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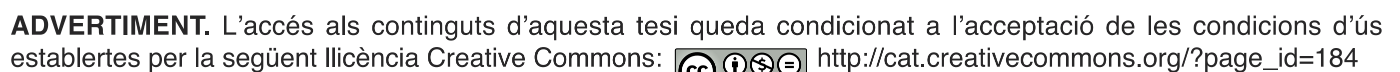
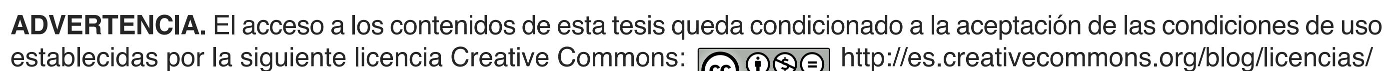
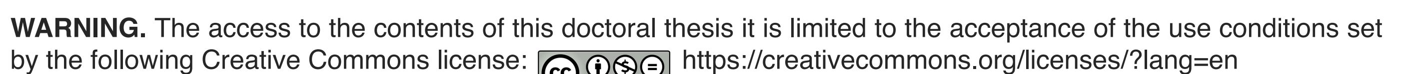
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Towards a universal influenza vaccine: generation and evaluation of vaccine formulations based on HA-epitopes in different influenza hosts.

Marta Sisteré i Oró

PhD Thesis

Bellaterra, 2019

UAB
Universitat Autònoma de Barcelona

Towards a universal influenza vaccine: generation and evaluation of vaccine formulations based on HA-epitopes in different influenza hosts.

Tesi doctoral presentada per **Marta Sisteré Oró** per accedir al grau de Doctor en el marc del programa de Doctorat en Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció del **Dr. Ayub Darji i la tutoria del Dr. Mariano Domingo.**

Bellaterra, 2019

CReSA
Centre de Recerca en Sanitat Animal

IRTA
RESEARCH & TECHNOLOGY
FOOD & AGRICULTURE

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El Dr. **Ayub Darji**, investigador de l'Institut de Recerca i Tecnologia Agroalimentàries- Centre de Recerca en Sanitat Animal (IRTA-CReSA) i el Dr. **Mariano Domingo**, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit a IRTA-CReSA,

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Que la memòria titulada "**Towards a universal influenza vaccine: generation and evaluation of vaccine formulations based on HA-epitopes in different influenza hosts**" presentada per **Marta Sisteré i Oró** per a l'obtenció del grau de Doctora en Medicina i Sanitat Animal, s'ha realitzat sota la seva supervisió i tutoria, i n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent.

I per tal que consti a efectes oportuns, signen el present certificat a Bellaterra (Barcelona), a 24 d'abril del 2019.

Dr. Ayub Darji	Dr. Mariano Domingo	Marta Sisteré i Oró
Director	Tutor	Doctoranda

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"The person with big dreams

is more powerful than the one with all the facts"

H. Jackson Brown

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Abbreviations

Aa: amino acid

Ab: antibody

ADCC: antibody-dependent cellular cytotoxicity

AcMNPV: *Autographa californica* multicapsid nucleopolyhedrovirus

AIV(s): avian influenza virus(es)

ALGENEX: Alternative Gene Expression

AP-1: activator protein-1

APC(s): antigen-presenting cell(s)

ASC(s): Ab-secreting cell(s)

ASFV: African swine fever virus

BacNi: recombinant baculovirus with no foreign gene

BALF(s): broncho alveolar fluid(s)

BALT: bronchial-associated lymphoid tissue

BEST: baculovirus expressions system technology

BEVS: baculovirus expression vector system

BIP: broncho-interstitial pneumonia

Bp: base-pairs

BSA: bovine serum albumin

BSL-2: biosafety level 2

BSL-3: biosafety level 3

CD4/CD8: cluster of differentiation 4 or 8

cDNA: complementary DNA

CALT: conjunctiva-associated lymphoid tissue

CCR-5: C-C chemokine receptor type 5

$C_{H:}$ constant regions for heavy chains

CHMP: Committee for Medicinal Products for Human use

CIS: consensus informational spectrum

CMI: cell-mediated immunity

CO_2: carbon dioxide

CPE: cytophatic effect

CPSF30: cleavage and polyadenylation specificity factor

cRBC(s): chicken red blood cell(s)

cRNA: complementary RNA

CS: cloacal swabs

CTL(s): cytotoxic T lymphocyte(s)

