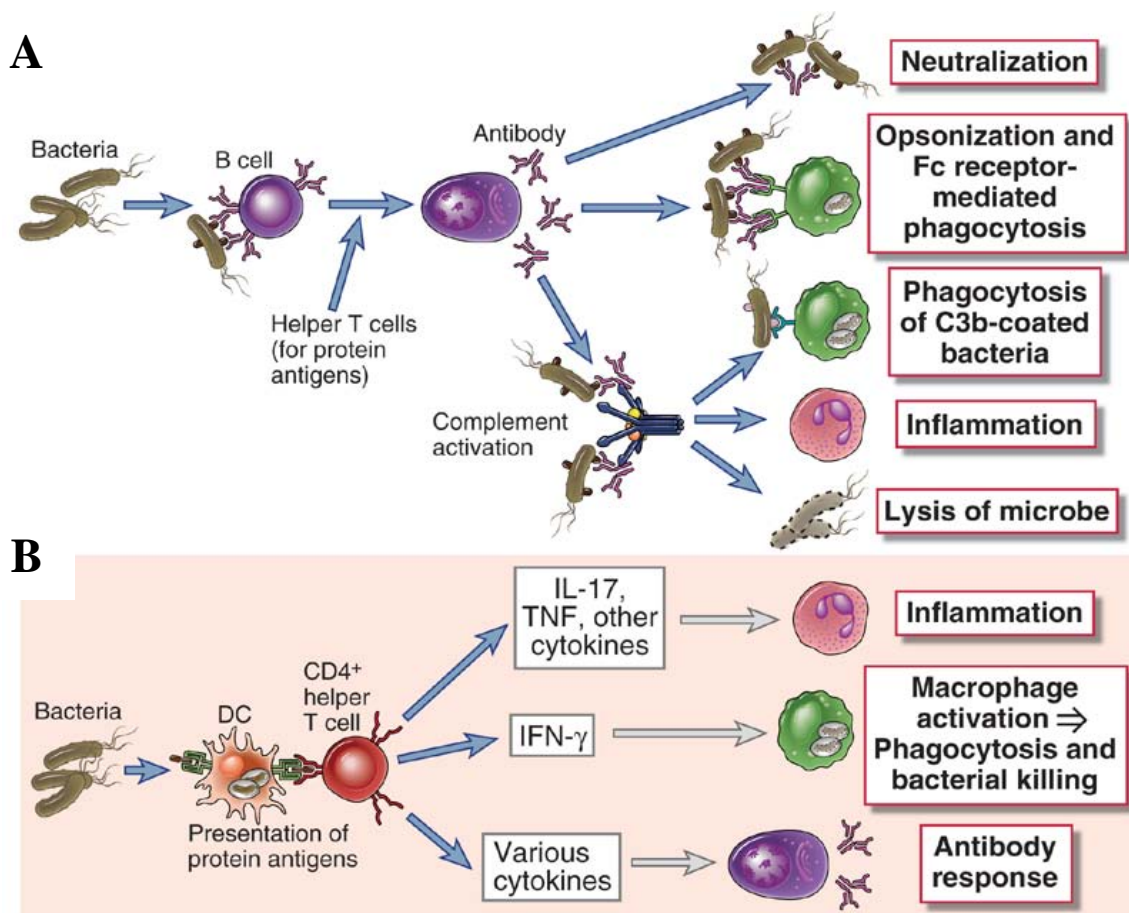
Figure 7. Adaptive immune response to extracellular bacteria. **(A)** Humoral immunity is a major protective immune response against extracellular bacteria and it fuctions to block infection, to eliminate the microbe and neutralize their toxins. **(B)** The proteins of extracellular bacteria also activate CD4^{+} Th cells, which produce cytokines that induce local

inflammation, enhance the phagocytic and microbicidal activities of macrophages and neutrophils, and stimulate antibody production. Figures from reference [202].

Until now few studies on porcine immune response against *H. parasuis* have been done. However, from the few studies performed *in vitro* or *in vivo*, in which *H. parasuis* was used, some knowledge on the immune response against these pathogens has been unravelled. For example, it was shown that *H. parasuis* strains isolated from the nose of healthy animals, were efficiently phagocytosed by porcine alveolar macrophages (PAMs), while the ones isolated from systemic lesions were resistant to this interaction [112]. Although the phagocytosis of suceptible strains proceeded through mechanisms independent of specific receptors and involved actin filaments and microtubules, the resistence to phagocytosis observe with systemic strains, could be related to the bacterial caspule as a different capsule was observed after the passage of systemic strain in PAMs [112].

In vivo, genes related to the immune response were found differentially expressed in spleen of *H. parasuis* infected pigs. Among them, genes for inflammasomes, adhesion molecules, transcription factors, acute-phase proteins and complement, differentiation genes for epithelial cells and keratinocytes, and genes related to antigen processing and presentation were described [230]. Also regarding to *in vivo* studies, it was shown that controls and immunized animals (with bacterin or an outer-membrane-protein-vaccine or a recombinant transferin binding protein B) or pre-immunized with a sub lethal dose of 10^5 CFU, showed no significant differences in PBMCs subsets. However, after challenge with *H. parasuis,* minor changes were found on blood cell populations. These changes consisted mainly in significant increases in the proportion of monocytes, granulocytes (SWC3^{+}) and B cells (IgM^{+}) as well as significant reduction of CD3^{+} cells [231]. Moreover, IL-1α was found in significant higher levels in spleen, lymphonodes and brain of dead pigs,

while IL-4, IL-10, TNF-α and IFN-γ were detected in significant higher levels by survivors [232].

Related to porcine DC interaction with a bacterial pathogens of pigs repiratory tract, Lecours *et al.*, [159] by using BMDCs evaluated the ability of *S. suis* to interact with swine DCs and the role of *S. suis* capsular polysaccharide (CPS) in modulating DCs functions [159]. Authors observed that the CPS was interfering with the capacity of DCs in phagocyte *S. suis*. The bacteria cell wall was mainly responsible for the DC activation, since the CPS negative mutant induced higher cytokine levels than the wild-type strain, confirming the role of *S. suis* CPS as a critical virulence factor [159].

Presently, there are no published studies in which <u>porcine DCs</u> were used as model to understand their role in *H. parasuis* infection. Although few *in vivo* co-infections were performed with the PRRSV as the main viral pathogen used with *H. parasuis* [233-235]; recently was observed that PRRSV infection accelerated the infection of conventional pigs with *H. parasuis* [236].

2. OBJECTIVES

Everything that is new or uncommon raises a pleasure in the imagination, because it fills the soul with an agreeable surprise, gratifies its curiosity, and gives it an idea of which it was not before possessed.

Joseph Addison

Three main objectives were determined for this Thesis:

1. To study *in vitro* the interaction of a circulating strains of porcine influenza virus with porcine bone marrow DCs (poBMDCs).

2. To study *in vitro* the immune response of poBMDCs generated against porcine, human and avian influenza viruses.

3. To study *in vitro* the interaction and immune response of poBMDCs against influenza virus and *H. parasuis* co-infection.

3. STUDY I:

Interaction of porcine conventional dendritic cells with swine influenza virus

Man's mind, once stretched by a new idea, never regains its original dimensions.

Oliver Wendell Holmes

3.1. Introduction.

Swine influenza virus (SwIV) causes sub-acute or acute respiratory infections in swine farms and pigs can act as "mixing vessels" for new influenza strains. Knowledge of the immune response against SwIV in its natural host, pigs, is very limited. DCs mediate the induction of immunity to pathogens, but their interaction with SwIV has not been fully characterized. Extensive studies on DCs have been done in mice and humans and although the knowledge on swine immunology has been developing quickly in recent years, it is rather scarce. Lytic viruses such as influenza virus do not form a stable, long-term host-virus relationship within the infected host. Moreover, most of the studies of influenza viral particles formation have been performed on polarized epithelial cells [9].

Given the pivotal role of DCs in triggering and directing the immune responses and the anatomical location of cDCs at the entry site of the body a set of experiments were designed to study the interaction between a circulating strain of porcine influenza virus (H3N2 SwIV) with swine cDCs *in vitro*.

3.2. Materials and Methods.

3.2.1. Cells.

Bone marrow hematopoietic cells, were obtained from femurs of healthy Large White X Landrace pigs of eight weeks of age, negative for porcine reproductive and respiratory syndrome virus (PRRSV) and for type-2 porcine circovirus (PCV2) by RT-PCR as previously described [237,238]. These

animals were also negative by enzyme linked-immunosorbent assay (ELISA) for influenza virus and Actibobacillus (HIPRA, Amer Spain), for Mycoplasma (OXOID, Cambridge, UK), for Parvovirus, Adenovirus and Aujeszky´s disease virus (INGENASA, Madrid, Spain), and Salmonella (SVANOVA Biotech AB, Uppsala, Sweden). Bone marrow dendritic cells were generated in an eight day protocol as previously described [150] with some modifications from reference [239]. Briefly, BMHCs were resuspended in RPMI-1640 (Lonza, Walkesville, USA) culture medium containing 2 mM of L-glutamine (Invitrogen®, Barcelona, Spain), 100 U/ml of Polymixin B (Sigma-Aldrich, Madrid, Spain) 10 % of fetal calf serum (FCS) (Euroclone, Sziano, Italy) and 100 μg/ml of penicillin with 100 U/ml of streptomycin (Invitrogen®, Barcelona, Spain). This medium will be named RPMI-DC in this study. One hundred nanograms per millilitre (100 ng/ml) of recombinant porcine GM-CSF (R&D Systems®, Madrid, Spain) was added to the cells three times during the culture within 2 days intervals. MDCK cells were maintained in DMEM (Lonza, Walkesville, USA) containing 8 mM of L-glutamine and 200 μg/ml of penicillin with 200 U/ml of streptomycin and 5% of FCS. This medium will be referred as DMEM-MDCK in this study.

3.2.2. Porcine BMDCs phenotype.

Flow cytometry was performed using indirect labelling for CD172a, SLA-I, SLA-II, CD4, CD11R1, CD40, CD80/86 and CD163 and direct labelling for CD14 and CD16. Commercially available purified monoclonal antibodies (mAbs) anti-porcine CD14, CD16 and the human CD152 (CTLA4)-muIg fusion protein for CD80/86 were used while the rest of markers were detected by hybridoma supernatants. The secondary antibody was R-Phycoerythryn anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK). Briefly, 2.5×10^5 cells/50 μl/well were labelled for 1h at 4ºC for each CD

marker, using 50 μl anti-CD172a (SWC3, BA1C11), 50 μl anti-SLA-I (4B7/8), 50 μl anti-SLA-II (1F12), 50 μl anti CD1 (76-7-4) 50 μl anti-CD4 (76-12-4), 50 μl anti-CD163 (2A10/11) and for the anti-CD11R1 (MIL4, IgG1, Serotec), anti-CD14 (MIL2, IgG2b, Serotec, bioNova cientifica, Madrid, Spain), anti-CD16 (G7, IgG1, Serotec, bioNova cientifica, Madrid, Spain), purified anti-human CD40 (G28.4, IgG1ϰ, Biolegend, San Diego CA, USA), CTLA4-muIg (Ancell, Minnesota, USA) the manufacturer´s instructions were followed. After 1h of incubation at 4ºC, cells were washed with cold PBS with 2% FCS by centrifugation at 450xg, 4ºC for 10 minutes. Then, the secondary antibody R-Phycoerythryn diluted 1:200 was added when required. Cells were incubated for 1h at 4ºC, and then they were washed as before and resuspended in PBS with 2% FCS. In order to determine if poBMDCs possess α-2,3-sialic acid or α-2,6 sialic acid linked to galactose, two lectins, MAA-II (*Maackia amurensis* lectin II) and SNA (*Sambucus nigra* lectin) (both from Vector Laboratories, Peterborough, UK) with known capacities to bind α-2,3 or α-2,6 sialic acid respectively were chosen. Stained cells were acquired using a Coulter® EPICS XL-MCL cytometer and analysed by EXPO 32 ADC v.1.2 program. A gate strategy was applied in 80% of living cells using the forward and side scatter (FS/SS) characteristic.

3.2.3. Porcine BMDCs ultrastructure.

At day eight, for conventional and immunogold labelling electron microscopy (EM) studies, poBMDCs were fixed with 2% (w/v) PFA and 2.5% (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB; Sigma-Aldrich, Steinheim, Germany), pH 7.4 and with 4% (w/v) PFA and 0.1% (v/v) glutaraldehyde in PB pH 7.4 respectively. For conventional EM procedures, cells were embedded in Eponate 12TM resin (Ted Pella, Inc, Redding, CA, USA). For immunogold labelling studies, after cryoprotection with sucrose (Sigma-Aldrich, Steinheim, Germany) solutions in

PB at 4ºC, the pellet-cells were embedded in Lowicryl HM20 resin (Polysciences Inc., Warrington, USA). Briefly, the immunogold labelling was performed as follows: after blocking in 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Steinheim, Germany) in PBS (BSA/PBS), the grid samples were incubated with the mAb to influenza A virus (Biodesign International, Saco, USA) at a dilution of 1:5 in 1% (w/v) BSA/PBS at 4ºC overnight in a humidified chamber. The secondary anti-mouse antibody was coupled to 10 nm-gold particles (British BioCell International, Cardiff, UK) in 1% (w/v) BSA/PBS for 40 min at RT. Sections treated with PBS/BSA instead of primary antibody served as negative controls. All grid samples, from conventional and immunogold labelling studies, were contrasted with conventional uranyl acetate and Reynolds lead citrate solutions, and evaluated using a Jeol microscope 1400 (Jeol LTD, Tokyo, Japan) and photographed with a Gatan Ultrascan ES1000 CCD Camera 2048 x 2048 pixels as previously described by Rodríguez-Cariño *et al.,* [240,241].

3.2.4. H3N2 SwIV preparation and infection.

Porcine *A/Swine/Spain/SF32071/2007(H3N2)* SwIV strain was isolated from a natural outbreak on a conventional farm in Spain. Viral isolation was performed on embryonated specific pathogen free (SPF) eggs and subsequently multiplied on MDCK following the procedures of International Organization of Epizooties (OIE).

Porcine BMDCs and MDCK were infected using a modified procedure, of previously described methodology [242]. Briefly, 10^6 poBMDCs were infected with 10^4 $TCID_{50}$ of previous porcine trypsin type IX (Sigma-Aldrich, St. Louis, USA) treated H3N2 10^7 $TCID_{50}$/ml. Then, cells were incubated for 1h at 37ºC 5% CO_2 for virus adsorption. After this time, cells were thoroughly washed with PBS with 2% FCS and 400 µl of RPMI-DC were added. For MDCK infection, DMEM medium supplemented with 8 mM of L-glutamine

and 200 µg/ml of penicillin with 200 U/ml of streptomycin and 2µg/ml of porcine trypsin type IX was added in the post infection DMEM (DMEM-PI). Mock and H3N2 SwIV infected cells were incubated for 1, 4, 8, 16 and 24h at 37ºC and 5% CO_2. As control, poBMDCs were stimulated with 50 µg/ml of Polyinosinic-Polycytidilic acid salt (Poly: IC) (Sigma-Aldrich, St. Louis, USA) for 24h.

3.2.5. Percentage viability and intracellular staining for influenza nucleoprotein.

Mortality 24h after infection was determined staining $2.5x10^5$ of mock or H3N2 SwIV infected cells with 5 µl of annexin V and after washing, adding 10 µl of propidium iodide (PI) (AB Serotec, Oxford, UK) following the manufacturer´s procedures.

Twenty-four hpi, intracellular staining for NP in poBMDCs was performed using $2.5x10^5$ cells fixed with 4% of PFA (Electron Microscopy Science, Hatfield, PA, USA) for 30 minutes 4ºC. After washing (centrifugation 450x*g* for 5 minutes 4ºC), cells were permeabilized with PBS with 0.2%v/v Tween 20 (Merck, Darmstadt, Germany) for 15 minutes at 37ºC. Then, cells were washed with PBS containing PBS+0.1% v/v Tween 20 and 100 µl of primary antibody HB 65 (H16-L10-4R5-IgG2a) (ATCC® Manassas, USA) diluted 1:1000 in staining buffer (PBS with 0. 1% w/v NaN3 and 1% w/v BSA) were added and incubated for 1h at 4ºC. After washing, cells were incubated with 1:200 diluted fluorescein isothiocyanate (FITC) conjugated affinity pure F(ab´)2 fragment goat anti mouse IgG (Jackson Immunoresearch, Suffolk, UK), for 1h at 4ºC. Then, after washing, cells were resuspended in staining buffer and analysed by FACSaria (Becton Dickinson). After 24h of infection or Poly:IC stimulation, poBMDCs were harvested and stained as described

elsewhere for SLA-I, SLA-II and CD80/86 using monoclonal antibodies or immunoglobulin fusion protein.

3.2.6. Immunofluorescence of influenza virus nucleoprotein.

The presence of virla NP in poBMDCs was visualized by indirect immunofluorescence in infected cells. Twenty four hpi, mock or infected poBMDCs were placed on a circular glass cover slip (VWR International, Spain) and left to adhere for 1h at 37ºC using 40 µl of Fibronectin from human plasma at 20 µg/ml (Sigma Aldrich). After that, cells were fixed with ethanol (Panreac, Barcelona, Spain) for 10 min at 4ºC, dehydrated with acetone and permeabilized with 0.1% of Triton X-100 for 15 min at 37ºC. Then, cells were washed with PBS 2% FCS and 100µl of primary antibody HB 65 (H16-L10-4R5-IgG2a) (ATCC®, Manassas, USA) diluted 1:500 were added. Cover slips were incubated at 4ºC for 1h and after two rounds of washes, 100 µl of Cy2-goat anti-mouse IgG (Jacson Jackson Immunoresearch, Suffolk, UK) diluted 1:200 were added for a further 1h of incubation at 4ºC. Finally and after several washes, nuclei were counterstained with DAPI. Cover slips were dried and mounted using 1 drop of Fluoprep (BioMérieux, France). To detect autofluorescence, mock or infected-poBMDCs were stained as controls with the primary and/or secondary antibody. Treated cells were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). Pictures were merged using Adobe®Photoshop®CS version 8 (Adobe System Incorporated, USA).

3.2.7. H3N2 SwIV replication in poBMDCs and MDCK cells.

Virus replication in infected cells was assessed by titration of supernatants on MDCK cells with the aid of trypsin in the post-infection media. Virus titre

was calculated by the Reed and Muench method [243]. Viral threshold cycles [244] values in MDCK or poBMDCs cells were assessed following a TaqMan one-step quantitative RT-PCR (RT-qPCR) in Fast7500 equipment (Applied Biosystems, Foster City, CA). RT-qPCR was performed using 60µl of eluted RNA extracted from mock or infected cells using TRIZOL® reagent (Invitrogen®, San Diego, USA). The primers and probe and the amplification conditions used to perform the RT-qPCR were previously described by Busquets *et al.*, [227]. The amplification profile was as follows: reverse transcription at 48ºC for 30 minutes; initial denaturation reaction at 95ºC for 15 min and 40 PCR-cycles of 95ºC 15 seconds and 60ºC 1 min. Serial 10-fold dilutions of H3N2 RNA, obtained from H3N2 infected MDCK of known concentration, were made and a standard curve generated. The limit of detection was 10^3 $TCID_{50}$ /ml corresponding to the Ct 30.

3.2.8. Infectious center assay and transwell assay.

Twenty-four hpi, mock or H3N2 SwIV infected-DCs ranging from 10^6 to 10 cells were co-cultured with MDCK in presence of DMEM-PI in 96 (Nunc® Kamstrupvej, Denmark) or in BD Falcon cell culture 24-well plate with or without inserts with 0.4 µm pores (Becton Dickinson) respectively. Then as positive control, 100 µl of trypsin treated H3N2 $5x10^5$ $TCID_{50}$ was added in the transwell (TA) assay. In addition, poBMDCs or MDCK were irradiated for 5 minutes with 30 Gy and 60 Gy respectively in an IBL 437C type H irradiator (CIS, Biointernational, Nice, France) before infection. After irradiation, cells were washed and infected with H3N2 SwIV for 1h at 37ºC (for virus adsorption). Then, cells were washed and incubated for 24h. After 24hpi, cells were washed, counted and co-cultured with MDCK for 7 days in an infectious center assay (ICA). At days four and eight after ICA or TA assays cytopathic effect (CPE) in MDCK was evaluated. Each condition in the co-culture had 8 replicas in the 96-well plate. Then, CPE was quantified as

being positive when 70% to 100% of monolayer disruption was observed in the wells from the 96-well plate with MDCK. When less than 70% was observed, the well was considered negative.

3.2.9. Statistical analysis.

All statistical analyses were carried out using the SAS system V.9.1.3 (SAS institute Inc, Cary, NC, USA). The significance level (α) was set at 0.05 with statistical tendencies reported when $p<0.10$. A non-parametric test (Mann–Whitney) was used to compare any variable response between experimental groups. In the particular case of the cytopathic effect in MDCK cells, an ANOVA test was carried out using number of infected poBMDCs and cell irradiation as independent variables.

3.3. Results.

3.3.1. Ultrastructure and phenotype of poBMDCs.

During the culture of porcine BMHCs with rpGM-CSF, cells grew in size, formed clusters and developed dendritic processes confirmed by electron microscopy at day eight **(Fig. 6A, B, C).** Porcine BMDCs in culture were semi-adherent cells, with some dendritic processes observed at day three which became noticeable as the culture progressed. At day eight of culture, the phenotype of poBMDCs was CD172a$^+$, SLAI$^+$, SLAII$^+$, CD1$^+$, CD4$^-$, CD11R1$^-$, CD14$^+$, CD16$^+$, CD40$^-$, CD80/86$^+$ and CD163^{low} **(Fig. 6D)** which was consistent with previous reports of Carrasco *et al.*, and Kekarainen *et al.*, [150,239]. At this time, the population of poBMDCs was rather homogenous with semi-mature cells and they constituted our starting culture for further experiments. Porcine BMDCs were also positive for α-2, 3 and α-2, 6 sialic acid.
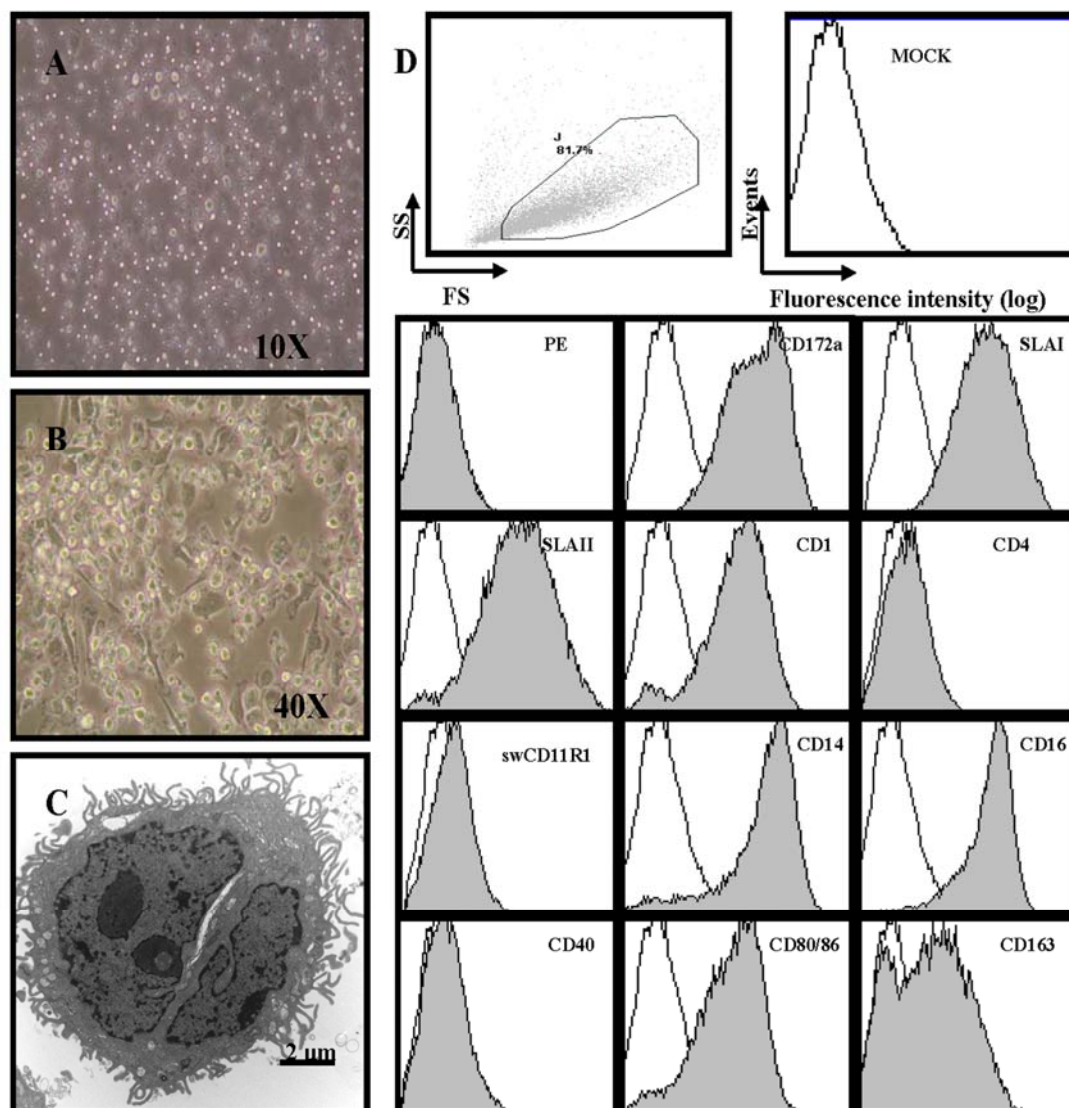
Figure 6. **Morphology and phenotype of poBMDCs**. (**A**) poBMDCs seen by optical microscopy at day 3, 10x magnification. (**B**) Day 8 of generation 40x magnification and (**C**) electron microscopy at day 8 of generation. Bar=2 μm. (**D**) Gate strategy and phenotype of poBMDCs at day 8 of generation with rpGM-CSF. White histograms show isotype control stained cells and grey histograms represent the CD marker stained cells. Mock are poBMDCs only and isotype control are cells stained with the secondary antibody only.

3.3.2. Infectious rate and viability of infected poBMDCs.

Influenza viruses efficiently infect and replicate *in vitro* in epithelial cells for e.g. MDCK cells but no data was available concerning the viability of poBMDCs after H3N2 SwIV infection. Thus, levels of apoptotic versus necrotic cells were evaluated using different amounts of H3N2 SwIV in *in vitro* experiments to set up the experimental conditions for further analysis. Annexin V and propidium iodide staining was performed on poBMDCs 24h after H3N2 SwIV infection. The overall mortality caused by H3N2 SwIV at 10^4 $TCID_{50}$ per 10^6 cells was similar to that observed in mock treated cells, 16.5% and 12.5% respectively **(Fig. 8A)**. When higher H3N2 SwIV doses were used, 10^5 $TCID_{50}$ and 10^6 $TCID_{50}$ per 10^6 cells, the percentage of dead cells increased to values around 30% of total cells. Also, the percentage of apoptotic cells was similar to that of necrotic cells when 10^4 $TCID_{50}$ was used **(Fig. 8A)**.

In order to evaluate the percentage of poBMDCs actually infected by H3N2 SwIV when 10^4 $TCID_{50}$ was used, intracellular NP staining of poBMDCs was studied. Data in **figure 8B** shows that around 34% of poBMDCs were positive for influenza virus NP. Also, only cytoplasmic influenza virus NP staining was observed 24 hpi in poBMDCs by immunofluorescence **(Fig. 8C)** whereas this staining was nuclear and cytoplasmic in the case of MDCKs at 24hpi **(Annexe 1)**. However, at 6hpi both cell types exhibited nuclear staining for viral NP **(Annexe 2)**. Subsequently, the amount of H3N2 SwIV used in the following experiments was 10^4 $TCID_{50}$ for 10^6 poBMDCs in the cultures.
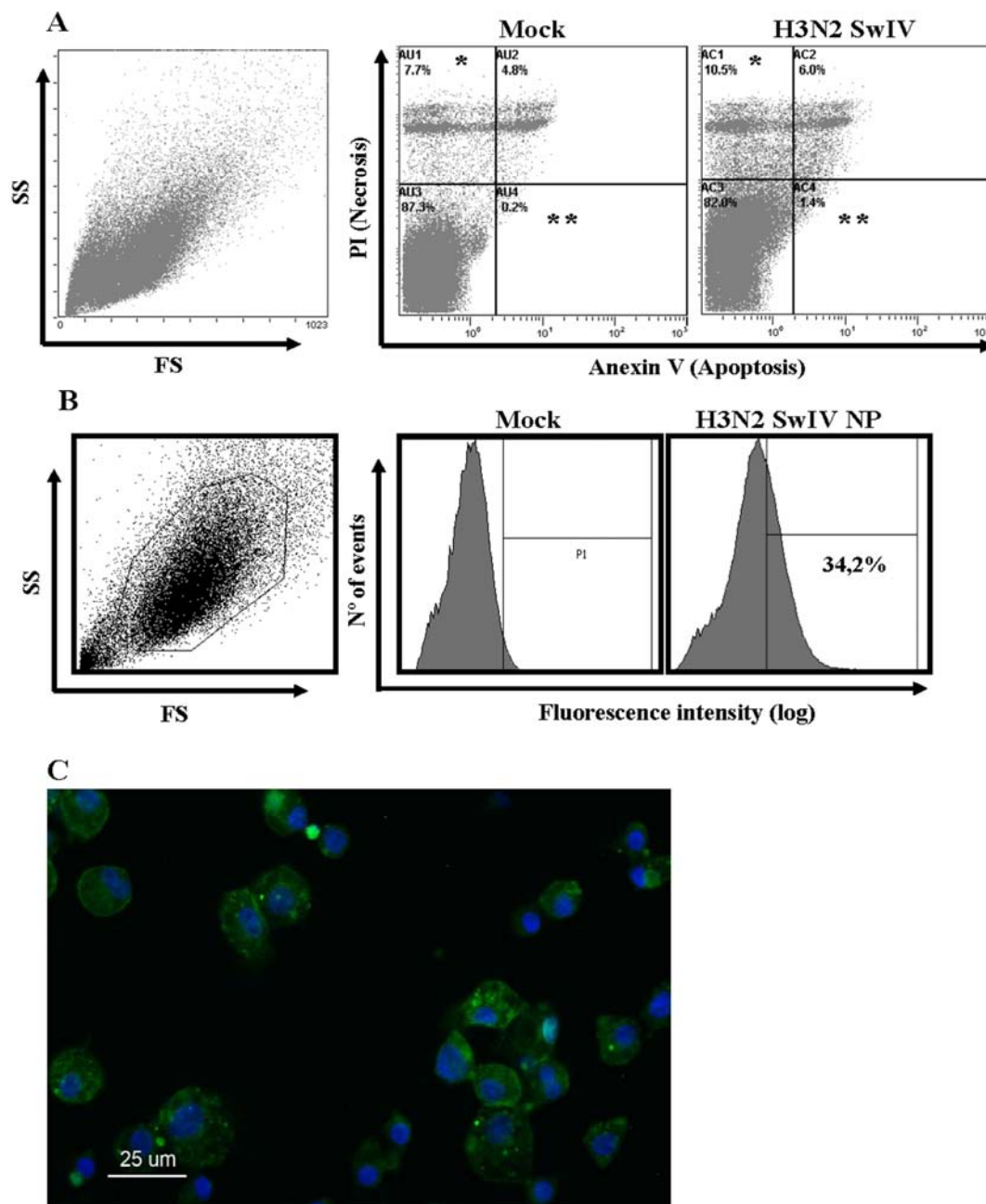
Figure 8. Apoptosis/necrosis (A) and infectivity (nucleoprotein) (B) staining of poBMDCs after 24h of infection with 0.01 MOI of H3N2 SwIV. Mock or infected poBMDCs after 24h were stained with (**A**) annexin V and/or propidium iodide, percentages in mock and infected cells were compared, *p=0.04, **p=0.01; n=5. The data was obtained without any gate strategy. (**B**) Anti-influenza NP staining in poBMDC by flow cytometry and (**C**) anti-influenza NP antibody (green) plus DAPI (blue) immunostaining of poBMDC. Bar=25 μm. FS (forward scatter) and SS (side scatter).