CTLA4: cytotoxic T lymphocyte-associated antigen

CVV(s): candidate vaccine virus(es)

CXCR4: C-X-C chemokine receptor type 4

DC(s): dendritric cell(s)

DEV: duck enteritis virus

DH10Bac™: *E. coli* competent cells containing a parent bacmid and a segment of the lacZα gene.

DIVA: differentiating infected from vaccinated animals

DMEM: dulbecco's modified eagle medium

DNA: deoxyribonucleic acid

DP: double positive

Dpi: days post-inoculation

dsRNA: double stranded RNA

DTT: 1,4-dithiothreitol

E: glutamic acid

ECL: enhance chemioluminiscence

E.coli: *Escherichia coli*

EIIP: electron-ion interaction potential

ELD_{50}: embryo lethal dose 50%

ELISA: enzyme-linked immunosorbent assay

FAO: Food and Agriculture Organization

FBS: fetal bovine serum

FITC: fluorescein isothiocynate

FliC: gene coding for flagellin from *Salmonella typhimurium*

GALT: gut-associated lymphoid tissue

GEC: genomic equivalent copies

GISRS: Global Influenza Surveillance and Response System

GMT: geometric mean titers

Gp: glycoprotein

HA: hemagglutinin

HA0: uncleaved precursor haemagglutinin

HA1: hemagglutinin subunuit 1

HA2: hemagglutinin subunit 2

Hbc: hepatitis B virus core protein

HEPA: high efficiency particulate air

HI: hemagglutination inhibition

His: histidine

HIV-1: human immunodeficiency virus type 1

HPAIV: highly pathogenic avian influenza virus(es)

HRP: horseradish peroxidase

HVT: herpesvirus of turkeys

IA: image analysis

IAV(s): influenza A virus(es)

IBV(s): influenza B virus(es)

ICV(s): influenza C virus(es)

Id: identification

IDAL: intra dermal application of liquids

IDV(s): influenza D virus(es)

IFN: interferon

IFNAR: interferon α/β receptor

IFNL: interferon-λ-receptor

Ig: immunoglobulin

IHA: inhibition of the hemagglutination assay

IL: interleukin

IRF: interferon regulatory factor

IS: informational spectra

ISG: interferon-stimulated gene(s)

ISM: informational spectrum method

IVs: influenza virus(es)

IVPI: intravenous pathogenicity index

IZSLER: Instituto Zooprofilattico Sperimentalle della Lombardia e dell'Emilia Romagna

JAK-STAT: Janus kinase-signal transducer and activator of transcription

K: lysine

Kb: kilobase

LAIV(s): live attenuated influenza vaccine(s)

Log: logarithmic

LRT: lower respiratory tract

M: matrix

M1: matrix protein 1

M2: matrix protein 2

mAb(s): monoclonal antibody(ies)

MAD: mucosal atomizing device

MAVS: mitochondrial antiviral signaling protein

MDA: maternally derived antibodies

MDA-5: melanoma differentiation associated gene 5

MDCK: madin-darby canine kidney

MHC: major histocompatibility complex

mRNA: messenger RNA

MSD: Merck Sharp & Dohme Corp.

MVA: vaccinia virus Ankara

MW: molecular weight

NA: neuraminidase

NDV: Newcastle disease virus

NEP: nuclear export protein

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK: natural killer(s)

NLR: NOD-like receptor family

NLS: nuclear localization signal(s)

NOD: nucleotide binding oligomerization domain

NP: nucleoprotein

NS: nasal swabs

NS2: nonstructural protein 2

OAS: 2'-5'-oligoadenylate synthetase

OD: optical density

OIE: World Organization for Animal Health

OS: oropharyngeal swabs

PA: polymerase acid protein

PABPII: poly(A)-binding protein II

PAMP(s): pathogen associated molecular pattern(s)

PB1: polymerase basic protein 1

PB2: polymerase basic protein 2

PBMC(s): peripheral blood mononuclear cell(s)

PBS: phosphate buffered saline

PBST: PBS-0.1% tween20

Pfu: plaque-forming units

PKR: protein kinase R

PMSF: phenylmethylsulfonyl fluoride

PRR(s): pathogen recognition receptor(s)

pTB3.2: plasmid Top Bac 3.2.®

pCDNA3.1.: commercial vector to express genes in distinct mammals cell lines.

pCMV: commercial vector to express genes in eukaryotic systems under the cytomegalovirus promoter

PCR: polymerase chain reaction

pDC(s): plasmacytoid dendritic cell(s)

pGEM-T: commercial vector for the direct cloning of PCR products

pH1N1: pandemic swine-origin H1N1 IV

PVD: post-vaccination day

QIV(s): quadrivalent influenza vaccine(s)

rBac: recombinant Baculovirus

RBS: receptor binding site

RG: reverse genetics

RIG-1: retinoic acid induced gene protein-I

RdRp: RNA-dependent RNA polymerase

RNA: ribonucleic acid

RP: replicon particles

RT: room temperature

RT-qPCR: reverse transcription-quantitative PCR

SA: sialic acid(s)

SDS: sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SEM: standard error mean

Sf: *Spodoptera frugiperda*

SIV: swine influenza virus(es)

SN: serum neutralization

SNT: serum neutralization test

SP: single positive

SPF: specific pathogen free

ssRNA: single-stranded RNA

TB3.2-Ni: recombinant TopBac3.2® baculovirus without a gene of interest

$TCID_{50}$: median tissue culture infectious dose

TCR: Tcell receptor

T_{EM}: effector memory T cells

Tfh: T follicular helper

Th: T helper

TIV(s): trivalent influenza vaccine(s)

TMB: 3, 3', 5, 5'-tetramethylbenzidine

Tn7: transposon 7

TNF-α: tumor necrosis factor alpha

T. ni: Trichoplusia ni

TNM-FH: *T.ni* medium formulation hink

TLR(s): Toll-like receptor(s)

TP: total protein

T reg(s): regulatory T cell(s)

TSP: total soluble protein

UK: United Kingdom

URT: upper respiratory tract

US: United States

USDA: United States Department of Agriculture

VAERD: vaccine associated enhanced respiratory disease

VLP(s): virus like particle(s)

vRNA: viral ribonucleic acid

vRNP: viral ribonucleoprotein

WHO: World Health Organization

Abstract

Influenza A viruses (IAVs), members of the *Orthomyxoviridae* family, have been implicated in five pandemics throughout the history and are the cause of seasonal epidemics year after year. They are characterized for their high zoonotic potential, affecting a wide range of hosts. Apart from having great impact on human and animal health, IAVs are also involved in huge economic loses.

IAVs are characterized for having a lipidic bilayer and consist of 8 segments of RNA (single chain and negative sense) which codify for viral proteins like the virus surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA) among others. Evolution of IAVs is abrupt and rapid, mainly due to the function of antigenic drift that leads to mutations (mainly in the HA) and emergence of new variants, and antigenic shift that gives rise to reassortant influenza virus progeny.

Wild aquatic birds are the main reservoirs of IAVs that transmit the virus to domesticated birds and may spread further to pigs and humans. Moreover, pigs are considered intermediate hosts, susceptible to infection for both humans and avian influenza viruses. The fact of being able to infect and transmit to different hosts is mainly due to changes in the HA preference for the receptors in the target cells (which varies between avian and mammalian viruses) but also to other mutations in some viral proteins.

The most used and effective countermeasure against IAVs are the vaccines. Most of them, both in human and in pigs, are inactivated

vaccines containing strains of the most common circulating subtypes of IAVs. However, they present several inconveniences like limiting cross-protection capacity against heterovariant or heterosubtypic strains. In contrast, avian influenza vaccines are usually composed of HA (or NA) obtained from particular influenza virus strain of interest. These limitations generally require reformulation of vaccines matched to the genetic changes of the virus and demand continuous vigilance/constant alert for possible pandemics. To overcome these hurdles, current research focuses on seeking for a universal influenza vaccine, implementing several vaccine/immunization strategies by using different immunomodulators and highly conserved IAV epitopes in vaccine formulations.