One of the hallmarks of DCs is their ability to up-regulate activation molecules after stimulation or infection. Consequently, it was investigated whether H3N2 SwIV-infected poBMDCs exhibited any alteration in surface molecules such as SLA-I, SLA-II and CD80/86. A slight up-regulation with statistical tendency ($p=0.06$) in SLA-I, II and CD80/86 expression was detected when values were compared with mock treated cells **(Fig. 9).** Values of mean fluorescence intensity were 22.7 for SLA-I mock treated cells compared with 40.7 for H3N2 SwIV infected cells; for SLA-II values were 40.6 compared to 64.6 and for CD80/86 values were 8.5 compared to 11.8 respectively. Similar results of slight up-regulation, with statistical tendency ($p=0.08$) were observed in poBMDCs stimulated for 24h with Poly:IC compared to mock treated cells **(Fig. 9).**
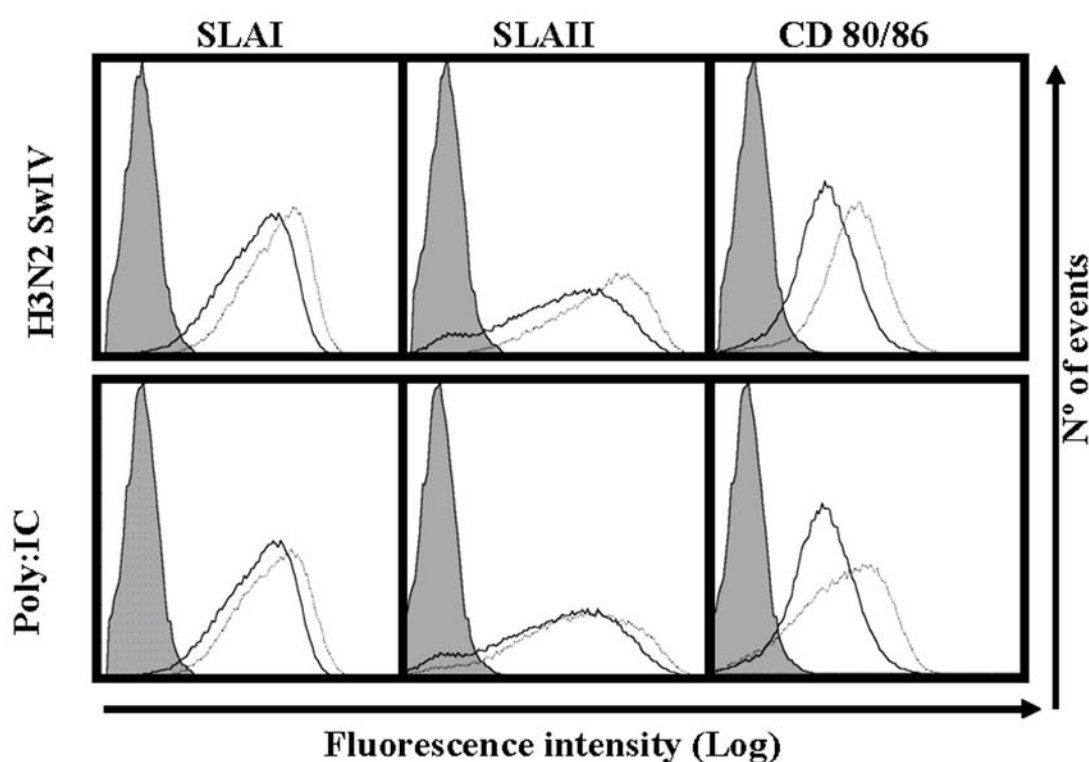
Figure 9. **Expression of SLA-I, SLA-II and CD80/86 24h after H3N2 SwIV infection or 24h after Poly:IC stimulation**. Infected/Poly:IC stimulated-poBMDCs (dotted line) or uninfected/unstimulated cells (continuous lines) were stained for SLA-I, SLA-II and CD80/86. Grey histograms represent the isotype control stained cells. Mean fluorescence of mock cells was compared with infected or Poly:IC stimulated cells and a statistical

tendency was found with *p*=0.06; *n*=3. The data was obtained with the same gate strategy shown in figure 6D.

3.3.3. Interaction of H3N2 SwIV with poBMDCs versus MDCK.

The replication cycle of influenza virus is composed of important steps such as fusion, endocytosis, replication, assembly and budding, which have been thoroughly investigated on permissive epithelial cells, like MDCK cells. Given the importance of DCs in triggering immune responses and once the conditions for H3N2 SwIV infection of poBMDCs were established, the H3N2 SwIV interaction with poBMDCs was analysed and compared with MDCK cells.

In the case of poBMDCs, structures resembling H3N2 SwIV virions were observed inside vesicles next to cellular membranes **(Fig. 10A).** Also, multiple vesicles were surprisingly observed in the cytoplasm, with 0.1 to 1 μm of diameter, containing several H3N2 SwIV like-particles with diameters of 80-100 nm. Most of them were surrounded by double membranes presenting H3N2 SwIV like-particles inside **(Fig. 10B)** which may reflect H3N2 SwIV particles entering the cell. Of note were several H3N2 SwIV like-particles observed free in the cytoplasm in close contact with Golgi complex. They may resemble virion structures budding from internal cistern of the Golgi complex membrane to the trans-Golgi network **(Fig. 10C)**. Furthermore, large vesicles with several immature H3N2 SwIV like-particles, 70-80 nm in diameter, were observed in infected cells, some of them without capsids **(Fig. 10D)**. In contrast with H3N2 SwIV-infected poBMDCs, in MDCK, H3N2 SwIV like-particles were observed in the extracellular space next to the cellular membrane **(Fig. 11A)** and mature particles were observed in the extracellular space, next to and attached to the cellular membrane **(Fig. 11B)**. In the cytoplasm of MDCKs, small and large vesicles were observed of less than 100

nm to 400 nm in diameter. These vesicles had simple or double membranes surrounded by immature (± 80 nm) and mature (± 100 nm) H3N2 SwIV like-particles in the cytosol **(Fig. 11C).** No H3N2 SwIV-like particles were detected freely in the cytoplasm of MDCK infected cells. The structures inside poBMDCs resembled H3N2 SwIV but in order to assess whether they exhibited H3N2 SwIV proteins an immunogold labelling for H3N2 SwIV nucleoprotein coupled with 10 nm gold particles was performed on poBMDC and MDCK infected cells. Those vesicles in the cytoplasm showed electron dense round and large structures consistent with H3N2 SwIV like-particles of 80-100 nm in diameter in poBMDCs **(Fig. 10E, F and 11E, F).**
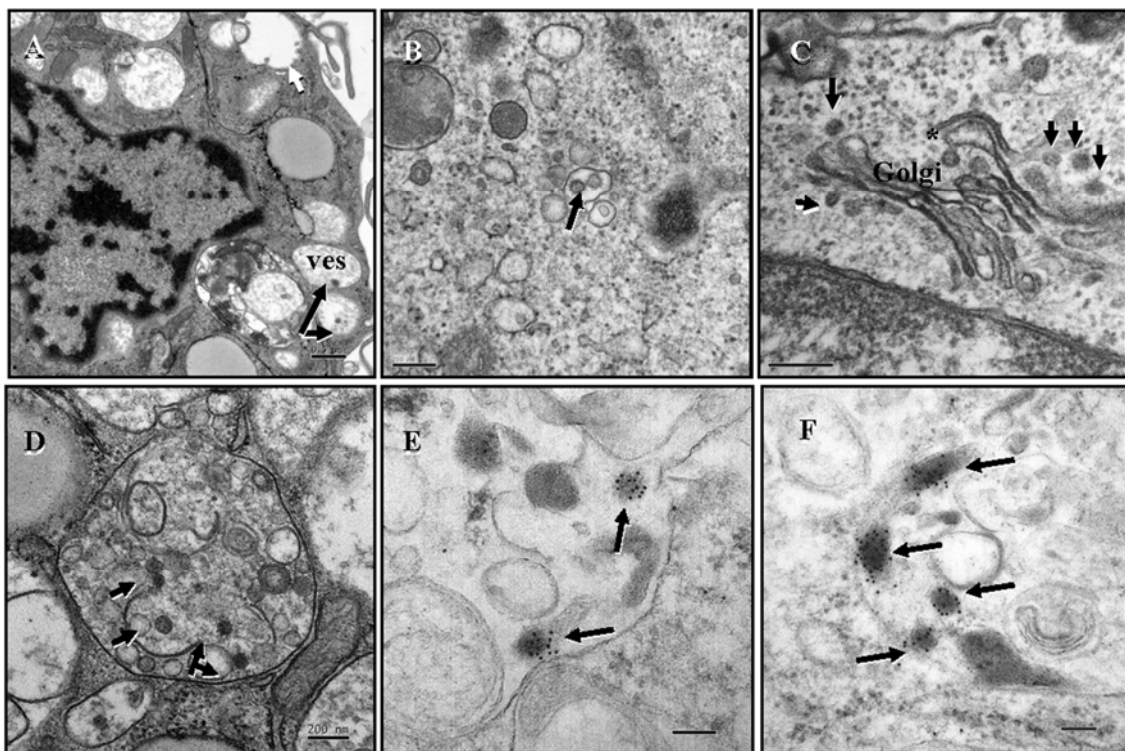
Figure 10. H3N2-infected poBMDCs, 24 hpi. (**A**) Virions were located next to cellular membrane (black-arrows), inside vesicles (ves). A lot of vesicles were observed in the cytoplasm, with diameters of 0.1 to 1 μm (approx), containing several H3N2 SwIV like-particles of 80–100 nm in diameter (white-arrow). Bar=0.5 μm. (**B**) Most of the vesicles have double membranes surrounding H3N2 SwIV like-particles (arrow), bar=200 nm. (**C**) Next to the Golgi complex, several H3N2 SwIV like-particles were observed (arrow). Virion budding from internal cisternae of the Golgi complex membrane (*), to the trans-

Golgi network are shown. Bar=200 nm. (**D**) Large vesicle has several immature H3N2 SwIV like-particles with 70–80 nm in diameter, and some of them without capsids (arrows). Bar=200 nm. (**E, F**) Immunogold labelling for H3N2 SwIV nucleoprotein coupled with 10 nm gold particle. Vesicles in the cytoplasm showing electron dense round and large structures, consistent with H3N2 SwIV like-particles with 80–100 nm in diameter, were heavily and specifically labelled (arrows). Bar=200 nm. Uranyl acetate and Reynolds lead citrate solution.

More importantly, these particles were heavily labelled. However, neither budding nor released virion particles were detected in poBMDCs. In MDCK, electron dense structures with different size and irregular shape, surrounding nucleoli and chromatin were observed and labelled. Budding and released virions were labeled **(Fig. 11F)**.
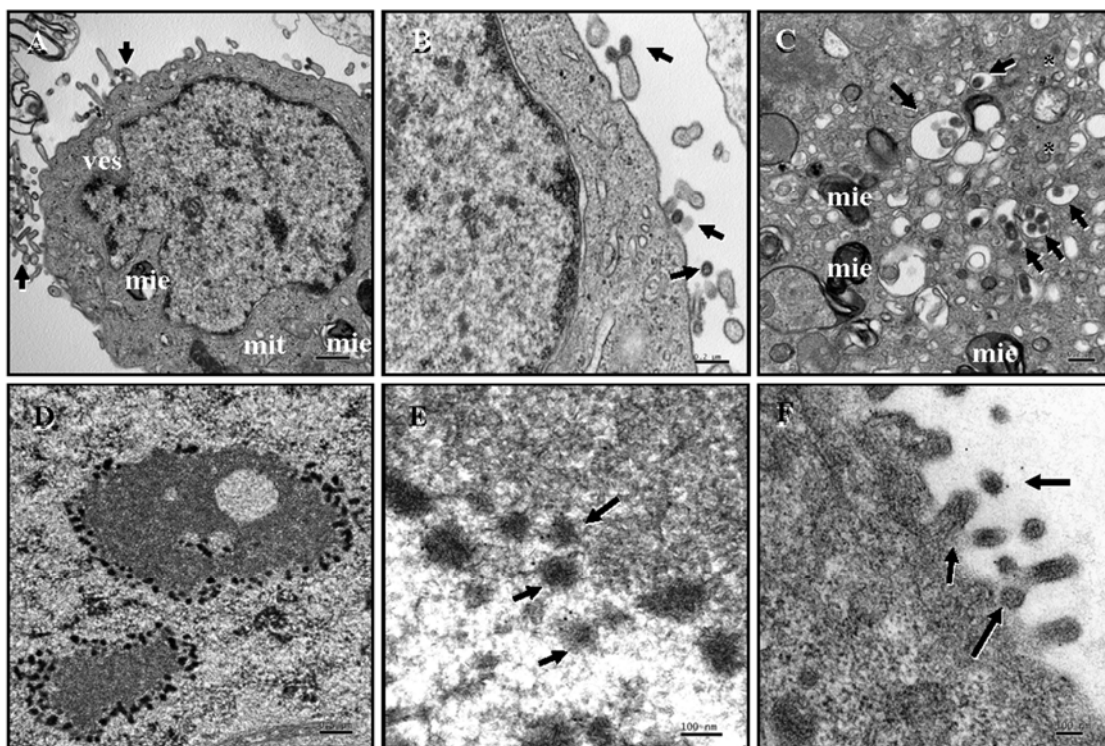
Figure 11. H3N2-infected MDCK, 24 hpi. (**A**) These cells are characterized by large nuclei compared to their cytoplasm, where several large myelinoid figures (mie) and some vesicles were observed. H3N2 SwIV like-particles were detected in the extracellular space and next to cellular membrane (arrows). Bar=0.5 μm. (**B**) Close-up of figure A, mature H3N2 SwIV like-particles were observed in the extracellular space next to and attached to

the cellular membrane (arrows). Bar=0.2 μm. (**C**) In the cytoplasm, small and large vesicles were observed of less than 100 nm to 400 nm diameter (approx). These vesicles contain SwIV like-particles surrounded by simple or double membranes (±80 nm, arrows) and mature ones (±100 nm) in the cytosol (*). Bar=0.2 μm. (**D, E**) and (**F**) Immunogold labelling for H3N2 SwIV nucleoprotein coupled with 10 nm gold particle. Electron dense structures with irregular shape surrounding nucleoli and chromatin were observed and labelled (arrows). Budding release (**F**) (arrows) virions were labelled. Bar=0.5 μm, 100 nm and 100 nm respectively. Uranyl acetate and Reynolds lead citrate solution.

3.3.4. H3N2 SwIV infection of poBMDCs.

Generally, to evaluate influenza virus replication and generation of viral progeny, supernatant from infected cells is titrated on MDCK cells. Supernatant from H3N2 SwIV infected-poBMDCs was assessed for viral progeny generation but no increase in viral titre was detected in poBMDCs compared with H3N2 SwIV-infected MDCK at different time points. However, statistically significant differences between poBMDCs and MDCK infected cells were observed at all time points with $p<0.05$ **(Fig. 12)**.
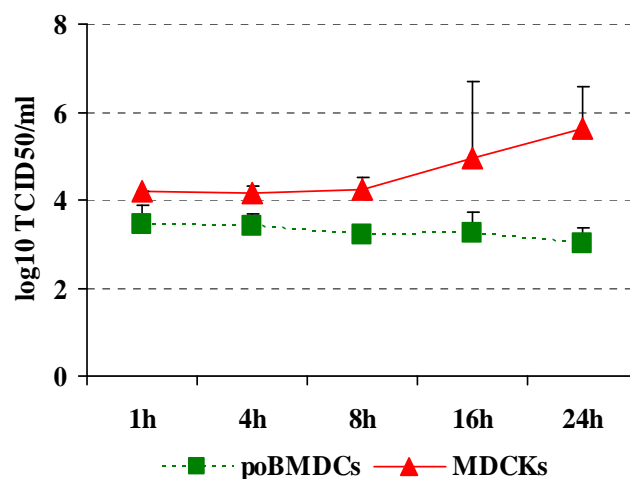
Figure 12. H3N2 SwIV progeny in the supernatant of poBMDCs and MDCK infected cells. Supernatants from infected cells were collected at different time points after infection to titrate viral progeny on MDCK cells. Statistically significant differences between poBMDCs and MDCK infected cells were observed at all time points with *p*<0.05. Bars represent the mean value plus one standard deviation; *n*=5.

Therefore, the question was whether or not H3N2 SwIV was able to replicate in poBMDCs as compared with MDCK cells. Thus, 10^6 MDCK cells and 10^6 poBMDCs were infected in parallel using 10^4 $TCID_{50}$, and viral RNA was evaluated by RT-qPCR at different time points. In MDCK cells, an increase in viral RNA was detected rising from 1hpi to 24h. In contrast, viral RNA in poBMDCs showed a limited increase between 1 and 8hpi and later decayed with time. The increase in viral RNA was observed by comparing inverted Ct values. No significant differences were observed between poBMDCs and MDCK infected cells **(Fig. 13)**. The limit of detection in this assay was set at 30 Ct which corresponded to 10^3 $TCID_{50}$ of H3N2 virus. Ct values for 10^7, 10^6, 10^5 and 10^4 $TCID_{50}$ were 17, 19, 23 and 26 respectively.
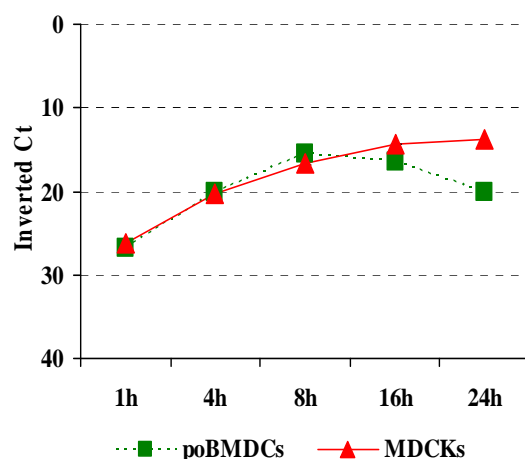
Figure 13. SwIV H3N2 RNA quantification in poBMDCs and MDCK infected cells. RT-qPCR was performed using viral RNA extracted from poBMDCs and MDCK infected cells at different times. No significant differences were observed between poBMDCs and MDCK infected cells. Bars represent the mean value plus one standard deviation; n=3.

3.3.5. H3N2 SwIV infected poBMDCs are able to infect permissive cells by cell-to-cell contact.

Once a limited increase of H3N2 SwIV RNA was detected in poBMDCs, the ability of H3N2 SwIV infected-poBMDCs to transmit the virus to other susceptible cells was evaluated. Thus, different amounts of H3N2 SwIV infected-poBMDCs were co-cultured with MDCK cells, in an ICA, in the absence or presence of a TA. When the TA was inserted and cell-to-cell contact was prevented, no cytopathic effect (CPE) was detected. In the absence of a TA insert, CPE on MDCK was observed in 100% of the wells when 10^6 to 10^5 infected poBMDCs were used. Subsequently, CPE levels fell when cell number decreased **(Fig. 14A)**. One hundred microliters of trypsin treated H3N2 $5x10^5$ $TCID_{50}$ were also placed in the upper chamber of the transwell during the assay as a positive control. CPE was observed in 100% of the wells in this control **(Fig. 14A)**. Moreover, high titers of virus were detected when supernatants from the 100% CPE positive wells in the ICA assay were titrated on MDCK cells. In these ICA positive wells, viral titres

ranged from 10^5 to 10^6 TCID$_{50}$/ml. The question of whether influenza particles infecting susceptible cells originated from viral progeny in DCs or they were particles attached to the poBMDCs arose. In order to answer this question, poBMDCs and MDCK were irradiated with 30 Gy and 60 Gy respectively for 5 minutes before infection. Irradiation of MDCK before H3N2 SwIV infection was enough to prevent generation of infectious viral progeny in the supernatant. After 24h of infection, an ICA was performed. After 7 days in co-culture, either irradiated or non-irradiated poBMDCs cells induced CPE on MDCK **(Fig. 14B)**. The same result was obtained when irradiated MDCK, infected with H3N2 SwIV, were tested, which was not surprising considering the ability of SwIV viral particles to attach to the MDCK cell surface. Therefore, all the data suggested that viral particles attached to cDCs were able to infect susceptible cells only when cell-to-cell contact occurred.

A

B
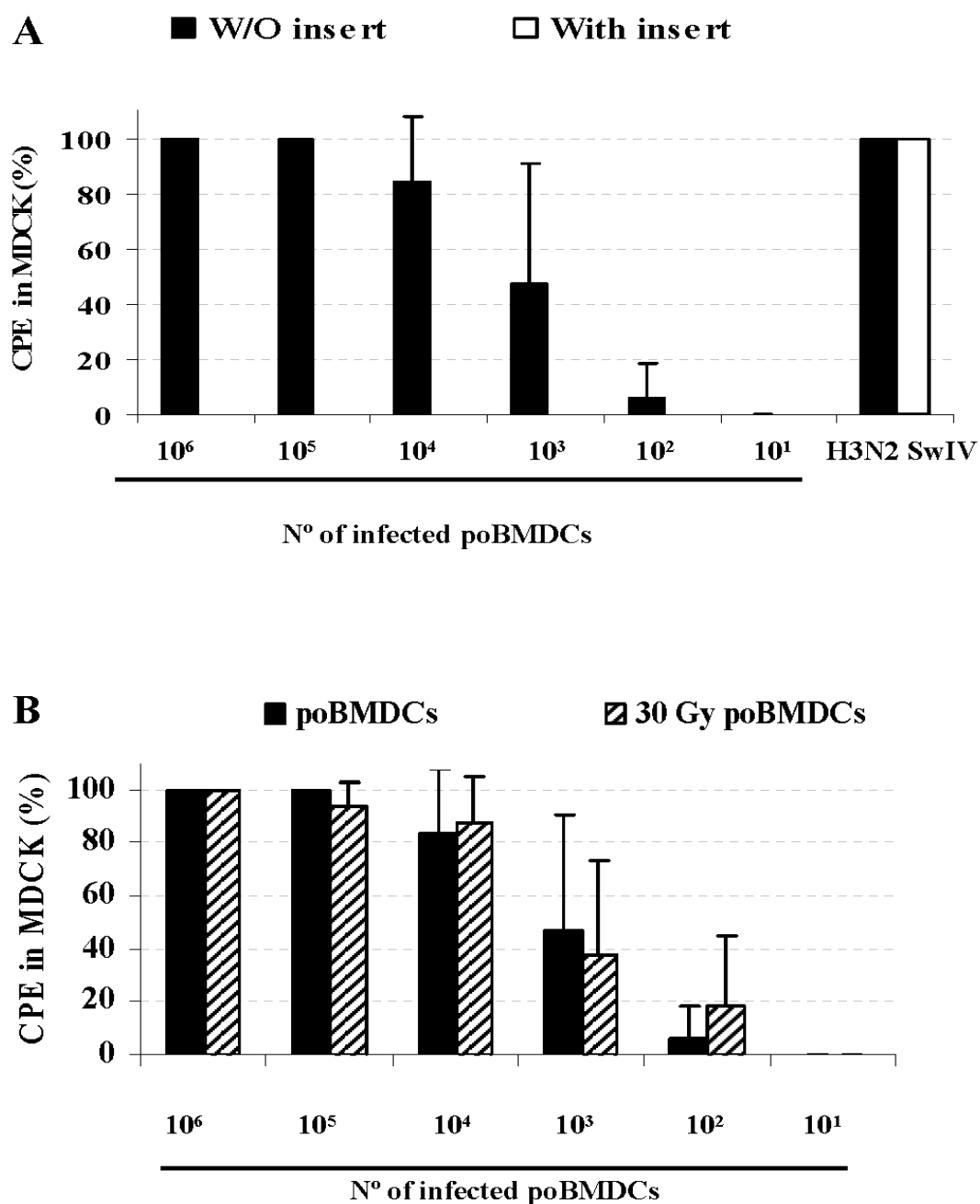
Figure 14. Infectious centre and transwell assay. (**A**) At 24hpi poBMDCs were co-cultured directly or indirectly with MDCK in 96 flat and 24 well plates respectively. As a positive control, 100 µl of trypsin treated H3N2 SwIV $5x10^5$ $TCID_{50}$ was added. Bars represent the mean value plus one standard deviation; $n=4$. (**B**) Normal or irradiated poBMDCs with 30 Gy were infected for 24h before the co-culture with MDCK in an ICA. CPE was evaluated at days 4 and 7 after ICA assay. No significant differences were observed between irradiated or not irradiated poBMDCs. Bars represent the mean value plus one standard deviation; $n=3$.

4. STUDY II:

Infection by swine, human or avian influenza viruses activate different cytokine profile in poBMDCs

We must accept finite disappointment, but never lose infinite hope.

Martin Luther King, Jr.

4.1. Introduction.

Pigs play a crucial role in the interspecies transmission of influenza viruses [245-247]. They are susceptible to experimental infection by virtually any avian influenza strain [248], including viruses of the H5N1 subtype [249]. By having receptors for human viruses, they are also susceptible to the strains circulating in humans, since porcine and human viruses are phylogenetically related, and they can easily cross the species barrier, as has happened with the new type A (H1N1) 2009 pandemic virus [46].

The goal of this study was to characterize *in vitro* the immune response of poBMDCs as a results of an interaction with influenza virus that may be encountered by the porcine immune system, such as: SwIV *A/Swine/Spain/SF32071/2007(H3N2)*, the human 2009 pandemic *A/Catalonia/63/2009(H1N1)*, the low pathogenic avian influenza virus (LPAIV) *A/Anas plathyrhynchos/Spain/1877/2009(H7N1)* and the high pathogenic avian influenza virus (HPAIV) *A/Chicken/Italy/5093/1999(H7N1)*; and to evaluate the role of NF-κB pathway on the cytokine response.

4.2. Material and Methods.

4.2.1. Cells.

The cells for this study were generated as described in Study I.

4.2.2. Viruses.

The *A/Swine/Spain/SF32071/2007(H3N2)* (here after referred as H3N2 SwIV) strain was isolated from a porcine influenza virus outbreak in Spain on

SPF eggs and subsequently multiplied on MDCK following the procedures of the International Organization of Epizooties (OIE) [101]. Eight sequences of this H3N2 SwIV virus, corresponding to HA, NP, PA, PB2, NA, PB1/ PB1-F2, NS1/NS2 and M1/M2 genes were submitted to GenBank (accession numbers: HE774666, HE774667, HE774668, HE774669, HE774670, HE774671, HE774672 and HE774673). The *A/Catalonia/63/2009(H1N1)* (here after referred as hH1N1) influenza virus was isolated from a patient in Hospital Clinic, Barcelona, Spain, during the 2009 pandemic and the complete genome was sequenced and submitted to GenBank (accession numbers: GQ464405-GQ464411 and GQ168897). The *A/Catalonia/63/2009(H1N1)* strain was propagated at 37°C in the allantoic cavities of 11-day-old embryonated chicken eggs originating from a commercial SPF flock (GDdeventer) following the same procedures of OIE. Two avian viruses, the LPAIV *A/Anas plathyrhynchos/Spain/1877/2009(H7N2)* (hereafter referred as aH7N2) and HPAIV *A/Chicken/Italy/5093/1999(H7N1)* (hereafter referred as aH7N1) were used. The aH7N2 was isolated from samples of avian influenza virus surveillance in Catalonia (North-eastern of Spain) and the aH7N1 was isolated from a poultry outbreak in Italy in 1999, and was kindly provided by Dr. Ana Moreno from *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna*, Brescia, Italy. The deduced amino acid sequence of the region coding for the cleavage site of the precursor of the haemagglutinin molecule (HA0) were PEIPKGSRVRR*GLF for the aH7N1 and PEIPKGR*GLF for the aH7N2, being typical of HPAIV and LPAIV, All the four viruses were previously used in experimental infections. All the four viruses were previously used in experimental infections [227,250-253] and in study I. For all viruses, virus titre was calculated by titration in MDCK cells, using the Reed and Muench method [243]. When required, H3N2 SwIV and aH7N1 influenza virus were inactivated by heat at 70ºC for 5 minutes in a termoblocker.

4.2.3. Sialic acid detection and poBMDC infection.

At day eight of generation porcine BMDCs or MDCK were stained for α-2,3 and α-2,6 sialic acids using 20 µg/ml of *Maackia amurensis* type II (MAA-II) or *Sambucus nigra* (SNA) (both from Vector Laboratories INC, CA, USA) and analysed as described in study I.

Influenza virus infection was performed in a 24 wells plate (Nunc, Kamstrepvej, Denmark) in which 10^6 cell were infected with 10^4 $TCID_{50}$ of virus for 4, 8, 16 and 24h following the procedures previously described in study I. In this study, 10^4 $TCID_{50}$ of previous porcine tripsin IX (Sigma-Aldrich, St. Louis, USA) treated H3N2 SwIV, hH1N1, aH7N2 or aH7N1 were added to the cells. When required, for RT-qPCR standard curves, poBMDCs were stimulated with 50 µg/ml of Poly:IC (Sigma-Aldrich, St. Louis, USA) for 8 and 16h. After each time, supernatants were collected and frozen for IFN-α, TNF-α, IL-12 and IL-18 detection by ELISA, while cells were collected in TRIZOL reagent (Ambion-Life Technologies, CA, USA) for *RPL19, β2M, IFN-β, NF-κB, TGF-β* and *IL-10* gene expression by RT-qPCR.