This doctoral thesis evaluates distinct approaches both in improving vaccine formulation and its application/evaluation to different IAV natural hosts with the objective to achieve an efficient and effective immune response against IAV infection. The work has been divided into 3 parts with the corresponding chapters:

In the Part I, the general introduction (Chapter I) and the general objective of the thesis with specific objectives for each of the studies (Chapter II), are described. The introduction part explains (i) the influenza virus in general and, in particular; (ii) the viruses of the genus A (IAVs); (iii) the host's immune response against the virus; (iv) a summary of the commercial influenza vaccines available against influenza in human, avian and porcine; (v) the concept and approaches in process to obtain a universal vaccine and the (vi) ISM, an *in silico* tool which has been used in the thesis to predict conserved peptides. Chapter II

describes the rationale and the main objective of this thesis. Overall, it is intended to create a universal prototype vaccine against different subtypes of influenza virus using techniques that incorporate *in silico* predicted conserved HA-epitopes introduced in plasmid or soluble form along with different adjuvants.

In Part II (Chapters III-V), three studies that have been carried out during the thesis that are either published or submitted to international peer-reviewed scientific journals, are included. The design of vaccine prototypes with possible multivalent character and its subsequent application in pigs and birds, important hosts to prevent IAVs infection and transmission (for both zoonosis and animal health), is evaluated.

In the first study, one of the conserved HA epitopes NF-34, predicted by ISM, was modified and formulated in a plasmid with the CTLA-4 molecule (pCMV-CTLA4-Ig-NG34), promoter of the adaptive response. The vaccine approach was used in swine influenza seronegative and seropositive pigs and challenged against heterologous H3N2 virus. Vaccinated pigs secreted fewer viruses, cleared the virus in the respiratory airways, presented humoral response to the most relevant circulating subtypes and also elicited neutralizing antibodies. However, there were no differences in the degree of pulmonary lesions and clinical signs. In addition, maternal antibodies did not interfere with the effect of the vaccine.

In the second study, a cocktail of epitopes of HA combined with flagellin (VC-4 flagellin), a promoter of the innate response, was used in

seronegative and seropositive swine influenza pigs and were challenged with homologous and heterologous influenza virus strains. Vaccinated pigs reduced the excretion of the virus, produced humoral response to subtypes H1 and H3, and neutralizing antibodies against both viruses. Again, maternal antibodies were not an obstacle but, clinical signs and pathology in the lungs were not reduced.

Finally, in the third study baculovirus expression vector system (BEVS) was used to obtain protein extracts containing HA-peptides linked with flagellin. The formulation was used to immunize chickens that were further challenged with H7N1, a highly pathogenic virus. Vaccinated animals survived without showing any clinical sign and fewer or no virus secretion. The protection mechanism is still under investigation.

The general discussion is described in part III (Chapters VI-VII) with possible significance of the results obtained and the relation of conclusions drawn from each one of the studies. In addition, the bibliographical references (Chapter VIII) and appendices with additional information of the chapters are also included in the last sections of the thesis.

Resum

Els virus de la influença tipus A (VIAs), membres de la família *Orthomyxoviridae*, han estat implicats en cinc pandèmies al llarg de la història i segueixen ocasionant, any rere any, epidèmies estacionals. Es caracteritzen pel seu elevat potencial zoonòtic, afectant un ampli ventall d'hostes. A banda d'impactar greument en la salut humana i l'animal, els VIAs també repercuteixen en l'economia provocant enormes pèrdues econòmiques.

Els VIAs es caracteritzen per tenir envolta lipídica i consisteixen en 8 segments de RNA (de cadena senzilla i sentit negatiu) que codifiquen per proteïnes virals com ara: hemaglutinina (HA) i la neuraminidasa (NA), entre d'altres. L'evolució dels VIAs és abrupta i ràpida, sobretot degut a la funció de "*antigenic drift*" que deriva en mutacions (principalment en la HA) i en la emergència de noves variants, i "*antigenc shift*" que dóna lloc a una progènie de virus amb reagrupaments en el genoma.

Els principals reservoris dels VIAs són les aus salvatges aquàtiques, les quals poden transmetre el virus a les aus domèstiques, i aquestes, poden transmetre el virus principalment a porcs i a humans. A més, els porcs són considerats hostes intermediaris, susceptibles d'infecció tant per virus humans com aviars. La capacitat dels VIAs de poder infectar i transmetre's en diferents hostes és majoritàriament degut a canvis en la preferència de la HA pels receptors en les cèl·lules diana (la qual varia entre virus aviars i mamífers), però també a altres mutacions en algunes proteïnes víriques.

La mesura preventiva més utilitzada contra els VIAs són les vacunes. La majoria d'elles, tan en humans com en porcs, són vacunes inactivades que contenen soques dels subtipus circulants més habituals de virus de la grip. No obstant això, presenten diversos inconvenients, com ara una limitada capacitat de cross-protecció enfront soques heterovariants o heterosubtípiques. En canvi, les soques contra la grip aviar solen estar formades per HA (o NA) obtingudes a partir de la soca d'influenza d'interès a combatre. Aquestes limitacions, generalment, requereixen una reformulació de les vacunes per tal que s'ajustin als canvis genètics del virus i exigeixen una vigilància continua/alerta constant davant possibles pandèmies. Per tal de superar aquests obstacles, la investigació actual es centra en cercar una vacuna universal contra la grip, implementant diverses estratègies de vacuna/immunització utilitzant diferents immunomodul·ladors i epítops altament conservats dels VIAs en les formulacions vacunals.

Aquesta tesi doctoral avalua diverses estratègies enfocades en la millora de les formulacions vacunals, com en la seva aplicació/avaluació en diferents hostes naturals de la grip amb l'objectiu d'aconseguir una resposta immune eficient i eficaç contra la infecció de la grip. Per això, aquest treball, s'ha dividit en tres parts amb els capítols corresponents:

A la primera part, s'hi descriu la introducció general (Capítol I) i l'objectiu general de la tesi amb els objectius específics per a cadascun dels estudis (Capítol II). La introducció general explica (i) el virus de la

grip en general i, en particular; (ii) els virus del gènere A (VIAs); (iii) la resposta immune de l'hoste contra el virus; (iv) un resum de les vacunes comercials contra la grip disponibles en humans, aviar i porcí; (v) el concepte i les estratègies en procés per obtenir una vacuna universal i (vi) l'ISM, una eina *in silico* que s'ha utilitzat en la tesi per predir els pèptids conservats. El capítol II descriu la justificació i l'objectiu principal d'aquesta tesi. En general, es pretén crear un prototip de vacuna universal contra diferents subtipus del virus de la grip utilitzant tècniques que incorporen epítops conservats de la hemaglutinina, predits *in silico*, introduïts en plàsmids o en forma soluble juntament amb adjuvants diferents.

En la part II (Capítols III-V), s'hi inclouen els tres estudis realitzats durant la tesi i què s'han publicat o enviat a revistes científiques internacionals revisades per experts. El disseny de prototips vacunals amb possible caràcter multivalent i la seva posterior aplicació en porcs i aus, hostes clau a prevenir la infecció i transmissió per VIAs (tant per zoonosis com per salut animal), és avaluada.

En el primer estudi, un dels epítops conservats de la HA: el NF-34, predit per ISM, va ser modificat i formulat en un plàsmid amb la molècula CTLA-4 (pCMV-CTLA4-Ig-NG34), promotora de la resposta adaptativa. Les estratègies vacunals es van utilitzar en porcs seronegatius i seropositius contra la grip porcina, els quals es van desafiar contra el virus heteròleg H3N2. Els porcs vacunats van secretar menys virus, van eliminar el virus a les vies respiratòries, van presentar una resposta humoral als subtipus circulants més rellevants i van mostrar anticossos

neutralitzants. No obstant això, no hi va haver diferències en el grau de lesions pulmonars ni en els signes clínics. A més, els anticossos materns no van interferir amb l'efecte de la vacuna.