4.2.4. Phenotype of infected cells and sialic acid detection.

Flow cytometry was performed using indirect labelling for SLA-I, SLA-II and CD80/86 as described in study I except that the stained cells were acquired using FACSaria I (Becton Dickinson®) and the MFI analysed by FACSDiva software v.6.1.2, while for sialic acids, Coulter® EPICS XL-MCL cytometer and an EXPO 32 ADC v.1.2 program were used for analysis. In both cytometers, a gate strategy was applied in 80% of living cells using the forward and side scatter (FS/SS) characteristic.

4.2.5. RNA extraction, DNAse treatment and Reverse Trancription.

In this study, approximately 10^6 infected cells were harvested with 1ml of Trizol reagent. Then, samples were frozen at -80ºC before RNA extraction. Total RNA from mock or infected poBMDCs was extracted using the RiboPure™ kit (Ambion-Life Technologies, CA, USA) following the manufacturer's instruction. For *IFN-β* gene analysis, contaminating DNA was removed from the RNA preparations using a Turbo DNA-Free™ kit (Ambion-Life Technologies, CA, USA). After that, the isolated RNA was reverse transcribed into cDNA using the High capacity cDNA Reverse Transcription kit (Applied Biosystems-Life Technologies, CA, USA) following the manufacturer's instructions. Negative RT control was performed using sterile water instead of MultiScribe® Reverse Transcripstase.

4.2.6. Quantitative Real Time PCR (RT-qPCR).

A qPCR assay using SYBR Green chemistry (Life Technology, Carlsbad, CA, USA) and the $2^{-\Delta\Delta CT}$ method [254] was performed in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Primers for amplification of *NF-κB, TGF-β, IL-10* and *IFN-β* mRNA were designed using the Primer Express 2.0 software (Applied Biosystems) and are shown in **Table 1**. Two genes, *β2M* and *RPL19*, previously validated as stable expressed control genes were used as endogenous controls (Corominas *et al.,* unpublished data; and [255]). In order to validate our designs to use the $2^{-\Delta\Delta CT}$ method, standard curve for all the genes were generated using cDNA extracted from poBMDCs stimulated with Poly:IC for 8h and 16h. Then, the log input amount of cDNA (dilutions of 1:20, 1:200, 1:2,000, 1:20,000) was plotted versus the ΔCt, obtaining absolutes slopes <0.1 in all cases that allowed the use of the $2^{-\Delta\Delta Ct}$ method. All samples were run in triplicate in a 20 µl reaction volume

containing 5µl of cDNA sample diluted 1:20 for *IL-10* and *IFN-β* amplification or 1:200 for the rest of genes. In all tested genes primers were used at 900 nM. Thermal cycle was: 2 min at 50ºC, 10 min at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C. A dissociation curve was drawn for each primer pair in order to assess that there were not primer dimer formation. Data was analyzed using the RQ manager v1.2.1 and the DataAssist^{TM}v3.0 softwares (Applied Biosystems). All the results are expressed as fold difference respect to mock cells ($2^{-\Delta\Delta CT}$).

4.2.7. Quantitation of IFN-α, TNF-α, IL-12 and IL-18 by ELISA.

For each ELISA three wells with 50 µl/well of mock or virus-infected supernatants were evaluated for cytokine production by ELISA. All ELISA were read with KC Junior Program (Bio Tek instruments) using the filter PowerWave XS reader. For TNF-α and IL-1β the Duo Set Developed system ELISA from R&D System® was used following the manufacturer's instructions while for IFN-α, an in-house ELISA using antibodies purchased from PBL interferon source were used according to reference [239]. To detect IL-18, the pig IL-18 Module Set BMS672MST (Bender Med Systems, Vienna, Austria) was used following manufacturer's instructions. Finally IL-12 secretion was analysed using mAbs purchased from R&D Systems®, following the manufacturer's instructions, with these amounts of antibodies: 2 µg/ml of anti-IL-12/IL-23 monoclonal antibody were used to coat 96 well plate (Costar, NY, USA) overnight at room temperature (RT). After washes recombinant porcine IL-12 starting from 10,000 pg/ml was used. Then 125 ng/ml of biotinylated anti-porcine IL-12/IL-23 p40 antibody was used. Finally, 0.05 µg/ml of peroxidase-conjugated streptavidine (Jackson ImmunoResearch, Suffolk, UK) was added. The reaction was revealed using

TMB (Sigma Aldrich, Madrid, Spain) and stopped using H_2SO_4 (0.5M). The limits of detection were 3.9 U/ml of IFN-α and 78.1 pg/ml of IL-12.

4.2.8. NF-ϰB inhibition.

To analyse the role of NF-ϰB pathway in the synthesis of the cytokines detected in the supernatants of H3N2 SwIV-infected cells, poBMDCs were treated for 1h at 37ºC with 10 µg/ml of caffeic acid phenethylester (CAPE) (Sigma-Aldrich, Madrid Spain) before infection with H3N2 SwIV. After the treatment, cells where infected with 10^4 $TCID_{50}$ for 24h as described in the section 2.3 or stimulated with 50µg/ml of Poly:IC. Then, supernatants were collected for IFN-α and IL-12 screening by ELISA.

4.2.9. Statistical analysis.

The Mann-Whitney test was used to compare parameters between the different experimental groups. All analyses were performed with NCSS 2004 and PASS 2005 software (Kavysville, Utah, USA). The significance level was set at 0.05 with statistical tendencies reported when $p \leq 0.10$.

Gene	Forward primer (5´to 3´)	Reverse primer (5´to 3´)	Ensembl ID or accession nº	Amplicon size (pb)
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG	AF435591	147
β2M	ACCTTCTGGTCCACACTGAGTTC	GGTCTCGATCCCACTTAACTATCTTG	ENSSSCG00000004682	108
NF-ϰB	CTGGCAGCTCTCCTCAAAGC	CACGAGTCATCCAGGTCATACAG	ENSSSCG00000009168	80
TGF-β	GCTTCAGCTCCACGGAGAAG	TGGTAGCCCTTGGGTTCATG	ENSSSCG00000003017	99
IL-10	AGGATATCAAGGAGCACGTGAAC	CACAGGGCAGAAATTGATGACA	ENSSSCG00000015652	89
IFN-β	TCCAGCAGATCTTCGGCATT	CCAGGATTGTCTCCAGGTCATC	GQ415073	120

Table 2. Primer sequences used in this study.

4.3. Results

4.3.1. Expression of α-2, 3 and α-2, 6 sialic acids receptors on poBMDCs.

Pigs have sialic acid receptors on the cells of respiratory organs [256] and the presence of those receptors was reported as being one of the mechanisms by which avian and human origin viruses could interact with porcine cells [257]. Thus, we evaluated the presence of these receptors on poBMDCs by flow cytometry using *MAA-II* and *SNA* lectins, which binds to α-2,3 and α-2,6 sialic acid respectively, by comparison with expression on MDCK cell line. After 8 days of generation, uninfected DCs were positive for α-2, 3 and α-2, 6 sialic acids with similar to those of MDCKs (**Fig.15**).
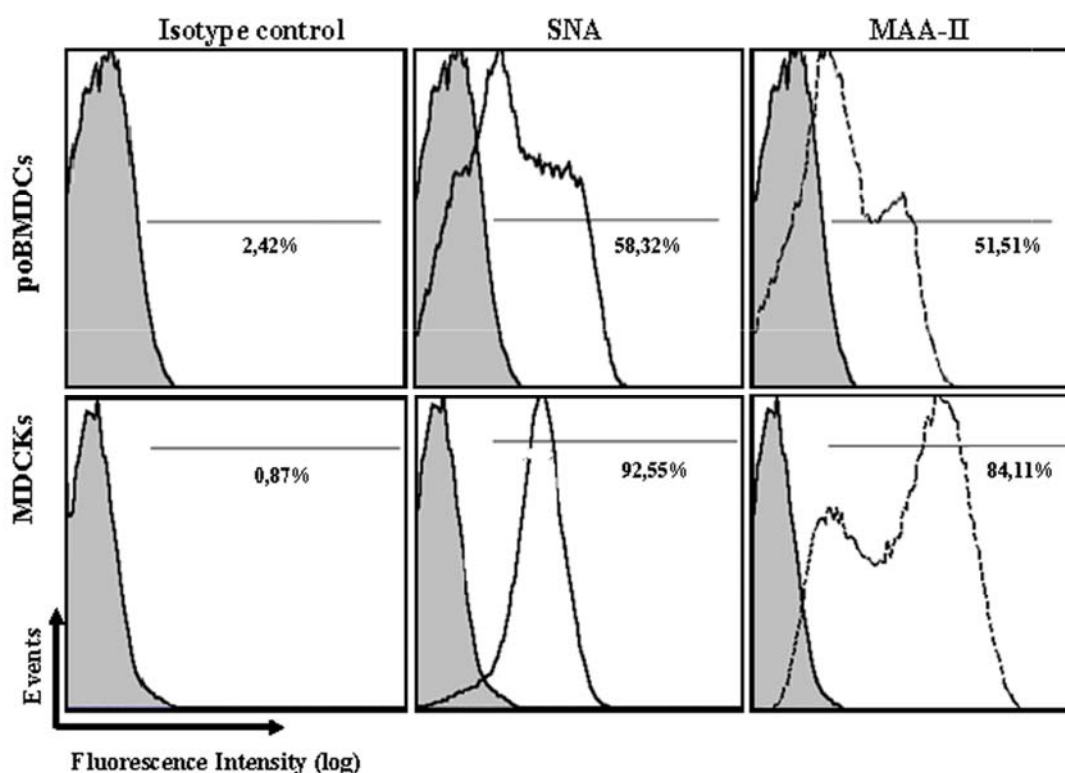
Figure 15. Expression of α-2, 3 and α-2, 6 sialic acids receptors on poBMDCs. Porcine BMDCs (at day 8 of generation) and MDCK were stained with *Macckia amurensis* (continuous line) and *Sambucus nigra* (dotted line) lectins. Then FITC-conjugated-

streptavidin was applied to reveal the binding. Gray histogram (isotype control). Representative results of two experiments.

4.3.2. Expression of SLA-I, SLA-II and CD80/86 by infected-poBMDCs.

To complete antigen presentation, DCs have to present antigen peptides (signal one) by means of SLA molecule and express co-stimulatory molecules (signal two) to T cells. This interaction also requires signal 3, which involves secretion of soluble mediators such as cytokines/chemokines for T cells undergoing activation/differentiation to initiate adaptive immune response. To assess the presenting capacity of poBMDCs after influenza infection with the set of viruses, SLA-I, SLA-II and CD80/86 molecules were analysed by flow cytometry. Surprisingly, only H3N2 SwIV was able to increase the expression of SLA-I and CD80/86 as compared with mock-infected DCs (tendency, $p=0.08$) whereas the rest of influenza viruses did not increase these cell markers with respect to mock infected DCs under the conditions of the study **(Fig. 16).**
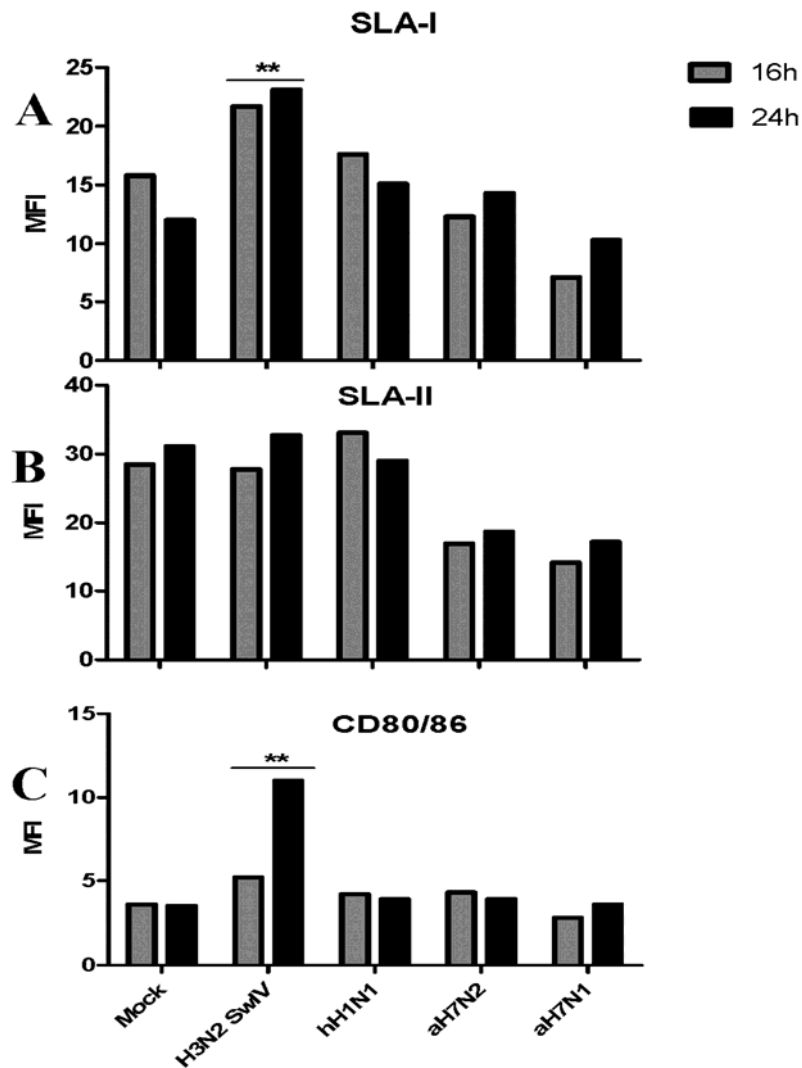
Figure 16. Mean fluorescence intensity (MFI) of SLA-I (A), SLA-II (B) and CD80/86 (C) expression by poBMDCs infected with *A/Swine/Spain/SF32071/2007(H3N2)*, *A/Catalonia/63/2009(H1N1)*, *A/Anas plathyrhynchos/Spain/1877/2009(H7N1)* or *A/Chicken/Italy/5093/1999(H7N1)* at 16 and 24hpi. After infection, cells were stained with anti-SLA-I (4B7/8), anti-SLA-II (1F12) and CTLA4-muIg followed by Phycoerythrin anti-mouse IgG. Statistical tendencies **p=0.08 were found in H3N2 SwIV infected cells compared to mock. Representative results of three independent experiments.

4.3.3. Expression of *NF-κB, TGF-β, IL-10* and *IFN-β* mRNA by infected-poBMDCs.

Activation of DCs by PAMPs may result in activation of several intracellular pathways that induce an activation of transcription factors or genes responsible of cytokine synthesis. Thus, we analysed the expression of *NF-κB, TGF-β, IL-10* and *IFN-β* genes by RT-qPCR on mock and infected cells with the set of influenza virus. All viruses used in this study induced *NF-κB* expression in a time dependent manner. The highest level was observed at 4h followed by decrease with the exception of hH1N1 in wich the highest level was at 8h and then decreased **(Fig. 17A)**. Also, all viruses induced expression of *TGF-β* mRNA which decreased. When H3N2 SwIV, hH1N1 and aH7N2 were used, an increase of *TGF-β* expression was observed from 4 to 8h followed by decrease whereas; in aH7N1 infected cells the highest expression was seen at 4h and then decreased **(Fig. 17B).** All viruses induced the expression of *IL-10*, which was time dependent, rising from 8h, reaching a peak at 16h and then decreased with the exception of aH7N2-infected cells in which the peak was at 24h (**Fig. 17C**); however, no *IL-10* protein was detected in the supernatants of non-mock or infected cells. Finally, the highest level of *IFN-β* expression was observed in H3N2 SwIV infected cells at 8h. hH1N1 induced the expression of *IFN-β* at 8 and 16hpi while the avian viruses induced little or no expression (**Fig. 17D**).
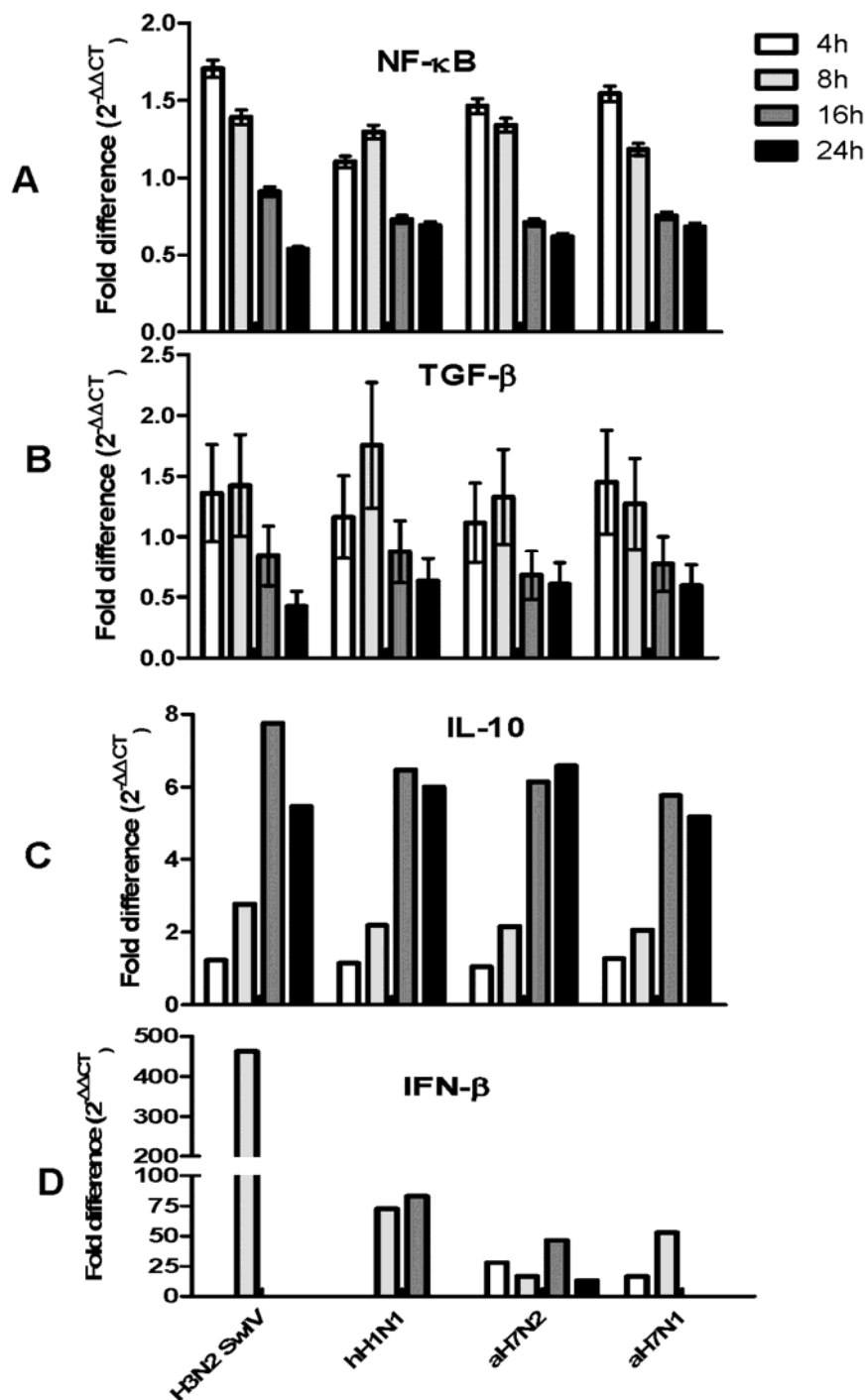
Figure 17. Relative quantitation of mRNA levels of *NF-κB* (A), *TGF-β* (B), *IL-10* (C) and *IFN-β* (D) in poBMDCs infected at different time points (4, 8, 16 and 24h) with *A/Swine/Spain/SF32071/2007(H3N2), A/Catalonia/63/2009(H1N1), A/Anas plathyrhynchos/Spain/1877/2009(H7N1)* or *A/Chicken/Italy/5093/1999(H7N1)* influenza

viruses. Bars represent the mean plus one standard deviation of three replicas. Representative results from two independent experiments.

4.3.4. Secretion of IFN-α, TNF-α, IL-12 and IL-18 by infected-poBMDCs.

IFN-α secreted by other viruses was under the limit of detection. The kinetics of IFN-α secretion by H3N2 SwIV-infected cells showed that the maximum level was reached at 24h post-infection **(Fig. 18A)**. Heat-inactivated H3N2 SwIV or aH7N1 viruses did not induce IFN-α secretion. H3N2 SwIV and aH7N1 induced more TNF-α (p=0.08) compared to hH1N1 and aH7N2 influenza viruses. However, the amount of TNF- induced by H3N2 SwIV was higher (p=0.08) than that induced by aH7N1 in all tested times **(Fig. 18B)**. Heat inactivated H3N2 SwIV induced TNF-α but at lesser extent compared to untreated virus whereas inactivated aH7N1 did not induce detectable TNF-α during the first 24 hours after infection (**Annexe 3**). H3N2 SwIV, aH7N2 and aH7N1 induced IL-12 in a time-dependent manner. An increase of IL-12 was observed from 16 to 24 hpi with all viruses and the amount induced by aH7N1 and aH7N2 at 24 hpi was higher (p=0.08) compared to H3N2 SwIV and hH1N1 induction (**Fig. 18C**). However, IL-12 was not detected when H3N2 SwIV or aH7N1 was heat-inactivated. Finally, all viruses used in this study induced IL-18 secretion in a time-dependent manner. Thus, H3N2 SwIV, hH1N1 and aH7N2 induced IL-18 secretion rising from 4h till 16h which later decreased while aH7N1-infected cells showed a very fast secretion of IL-18 at 4hpi followed by a decrease afterwards **(Fig. 18D)**. Moreover, the amount of IL-18 produced by aH7N2 and aH7N1 was higher (p=0.08) than that of H3N2 SwIV and hH1N1 in all the tested times. Inactivation of H3N2 SwIV resulted in IL-18 secretion but at lower levels compared to untreated virus while the inactivated aH7N1 did not induce IL-18 secretion **(Annexe 4).**
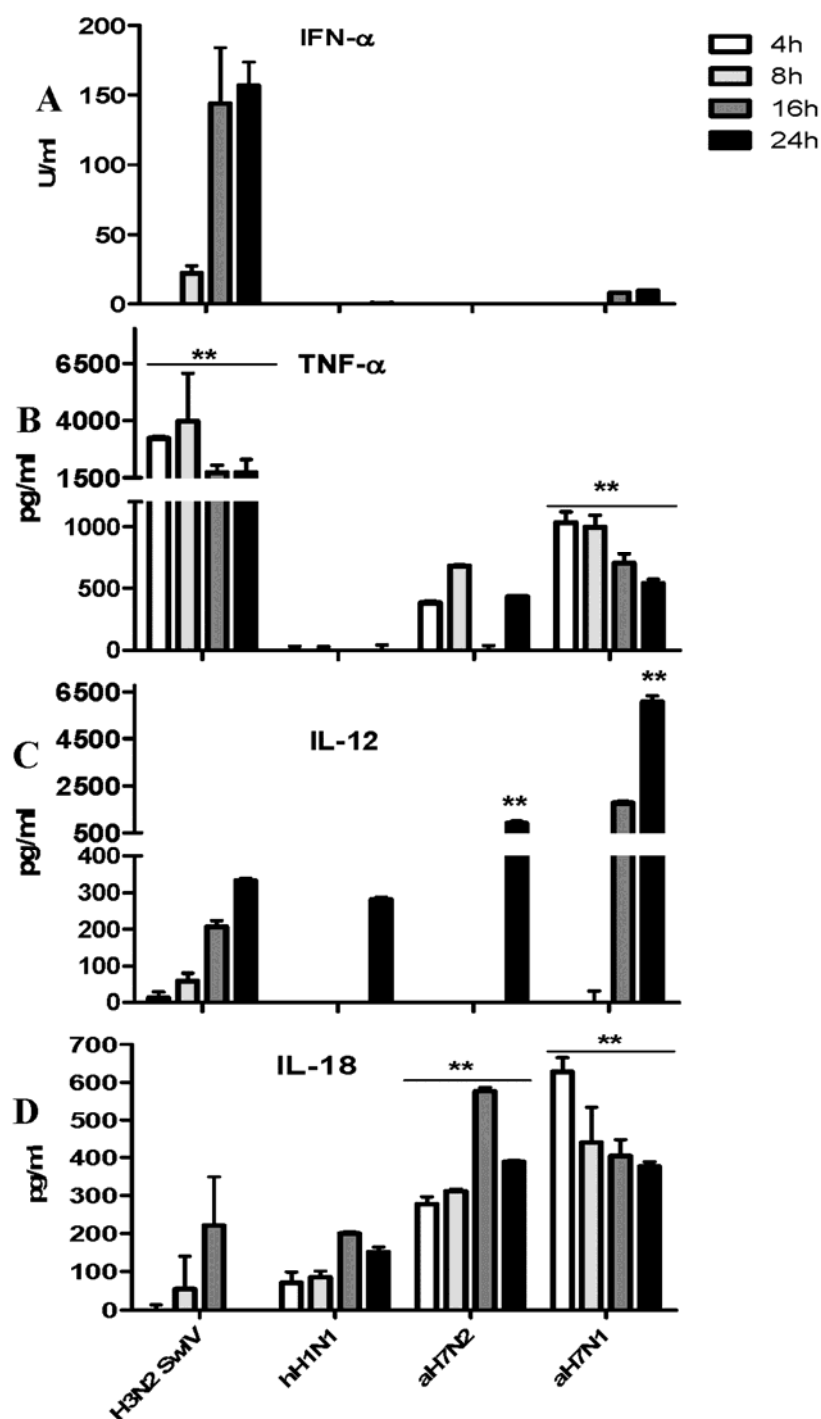
Figure 18. IFN-α (A), TNF-α (B), IL-12 (C) and IL-18 (D) cytokine secretion by poBMDCs at 4, 8, 16 and 24hpi with *A/Swine/Spain/SF32071/2007(H3N2)*, *A/Catalonia/63/2009(H1N1)*, *A/Anas plathyrhynchos/Spain/1877/2009(H7N1)* or *A/Chicken/Italy/5093/1999(H7N1)* influenza viruses. The secretion of mock cells was subtracted from the infected cells. Bars indicate mean plus one standard deviation.

Statistical tendencies ** p=0.08 were observed within the groups. Representative results of three independent experiments.

4.3.5. Inhibition of NF-ϰB pathway

As influenza virus NS1 protein was shown to be a suppressor of IFN-β and NF-ϰB activation [258] we wondered whether influenza virus may elicit cytokine responses through this pathway by using an inhibitor of NF-ϰB. Porcine DCs were infected with H3N2 SwIV and treated with Poly:IC to study IFN-α secretion. H3N2 SwIV-infected cells secreted more IFN-α (p=0.06) compared to Poly:IC treated cells after 24h and in both cases, IFN-α levels were reduced (p=0.06) when cells were previously treated with CAPE **(Fig. 19A)**. Also, H3N2 SwIV and Poly:IC induced IL-12 secretion which was reduced (p=0.06) after pre-treatment with CAPE **(Fig. 19B)**. Similar results were obtained for TNF- α secretion and IL-1β **(Annexe 5)**.
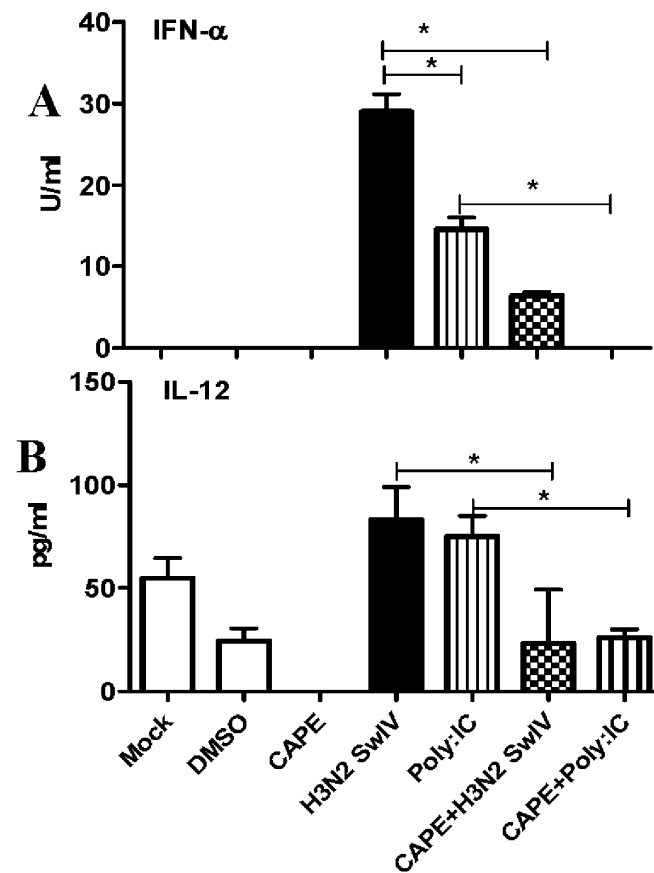
Figure 19. IFN-α (A), IL-12 (B) secretion in mock, DMSO and *A/Swine/Spain/SF32071/2007(H3N2)* infected-poBMDCs at 24h. Porcine BMDCs were treated with CAPE for 1h before H3N2 SwIV/Poly:IC infection/stimulation for 24h. Bars represent the mean plus one standard deviation. Statistical tendencies *p*=0.06 were observed within the groups. Representative results from two independent experiments.

5. STUDY III:

Differential dendritic cell interaction with virulent and non-virulent *Haemophilus parasuis* strains is immunomodulated by a pre-infection with H3N2 swine influenza virus.

Experience is not what happens to a man. It is what a man does with what happens to him.

Aldous Huxley

5.1. Introduction.

Respiratory disease is common in modern pig production worldwide and is often referred as porcine respiratory disease complex. This disease complex results from infection with diverse combinations of primary and secondary respiratory pathogens such as *Mycoplasma hyopneumoniae, Pasteurella multocida, Actinobacillus pleuropneumoniae,* **Haemophilus parasuis** and *Streptococcus suis.* Also, PRRSV, SwIV, PCV2 are frequent players in the respiratory complex disease. Indeed, combined infection of pigs with typically low pathogenic organisms like PCV2 and *M. hyopneumoniae* results in severe respiratory disease [104].

Here, the main objective was to study the immune response of dendritic DCs in an *in vitro* co-infection with H3N2 SwIV and non-virulent *H. parasuis* serovar 3 (SW114) and virulent serovar 5 (Nagasaki).

5.2. Materials and Methods.

5.2.1. Cells.

The cells for this study were generated as described in Study I with the exception that the culture medium had no antibiotic.