En el segon estudi, es va utilitzar un còctel d'epítops de HA combinats amb flagel·lina (VC-4-flagellin), promotor de la resposta innata, en porcs seronegatius i seropositius a la grip porcina i es van desafiar amb soques homòlogues i heteròlogues del virus de la grip. Els porcs vacunats van reduir l'excreció del virus, van produir resposta humoral front els subtipus H1 i H3 i, van produir anticossos neutralitzants contra ambdós virus. De nou, els anticossos materns no varen ser un obstacle, no obstant, els signes clínics i la patologia dels pulmons no es van reduir.

Finalment, en el tercer estudi, es va utilitzar el sistema vectorial d'expressió de baculovirus (BEVS) per obtenir extractes de proteïnes que contenien pèptids de la HA units a la flagel·lina. La formulació es va utilitzar per immunitzar els pollastres que es van desafiar amb H7N1, un virus altament patogènic. Els animals vacunats van sobreviure sense mostrar cap signe clínic i sense secretar o, en menor quantitat, el virus. El mecanisme de protecció encara està sota investigació.

En la part III (capítols VI-VII), es descriu la discussió general amb la possible importància dels resultats obtinguts i la relació de conclusions extretes de cadascun dels estudis. A més, en les darreres seccions de la tesi també s'hi inclouen les referències bibliogràfiques (Capítol VIII) i els annexos amb informació addicional dels capítols.

Results presented in this thesis dissertation have been published or submitted for publication in international scientific peer-review journals:

Sisteré-Oró M, Vergara-Alert J, Stratmann T, López-Serrano S, Pina-Pedrero S, Córdoba L, Pérez-Maillo M, Pleguezuelos P, Vidal E, Veljkovic V, Segalés J, Nielsen J, Fomsgaard A, Darji A. Conserved HA-peptide NG34 formulated in pCMV-CTLA4-Ig reduces viral shedding in pigs after a heterosubtypic influenza virus SwH3N2 challenge. PLoS One, 2019; 14(3):e0212431.

Sisteré-Oró M, López-Serrano S, Veljkovic V, Pina-Pedrero S, Vergara-Alert J, Córdoba L, Pérez-Maíllo M, Pleguezuelos P, Vidal E, Segalés J, Nielsen J, Fomsgaard A, Darji A. DNA vaccine based on conserved HA-peptides induces strong immune response and rapidly clears influenza virus infection from vaccinated pigs. Submitted for publication.

Sisteré-Oró M, Martínez-Pulgarín S, Solanes D, Veljkovic V, López-Serrano S, Córdoba L, Cordón I, Escribano JM, Darji A. Conserved HA-peptides expressed along with flagellin in *Trichoplusia ni* larvae protects chicken against intranasal H7N1 HPAIV challenge. Submitted for publication.

PART I:

General Introduction, Hypothesis and Objectives

"Scientific knowledge is in perpetual evolution;

it finds itself changed from one day to the next"

Jean Piaget

CHAPTER I

General Introduction

1. Introduction

1.1. Influenza viruses (IVs)

The *Orthomyxoviridae* family encompasses seven distinct genera of RNA viruses: Influenza viruses (IVs) classified into four types (A, B, C and D), Isaviruses, Thogotoviruses and Quaranjaviruses.

IVs are causative agents of the contagious respiratory disease named influenza. They are divided according to the antigenic variations within their nucleoprotein (NP) and matrix 1 (M1) proteins and are considered from different genera when their intergenic homology is low (20-30%) and the intragenic one is high (>85%) [1–3]. Additionally, such genetic divergences may promote reassortment solely within intragenic strains and subsequent viable progeny could occur [4,5].

Influenza A viruses (IAVs) are recurrently evoking seasonal epidemics and on several occasions have been responsible of global pandemics with high rates of morbidity and mortality. These IAVs mainly infect birds, particularly in avian species such as wild waterfowl and shorebirds that live in aquatic environments [6,7]. Nevertheless, due to the IAVs zoonotic potential they have also been isolated from a wide variety of avian and mammalian animals and, in some instances, their adaptation promoted efficient replication and maintained transmission [8,9].