5.2.2. *Haemophilus parasuis.*

Two reference strains of *H. parasuis,* SW114 and Nagasaki, belonging to the nasal and systemic clade respectively on the MLST were used. SW114 is the reference strain of serovar 3 and it is non-virulent; Nagasaki is the reference strain of serovar 5 and it is highly virulent. Before infecting DCs, bacteria

were grown overnight as described by Olvera *et al.,* [112]. Briefly, strains were cultured on chocolate agar plates (BioMérieux, Madrid, Spain) overnight at 37ºC with 5% CO_2. The following day, fresh colonies were collected and resuspended in PBS at the appropriate concentration. Bacterial counts were confirmed by plating serial dilutions on chocolate agar plates [112].

5.2.3. Influenza virus.

The porcine *A/swine/Spain/SF32071/2007(H3N2)* SwIV strain was the same used in studies I and II. Virus isolation and cell infection was performed as previously described in study I and II except for the fact that the post infection medium has no antibiotics.

5.2.4. poBMDC infection or stimulation with toll like receptor 3 agonist.

After 8 days of generation, poBMDCs were harvested washed with RPMI (LONZA) with neither antibiotic nor serum, counted and plated on 24 well plates. Then, 10^7 CFU (MOI 10) of SW114 or Nagasaki were added to each respective well. Cells were incubated with the bacteria at 37ºC for 1h and after that they were washed thrice with RPMI by centrifugation at 450x*g* for 5 minutes. After the third wash the supernatants were discarded, 500 µl of RPMI containing L-glutamine and 10% FCS were added and cells were left for a further 7h at 37ºC 5% CO_2. One hour after bacteria addition, cells were washed, harvested and stained. At 8hpi, plates were centrifuged, the supernatants frozen at -20ºC for cytokine detection by ELISA and cells harvested for staining. When required, infection with H3N2 SwIV was performed as follows: 100 µl with 10^5 $TCID_{50}$ of swine influenza H3N2 virus were added and left to adhere for 1h at 37ºC 5%CO_2. After that, cells were washed once with RPMI and incubated with medium only or with *H. parasuis*

as stated previously. When required, cells were stimulated with 50µg/ml of Poly: IC for 4h followed by infection with *H. Parasuis* for a further 6h.

5.2.5. Staining of *Haemophilus parasuis.*

Staining of *H. parasuis* was assessed by flow cytometry or by immunofluorescence at 1h and at 8hpi, as follows. For flow cytometry, poBMDCs were fixed with 4% PFA (EMS, Hatfield, PA, USA) for 10 min at RT. After washing (centrifugation 450x*g* for 5 min at 4ºC), cells were permeabilized with 0.1% Triton-X100 in PBS for 15 min at 37ºC. Then, 1h incubation at 4ºC for each antibody was used. A polyclonal rabbit anti-SW114 or anti-Nagasaki serum (10 µl serum /490 µl PBS 2% FCS) was used as primary antibody and as secondary antibody the monoclonal anti-rabbit Ig-FITC (Clone RG-16, Sigma Aldrich, Madrid, Spain) diluted 1:100. For immunofluorescence, cells were infected on a circular glass cover slip (VWR International, Barcelona, Spain) inserted in a 24 well plates (Nunc®, Kamstrupvej, Denmark). After infection, supernatants were recovered for ELISA. Then, cells were fixed with ethanol (Panreac, Barcelona, Spain) for 10 min at 4ºC, dehydrated with acetone and then permeabilized with 0.1% Triton X-100 for 15 min at 37ºC. Next, cells were washed with PBS with 0.1% BSA and 100 µl of polyclonal rabbit anti-SW114 or anti-Nagasaki serum diluted as before, were added and slides incubated for 1h at 4ºC. After three washes a secondary antibody anti-rabbit Ig-FITC diluted 1:50 was added and left to incubate for further 1h at 4ºC. Phalloidin-Tetramethylrhodamine B isothiocyanate (TRITC) (Sigma-Aldrich, Madrid, Spain) at 5 µg/ml was used to stain the cytoplasm and finally after several washes, nuclei were counterstained with DAPI. Cover slips were dried and mounted using 1 drop of Fluoprep (BioMérieux, Madrid, Spain). To detect unspecific binding, mock or infected-poBMDCs, were stained as controls with the primary and/or

secondary antibody. Treated cells were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporate, Japan). To assess the association of bacteria with poBMDCs, image stacks were captured using a Leica TCS SP2 confocal microscope, with an objective of 63x. Z stack images were acquired at intervals of 0.3 μm. Images were processed by using the ImageJ v1.42k software (http://rsb.info.nih.gov/ij).

5.2.6. Electron microscopy.

Mock and infected poBMDCs were fixed with 2% (w/v) PFA and 2.5% (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) in 0.1 M PB and processed following conventional procedures as previously described in detail by Rodríguez-Cariño *et al.,* [240,241] and in Study I.

5.2.7. Cell invasion assay.

Cells were infected as described above, and after 1h of *H. parasuis* infection (time zero), RPMI containing L-glutamine, 10%FCS, penicilin G (5 μg/ml) and gentamicin (100 μg/ml) (both from Sigma-Aldrich, Madrid, Spain) were added and poBMDCs left at 37ºC and 5%CO_2 for a further two or four hours. After these times, poBMDCs were centrifuged and 100 μl supernatants were plated on chocolate agar (Biomérieux, Madrid, Spain) to check if *H. parasuis* were efficiently killed by antibiotics. Then, after three washes with PBS, poBMDCs were disrupted using sterile water, and serial 10 fold dilution of lysate were plate on chocolate agar for 48h. The results were expressed as log CFU/ml.

5.2.8. Activation markers.

The activation marker of mock or infected cells either with H3N2 SwIV or *H. parasuis* or co-infected cells, was performed as described in Study I, by using hybridoma supernatants containing anti-SLA-I (4B7/8) or anti-SLA-II (1F12), and the human CD152 (CTLA4)-muIg fusion protein as primary Abs and the R-phycoerythryn anti-mouse IgG as secondary Ab. Stained cells were acquired using a Coulter® EPICS XL-MCL cytometer and analysed by EXPO 32 ADC v.1.2 program. The MFI of each sample were analysed.

5.2.9. ELISA.

The supernatants of 8h infected-poBMDCs were defrosted only once for cytokine screening by ELISA. All ELISAs were read with KC Junior Program (BioTek, Potton, UK) using the filter PowerWave XS reader. For IL-6, IL-1β, IL-10 and TNF-α the Duo Set Developed system ELISA from R&D System® was used following the manufacturer's instructions. To detect IL-8 and IL-18, swine IL-8 (CXCL8) VetSet™ ELISA development kit (Kingfisher Biotek, MN, USA) and pig IL-18 Module Set BMS672MST (Bender Med Systems, Vienna, Austria) were used following the manufacturer's instructions. For IFN-α and IL-12 an in house ELISA using antibody purchased from PBL Interferon Source and R&D System® respectively and the procedure from study II were followed.

5.2.10. Statistical analysis.

All statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). For statistical comparisons, pig (as source of cells) was used as the experimental unit. The significance level (α) was set at 0.05. A non-parametric test (Mann-Whitney) was chosen to compare the different

values obtained for all the immunological parameters between groups at all sampling times.

5.3. Results.

5.3.1. Differential interaction of *H. parasuis* Nagasaki and SW114 individually or in SwIV co-infection with poBMDCs.

Measuring *H. parasuis* interaction with swine DCs could be a good indicator of virulence. Also, DCs ability to capture antigens may be important in the interaction that DCs may have with a particular pathogen to elicit a specific immune response. Therefore, *H. parasuis* interaction with poBMDCs was studied by flow cytometry after staining infected cells with specific serum against SW114 or against Nagasaki at two different time points. Porcine BMDCs infected with the non-virulent SW114 strain presented higher fluorescence intensity compared to Nagasaki infected cells (**Fig. 20A)** at 1hpi, whereas at 8hpi both strains showed a similar pattern (**Fig. 20B)**. This result indicate that, at early time points, non-virulent SW114 strain interact in higher numbers with poBMDCs than the virulent Nagasaki counterpart.
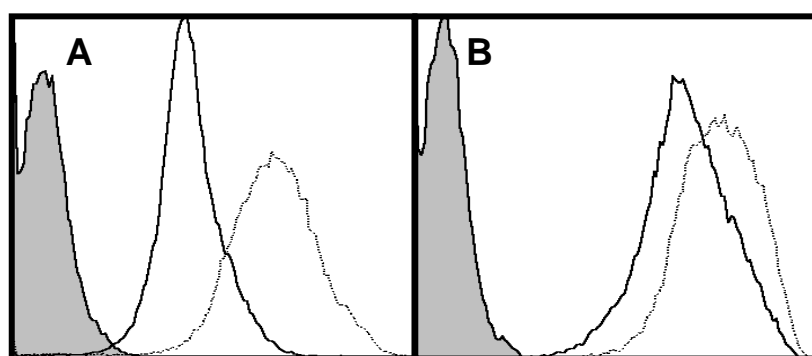
Figure 20. Staining of *H. parasuis* SW114 or Nagasaki at 1h (A) and 8h (B) after infection. PoBMDCs were infected and stained using anti-SW14 or anti-Nagasaki rabbit serum for 1h at 4ºC, and then with the anti-rabbit IgG-FITC antibody. Mock (grey histograms), SW114 (dotted line), Nagasaki (continuous line). Representative results from four experiments.

The ability of poBMDCs to interact with and internalize *H. parasuis* was confirmed by confocal and electron microscopy for SW144 and Nagasaki. Confocal microscopy was performed using serum against each particular strain. One hour after infection, SW114 strain was observed attached and inside poBMDCs (**Fig. 21, I and I.a**) and bacterial numbers increased at a later time point inside the cell (**Fig. 21, II and II.a-c**). Conversely, Nagasaki strain was mostly attached to the cells 1 hpi, though in small numbers (**Fig. 22, I**). At 8hpi, Nagasaki was observed not only attached but also inside the DCs (**Fig. 22, II.a-e)**.
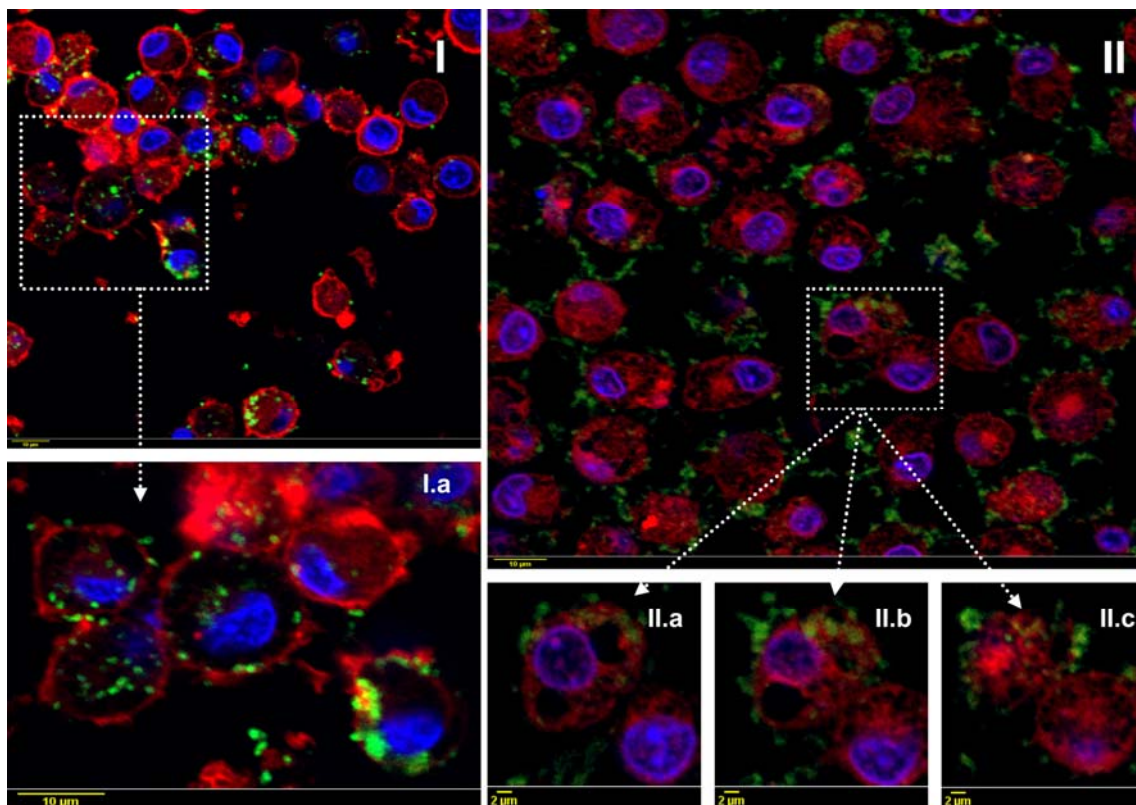
Figure 21: **Confocal images of poBMDCs infected with SW114 1h (I) and 8h (II).** Porcine BMDCs were stained with polyclonal anti-SW14 rabbit serum and anti-rabbit IgG-FITC antibody. Cytoplasm was stained with rhodamine-phalloidin and nuclei with DAPI. Green (SW114), red (cytoplasm), blue (nuclei). Bars I, I.a, and II = 10 μm, while bars II.a-c = 2 μm. **I.a, II.a-c** are representative results of Z stacks sections.
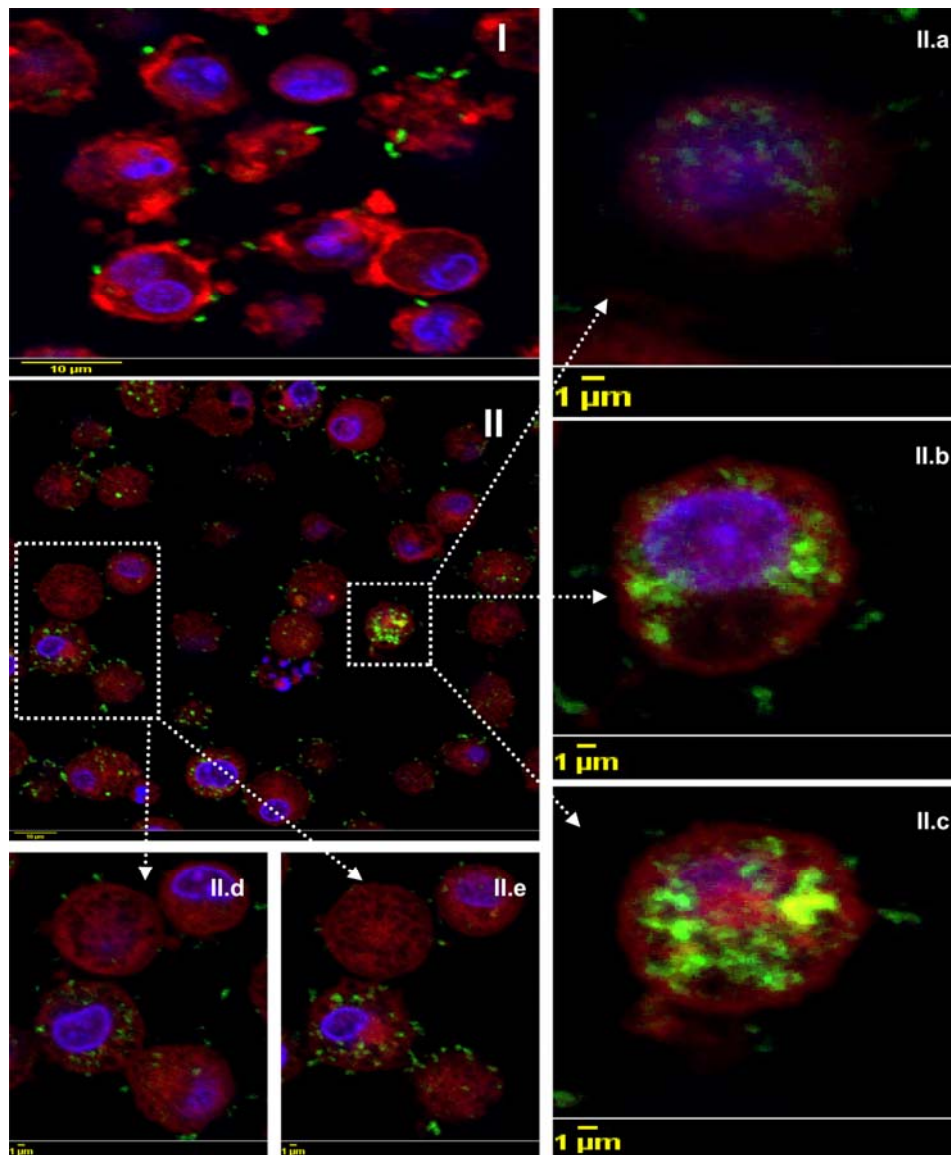
Figure 22. Confocal images of poBMDCs infected with Nagasaki 1h (I) and 8h (II). Porcine BMDCs were stained with polyclonal anti-Nagasaki rabbit serum and anti-rabbit IgG-FITC antibody. Cytoplasm was stained with rhodamine-phalloidin and nuclei with DAPI. Green (Nagasaki), red (cytoplasm), blue (nuclei). Bars I and II = 10 μm, while bars II.a-II.e = 1 μm. **II.a-e** are representative results of Z stacks sections.

Once it had been observed by immunofluorescence that the two strains of *H. parasuis* have different abilities to interact with poBMDCs, we went on to study this interaction at the ultrastructural level. At 1 or 8hpi, mock poBMDCs showed normal morphology (**Fig. 23A** and **23A'**). In agreement with the fluorescence microscopy results, differential interaction with

poBMDCs was observed after SW114 or Nagasaki infection: higher numbers of SW114 bacteria were found inside phagolysosome-like structures as compared with Nagasaki infected cells (**Fig. 23B**). At 8hpi, many SW114 were also in the extracellular space (**Fig. 23B'**). Moreover, SW114 showed different levels of degradation (**Fig. 23B´close up**). Infection of poBMDCs with Nagasaki induced higher subcellular changes. However, few bacteria were observed inside the poBMDCs after the first hpi but an important number of vesicles were observed (**Fig. 23C**). These changes were more evident at 8 hpi. At this time, larger vesicles containing more than one Nagasaki bacteria were observed (**Fig. 23C'**).
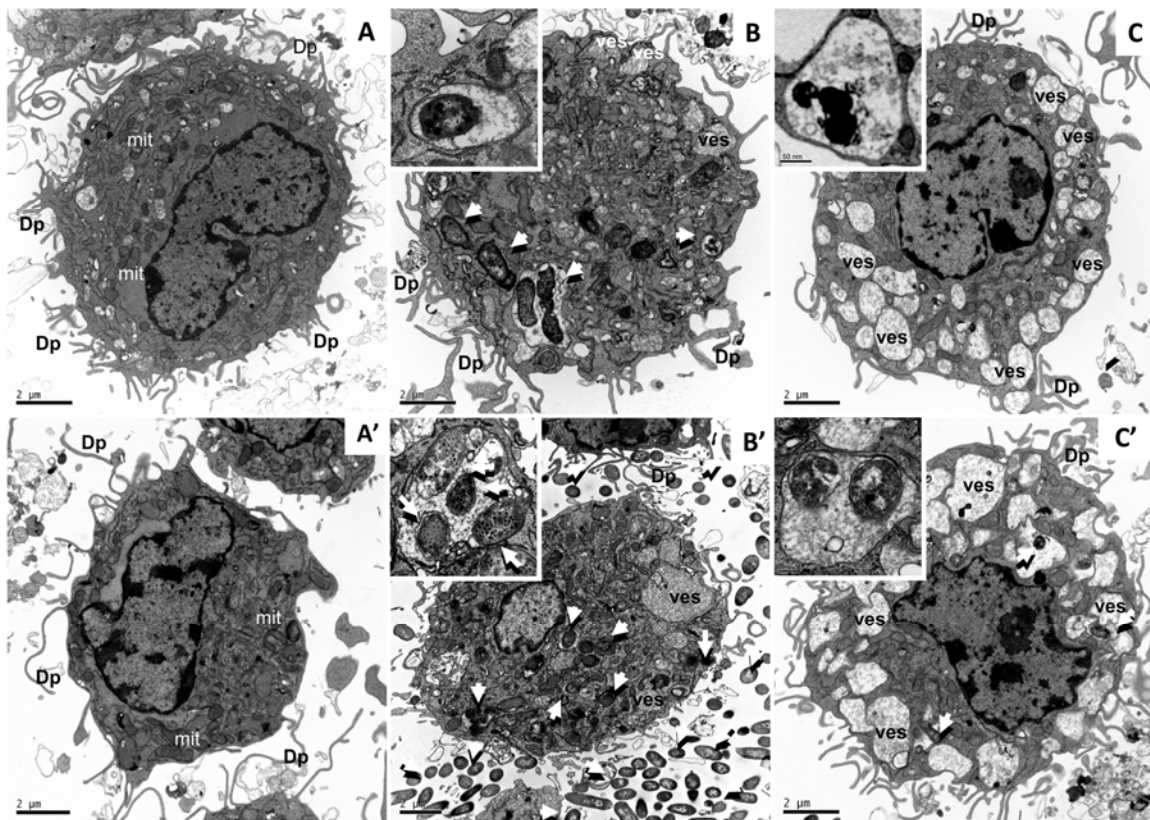
Figure 23. EM of mock poBMDCs or *H. parasuis* infected-poBMDCs. A, B and **C** 1hpi, **A', B'** and **C'** 8hpi. **A** and **A'**: Mock poBMDC with normal morphology of DC, dendritic processes (Dp) as well as some organelles such as mitochondria (mit). **B** and **B'**: SW114-infected-poBMDC. **B**: poBMDC with several vesicles in the cytoplasm (ves) some of them containing bacteria (white-arrows indicate some of the bacteria). Bacteria are inside

phagolysosomes and some had a degenerate aspect (more electron dense); in the upper-left box, details of bacteria can be observed with phagosome membrane bound. **B'**: 8h later, more SW114 bacteria were appreciated inside and outside the poBMDCs (white-arrows). **C** and **C'**: Nagasaki-infected DCs. **C**: 1hpi, there were several vesicles (ves) observed in the cytoplasm, of different sizes and, some contained bacteria (white-arrow) as can be noted in the upper-left box. **C'**: 8h later, the number and size of vesicles (ves) had increase and also the Nagasaki bacteria were found inside vesicles (white-arrow) within the cell (close-up). Bar = 2 μm.

To get insight into the immune modulation of DCs in a previous infection with SwIV followed by *H. parasuis* infection, *in vitro* co-infection experiments were performed. The behaviour of poBMDCs to *H. parasuis* infection when a previous infection with H3N2 SwIV took place was analyzed by flow cytometry (**Annexe 6)**, fluorescence microscopy (**Annexe 7**) and by EM (**Fig. 24**). As previously observed, both *H. parasuis* strains showed differences in their interaction with poBMDCs, even when SwIV pre-infection was present: H3N2 SwIV plus SW114 co-infected cells, showed a higher level of interaction with poBMDCs than H3N2 SwIV plus Nagasaki at 1hpi (**Annexe 6**) and at 8hpi (**Fig. 24A1, 2 and 24B1, 2**). In these experiments, poBMDCs infected with both *H. parasuis* strains presented SwIV virus-like particles inside vesicles and on some occasions SW114 or Nagasaki were in the same phagolysosome-like structure as these SwIV virus-like particles (**Fig. 24A3 and 24B3**). At 8hpi, SW114 co-infected cells showed many bacteria inside phagolysosome-like structures with different levels of degradation (**Annex 8: D-G**). Moreover, the organelles of Nagasaki co-infected cells showed important changes such as dilation of the Golgi complex (**Annexe 9: F, H**) and degraded bacteria inside phagolysosome-like structures (**Annexe 9: G´**).
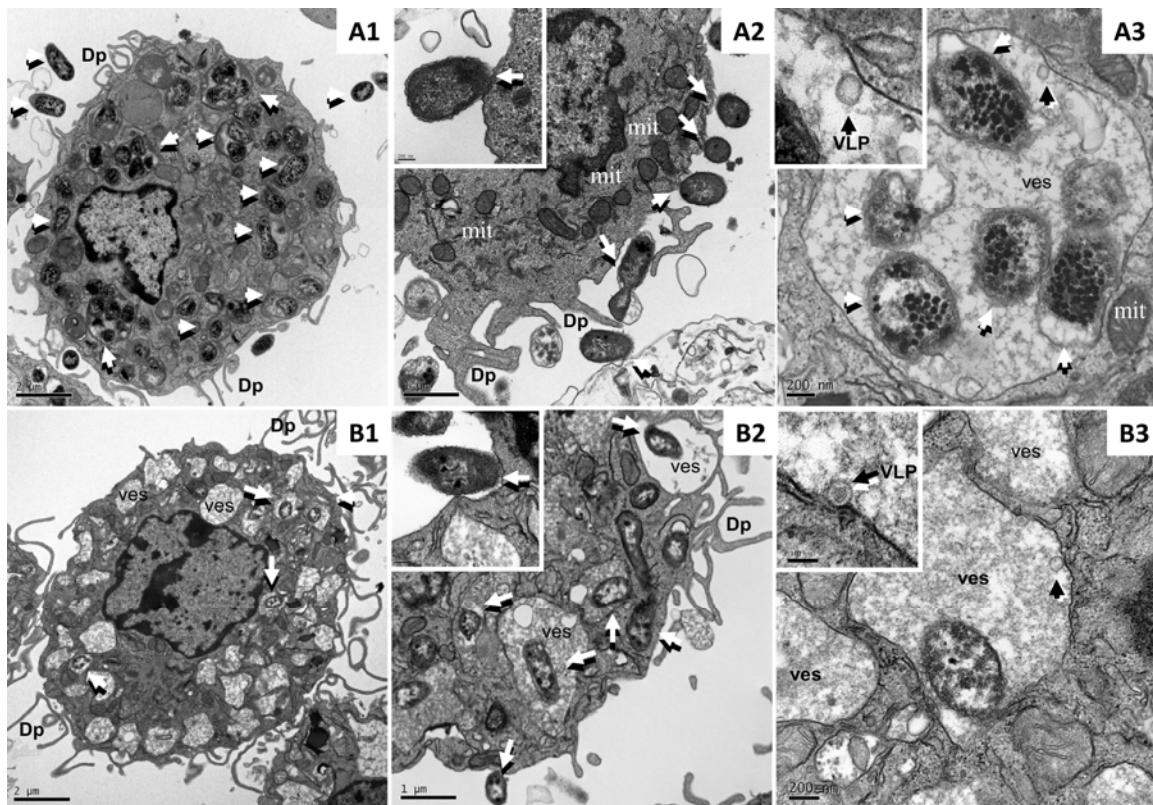
Figure 24. **EM of poBMDCs co-infected with H3N2 SwIV and *H. parasuis* at 8hpi**. **A1**, **A2** and **A3**: H3N2 SwIV plus SW114 strain. **A1**: Several bacteria were observed inside the poBMDCs (white-arrow) occupying phagolysosomes, and few vesicles, Bar = 2 μm. **A2**: Many SW114 bacteria were observed attached to the cellular membrane (white-arrow), as can be observed in the close-up (left-up box), Bar = 1 μm. **A3**: In some phagolysosomes which contained bacteria (white-arrow) also virus-like particles (VLPs) were observed, (black-arrow) 100 nm in diameter, Bar = 0.5 μm. B1, B2 and B3: H3N2 SwIV plus Nagasaki strain. **B1**: Few Nagasaki bacteria (white-arrow) were seen in poBMDCs. However, many vesicles of different sizes which occupied large sub-cellular space were observed; less organelles were seen, Bar = 2 μm. **B2**: Some poBMDCs showed a large number of bacteria (white-arrow) inside and outside. Nagasaki was also, to a lesser extent, attached to the cellular membrane, Bar = 1 μm. **B3**: This figure shows a phagolysosome-like structure with Nagasaki strain and VLPs (black-arrow) inside, in the upper-left box VLPs can be observed. Bar = 0.2 μm.

5.3.2. Survival of *H. parasuis* after incubation with poBMDCs.

To study the capacity of these poBMDCs in internalizing and processing antigens, a bacteria survival assay was performed. Thus, 1h after *H. parasuis* infection, unbound bacteria were removed and poBMDCs were incubated in medium containing penicillin G and gentamicin for two or four extra hours. After these times, the number of internalized viable bacteria decreased when compared to the time zero (**Fig. 25**). The differences between SW114 and Nagasaki in their interaction with poBMDCs were noteworthy, with the number of SW114 being higher than the number of Nagasaki.
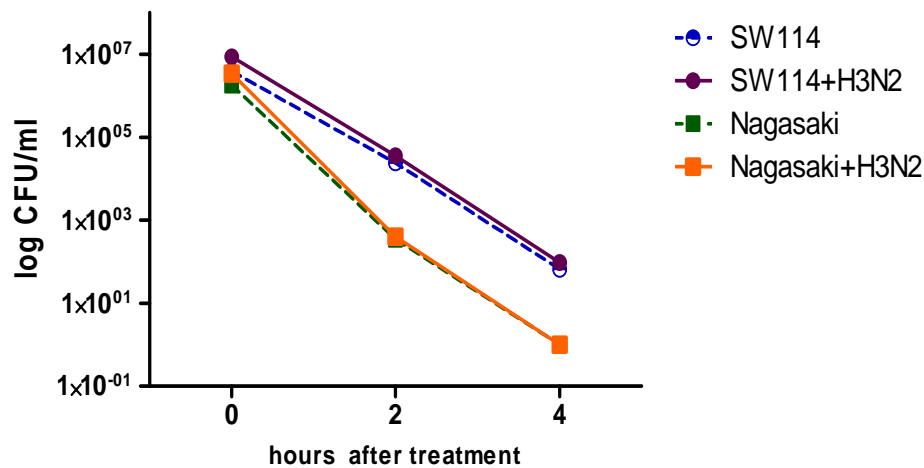
Figure 25. Invasion/adhesion of *H. parasuis*. Porcine BMDCs were infected or co-infected with H3N2 SwIV and SW114 or Nagasaki. After 1h (time zero) cells were incubated with gentamicin and penicilin G for 2 or 4h. After each time, cells were lysed and 100 µl plated on chocolate agar for 48h. Two replicas of each condition were processed in each experiment. Bars indicate mean plus one standard deviation. Representative result from three independent experiments.

5.3.3. Activation profile of *H. parasuis* or *H. parasuis* and SwIV-infected poBMDCs.

One of the hallmarks of DCs is the triggering of an effective immune response by capture, processing and presenting antigens to T cells. The antigen presenting process needs a complete signalling, where the MHC molecules presentation (signal one), activation or co-activation molecules (signal two) and the soluble mediators such as cytokines (signal three) participate. Therefore, to evaluate the level of expression of activation markers, poBMDCs were stained for SLA-I, SLA-II and CD80/86 after infection with SW114 or Nagasaki. After 1h of infection with *H. parasuis*, no significant differences in SLA-I, SLA-II or CD80/86 expression were found (**Fig. 26**). Statistically significant decreases ($p<0.05$) were found at 8hpi in SLA-I and SLA-II of SW114-infected poBMDCs (**Fig. 26A, B**). No significant differences were observed in CD80/86 expression (**Fig. 26C**).

Also, expression levels of activation markers and cytokine patterns after co-infection were analyzed. One hour after H3N2 SwIV single infection or H3N2 SwIV-SW114 and H3N2 SwIV-Nagasaki co-infections, no significant differences ($p>0.05$) were observed in SLA-I and CD80/86 expression. However, at this time, significant decreases ($p<0.05$) of SLA-II were observed in H3N2 SwIV plus SW114 or Nagasaki co-infected cells when compared to mock cells (**Fig. 26B**). At 8hpi, H3N2 SwIV single infection or co-infection with Nagasaki induced a statistically significant increase of SLA-I when compared to SW114 single infection (**Fig. 26A**). Also, at 8hpi, co-infection of poBMDCs with H3N2 SwIV and SW114 or Nagasaki resulted in a significant increase in the secretion of IL-12 compared to mock control cells (**Fig. 27IV**). IL-12 production in Nagasaki infected-poBMDCs was modulated by a previous virus infection, while induction of IL-12 by SW114 was not affected. H3N2 SwIV induced higher levels of IFN-α compared to mock control cells. Surprisingly, significant decreases of this cytokine were observed in H3N2

SwIV-SW114 co-infected cells compared to H3N2 SwIV-Nagasaki co-infected cells (**Fig. 27VIII**).
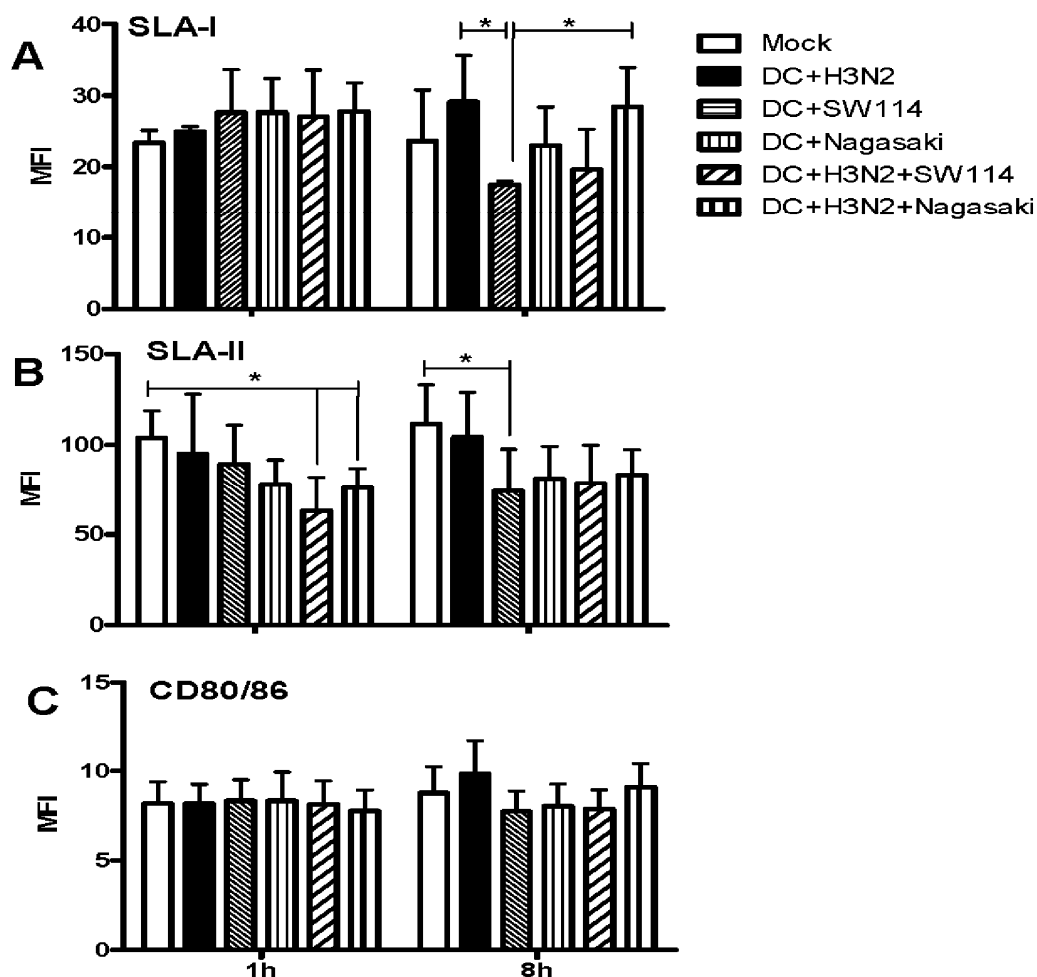
Figure 26. Expression of (A) SLA-I, (B) SLA-II and (C) CD80/86 at 1h and 8hpi. After each analysed time, mock and infected-poBMDCs were stained using mAb anti-SLA-I, anti-SLA-II or anti-human fusion protein (CTLA4-muIg) for CD80/86. Mean fluorescence of each markers were analysed. Asterisk (*) indicates significant difference between the compared groups $p < 0.05$. Representative results from three independent experiments.

5.3.4. Cytokines pattern of *H. parasuis* infected poBMDCs and SwIV immunomodulation.

Once the profile of poBMDCs activation after *H. parasuis* infection was described, we analysed the cytokine profile to figure out the type of immune response that these pathogens may induce in poBMDCs. Consequently, IL-1β, TNF-α, IL-6, IL-10, IL-8, IL-12, IL-18 and IFN-α were analysed 8 hpi. Statistically significant differences ($p<0.05$) were found in the secretion of IL-1β, IL-6, TNF-α and IL-10 between SW114 and Nagasaki-infected poBMDCs compared to mock cells whereas IL-12 was secreted significantly in higher levels by SW114-infected cells compared to Nagasaki-infected cells and to mock cells (**Fig. 27**). No significant differences were found in IL-8, IL-18 or IFN-α secretion from SW114 or Nagasaki infected cells compared to the mock controls (**Fig. 27V, VI, and VIII**).
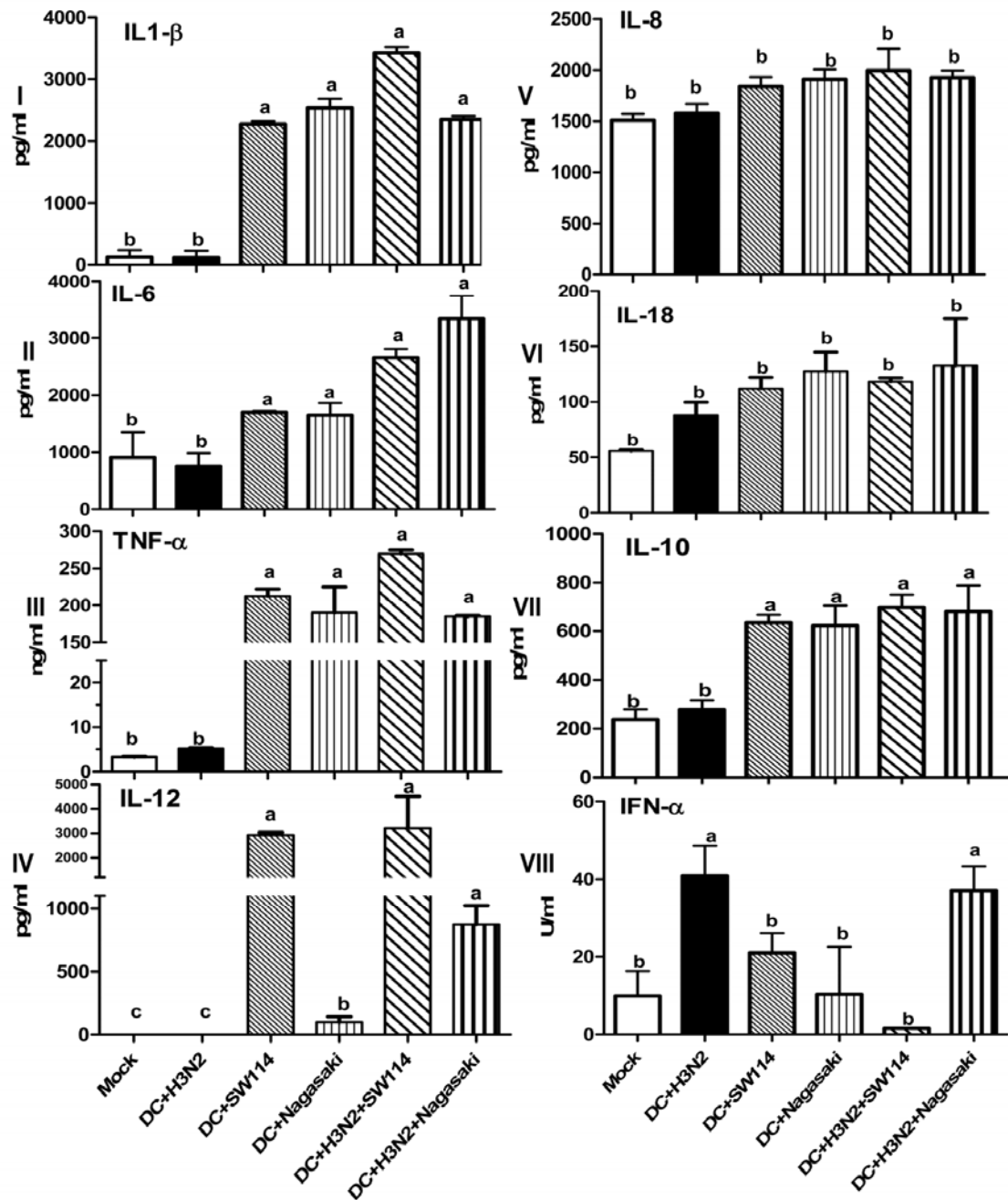
Figure 27. Cytokine secretion by poBMDCs at 8hpi. Porcine BMDCs were infected or co-infected with H3N2 SwIV and SW114 or Nagasaki. Supernatants of mock, single or co-infection were analysed for cytokine secretion using ELISA. Bars are mean plus one standard deviation. The presence of significant differences ($p<0.05$) between groups are represented by (a, b or c). Representative results from three independent experiments.

5.3.5. Toll like receptor 3 immunomodulation on *H. parasuis* infected poBMDCs.

The differences in IL-12 and IFN-α secretion by cells infected with SW114 or Nagasaki strain and the modulation of both cytokines in SwIV-*H. parasuis* co-infected DCs led us to investigate whether other stimuli, like an agonist of TLR3 could alter this cytokine pattern in the same way. Hence, DCs where pre-treated with Poly:IC, mimicking dsRNA virus infection, before infection with *H. parasuis*. Lower levels of IL-12 and IFN-α induced by Nagasaki infection, shown in **figure 27**, were overcome by pretreatment with Poly:IC, levelling up IL-12 and IFN-α between both *H. parasuis* strains **(Fig. 28)**.
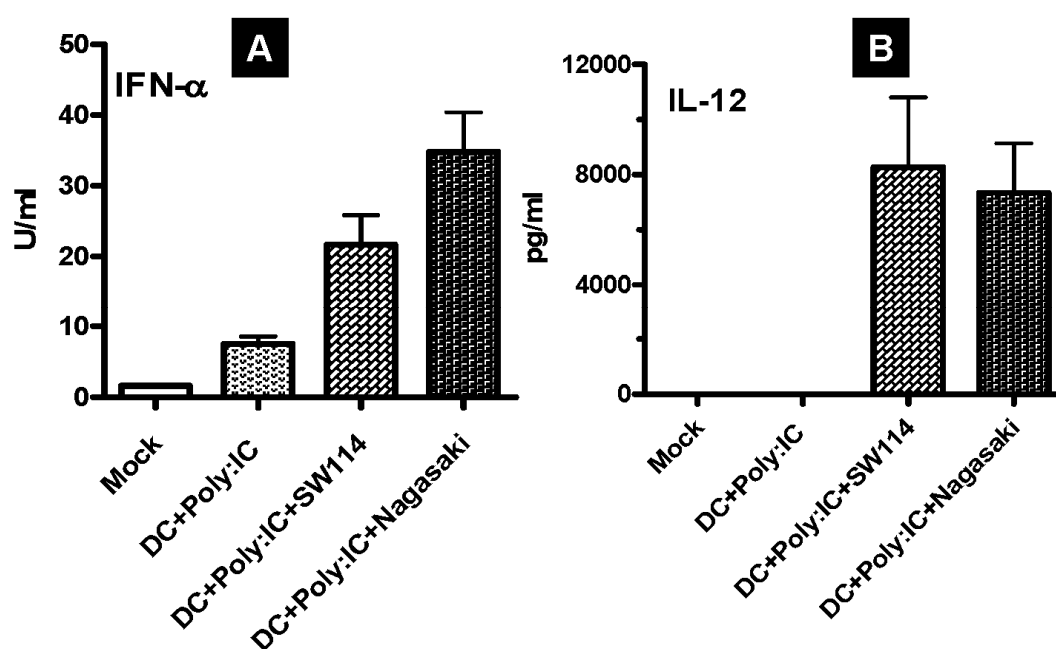
Figure 28. IFN-α and IL-12 secretion by poBMDCs stimulated with TLR 3 agonist before *H. parasuis* infection. Porcine BMDCs were stimulated with Poly:IC for 4h and then infected with SW114 or Nagasaki. Supernatants of mock and stimulated samples were analysed for **(A)** IFN-α and **(B)** IL-12 secretion using ELISA. Bars are mean plus one standard deviation. Representative results from two independent experiments.

6. DISCUSSION

We think in generalities, but we live in details.

Alfred North Whitehead

Several aspects of influenza virus replication cycle have been studied, especially the molecular events that occur during the infection [4,6-11,24,26,41,259,260] or the immune response as result of infection [155-157,165,188,190,191,261-265]. *In vitro*, studies related to the replication cycle were performed in epithelial cells [9,38,266-268], using respiratoy explant [256] or pulmonary organ [269]. The majority of studies regarding to the immune responses were mostly handled in mice [213,262,270-272] and there is an emerging interest on studying the immune response of influenza virus using porcine DCs [155,157] and macrophages [189] *in vitro*. During virus or bacteria infection of the respiratory tract, diverse cells such as macrophage, DCs, NK and T cells with a specialized function are recalled to eliminate pathogens. Among these cells DCs are strategically located underneath the epithelium of respiratory organs and from theses sites they scan for pathogens or danger signals by using dendritic processes. Although the knowledge on swine immunology has been developing quickly in recent years, studies to highlight the interaction of pathogens with the porcine immune system are scarce. With the aim to understand at cellular level the interaction of influenza virus and *H. parasuis* as common pathogens of pig's respiratory tract, three studies were undertaken and are presented in this thesis.

Study I: Interaction of porcine cDCs with swine influenza virus.

The aim of this study was to evaluate the interaction between porcine DCs and a circulating SwIV and the possible role by the former in being carriers of porcine influenza virus. We used poBMDCs to strengthen our understanding of the interaction of SwIV with the host immune response. There is convincing evidence that cDCs play a critical role *in vivo* in immunity to acute infections [261,273,274]. Based on these studies, our working hypothesis in this study was that SwIV infection of respiratory epithelial cells induces

inflammation, resulting in recruitment of monocytes that differentiate into DCs and in activation of sentinel DCs. Then, resident DCs in the respiratory epithelial tissue would encounter SwIV during influenza virus infection. Indeed, DCs and macrophages reside beneath the epithelium of the respiratory organs, and these cells are potential targets for influenza viruses [275]. Thus, DCs would be exposed to virus before leaving the inflamed tissue to stimulate the adaptive immune response in the regional lymph nodes. Porcine BMDCs are considered an experimental model for porcine cDCs, a group which includes monocyte derived DCs as well as resident DCs. Therefore, we focused this study on understanding the nature of the interaction between H3N2 SwIV and cDCs in an *in vitro* system. In a natural setting of influenza infection, humans and animals are usually infected at very low multiplicities of infection [276]. Thus, our experimental system was set up using a rather low MOI with the intention of both, mimicking the natural setting of the infection and preserving cellular integrity during the experimental procedure. Interaction of DCs with virus has been proven to be extremely dependent on the type of virus and the DCs being studied. Our general knowledge on this interaction has been mainly obtained from humans and mice where there are numerous examples ranging from functional inhibition of DCs or weak response after viral infection [275] to the exploitation of DC functions for the dissemination of virus infection [277]. Other experimental systems have been used to study influenza infection and interaction with DCs in other natural hosts, like horses. In an *in vitro* equine MoDCs model, equine influenza virus successfully infected and initiated transcription of its viral genome in DCs, although only limited viral protein synthesis and progeny was achieved [278]. In pigs, a study on porcine DCs has recently been published [155] but the authors focus on the ability of pDCs to respond to different influenza virus strains. Therefore, our data on poBMDCs interaction with a circulating H3N2 SwIV are, to our knowledge, the first one

describing that porcine DCs support a limited increase in viral H3N2 SwIV RNA, and showing that influenza infected DCs are able to infect susceptible cells by cell-to-cell contact.

In vitro, for different purposes including vaccine development, studies of influenza virus are mainly carried out in polarized cells [12]. Thus, MDCK were used for many years as a model to study the replication cycle of influenza virus *in vitro*. A body of accumulating evidence indicates that complete influenza viral particles are not found inside the infected cell and the process of assembly, morphogenesis, budding and release of progeny virus particles takes place at the plasma membrane of the infected cells [7]. These events are crucial for the production of infectious virions and pathogenesis of influenza virus. However, our data indicates that H3N2 SwIV like structures are detected freely in the cytoplasm of poBMDCs, surprisingly located near intracellular membranes (Golgi and reticulum). Similar experiments were conducted with other influenza viruses different from the H3N2 SwIV selected for this study to analyse whether this interaction was a particular feature of the H3N2 chosen: and the results were identical to the ones shown previously with H3N2 SwIV. As defective particles are produced during RNA viruses replication [13] probably they could profoundly compromise the course of infection and the replication in porcine DCs. Moreover, assembly is a very inefficient process in which more that 90% of virus particles are not infective as they have packed few or excessive viral segments [37]. As a consequence of failure in replication, defective particles can cause restricted gene expression and results in persistent infection [13]. Althought, DCs are susceptible to influenza virus infection, the lack of progeny is not a particular signature of porcine DCs; as in equine DCs an equine influenza virus induced limited synthesis and progeny [278]; or in porcine DCs although replication was detected by RT-qPCR no virus progeny was found in avian H5N1 or H1N1 SwIV-infected cells [157]. Additionally in mouse DCs, although

replication of each gene segment and viral NA and HA had occurred, assembly was defective [279]. These results strongly suggest that replication of influenza A virus in porcine DCs is defective or abortive; differently from the process taking place in polarized cells. Seen these results one question would be whether the fail in influenza virus replication in DCs is related to the cell type in terms of ultra structure. A study of influenza virus entry involving tracking of an individual, *1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine* (DiD) labelled virus particles in living CHO cells showed that actin filaments and microtubules were involved in a stepwise manner during viral endocytosis [259]. However, this study was carried in non-polarized cells and it is possible that in non polarized cells, influenza virus trafficking between membrane compartments can be facilatated by the existence of intact cytoskeleton structure which is not absolutely required for virus infection, in contrast to the situation in polarized cells [280]. Thus influenza virus infection is actin dependent in polarized cells but not in non-polarized cells [280]. Pioneering studies by Winkler *et al.* (1986), demonstrated that budding of pleomorphic virus particles from the alveolar epithelial cells and accumulation of viral protein within the nucleus and cytoplasm of epithelial cells were detected in lung sections from H1N1 SwIV infected pigs [269]. Our studies *in vitro* also confirm that budding of influenza particles is mainly seen in polarized epithelial cells when compared with porcine DCs.

In vivo, the involvement of other immune cells against influenza virus infection such as DCs has been less studied. During influenza virus infection, the role of DCs in priming an effective T cell response is crucial in generating adaptive responses. For influenza virus, RNA replication might be less effective on DCs than in other cell types. In fact, H3N2 SwIV infected-poBMDCs and MDCKs exhibited marked differences in RNA viral production and replication, suggesting that this difference would probably account for such levels *in vivo*.

The NP is primarily nuclear when expressed alone and at early times post infection NP is localized predominantly to the nucleus, whereas at later times it is found in the cytoplasm, which reflects the trafficking of ribonucleoproteins during the virus life cycle [281]. In our study, at 6hpi poBMDCs and MDCK infected cells exhibited nuclear and cytoplasmic staining of influenza virus NP. On the other hand, poBMDCs were positive for NP at 24hpi but this staining was mainly detected in the cytoplasm of infected cells whereas H3N2 SwIV-infected MDCK exhibit NP staining in the nucleus as well as cytoplasm at this time point. Also, infectious particles were not detected in the supernatant of poBMDCs cultures and neither budding nor viral particle release was observed in poBMDCs by EM. This evidence would suggest that the limited increase in viral RNA is due to a default replication or an increase in influenza viral particles inside poBMDCs but no functional H3N2 SwIV replication was taking place in poBMDCs. Additionally, infected poBMDCs were able to transmit H3N2 SwIV to susceptible cells in the ICA assays, indicating that virus transmission was efficient between both cells. However, when cell-to-cell contact was prevented between H3N2 infected poBMDCs and MDCKs, no CPE was observed. H3N2 SwIV alone was able to induce CPE when an insert was used. The same behaviour was observed when H1N1 SwIV was used, indicating that the spread by cell-to-cell contact was not a particular signature of this H3N2 SwIV strain. Furthermore, when the cell replication machinery was inhibited by gamma-irradiation, H3N2 SwIV transmission from poBMDCs took place when susceptible cells were in close contact, indicating that H3N2 SwIV replication was not required for transmission of H3N2 SwIV from poBMDCs.

In humans, DCs serves as carriers of Human Immunodeficiency Virus (HIV) to the T cell area in the lymph nodes where T CD4$^+$ cells are infected through CD4 and CCR5 receptors [277]. Based on the experimental data on HIV, DCs

have been suggested to act as a "Trojan horses" for HIV in humans. In the porcine system, the fact that H3N2 SwIV infected poBMDCs were able to infect other susceptible cells when they are in close contact open the possibility of speculation about the role of DCs carrying H3N2 SwIV infectious particles to other tissues than the respiratory organs. Dissemination of influenza virus in tissues outside the respiratory system has been described in experimentally infected mice [282] as well as in human patients suffering from severe influenza infection [283], however there is no evidence that SwIV could disseminate systemically in pigs. Alternatively, a plausible explanation for this finding would be that DCs would carry the virus to secondary lymphoid organs such as lymph nodes where it could be detected by or possibly pass it on to B cells or other DCs for induction of specific immune responses (e.g. antibodies, CD8$^+$T cells...). This could be a beneficial effect to mount a specific immune response and thus contribute to viral clearance.

Finally, given the role of DCs in priming an effective and long lasting immune response, we report for the first time the interaction of porcine influenza viruses with poBMDCs. These data may help in understanding the role of DCs as important APCs in the pathogenesis and epidemiology of influenza virus and the role of pigs as virus reservoirs. The data presented in this first study, shed new light on H3N2 SwIV interaction with poBMDCs. In contrast to earlier findings in other systems, an ability to infect susceptible cells by close contact was described. This opens new opportunities for the virus with respect to its dissemination inside the host and the interference with the host's immune system at various sites besides the respiratory tract. Further studies have to be performed to elucidate the cause for this inefficient replication in porcine DCs and to define the consequences of influenza virus interaction with the immune system most potent APCs.

The observation that DCs may be infected by a porcine influenza virus and then infected cells can transmit the virus particles to susceptible cells; highlighted the need of understanding the role of these cells on porcine immune response against other influenza virus. The emergence in 2009 of the swine origin H1N1 influenza virus that caused the last pandemic in humans reinforced the concept of pigs as "mixing vessels".

Study II: Infection by swine, human or avian influenza viruses differentially activates porcine DC cytokine profile.

The main objective of this study was to analyse *in vitro* the innate immune response of DCs against influenza viruses that may be enter in contact with the porcine immune system in the nature. Thus, porcine bone marrow derived DCs were infected with a circulating strain of swine influenza (H3N2 SwIV), the 2009 human pandemic (hH1N1), the LPAIV (aH7N2) or the HPAIV (aH7N1) viruses. Nowadays, it is well accepted that pigs are considered "mixing vessels" as they posses α-2,3 or α-2,6 sialic acid receptors for both avian and human viruses respectively [256]; and they are susceptible to infection with LPAIV and HPAIV [65]. Sialic acids (SA, *N*-acetylneuraminic acid) are considered the primary receptors for virus attachment to cell surfaces, binding to a pocket at the distal tip of the HA of influenza A virus [284]. SA consist of nine carbon sugars frequently attached through α-2,3 or α-2,6 linkages to underlying sugar chains of glycoproteins in the cell membrane. It is well established that avian and human viruses differ in their SA binding affinity, since HA from human isolates usually bind SA α-2,6, whereas the avian isolates usually have affinity for SA α-2,3 [14]. In study I, we have shown that poBMDCs were infected with a circulating strain of SwIV suggesting the presence of SA receptors on porcine DCs. In the present study we observed that poBMDCs had SA receptors for both avian and human viruses supporting the idea that influenza A virus from different origins may

be able to easily interact with porcine DCs; but we did not detect virus progeny in the supernatants of hH1N1, aH7N2 and aH7N1 infected cells.

In the last years, defective or abortive replication of influenza viruses in DCs has been described [157,278,279]; also, numerous studies have provided increasing evidence for a role of the viral HA-SA interaction in the induction of innate immunity and pathogenesis. SA are structural components of the cell surface, and they have important implications in the immune responses against pathogens [15,285,286]. The H3N2 SwIV virus was isolated from an outbreak in Spain in 2007 and its HA sequence has been shown to be human-like origin while the internal gene segments are avian-like (Baratelli *et al*, manuscript in preparation). The last pandemic was caused by a triple reassortant virus with a capacity of human-to-human transmission. This novel virus was shown to be different from the seasonal virus as it posses six genomic RNA segments from triple reassortant swine viruses and acquired their HA and M segments from a Eurasian avian-like swine virus [46,47]. The other two avian viruses included in this study, aH7N1 and aH7N2, posses avian HA as reported elsewhere in materials and methods of this sudy. Thus, H3N2 SwIV posses human-like HA, as opposed to the hH1N1, aH7N2 and aH7N1 viruses which show an avian-like HA. This fact may explain the different activation pattern and cytokine mRNA or protein induction between H3N2 SwIV and the other viruses. However, Ramos *et al.,* (2012) suggested a model in which binding of avian influenza virus to a putative receptor containing SA α-2,3 would result in activation of signalling pathways leading to increased cytokine production and inflammation. The same authors suggest that human HA in influenza virus would result in delayed induction of cytokines [287]. Conversely, in the data presented in this study it is the H3N2 SwIV possessing human-like HA the one inducing higher activation and cytokine levels comparing with the virus with an avian-like HA. Additionally,

other viral components such as PB2 might be taken into account to explain the whole picture.

DCs are essential players in the induction of innate immunity, as well as in the initiation of adaptive responses. Upon sensing invading pathogens via several pattern recognition receptors (PRRs), they differentiate to mature DCs and migrate to secondary lymphoid organs, where they present the processed antigen to T-lymphocytes. It has been shown that human DCs express SA α-2,3 and SA α-2,6 on their surface [286,288], and the levels of expression of sialyltransferases and therefore, the SA levels on the surface, are modulated during differentiation and maturation processes [286,289]. The antigen presentation, co-stimulatory markers and the presence of soluble mediators are important at the time of priming an effective immune response [290]. The increase of CD80/86 and SLA-I observed in H3N2 SwIV-infected cells may suggest that poBMDCs interaction with H3N2 SwIV influenza virus resulted in an increase of SLA-I presentation. While in mice the infectivity of respiratory DCs by influenza A viruses may be influenced by the expression of MHC-II and the subtype of HA [291], in porcine DCs, MHC-II expression was induced in response to avian H5N1 and porcine H1N1 influenza viruses or to the reassortant containing exchanged polymerase complex genes [157]. However, in the present study SLA-II remained unaltered with H3N2 SwIV and hH1N1 infection while it decreased with the avian viruses compared to mock. During the first steps of virus infection, many are the mechanisms used by immune cells to eliminate pathogens. Signalling through TLRs leads to activation of intracellular pathways and transcription factors, and induces critical genes that regulate cell survival, proliferation, cytokine secretion and differentiation. Among these factors, the NF-κB is the most studied factor as it was shown to be involved in the regulation of many genes and also to play an important role in influenza virus infection [182,186,292]. Recently, Ocaña-Macchi *et al.,* (2012) reported that porcine DCs infected with a porcine H1N1

virus expressing the PB2 of H5N1 avian influenza virus, induced more nuclear translocation of NF-*κ*B compared to native porcine H1N1 virus [157]. In the present study, all viruses induced NF-*κ*B expression at 4hpi which decreased within time. The decrease of NF-*κ*B expression may be related to virus infection since it was shown that overexpression of viral proteins such as NS1 can block the expression of NF-*κ*B [186,187,258]; or it could be that NF-*κ*B is an early gene. But it could also be due to the increase of IL-10 mRNA observed in all virus-infected cells in a time dependent manner. IL-10 is an anti-inflammatory cytokine that can block the NF-*κ*B activation [293,294]. Thus, the peak of IL-10 mRNA was at 8 hpi, time in which the expression of NF-*κ*B was half of that observed at 4 hpi. CAPE has been described as a potent inhibitor of p65 phosphorylation, preventing the translocation of this protein to the nuclei [295] and in this way the synthesis of immune genes dependent of NF-*κ*B is prevented. We observed that CAPE treatment before H3N2 SwIV did not block the synthesis of IFN-α and IL-12 but decrease their secretion. This result led us to speculate that probably there is another pathway triggering the synthesis and secretion of these cytokines (for example the IRF pathway for IFN [296]). Triggering of TLRs results in production of different cytokines acting as antiviral, pro-inflammatory or anti-inflammatory molecules that interact with other cells (such as macrophages, DCs, NKs, T cell) to clear the infection or elicit adaptive response [201]. In human and mice, the role of IFN-α/β [297], TNF-α [268], IL-12 [262,263], IL-18 [257] and IL-10 [298] in response against influenza virus have been reported. In this study, infection of poBMDCs with H3N2 SwIV induced the production of IFN-α, TNF-α, IL-12 and IL-18. The presence of these cytokines correlates with an early response to *in vivo* influenza virus infection in swine as was previously reported [66,154,264]. The absence of IFN-α or the lower levels of TNF-α, IL-12 and IL-18 in poBMDCs treated with inactivated H3N2 SwIV or aH7N1 could be due to a virus entry requirement to induce cytokine

secretion, as was observed in human neutrophils where viral entry, endosomal acidification and viral uncoating, but not replication, were required for cytokine induction [299]. The activation of IFN-β promoter, results in secretion of a bioactive IFN-β that through autocrine or paracrine signalling induces the expression of genes that stimulate the secretion of IFNs [175,176]. In this study, the expression of IFN-β varies dependent on virus. However, further studies will analyse the presence of bioactive IFN-β at 4h of DCs infection. In swine, IFN-α played an important role in the secretion of IL-6 and IL-12; these cytokines were implicated in the symptoms caused by H1N1 SwIV infection [300]. However, *in vitro*, Östelund *et al.,* (2010), shown that human macrophages and DCs had weak cytokine responses against the pandemic H1N1 virus [275]; on the other hand, To *et al.,* (2010), reported that patients infected with the pandemic H1N1 showed a delayed viral clearance but a huge amount of cytokines [301]. The results in this study, are more in line with the work of Östelund *et al.,* [275] by showing that human pandemic H1N1 is not a high cytokine inducer, this time in pig DCs. Finally, it is worth mentioning that interaction of different influenza virus with porcine DCs induced sequential waves of cytokine production that are dependent on time and on the virus itself. This effect could account for the different immune responses generated by different influenza virus. On the whole, the results of this second study illustrate differential interactions influenza A viruses with porcine DCs, however, more studies have to be performed to get further insight into this interaction. Finally these results provide useful knowledge about the interaction between different influenza viruses, porcine DCs and the innate immune system altogether.

Study III: Differential DCs interaction with virulent and non-virulent H. parasuis strains is immunomodulated by a pre-infection with H3N2 SwIV.

In this last study, the immune response generated by porcine DCs against *H. parasuis* non-virulent serovar 3 (SW114) or virulent serovar 5 (Nagasaki) alone or after a previous infection with H3N2 SwIV were evaluated. The goals in this study were: *(i)* to establish an *in vitro* model to study the interaction of virulent and non-virulent *H. parasuis* strains that could give us some insight into the virulent factors associated with the immune response; *(ii)* to analyse whether SwIV infection could alter those immune responses.

A recent study evaluated the ability of *Streptococcus suis* to interact with swine DCs. Thus, *S. suis* capsular polysaccharide was shown to interfere with DCs phagocytosis and to be mainly responsible for DCs activation, addressing the role of *S. suis* CPS as a critical virulence factor [159]. Also, functional disruption of conventional swine DCs has been described by RNA virus such as classical swine fever virus, as an important strategy for viral pathogens to evade host defences [56,156,302]. A previous study of our collaborators has shown that phagocytosis resistance was a virulence mechanism of *H. parasuis*, on PAMs. The Nagasaki strain showed negligible association with PAMs although when it entered the cells they were greatly affected [112]. In the present study, we have described marked differences in the interaction of a virulent and a non-virulent strain of *H. parasuis* with porcine DCs. Firstly, SW114 was rapidly internalized by poBMDCs compared to Nagasaki and this ability was not affected by a previous infection with a H3N2 SwIV. The lower level of interaction of Nagasaki with poBMDCs, as the one observed with PAMs [112], could be a general evasion strategy from the immune system. Contrary to *H. ducreyi*, which persists in DCs without affecting the eukaryotic cell viability [303], Nagasaki was killed once internalized in poBMDCs. Although Nagasaki was internalized in lower numbers, it induced more

cellular lesions when compared to SW114, indicating that other mediators rather than bacterial numbers present in the cell are playing a role. One noteworthy observation was that cell death after *H. parasuis* infection evaluated by number of apoptotic and necrotic cells did not changed according to the *H. parasuis* strain used in the experiment. Also, this percentage of dead cells remained similar in the co-infection experiments with SwIV. These results suggested that the Nagasaki strain interaction with swine DCs altered cell morphology to a certain extent but did not induce increased cell death under the experimental conditions used in the study.

Regarding the antigen presentation function of DCs a reduction in SLA-II was observed in H3N2 SwIV plus SW114 or Nagasaki-infected cells and this could be due to a reduction of surface membrane as SW114 was attached in large numbers at one and 8hpi. However, after 8h when the interaction of both bacterial strains with DCs was similar, SLA-I expression decreased in H3N2 SwIV plus SW114-infected cells while increased in H3N2 SwIV plus Nagasaki infected cells. These differences in interaction of both pathogens with poBMDCs might suggest that they interfere in a different way with the transport and/or recirculation of MHC-I and II molecules. For example, it has been reported that *Helicobacter pylori* infect murine DCs and as consequence, MHC-II is retained within the *H. pylori* vacuoles and the export of MHC-II molecules to cell surface is blocked [221]. The activation of APCs is conditioned by the local environment in which they are primed, and this influences the way in which they control T helper type 1/type 2 (Th1/Th2) [304] or Th17 [305] cell development. In addition, adaptive immune response to micro-organisms is often characterized by the polarization of the cytokine response. Generally speaking, type I cytokines are known to suppress type II responses and *vice versa*. Pathogens that strongly polarize the immune response may modify the type I and type II cytokine balance and/or effectors and consequently the local environment in which immunity to a concurrent micro-

organism develops. For example, successive challenge of human DCs with influenza virus and *Streptococcus pneumoniae* resulted in production of pro-inflammatory cytokines such as TNF-α and IL-12 [306]. In our system, swine DCs infected with either *H. parasuis* strains tested showed a predominant inflammatory and Th1 pattern of cytokines with high levels of IL-1β, IL-8, IL-6, IL-18, IL-10 and TNF-α. Moderate levels of IFN-α were secreted in swine DCs infected with both strains of bacteria. However, there were significant differences regarding secretion of IL-12, the non-virulent strain being the high inducer. IL-12 is a cytokine which links both innate and adaptive immunity systems playing a critical role in inducing Th1 responses, which in turn leads to the production of a number of cytotoxic cytokines, as well as interferon-gamma (IFN-*γ*) by T cells [307]. Therefore, differential secretion of IL-12 might be considered a candidate of virulence in terms of immune responses to *H. parasuis*. Further studies will elucidate whether this will be the case in pigs. Interestingly, when H3N2 SwIV co-infection took place with Nagasaki, IL-12 secretion increased to levels comparable to cells infected with non-virulent SW114, suggesting that a change in IL-12 pattern was accomplished. IFN-α secretion may be due to viral PAMP through TLR3 or through bacterial PAMP due to TLR4. Nagasaki and SW114 strains differ in the nature of their lipooligosaccharide (LOS) [308] and capsule production [112], nevertheless, IFN-α secretion was similar when a single bacterial infection took place. However, these structural differences may account for the different cytokine pattern in the co-infection experiment as well as acting as a positive feedback loop for interferon upon influenza infection. In mice, Nakamura *et al.*, (2011) reported that co-infection with influenza virus and *S. pneumoniae* leads to synergistic stimulation of type I IFNs [309], which might also be the case in the experiments presented in this work; and recently also in mice, Neigishi *et al.*, (2012) demonstrated a modulated antibacterial T cell response as a result of cross-interference of RLR and TLR signalling pathways

[296]. In this study, we found a decrease in IFN-α level when SW114 was co-infected with H3N2 SwIV but it was not statistically significant. Previously, Bouchet *el al.*, (2008, 2009) reported that *H. parasuis* serotype 4 of moderate virulence, induced IL-8 and IL-6 secretion in higher levels compared to serotype 5 (high virulence) in porcine epithelial tracheal cells [118,119]. The data presented in this work indicated that there was no statistical difference in IL-1β, IL-8, IL-6, IL-18, IL-10, IFN-α and TNF-α levels after infection with SW114 (serovar 3, non-virulent) or with Nagasaki (serovar 5, virulent), at 8hpi in porcine DCs. Different cell types used in these studies may account for the abovementioned apparent discrepancies. Concurrent or sequential infections with viruses or bacteria and *vice versa*, influences the immune response to secondary unrelated pathogens [310], something that occurs in the porcine respiratory disease complex. For example, in pigs, it has been shown that co-infection of *H. parasuis* and highly pathogenic PRSSV resulted in more *H. parasuis* in heart, lung, spleen and lymph nodes compared to *H. parasuis* single infection [236]. Therefore, we pre-infected DCs *in vitro* with H3N2 SwIV before infection with SW114 or Nagasaki strains respectively. Morphologically, cells were not altered when SwIV infection took place, however, the pattern of IL-12 and IFN-α secretion was altered after co-infection with the Nagasaki strain. This result led us to speculate that if this pattern of cytokine was associated with pathogenesis, then any substance inducing a similar effect would change the immune reaction to this infection. In fact, when Poly:IC was used as a viral dsRNA surrogate, IL-12 and IFN-α secretion were elevated in the Nagasaki infected samples as compared with the infection with Nagasaki alone. It would be plausible to speculate that substances stimulating cytokines such as IL-12 and IFN-α secretion, like Poly:IC, would be promising candidates to complement a *H. parasuis* vaccine by biasing the pattern of secreted cytokines. Further experiments in infected pigs are required to establish whether this would be the case. Overall, the

results presented in this third suty, showed that *in vitro* DCs studies may help us understand the complex relationship of virulent and non virulent pathogens and the intimate relation among different pathogens in co-infections.

These *in vitro* analyses have allowed us to investigate new avenues to stimulate the immune system for better response to pathogens. These are also the first report of immunological differences among virulent versus non-virulent *H. parasuis* strains and their immunomodulation by SwIV co-infection.

In conclusion, pioneer studies describing the immune response of porcine DCs against respiratory pathogens such as influenza viruses and *H. parasuis* were presented in this thesis. However, further studies should be conducted to fully clarify the structural components involved in the defective replication of influenza virus and the synergistic effects of concurrent respiratory pathogens infection in pigs.

The lack of information on the interaction of DCs as important cells of immune system with influenza virus provide an excellent opportunity for future research that might lead to elucidate some of the mechanism involved in the resistance of RNA viruses to antiviral drugs.

7. CONCLUSIONS

Nature only shows us the tail of the lion. I am convinced, however that the lion is attached to it, even though he cannot reveal himself directly because of his enormous size.

Albert Einstein

1. Porcine bone marrow derived DCs (poBMDCs) were infected with 0.01 mutiplicity of infection (MOI) of a circulating strain of porcine influenza virus H3N2 resulting in limited replication within the first 8 hours after infection.

2. Porcine BMDCs infected with 0.01 MOI of H3N2 transmited the virus to permissive cells only when cell-to-cell contact was favoured, and this transmission did not required cellular machinery.

3. Infection of poBMDCs with the *A/Swine/Spain/SF32071/2007(H3N2), A/Catalonia/63/2009(H1N1), A/Anas plathyrhynchos/Spain/1877/2009(H7N1)* or *A/Chicken/Italy/5093/1999(H7N1)* induced variable levels of Th1 cytokine profile during the first 24h.

4. Infection of poBMDCs with *A/Swine/Spain/SF32071/2007(H3N2), A/Catalonia/63/2009(H1N1), A/Anas plathyrhynchos/Spain/1877/2009(H7N1)* or *A/Chicken/Italy/5093/1999(H7N1)* induced the expression of NF-κB, a central regulator of the immune responses, but also the expression of *TGF-β* and *IL-10* in a timelapsed way.

5. Inhibition of NF-κB expression before H3N2 SwIV affected the production of IL-12 and IFN-α two important cytokines in immune response against viral infection.

6. The *H. parsuis* non-virulent strain (SW114) interacted with poBMDCs in large numbers compared to the high-virulen strain (Nagasaki). However, Nagasaki infection resulted in a drastic cellular damage, even at low numbers, compared to SW114. Both strains elicited an immune response mainly directed to an inflammatory profile.

7. Infection with Nagasaki strain has a synergistic effect on IFN-α and IL-12 secretion when the poBMDCs were previously infected with the H3N2 SwIV.

8. Pre-treatment of porcine DCs with the TLR 3 agonists (Poly:IC) before infection with SW114 or Nagasaki, potentiated the IFN-α and IL-12 response.

8. REFERENCES

It is hard to fail, but it is worse never to have tried to succeed.

Theodore Roosevelt

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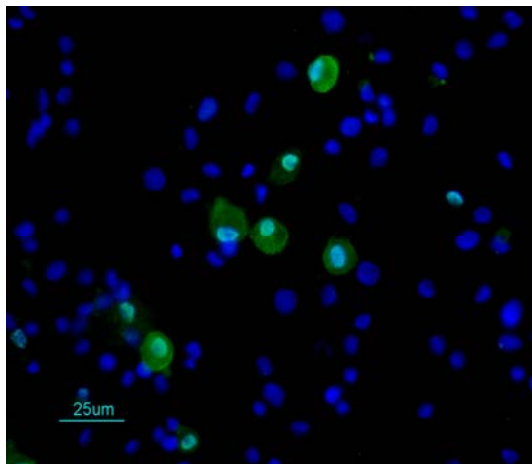
9. ANNEXES

A life spent making mistakes is not only more honorable,

but more useful than a life spent doing nothing.

George Bernard Shaw
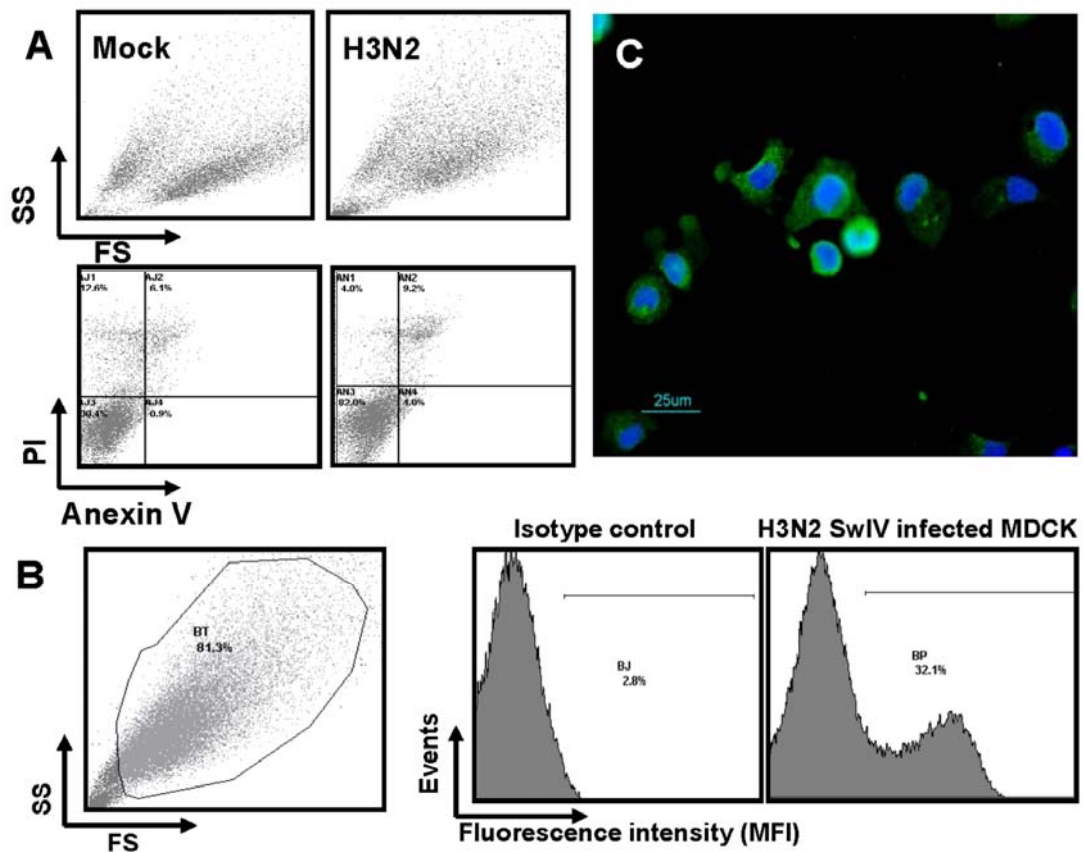
Annexe 1. MDCK cells 24h post infection with H3N2. **SwIV** (**A**) Mock or H3N2 SwIV infected MDCKs were stained with PI (necrosis) and Annexin V (apoptosis), or (**B**) with anti-influenza antibody for influenza virus NP. (**C**) Immunoflurescence staining to detect the NP influenza protein 24 hpi. NP (green), Nucleus (Blue). Bar= 25μm. Representative results of two independent experiments.
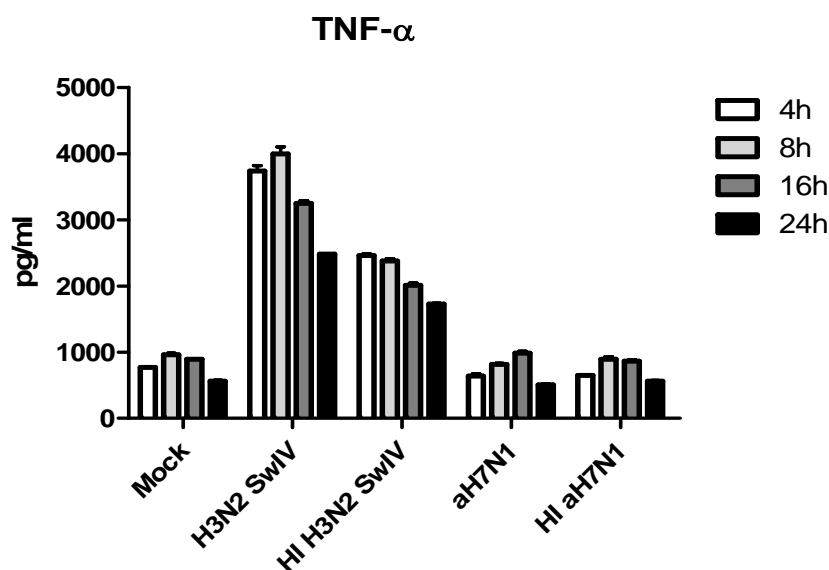
A. poBMDCs **B. MDCK**
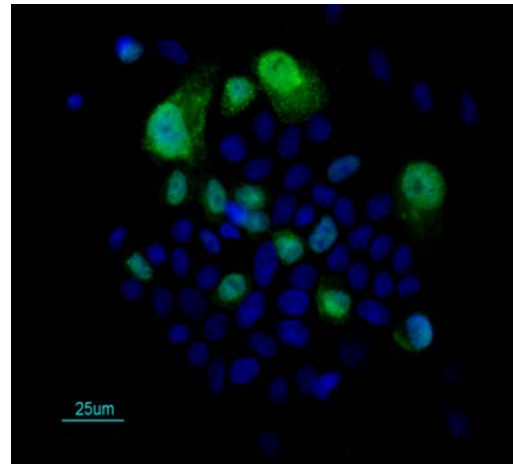
Annexe 2. Immunoflurescence of influenza virus NP 6hpi. (**A**) poBMDCs and (**B**) MDCKs were infected for 6h before staining. NP (green), nucleus (blue). Bar= 25μm. Representative results of two independent experiments.


Annexe 3. TNF-α secreted by poBMDCs treated with heat inactivated H3N2 SwIV and aH7N1 influenza virus. Porcine BMDCs were infected with 10^4TCID$_{50}$ of heat inactivated or normal SwIV *A/Swine/Spain/SF32071/2007(H3N2)* or HPAIV *A/Chicken/Italy/5093/1999(H7N1)* for 4, 8, 16 and 24h. Then supernatants were collected for ELISA. Representative results of two independent experiments.
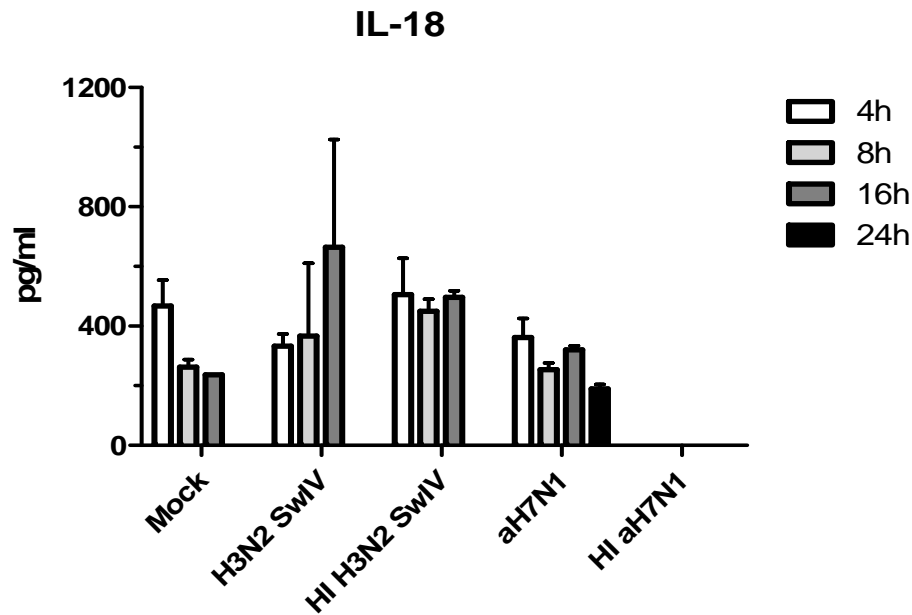
Annexe 4. IL-18 secreted by poBMDCs treated with heat inactivated H3N2 SwIV and aH7N1 influenza viruses. Porcine BMDCs were infected with 10^4 TCID$_{50}$ of heat inactivated or normal SwIV *A/Swine/Spain/SF32071/2007(H3N2)* or HPAIV *A/Chicken/Italy/5093/1999(H7N1)* for 4, 8, 16 and 24h. Then supernatants were collected for ELISA. Representative results of two independent experiments.
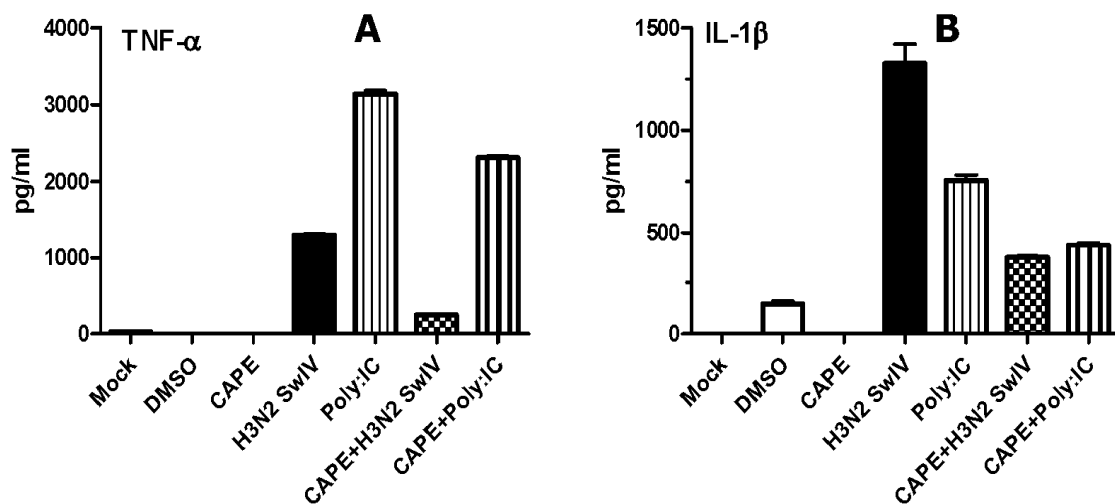

Annexe 5. TNF-α and IL-1β secreted by poBMDCs treated with CAPE before H3N2 SwIV infection or Poly:IC treatment. Porcine BMDCs were infected with 10^4TCID$_{50}$ of SwIV *A/Swine/Spain/SF32071/2007(H3N2)* for 24h. Then supernatants were collected for ELISA. Representative results of three independent experiments.
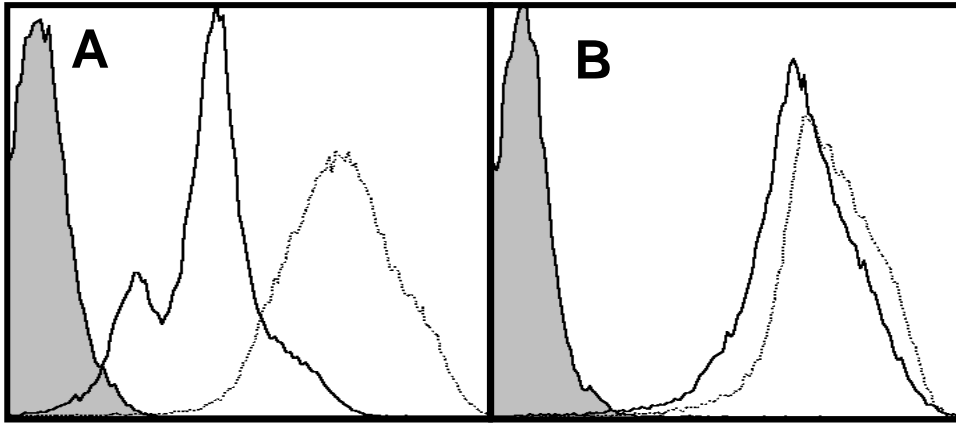
Annexe 6. H3N2 SwIV and _H. parasuis_ SW114 or Nagasaki co-infected-poBMDCs at (A) 1h and (B) 8hpi. Porcine BMDCs were infected and stained using anti-SW14 or anti-Nagasaki rabbit serum 1h at 4ºC, and then with the anti-rabbit IgG-FITC antibody. Mock (grey histograms), SW114 (dotted line), Nagasaki (continuous line). Representative results of two independent experiments.
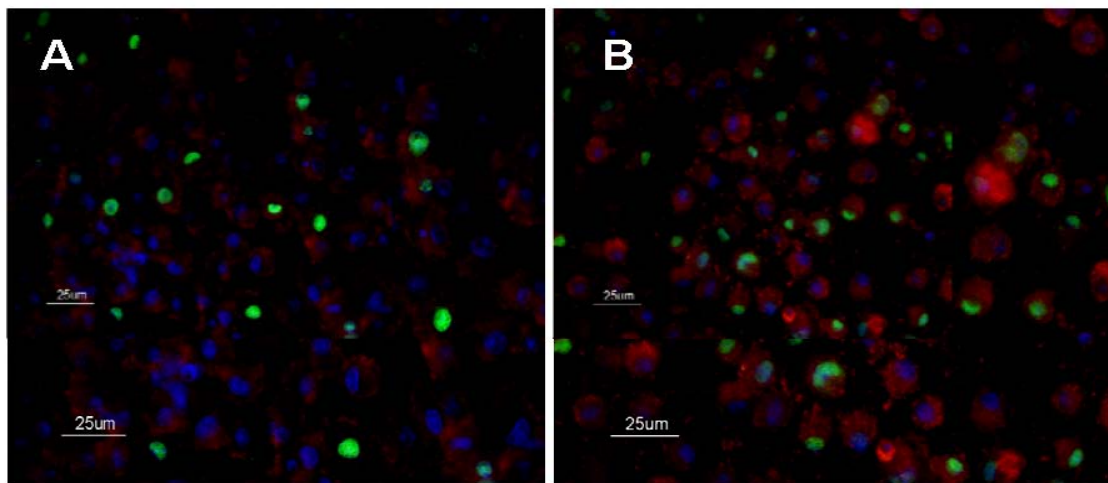
Annexe 7. Fluorescence images of co-infected-poBMDCs with H3N2 SwIV and SW114 or Nagasaki at 8hpi. poBMDCs were infected and stained using **(A)** anti-SW14 or **(B)** anti-Nagasaki rabbit serum, and then with the anti-rabbit IgG-Dye Light 549, and anti-mouse NP HB65 ATCC® antibody followed by an anti-mouse IgG-FITC. Therefore, nuclei were stained with DAPI (blue). SW114 or Nagasaki (red), SwIV NP (green). Bar = 25 μm. Representative results from two independent experiments.
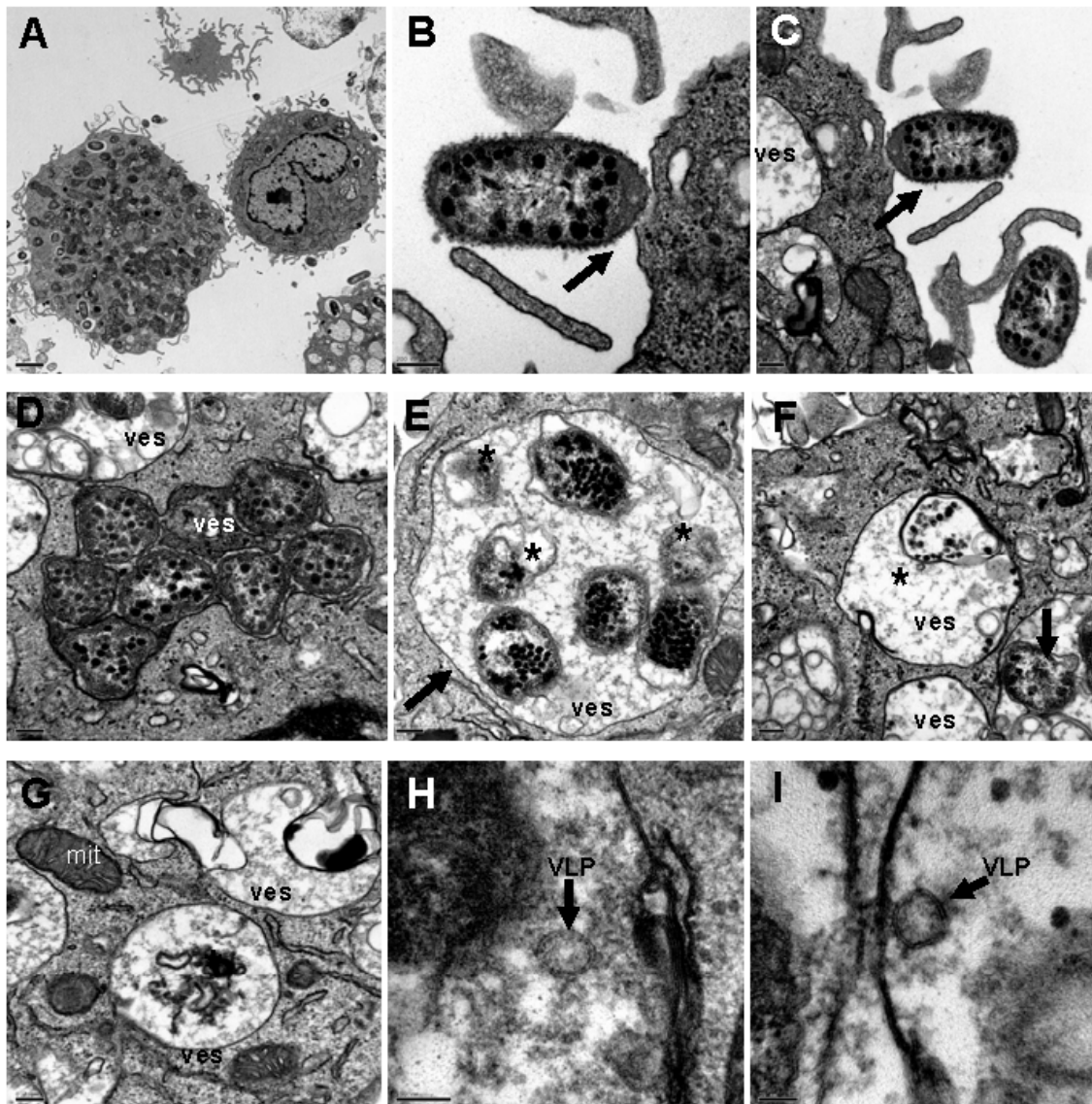
Annexe 8. EM of poBMDCs co-infected with H3N2 SwIV and SW114 at 8hpi. Porcine BMDCs showed many vesicles (ves) containing SW114 (**B, C**). Some vesicles had more than one bacteria (**D, E**). SW114 was found inside vesicles at different levels of degradation (asterisk, **E, F, G**). Also virus-like-particles (VLPs) were observed (**H, I**). Bars: A= 2 μm; B-G= 200 nm; H= 100 nm and I= 50 nm.
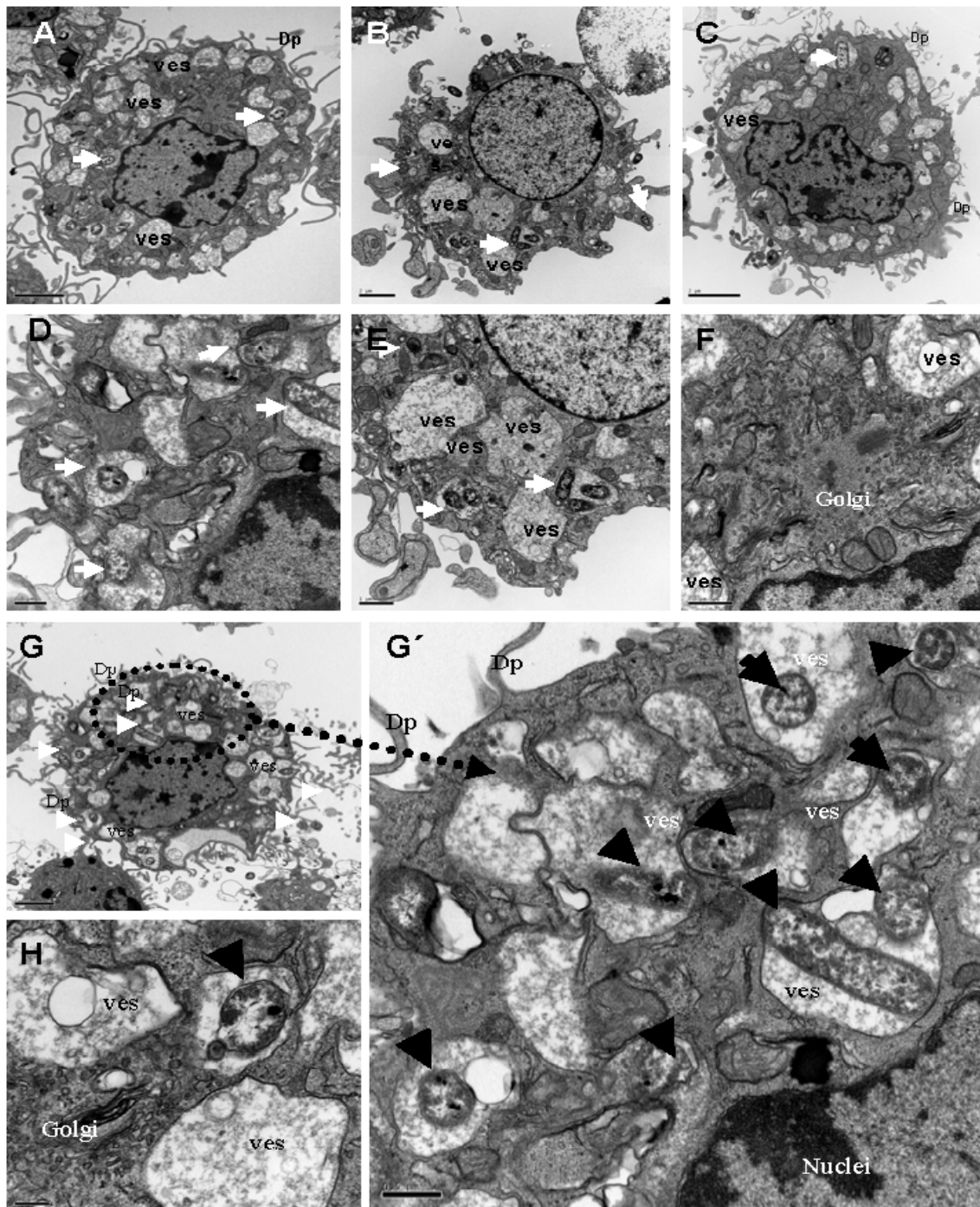
Annexe 9. EM of poBMDCs co-infected with H3N2 SwIV and Nagasaki at 8h.
Porcine BMDCs were infected with SwIV followed by Nagasaki. At 8hpi, poBMDCs
showed several vesicles (ves) (**A-E**). Few cells showed drastic cell damage (**B**). Some
vesicles contained more than one bacterium (**D, G**). The Golgi of Nagasaki-infected
poBMDCs was dilated (**F,** H). Different levels of Nagasaki degradation were observed
(black arrows) (**G´**). The Golgi of Nagasaki-infected poBMDCs was enlarged (**H**). Bars: A-
C, G= 2μm; D, F, G´= 500 nm, E = 1 μm and H = 200 nm.

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Interaction of porcine conventional dendritic cells with swine influenza virus

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ABSTRACT

Swine influenza virus (SwIV) causes sub-acute or acute respiratory infections on swine farms and pigs can act as "mixing vessels" for new influenza strains. Knowledge of the immune response of SwIV in its natural host, pigs, is very limited. Dendritic cells (DCs) mediate the induction of immunity to pathogens, but their interaction with SwIV has not been fully characterized. Thus, porcine bone marrow derived DCs (poBMDCs) were exposed to a circulating strain of H3N2 SwIV *in vitro*. Infection of poBMDCs resulted in structures resembling influenza virus inside poBMDCs in vesicles and also free in cytoplasm. Viral progeny was undetectable in supernatant but limited replication was detected in the first 8 h after infection. However, viral particles from infected-poBMDCs were able to induce cytopathic effect in susceptible cells only when cell-to-cell interaction was favoured. The data generated in our studies reveal the particular interaction of H3N2 SwIV with conventional DCs.

Introduction

Influenza viruses are enveloped, single stranded RNA viruses in the family *Orthomyxoviridae*. The genome of influenza A viruses consists of eight unique segments of single-stranded RNA, which are of negative polarity (Webster et al., 1992). The hemagglutinin (HA) and neuraminidase (NA) are very important for the induction of an antibody response in the host, but they are also highly variable whereas the nucleoprotein (NP) and matrix (M) proteins are highly conserved between different influenza A viruses (Van Reeth, 2007). Nevertheless, influenza viruses are genetically unstable due to drift and shift antigenic mechanisms (Webster et al., 1992; Yassine et al., 2009). Influenza virions are pleomorphic, although their shape is generally roughly spherical with a diameter <150 nm. However, larger (100–400 nm) influenza virions are also generated as filamentous forms (Fujiyoshi et al., 1994).

Pigs play a crucial role in the interspecies transmission of influenza viruses (Horimoto and Kawaoka, 2001; Lipatov et al., 2004). They are susceptible to experimental infection by virtually any avian influenza strain (Kida et al., 1994), including viruses of the H5N1 subtype (Choi et al., 2005), and they are also susceptible to the strains circulating in humans, since porcine and human viruses are phylogenetically related, and they can easily cross the species barrier, as has happened with the new type A (H1N1) 2009 pandemic virus (Garten et al., 2009). In fact, phylogenetic and sero-archaeological studies suggest porcine involvement in the appearance of the strains that caused the human pandemics of the 20th century (Horimoto and Kawaoka, 2001).

The pathogenicity of influenza virus lies in its ability to elude host anti-viral immune responses. In mice, protection against influenza A virus requires that memory CD8^{+} T cells be reactivated by antigen presented by bone marrow derived dendritic cells (BMDCs) in the lymph nodes draining the site of infection (Castiglioni et al., 2008; Tamura and Kurata, 2004), while in pigs, primary influenza virus infection induced long-lived increase of lung CD8^{+} T cells and local lymphoproliferative responses (Charley et al., 2006). Activation of cell mediated immunity or cytotoxic T lymphocytes, in turn, depends on efficient delivery of signals by antigen presenting cells (APC). Dendritic cells (DCs), a heterogeneous population of haematopoietic cells, are the most

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potent APC in the body (Steinman, 2006). They play a versatile role in orchestrating immune responses against an array of invading pathogens, including influenza virus. The interactions between DCs and naïve and memory T cells determine both the magnitude and quality of the immune response. According to their functionality and phenotype, DCs can be classified as conventional DCs (cDCs) known as professional presenting cells or plasmacytoid DCs (pDCs), which naturally produce high levels of interferon type-I (Summerfield and McCullough, 2009). Indeed, it is well known that both cDCs and pDCs have important antigen-presenting functions and they complement each other by particular cross-talk pathways (Perez-Cabezas et al., 2011). Conventional DCs are amongst the first cells encountered by most viruses, simply due to their availability at every possible entry site of the body (Freer and Matteucci, 2009).

Extensive studies on DCs have been done in mice and humans and although the knowledge on swine immunology has been developing quickly in recent years, it is rather scarce. In mice, pDCs regulate the accumulation of T cells in the bronchoalveolar space during early influenza virus infection, but are dispensable for the control of this disease (Wolf et al., 2009). On other hand, the ability of human pDCs to engulf viral antigens from virus-containing cells without being infected, allows them to acquire and present viral antigens, avoiding virus-induced subversion of their functions (Lui et al., 2009). However, a recent study evaluated the ability of swine pDCs to sense different influenza viruses. In this study, authors found that pDC in one side may play a role in the cytokine storm observed during severe disease. On the other hand they could participate in early antiviral responses limiting virus replication (Michael et al., 2011). Also, previous results showed that influenza virus alters DC interaction with naïve T cells *in vitro*. Influenza virus infection increased the capacity of cDCs to stimulate T cell proliferation and infection is not required to observe enhanced T cell proliferation (Oh and Eichelberger, 1999). Functional disruption of conventional swine DCs has been described with another RNA virus, classical swine fever virus, as an important strategy for viral pathogens to evade host defences (Carrasco et al., 2004). Recently, Goldwich et al. (2011), studied the interaction of herpes simplex virus type I (HSV-1) with human cDCs. Their results demonstrate that HSV-1 replicates in mature DCs but it can only be transferred to permissive cells in a cell-to-cell contact-dependent manner (Goldwich et al., 2011).

Lytic viruses such as influenza virus do not form a stable, long-term host–virus relationship within the infected host. Most of the studies of influenza viral particles formation have been performed on polarized epithelial cells (Nayak et al., 2009). In those studies, complete influenza virions were not present inside the infected cell and it could only be produced by budding from the plasma membrane. However, our knowledge about the roles of host factors in influenza infection is critically lacking, as well as the interaction of influenza viral particles with cells other than epithelial cells.

Given the pivotal role of DCs in triggering and directing the immune responses and the anatomical location of cDCs at the entry site of the body a set of experiments was designed to study the interaction between a circulating strain of porcine influenza virus (H3N2 SwIV) with swine cDCs *in vitro*. We found that influenza-like virions were located not only inside vesicles but also freely in the cytoplasm of influenza infected cDCs. Swine influenza virus interaction with cDCs did not induce viral budding nor detectable viral production in the supernatants. However, a limited increase in viral RNA was detected in cDCs by RT-qPCR. Surprisingly, H3N2 SwIV infected cDCs were able to infect susceptible cells when cell to cell contact was favoured. These new findings pave the way to elucidate the role of DCs in the course of influenza viral infection in its natural host.

Results

Ultrastructure and phenotype of poBMDCs

During the culture of porcine bone marrow haematopoietic cells with rpGM-CSF, cells grew in size, formed clusters and developed dendritic processes confirmed by electron microscopy at day eight (Figs. 1A, B, C). Porcine BMDCs in culture were semi-adherent cells, with some dendritic processes observed at day three which became noticeable as the culture progressed. At day eight of culture, the poBMDC phenotype was $CD172a^+$, $SLAI^+$, $SLAII^+$, $CD1^+$, $CD4^-$, $CD11R1^-$, $CD14^+$, $CD16^+$, $CD40^-$, $CD80/86^+$ and $CD163^{low}$ (Fig. 1D) which was consistent with previous reports (Carrasco et al., 2001; Kekarainen et al., 2008). At this time, the population of poBMDCs was rather homogenous with semi-mature cells and they constituted our starting culture for further experiments. Porcine BMDCs were also positive for α-2, 3 and α-2, 6 sialic acid (data not shown).

Infectious rate and viability of infected poBMDCs

Influenza viruses efficiently infect and replicate *in vitro* in epithelial cells for *e.g.* MDCK cells but no data was available concerning the viability of poBMDCs after H3N2 SwIV infection. Thus, levels of apoptotic *versus* necrotic cells were evaluated using different amounts of H3N2 SwIV in *in vitro* experiments to set up the experimental conditions for further analysis. Annexin V and propidium iodide staining were performed on poBMDCs 24 h after H3N2 SwIV infection. The overall mortality caused by H3N2 SwIV at 10^4 $TCID_{50}$ per 10^6 cells was similar to that observed in mock treated cells, 16.5% and 12.5% respectively (Fig. 2A). When higher H3N2 SwIV doses were used, 10^5 $TCID_{50}$ and 10^6 $TCID_{50}$ per 10^6 cells, the percentage of dead cells increased to values around 30% of total cells (data not shown). Also, the percentage of apoptotic cells was similar to that of necrotic cells when 10^4 $TCID_{50}$ was used (Fig. 2A).

In order to evaluate the percentage of poBMDCs actually infected by H3N2 SwIV when 10^4 $TCID_{50}$ was used, intracellular NP staining of poBMDC was studied. Data in Fig. 2B shows that around 34% of poBMDCs were positive for influenza virus NP. Also, only cytoplasmic influenza virus NP staining was observed 24 h post-infection (hpi) in poBMDCs by immunofluorescence (Fig. 2C) whereas this staining was nuclear and cytoplasmic in the case of MDCKs at 24 hpi (data not shown). However, at 6 hpi both cell types exhibited nuclear staining for NP viral protein (data not shown). Subsequently, the amount of H3N2 SwIV used in the following experiments was 10^4 $TCID_{50}$ for 10^6 poBMDCs in the cultures.

One of the hallmarks of DCs is their ability to up-regulate activation molecules after stimulation or infection. Consequently, it was investigated whether H3N2 SwIV-infected poBMDCs exhibited any alteration in surface molecules such as SLA I, SLA II and CD80/86. A slight up-regulation with statistical tendency ($p = 0.06$) in SLA I, II and CD80/86 expression was detected when values were compared with mock treated cells (Fig. 3). Values of mean fluorescence intensity were 22.7 for SLA I mock treated cells compared with 40.7 for H3N2 SwIV infected cells; for SLA II values were 40.6 compared to 64.6 and for CD80/86 values were 8.5 compared to 11.8 respectively. Similar results of slight up-regulation, with statistical tendency ($p = 0.08$) were observed in poBMDC stimulated for 24 h with poly:IC compared to mock treated cells (Fig. 3).

Interaction of H3N2 SwIV with poBMDCs versus MDCK

The replication cycle of influenza virus is composed of important steps such as fusion, endocytosis, replication, assembly and budding, which have been thoroughly investigated on permissive epithelial cells, like MDCK cells. Given the importance of DCs in triggering immune responses and once the conditions for H3N2 SwIV infection of
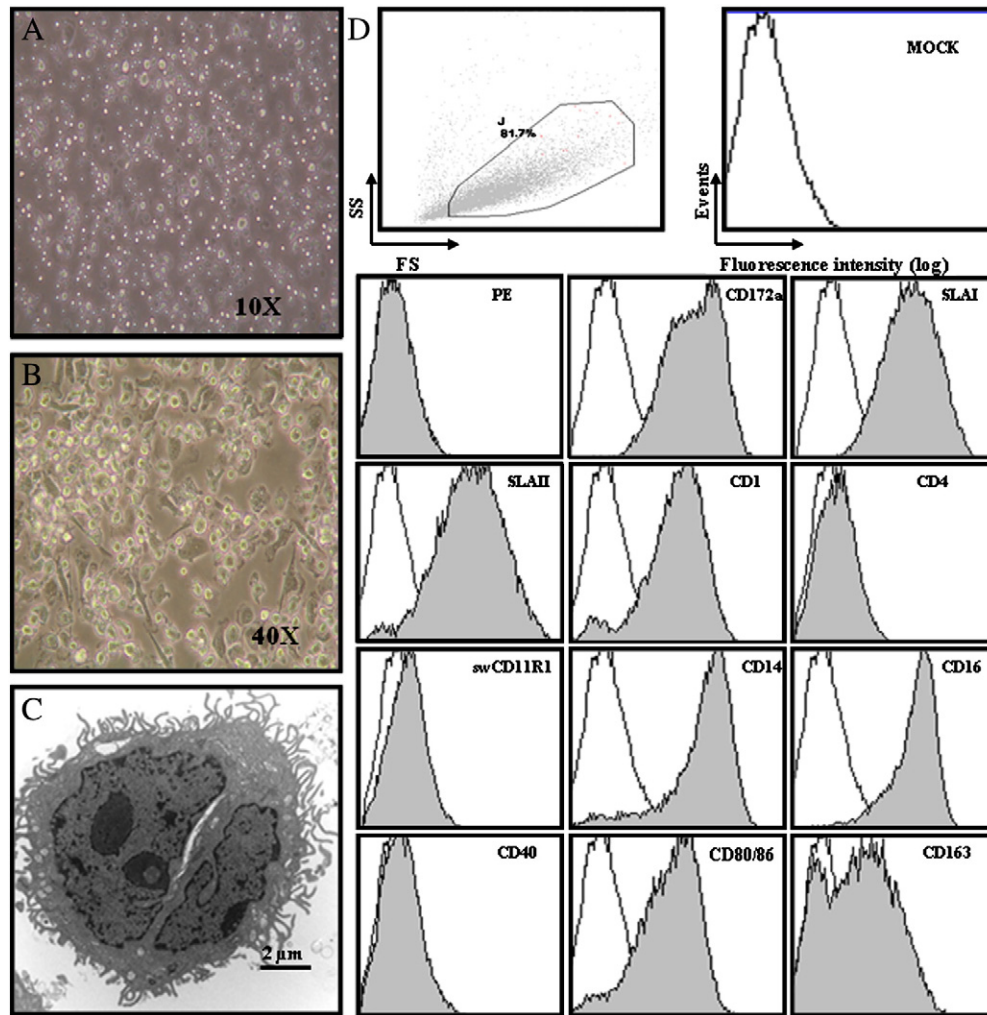
Fig. 1. Morphology and phenotype of poBMDCs. (A) poBMDCs seen by optical microscopy at day 3, 10× magnification. (B) Day 8 of generation 40× magnification and (C) electron microscopy at day 8 of generation. Bar = 2 μm. (D) Gate strategy and phenotype of poBMDCs at day 8 of generation with rpGM-CSF. White histograms show isotype control stained cells and grey histograms represent the CD marker stained cells. Mock are poBMDCs only and isotype control are cells stained with the secondary antibody only. Representative results from nine independent experiments.

poBMDCs were established, the H3N2 SwIV interaction with poBMDCs was analysed and compared with MDCK cells.

In the case of poBMDCs, structures resembling H3N2 SwIV virions were observed inside vesicles next to cellular membranes (Fig. 4A). Also, multiple vesicles were surprisingly observed in the cytoplasm, with 0.1 to 1 μm of diameter, containing several H3N2 SwIV like-particles with diameters of 80–100 nm. Most of them were surrounded by double membranes presenting H3N2 SwIV like-particles inside (Fig. 4B) which may reflect H3N2 SwIV particles entering the cell. Of note were several H3N2 SwIV like-particles observed free in the cytoplasm in close contact with Golgi complex. They may resemble virion structures budding from internal cistern of the Golgi complex membrane to the trans-Golgi network (Fig. 4C). Furthermore, large vesicles with several immature H3N2 SwIV like-particles, 70–80 nm in diameter, were observed in infected cells, some of them without capsids (Fig. 4D). In contrast with H3N2 SwIV-infected poBMDCs, in MDCK, H3N2 SwIV like-particles were observed in the extracellular space next to the cellular membrane (Fig. 5A) and mature particles were observed in the extra-cellular space, next to and attached to the cellular membrane (Fig. 5B). In the cytoplasm of MDCKs, small and large vesicles were observed of less than 100 nm to 400 nm in diameter. These vesicles had simple or double membranes surrounded by immature (±80 nm) and mature

(±100 nm) H3N2 SwIV like-particles in the cytosol (Fig. 5C). No H3N2 SwIV-like particles were detected freely in the cytoplasm of MDCK infected cells.

The structures inside poBMDCs resembled H3N2 SwIV but in order to assess whether they exhibited H3N2 SwIV proteins an immunogold labelling for H3N2 SwIV nucleoprotein coupled with 10 nm gold particles was performed on poBMDCs and MDCK infected cells. Those vesicles in the cytoplasm showed electron dense round and large structures consistent with H3N2 SwIV like-particles of 80–100 nm in diameter in poBMDC cells (Figs. 4E, F). More importantly, these particles were heavily labelled. However, neither budding nor released virion particles were detected in poBMDCs. In MDCKs, electron dense structures with different size and irregular shape, surrounding nucleoli and chromatin were observed and labelled. Budding and released virions were labelled (Figs. 5D, E and F).

H3N2 SwIV infection of poBMDCs

Generally, to evaluate influenza virus replication and generation of viral progeny, supernatant from infected cells is titrated on MDCKs. Supernatant from H3N2 SwIV infected-poBMDCs was assessed for viral progeny generation but no increase in viral titre
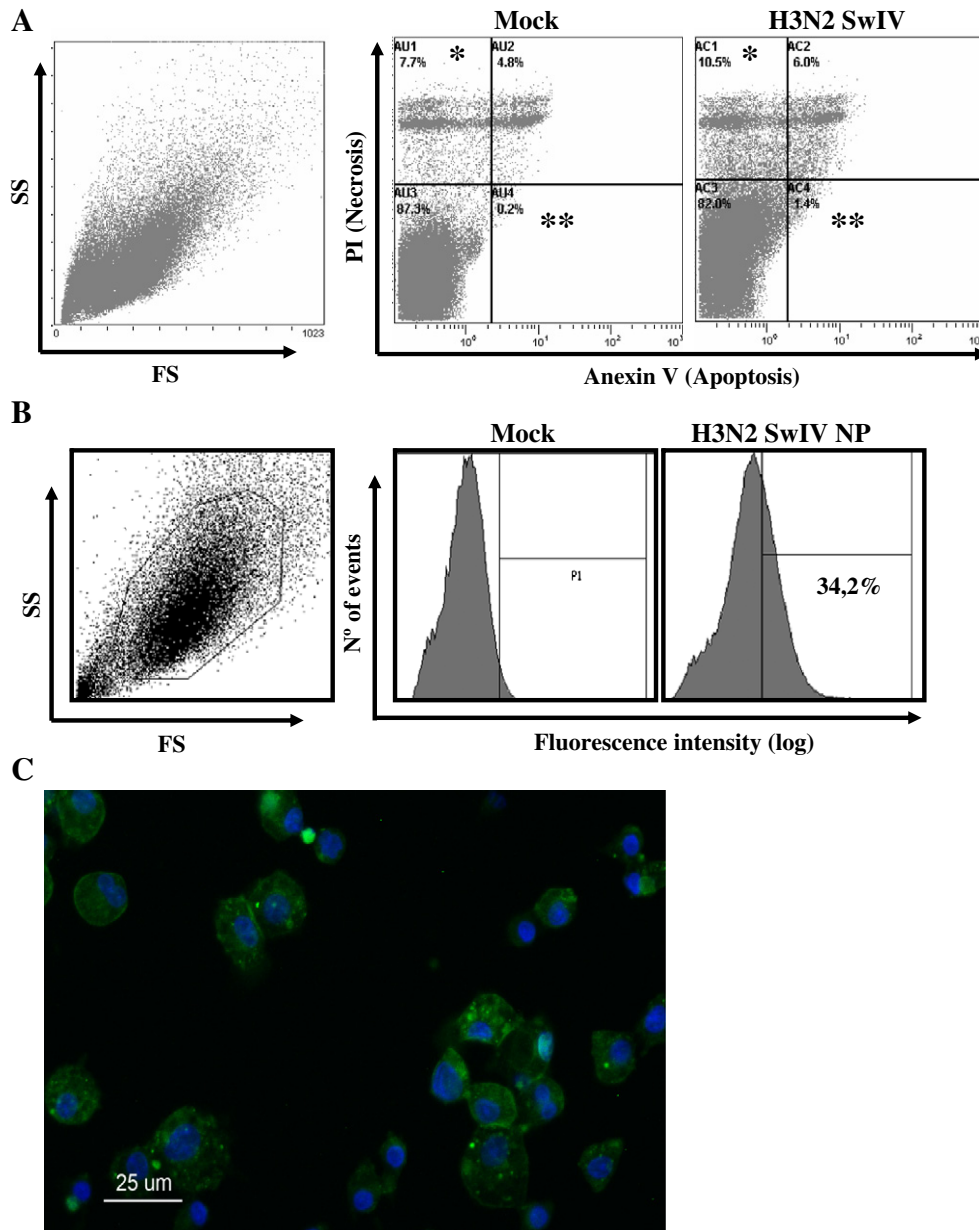
Fig. 2. Apoptosis/necrosis (A) and infectivity (nucleoprotein) (B) staining of poBMDCs after 24 h of infection with 0.01 MOI of SwIV H3N2. Mock or infected poBMDCs after 24 h were stained with (A) annexin V and/or propidium iodide, percentages in mock and infected cells were compared, $^*p = 0.04$, $^{**}p = 0.01$; $n = 5$. The data was obtained without any gate strategy. (B) Anti-influenza NP staining in poBMDC by flow cytometry and (C) anti-influenza NP antibody (green) plus DAPI (blue) immunostaining of poBMDC. Bar = 25 μm. FS (forward scatter) and SS (side scatter).

was detected in poBMDCs compared with H3N2 SwIV-infected MDCKs at different time points. However, statistically significant differences between poBMDC and MDCK infected cells were observed at all time points with $p < 0.05$ (Fig. 6). Therefore, the question was whether or not H3N2 SwIV was able to replicate in poBMDCs as compared with MDCK cells. Thus, 10^6 MDCK cells and 10^6 poBMDCs were infected in parallel using 10^4 TCID$_{50}$, and viral RNA was evaluated by RT-qPCR at different time points. In MDCK cells, an increase in viral RNA was detected rising from 1 hpi to 24 h. In contrast, viral RNA in poBMDCs showed a limited increase between 1 and 8 hpi and later decayed with time. The increase in viral RNA was observed by comparing inverted Ct values. No significant differences were observed between poBMDC and MDCK infected cells (Fig. 7). The limit of detection in this assay was set at 30 Ct which corresponded to 10^3 TCID$_{50}$ of

H3N2 virus. Ct values for 10^7, 10^6, 10^5 and 10^4 TCID$_{50}$ were 17, 19, 23 and 26 respectively.

H3N2 SwIV infected poBMDCs are able to infect permissive cells by cell-to-cell contact

Once a limited increase of H3N2 SwIV RNA was detected in poBMDCs, the ability of H3N2 SwIV infected-poBMDCs to transmit the virus to other susceptible cells was evaluated. Thus, different amounts of H3N2 SwIV infected-poBMDCs were co-cultured with MDCK cells, in an infectious centre assay (ICA), in the absence or presence of a transwell (TA). When the TA was inserted and cell-to-cell contact was prevented, no cytopathic effect (CPE) was detected. In the absence of a TA insert, CPE on MDCK was observed in 100% of
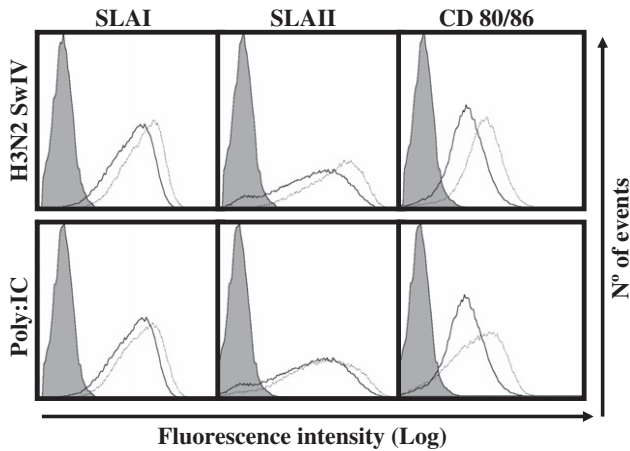
Fig. 3. Expression of SLA I, SLA II and CD80/86 24 h after H3N2 SwIV infection or 24 h after poly:IC stimulation. Infected/poly: IC stimulated-poBMDCs (dotted line) or uninfected/unstimulated cells (continuous lines) were stained for SLA I, SLA II and CD80/86. Grey histograms represent the isotype control stained cells. Mean fluorescence of mock cells was compared with infected or poly:IC stimulated cells and a statistical tendency was found with $p = 0.06$; $n = 3$. The data was obtained with the same gate strategy shown in Fig. 1.

the wells when 10^6 to 10^5 infected poBMDCs were used. Subsequently, CPE levels fell when cell number decreased (Fig. 8A). One hundred microlitres of trypsin treated H3N2 5×10^5 TCID$_{50}$ was also placed in the upper chamber of the transwell during the assay as a positive control. CPE was observed in 100% of the wells in this control (Fig. 8A). Moreover, high titres of virus were detected when supernatants from the 100% CPE positive wells in the ICA assay were titrated on MDCK cells. In these ICA positive wells, viral titres ranged from 10^5 to 10^6 TCID$_{50}$/ml (data not shown). The question of whether influenza particles infecting susceptible cells originated from viral progeny in DCs or they were particles attached to the poBMDCs arose. In

order to answer this question, poBMDCs and MDCK were irradiated with 30 Gy and 60 Gy respectively for 5 min before infection. Irradiation of MDCK before H3N2 SwIV infection was enough to prevent generation of infectious viral progeny in the supernatant (data not shown). After 24 h of infection, an ICA was performed. After 7 days in co-culture, either irradiated or non-irradiated poBMDC cells induced CPE on MDCK (Fig. 8B). The same result was obtained when irradiated MDCKs, infected with H3N2 SwIV, were tested (data not shown), which was not surprising considering the ability of SwIV viral particles to attach to the MDCK cell surface. Therefore, all the data suggested that viral particles attached to cDCs were able to infect susceptible cells only when cell-to-cell contact occurred.

Discussion

The aim of this study was to evaluate the interaction between porcine DCs and a circulating SwIV and the possible role by the former in being carriers of porcine influenza virus. We used poBMDCs to strengthen our understanding of the interaction of SwIV with the host immune response. There is convincing evidence that cDCs play a critical role *in vivo* in immunity to acute infections (Castiglioni et al., 2008; Langlois and Legge, 2010; Montoya et al., 2005). Based on these studies, our working hypothesis was that SwIV infection of respiratory epithelial cells induces inflammation, resulting in recruitment of monocytes that differentiate into DCs and in activation of sentinel DCs. Then, resident DCs in the respiratory epithelial tissue would encounter SwIV during influenza virus infection. Indeed, DCs and macrophages reside beneath the epithelium of the respiratory organs, and these cells are potential targets for influenza viruses (Osterlund et al., 2010). Thus, DCs would be exposed to virus before leaving the inflamed tissue to stimulate the adaptive immune response in the regional lymph nodes. Porcine BMDCs are considered an experimental model for porcine cDCs, a group which includes monocyte derived DCs as well as resident DCs. Therefore, our studies
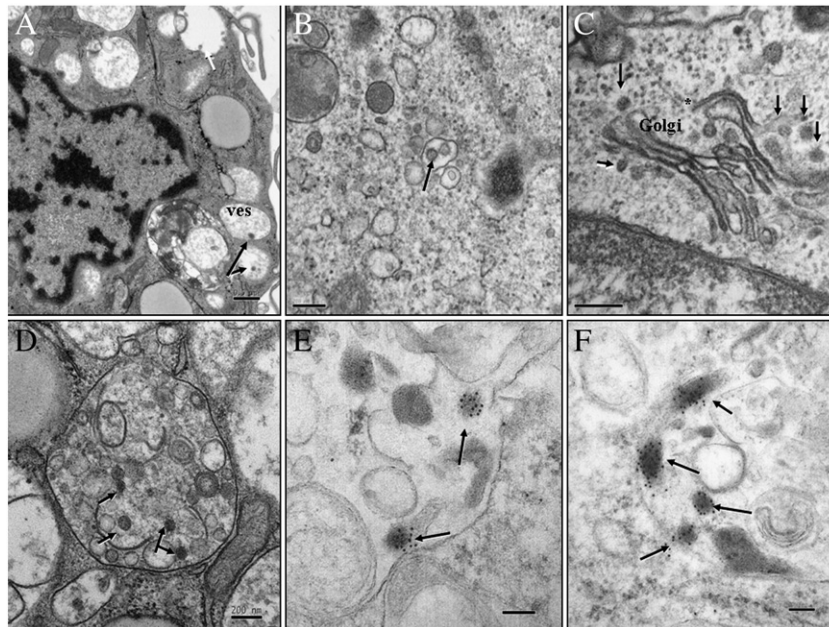

Fig. 4. H3N2-infected poBMDCs, 24 hpi. (A) Virions were located next to cellular membrane (black-arrows), inside vesicles (ves). A lot of vesicles were observed in the cytoplasm, with diameters of 0.1 to 1 μm (approx), containing several H3N2 SwIV like-particles of 80–100 nm in diameter (white-arrow). Bar = 0.5 μm. (B) Most of the vesicles have double membranes surrounding H3N2 SwIV like-particles (arrow), bar = 200 nm. (C) Next to the Golgi complex, several H3N2 SwIV like-particles were observed (arrow). Virion budding from internal cisternae of the Golgi complex membrane (*), to the trans-Golgi network. Bar = 200 nm. (D) Large vesicle has several immature H3N2 SwIV like-particles with 70–80 nm in diameter, and some of them without capsids (arrows). Bar = 200 nm. (E, F) Immunogold labelling for H3N2 SwIV nucleoprotein coupled with 10 nm gold particle. Vesicles in the cytoplasm showing electron dense round and large structures, consistent with H3N2 SwIV like-particles with 80–100 nm in diameter, were heavily and specifically labelled (arrows). Bar = 200 nm. Uranyl acetate and Reynolds lead citrate solution.
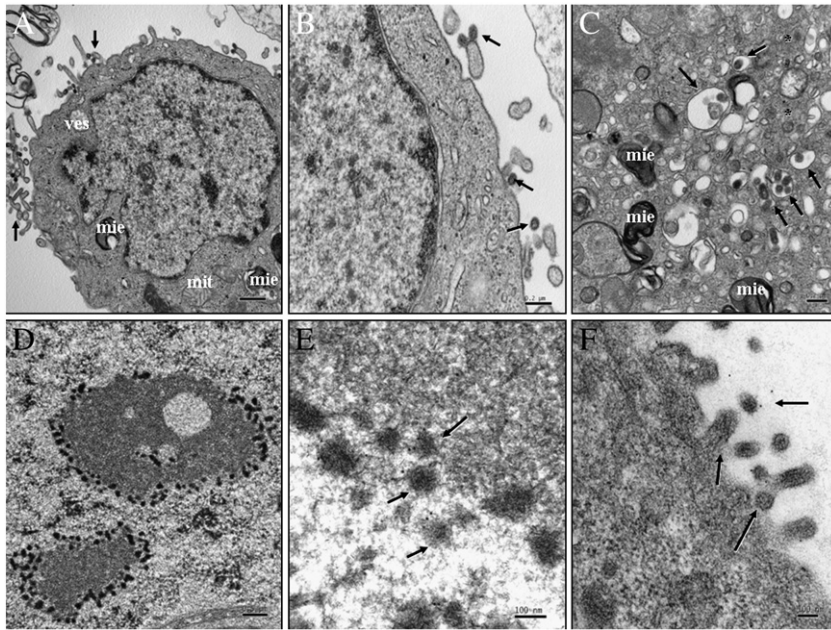
Fig. 5. H3N2-infected MDCK, 24 hpi. (A) These cells are characterized by large nuclei compared to their cytoplasm, where several large myelinoid figures (mie) and some vesicles were observed. H3N2 SwIV like-particles were detected in the extracellular space and next to cellular membrane (arrows). Bar = 0.5 μm. (B) Close-up of figure A, mature H3N2 SwIV like-particles were observed in the extracellular space next to and attached to the cellular membrane (arrows). Bar = 0.2 μm. (C) In the cytoplasm, small and large vesicles were observed of less than 100 nm to 400 nm diameter (approx). These vesicles contain SIV like-particles surrounded by simple or double membranes (± 80 nm, arrows) and mature ones (± 100 nm) in the cytosol (*). Bar = 0.2 μm. (D, E) and (F) Immunogold labelling for H3N2 SwIV nucleoprotein coupled with 10 nm gold particle. Electron dense structures with irregular shape surrounding nucleoli and chromatin were observed and labelled (arrows). Budding release (F) (arrows) virions were labelled. Bar = 0.5 μm, 100 nm and 100 nm respectively. Uranyl acetate and Reynolds lead citrate solution.

were focused on understanding the nature of the interaction between H3N2 SwIV and cDCs in an *in vitro* system.

In a natural setting of influenza infection, humans and animals are usually infected at very low multiplicities of infection (MOI) (Nayak et al., 2004). Thus, our experimental system was set up using a rather low MOI (Fig. 2) with the intention of both, mimicking the natural setting of the infection and preserving cellular integrity during the experimental procedure.

Interaction of DCs with virus has been proven to be extremely dependent on the type of virus and the DCs being studied. Our general knowledge on this interaction has been mainly obtained from humans and mice where there are numerous examples ranging from functional inhibition of DCs or weak response after viral infection (Osterlund et al., 2010) to the exploitation of DC functions for the dissemination of virus infection (Rowland-Jones, 1999). Other experimental systems have been used to study influenza infection and interaction with DCs in other natural hosts, like horses. In an *in vitro* equine monocyte-DC model, equine influenza virus successfully

infected and initiated transcription of its viral genome in DCs, although only limited viral protein synthesis and progeny was achieved (Boliar and Chambers, 2010). In pigs, a study on porcine DCs has recently been published (Michael et al., 2011) but the authors focus on the ability of pDCs to respond to different influenza virus strains. Therefore, our data on poBMDC interaction with a circulating H3N2 SwIV are, to our knowledge, the first one describing that porcine DCs support a limited increase in viral H3N2 SwIV RNA, and showing that influenza infected DCs are able to infect susceptible cells by cell-to-cell contact.

In vitro, for different purposes including vaccine development, studies of influenza virus are mainly carried out in polarized cells (Youil et al., 2004). Thus, MDCK was used for many years as a model to study the replication cycle of influenza virus *in vitro*. A body of accumulating evidence indicates that complete influenza viral particles are not found inside the infected cell and the process of assembly, morphogenesis, budding and release of progeny virus particles takes place at the plasma membrane of the infected cells
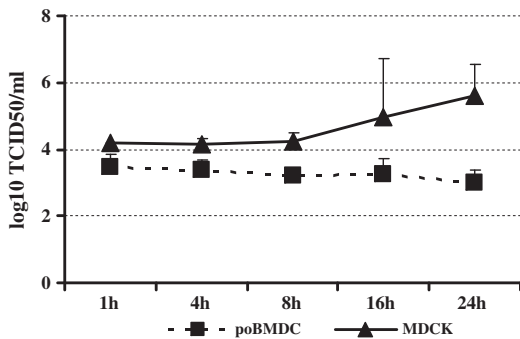

Fig. 6. H3N2 SwIV progeny in the supernatant of poBMDCs and MDCK infected cells. Supernatants from infected cells were collected at different time points after infection to titrate viral progeny on MDCK cells. Statistically significant differences between poBMDC and MDCK infected cells were observed at all time points with $p < 0.05$. Bars represent the mean value plus one standard deviation; $n = 5$.
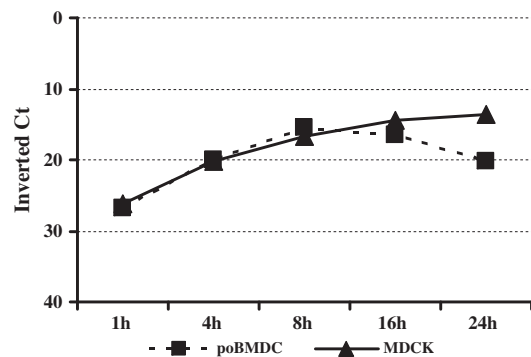

Fig. 7. SwIV H3N2 RNA quantification in poBMDCs and MDCK infected cells. RT-qPCR was performed using viral RNA extracted from poBMDCs and MDCK infected cells at different times. No significant differences were observed between poBMDC and MDCK infected cells. Bars represent the mean value plus one standard deviation; $n = 3$.
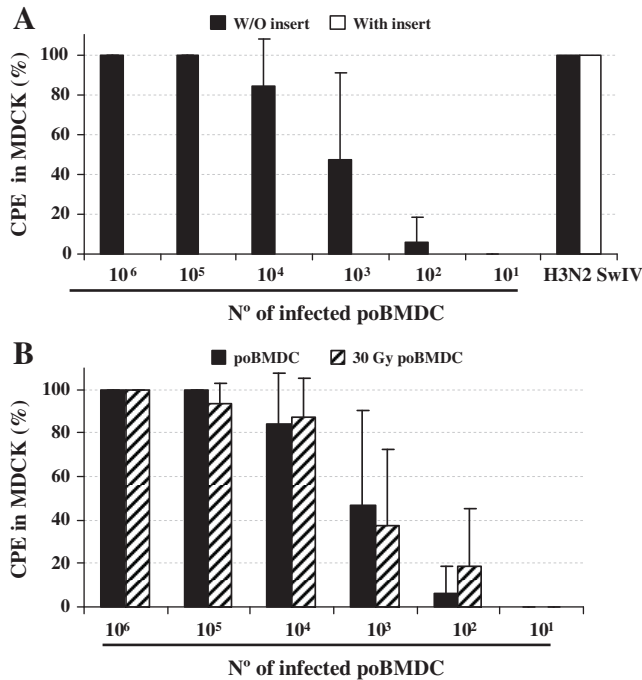
Fig. 8. Infectious centre and transwell assay. (A) Twenty-four hours post infection poBMDCs were co-cultured directly or indirectly with MDCK in 96 flat and 24 well plates respectively. As a positive control, 100 μl of trypsin treated H3N2 SwIV $5*10^5$ $TCID_{50}$ was added. Bars represent the mean value plus one standard deviation; $n = 4$. (B) Normal or irradiated poBMDCs with 30 Gy were infected for 24 h before the co-culture with MDCK in an ICA. CPE was evaluated at days 4 and 7 after ICA assay. No significant differences were observed between irradiated or not irradiated poBMDC. Bars represent the mean value plus one standard deviation; $n = 3$.

other hand, poBMDCs were positive for NP at 24 hpi but this staining was mainly detected in the cytoplasm of infected cells (Fig. 2C) whereas H3N2 SwIV-infected MDCKs exhibit NP staining in the nucleus as well as cytoplasm at this time point (data not shown). Also, infectious particles were not detected in the supernatant of poBMDC cultures (Fig. 6) and neither budding nor viral particle release was observed in poBMDCs by TEM (Fig. 4). All in all, this evidence would suggest that the limited increase in viral RNA is due to a default replication or an increase in influenza viral particles inside poBMDCs but no functional H3N2 SwIV replication was taking place in poBMDCs.

Additionally, infected poBMDCs were able to transmit H3N2 SwIV to susceptible cells in the ICA assays, indicating that virus transmission was efficient between both cells. However, when cell-to-cell contact was prevented between H3N2 infected poBMDCs and MDCK, no CPE was observed. H3N2 SwIV alone was able to induce CPE when an insert was used (Fig. 8A). The same behaviour was observed when H1N1 SwIV was used (data not show), indicating that the spread by cell-to-cell contact was not a particular signature of this H3N2 SwIV strain. Furthermore, when the cell replication machinery was inhibited by gamma-irradiation, H3N2 SwIV transmission from poBMDCs took place when susceptible cells were in close contact (Fig. 8B), indicating that H3N2 SwIV replication was not required for transmission of H3N2 SwIV from poBMDCs.

In humans, DCs serve as carriers of Human Immunodeficiency Virus (HIV) to the T cell area in the lymph nodes where T CD4+ cells are infected through CD4 and CCR5 receptors (Rowland-Jones, 1999). Based on the experimental data on HIV, DCs have been suggested to act as a "Trojan horses" for HIV in humans. In the porcine system, the fact that H3N2 SwIV infected poBMDCs were able to infect other susceptible cells when they are in close contact opens the possibility of speculation about the role of DCs carrying H3N2 SwIV infectious particles to other tissues than the respiratory organs. Dissemination of influenza virus in tissues outside the respiratory system has been described in experimentally infected mice (Sun et al., 2009) as well as in human patients suffering from severe influenza infection (Korteweg and Gu, 2008), however there is no evidence that SwIV could disseminate systemically in pigs. Alternatively, a plausible explanation for this finding would be that DC would carry the virus to secondary lymphoid organs such as lymph nodes where it could be detected by or possibly pass it on to B cells or other DCs for induction of specific immune responses (e.g. antibodies, CD8+ T cells…). This could be a beneficial effect to mount a specific immune response and thus contribute to viral clearance.

Finally, given the role of DCs in priming an effective and long lasting immune response, we report for the first time the interaction of porcine influenza viruses with poBMDCs. These data may help in understanding the role of DCs as important APC in the pathogenesis and epidemiology of influenza virus and the role of pigs as virus reservoirs. Taken together, our data shed new light on H3N2 SwIV interaction with poBMDCs. In contrast to earlier findings in other systems, an ability to infect susceptible cells by close contact was described. This opens new opportunities for the virus with respect to its dissemination inside the host and the interference with the host's immune system at various sites besides the respiratory tract. Further studies have to be performed to elucidate the cause for this inefficient replication in porcine DCs and to define the consequences of influenza virus interaction with the immune system most potent APCs.

Materials and methods

Cells

Bone marrow haematopoietic cells were obtained from femurs of healthy Large white×Landrace pigs of eight weeks of age, negative for porcine reproductive and respiratory syndrome virus (PRRSV) and to type-2 porcine circovirus (PCV2) by RT-PCR as previously

(Rossman and Lamb, 2011). These events are crucial for the production of infectious virions and pathogenesis of influenza virus. However, our data indicates that H3N2 SwIV like structures are detected freely in the cytoplasm of poBMDCs, surprisingly located near intracellular membranes (Golgi reticulum) (Figs. 4C, D, E and F). Similar experiments were conducted with other influenza viruses different from the H3N2 SwIV selected for this study to analyse whether this interaction was a particular feature of the H3N2 chosen. The results were identical to the ones shown previously with H3N2 SwIV in Fig. 4 (and data not shown). All these data suggest that SwIV interaction in porcine DCs might be different from the process taking place in polarized cells.

Pioneering studies by Winkler and Cheville (1986) demonstrated that budding of pleomorphic virus particles from the alveolar epithelial cells and accumulation of viral protein within the nucleus and cytoplasm of epithelial cells were detected in lung sections from H1N1 SwIV infected pigs (Winkler and Cheville, 1986). Our studies in vitro also confirm that budding of influenza particles is mainly seen in polarized epithelial cells (Fig. 5F) when compared with DCs in porcine cells.

In vivo, the involvement of other immune cells against influenza virus infection such as DCs has been less studied. During influenza virus infection, the role of DCs in priming an effective T cell response is crucial in generating adaptive responses. For influenza virus, RNA replication might be less effective on DCs than in other cell types. In fact, H3N2 SwIV infected-poBMDCs and MDCK (Figs. 6 and 7) exhibited marked differences in RNA viral production and replication, suggesting that this difference would probably account for such levels in vivo.

At early times post infection NP is localized predominantly to the nucleus, whereas at later times it is found in the cytoplasm, which reflects the trafficking of ribonucleoproteins during the virus life cycle. NP is primarily nuclear when expressed alone (Peter et al., 2007). At early times post infection (6 hpi) poBMDC and MDCK infected cells exhibited nuclear and cytoplasmic staining of influenza virus NP (data not shown). On the

described (Olvera et al., 2004; Sibila et al., 2004). These animals were also negative by enzyme linked-immunosorbent assay (ELISA) for influenza virus and Actinobacillus (HIPRA, Amer, Spain), for mycoplasma (OXOID, Cambridge UK), for parvovirus, Adenovirus and Aujeszky's disease virus (INGENASA, Madrid, Spain), and Salmonella (SVANOVA Biotech AB, Uppsala, Sweden). Bone marrow dendritic cells (BMDCs) were generated in an eight day protocol as previously described by Carrasco et al. (2001) with some modifications from Kekarainen et al. (2008). Briefly, bone marrow haematopoietic cells (BMHC) were resuspended in RPMI-1640 (Lonza, Walkesville, USA) culture medium containing 2 mM of L-glutamine (Invitrogen®, Barcelona, Spain), 100 U/ml of Polymyxin B (Sigma Aldrich Quimica S.A., Madrid, Spain), 10% of fetal calf serum (FCS) (Euroclone, Sziano, Italy) and 100 µg/ml of penicillin with 100 U/ml of streptomycin (Invitrogen®, Barcelona, Spain). This medium will be named RPMI-DC in this study. One hundred nanogrammes per millilitre (100 ng/ml) of recombinant porcine GM-CSF (R&D Systems, Spain) was added to the cells three times during the culture within 2 day intervals. Madin Darby Canine Kidney (MDCK) cells were maintained in a Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Walkesville, USA) containing 8 mM of L-glutamine and 200 µg/ml of penicillin with 200 U/ml of streptomycin and 5% of FCS. This medium will be referred as DMEM-MDCK in this study.

BMDC phenotype

Flow cytometry was performed using indirect labelling for CD172a, SLAI, SLAII, CD4, CD11R1, CD40, CD80/86 and CD163 and direct labelling for CD14 and CD16. Commercially available purified monoclonal antibodies (mAbs) anti-porcine CD14, CD16 and the fusion protein CD152 (CTLA4) for CD80/86 were used while the rest of the markers were detected by hybridoma supernatants. The secondary antibody was R-Phycoerythrin anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK). Briefly, $2.5*10^5$ cells/50 µl/well were labelled for 1 h at 4 °C for each CD marker, using 50 µl anti-CD172a (SWC3, BA1C11), 50 µl anti-SLA I (4B7/8), 50 µl anti-SLAII (1F12), 50 µl anti-CD1 (76-7-4), 50 µl anti-CD4 (76-12-4), and 50 µl anti-CD163 (2A10/11) and for anti-CD11R1 (MIL4, IgG1, Serotec), anti-CD14 (MIL2, IgG2b, Serotec, bioNova cientifica, Madrid, Spain), anti-CD16 (G7, IgG1, Serotec, bioNova cientifica, Madrid, Spain), purified anti-human CD40 (G28.4, IgG1κ, Biolegend, San Diego CA, USA), and CTLA4-mIg (Ancell, Minnesota, USA) the manufacturer's instructions were followed. After 1 h of incubation at 4 °C, cells were washed with cold PBS with 2% FCS by centrifugation at 450 g, 4 °C for 10 min. Then, the secondary antibody R-Phycoerythrin diluted 1:200 was added when required. Cells were incubated for 1 h at 4 °C, and then they were washed as before and resuspended in PBS with 2% FCS. In order to determine if poBMDCs possess α-2,3-sialic acid or α-2,6 sialic acid linked to galactose, two lectins, MAA II (Maackia amurensis lectin II) and SNA (Sambucus nigra lectin) (Vector Laboratories, Peterborough, UK) with known capacities to bind α-2,3 or α-2,6 sialic acid respectively were chosen. Stained cells were acquired using a Coulter® EPICS XL-MCL cytometer and analysed by EXPO 32 ADC v.1.2 programme. A gate strategy was applied in 80% of living cells using the forward and side scatter (FS/SS) characteristic.

DC ultrastructure

At day eight, for conventional and immunogold labelling electron microscopy (EM) studies, poBMDCs were fixed with 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde (EM grade, Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB, Sigma-Aldrich, Steinheim, Germany), pH 7.4 and with 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in PB pH 7.4 respectively. For conventional EM procedures, cells were embedded in Eponate 12™ resin (Ted Pella, Inc, Redding, CA, USA). For immunogold labelling

studies, after cryoprotection with sucrose (Sigma-Aldrich, Steinheim, Germany) solutions in PB at 4 °C, the pellet-cells were embedded in Lowicryl HM20 resin (Polysciences Inc., Warrington, USA). Briefly, the immunogold labelling was performed as follows: after blocking in 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Steinheim, Germany) in PBS (BSA/PBS), the grid samples were incubated with the monoclonal antibody to influenza A virus (Biodesign International, Saco, USA) at a dilution of 1:5 in 1% (w/v) BSA/PBS at 4 °C overnight in a humidified chamber. The secondary anti-mouse antibody was coupled to 10 nm-gold particles (British BioCell International, Cardiff, UK) in 1% (w/v) BSA/PBS for 40 min at room temperature. Sections treated with PBS/BSA instead of primary antibody served as negative controls. All grid samples, from conventional and immunogold labelling studies, were contrasted with conventional uranyl acetate and Reynolds lead citrate solutions, and evaluated using a Jeol microscope 1400 (Jeol LTD, Tokyo, Japan) and photographed with a Gatan Ultrascan ES1000 CCD Camera 2048 × 2048 pixels as previously described by Rodríguez-Cariño et al. (2010) and Rodríguez-Cariño and Segales (2011).

Swine H3N2 influenza virus preparation and infection

Porcine A/Swine/Spain/SF32071/2007 (H3N2) SwIV strain was isolated from a natural outbreak on a conventional farm in Spain. Viral isolation was performed on embryonated specific pathogen free (SPF) eggs and subsequently multiplied on MDCK following the procedures of International Organization of Epizooties (OIE, 2008).

Porcine BMDCs and MDCK were infected using a modified procedure, of previously described methodology by Rimmelzwaan et al. (1998). Briefly, 10^6 poBMDCs were infected with 10^4 TCID$_{50}$ of previous porcine trypsin type IX (Sigma-Aldrich, St. Louis, USA) treated H3N2 10^7 TCID$_{50}$/ml. Then, cells were incubated for 1 h at 37 °C 5% CO$_2$ for virus adsorption. After this time, cells were thoroughly washed with PBS with 2% FCS and 400 µl of RPMI-DC was added. For MDCK infection, DMEM medium supplemented with 8 mM of L-glutamine and 200 µg/ml of penicillin with 200 U/ml of streptomycin and 2 µg/ml of porcine trypsin type IX was added in the post infection DMEM (DMEM-PI). Mock and H3N2 SwIV infected cells were incubated for 1 h, 4 h, 8 h, 16 h and 24 h at 37 °C and 5% CO$_2$. As control, poBMDCs were stimulated with 50 µg/ml of Polyinosinic Polycytidylic Acid Salt (Poly: IC) (Sigma Aldrich) for 24 h.

Percentage viability and intracellular staining for influenza nucleoprotein (NP)

Mortality 24 h after infection was determined by staining $2.5*10^5$ of mock or H3N2 SwIV infected cells with 5 µl of annexin V and after washing, adding 10 µl of propidium iodide (PI) (AB Serotec, Oxford, UK) following the manufacturer's procedures.

At 24 hpi, intracellular staining for NP in poBMDCs was performed using $2.5*10^5$ cells fixed with 4% of paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) for 30 min at 4 °C. After washing (centrifugation at 450 g for 5 min at 4 °C), cells were permeabilized with PBS with 0.2% v/v Tween 20 (Merck, Darmstadt, Germany) for 15 min at 37 °C. Then, cells were washed with PBS containing PBS + 0.1% v/v Tween 20 and 100 µl of primary antibody of HB 65 (H16-L10-4R5-IgG2a) (ATCC® Manassas, USA) diluted 1:1000 in staining buffer (PBS with 0.1% w/v NaN3 and 1% w/v BSA) was added and incubated for 1 h at 4 °C. After washing, cells were incubated with 1:200 diluted fluorescein (FITC) conjugated affinity pure F(ab′)2 fragment goat anti-mouse IgG (Jackson ImmunoResearch, Suffolk, UK), for 1 h at 4 °C. Then, after washing, cells were resuspended in staining buffer and analysed by FACSaria (Becton Dickinson). After 24 h of infection or poly:IC stimulation, poBMDCs were harvested and stained as described elsewhere for SLA I, SLA II

and CD80/86 using monoclonal antibodies or immunoglobulin fusion protein.

Immunofluorescence of influenza virus NP

Presence of NP viral protein in poBMDCs was visualized by indirect immunofluorescence in infected cells. At 24 hpi, mock or infected poBMDCs were placed on a circular glass cover slip (VWR International, Spain) and left to adhere for 1 h at 37 °C using 40 µl of fibronectin from human plasma at 20 µg/ml (Sigma Aldrich). After that, cells were fixed with ethanol (Panreac, Spain) for 10 min at 4 °C, dehydrated with acetone and permeabilized with 0.1% of Triton X-100 for 15 min at 37 °C. Then, cells were washed with PBS 2% FCS and 100 µl of primary antibody HB 65 (H16-L10-4R5-IgG2a) (ATCC® Manassas, USA) diluted 1:500 was added. Cover slips were incubated at 4 °C for 1 h and after two rounds of washes, 100 µl of Cy™ 2-goat anti-mouse IgG (Jackson Immunoresearch, Suffolk, UK) diluted 1:200 was added for a further 1 h of incubation at 4 °C. Finally and after several washes, nuclei were counterstained with DAPI. Cover slips were dried and mounted using 1 drop of Fluoprep (BioMérieux, France). To detect autofluorescence, mock or infected-poBMDCs, were stained as controls with the primary and/or secondary antibody. Treated cells were viewed on a Nikon eclipse 90i epifluorescence microscope equipped with a DXM 1200F camera (Nikon Corporation, Japan). Pictures were merged using Adobe®Photoshop®CS version 8 (Adobe System Incorporated, USA).

H3N2 replication in BMDCs and MDCK cells

Virus replication in infected cells was assessed by titration of supernatants on MDCK cells with the aid of trypsin in the post-infection media. Virus titre was calculated by the Reed and Muench method (Reed and Muench, 1938). Viral threshold cycle (Ct) values in MDCK or BMDC cells were assessed following a TaqMan one-step quantitative RT-PCR (RT-qPCR) in Fast7500 equipment (Applied Biosystems, Foster City, CA). RT-qPCR was performed using 60 µl of eluted RNA extracted from mock or infected cells using TRIZOL® reagent (Invitrogen®, San Diego, USA). The primers and probe and the amplification conditions used to perform the RT-qPCR were previously described by Busquets et al. (2010). The amplification profile was as follows: reverse transcription at 48 °C for 30 min; initial denaturation reaction at 95 °C for 15 min and 40 PCR-cycles of 95 °C for 15 s and 60 °C for 1 min. Serial 10-fold dilutions of H3N2 RNA, obtained from H3N2 infected MDCK of known concentration, were made and a standard curve generated. The limit of detection was 10^3 TCID$_{50}$/ml corresponding to the Ct 30.

Infectious centre assay (ICA) and transwell assay (TA)

At 24 hpi, mock or H3N2 SwIV infected-DCs ranging from 10^6 to 10 cells were co-cultured with MDCK in the presence of DMEM-PI in 96 (Nunc® Kamstrupvej, Denmark) or in BD Falcon cell culture 24-well plate with or without inserts with 0.4 µm pores (Becton Dickinson) respectively. Then as positive control, 100 µl of trypsin treated H3N2 $5*10^5$ TCID$_{50}$ was added in the TA assay. In addition, poBMDCs and MDCK were irradiated for 5 min with 30 Gy and 60 Gy respectively in an IBL 437C type H irradiator (CIS, Biointernational, Nice, France) before infection. After irradiation, cells were washed and infected with H3N2 SwIV for 1 h at 37 °C (for virus adsorption). Then, cells were washed and incubated for 24 h. After 24 hpi, cells were washed, counted and co-cultured with MDCK for 7 days in an ICA. At days four and eight after ICA or TA assays cytopathic effect (CPE) in MDCK was evaluated. Each condition in the co-culture had 8 replicas in the 96-well plate. Then, CPE was quantified as being positive when 70% to 100% of monolayer disruption was observed in the

wells from the 96-well plate with MDCK. When less than 70% percentage was observed, the well was considered negative.

Statistical analysis

All statistical analyses were carried out using the SAS system V.9.1.3 (SAS Institute Inc, Cary, NC, USA). The significance level (α) was set at 0.05 with statistical tendencies reported when $p < 0.10$. A non-parametric test (Mann–Whitney) was used to compare any variable response between experimental groups. In the particular case of the cytopathic effect in MDCK cells, an ANOVA test was carried out using a number of infected poBMDCs and cell irradiation as independent variables.

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