IVs from type B also trigger seasonal global epidemics, also creating urgent public health problems worldwide. Influenza B viruses (IBVs) belong to only one subtype [10] and are circulating in humans and seals [11–14]. In humans, two antigenically distinct lineages (B/Victoria and B/Yamagata) have been recognized that distinctly spread periodically and

geographically across the world [15–20]. Though causing milder disease than IAVs in humans [10,14,21], IBVs may cause severe illness demanding hospitalization on occasions [22].

Types C and D influenza viruses (ICVs and IDVs, respectively), on the other hand, are not reported to cause epidemics. Type D affects only cattle and swine [3,23–25] but not humans, while type C infects humans [26–28] and has also been detected in pigs [29,30], dogs [31] and cattle [32]. In humans, ICVs generally induce mild respiratory disease or an asymptomatic infection mainly during childhood and adolescence [3,33,34].

1.2. Influenza A viruses (IAVs)

1.2.1. Antigenic types and nomenclature

The two major surface proteins of IVs, the hemagglutinin (HA) and the neuraminidase (NA), are used to classify IVs into different subtypes. Up to date, 18 HAs (H1 to H18) and 11 NAs (N1 to N11) have been recognized. Two subtypes (H17N10, H18N11) have been detected in the last decade and found exclusively in bats, dramatically expanding the host range of IAVs [35–37].

The standard nomenclature that IVs follow consists in: virus type (A/B/C/D), species from which it was isolated (if non-human), location where it was isolated, isolate number, isolate year; and in case it is type A, HA and NA subtypes are indicated in brackets. An example such as the 39th isolate of an IAV with H1 and N1 retrieved from a person in

Denmark during 2000 would be written as A/Denmark/39/2000 (H1N1).

1.2.2. Structure

IAVs are enveloped and characterized by a negative sense single-stranded (ss) RNA genome, divided into 8 segments. The virus is about 100 nm in diameter and approximately 300 nm long [38]. The envelope consists of a host cell-derived lipid bilayer harboring two transmembrane glycoproteins as spikes: HA and NA (in a ratio of 4 HAs to 1 NA) [4,38]. While HA, a rod-like shaped protein is a trimer, the NA protein forms a tetramer and is described as a mushroom-like shape [38–40]. Furthermore, a membrane protein with proton channel activity, the matrix-2 (M2) protein, consolidates also the envelope [41]. All these proteins overlay the matrix-1 (M1) protein, present in the inner layer of the capsid or virion core, maintaining IAVs shape and integrity. The virion core contains the nuclear export protein (NEP), also known as the nonstructural protein 2 (NS2), and the viral ribonucleoprotein (vRNP) complexes. vRNP complexes are formed by each of the eight viral RNA segments folded on the viral NP in association with a globular head, the heterotrimeric RNA-dependent RNA-polymerase (RdRp). Three subunits form the RdRp: the polymerase basic protein 2 (PB2), the polymerase basic protein 1 (PB1) and the polymerase acid protein (PA) [42] (*Figure 1-1*).

1.2.2.1. Genome

The IAVs genome size is of 13.6 kb [43], comprising 8 vRNA segments. Each segment is numbered to decrease length and encodes for at least

one protein [4]. IAVs can also make use of different mechanisms such as leaky ribosomal scanning, alternative splicing, ribosomal frameshifting or use of alternative start codons to encode more than one protein [44,45].

Segments 1, 2 and 3 consolidate the proteins forming the RdRp of IAVs: PB2, PB1 and PA respectively. Segments 4, 5 and 6 encode for the HA, the NP and the NA, respectively. Segment 7 encodes for both matrix proteins (M1 protein and, by RNA splicing, the M2). Finally, segment 8 encodes for both non-structural proteins (NS1 and, by RNA splicing, the NEP/NS2).

Nonetheless, the proteome of IAVs can be more complex as novel accessory proteins like M42, PB1-N40, PA-N155, PA-N182, PA-X, PB1-F2, PB2-S2 and NS3 have been discovered. PB2-S2 is encoded by a novel spliced mRNA in segment 1 [45]. In segment 2, ribosomal scanning produces two internal open reading frames (ORFs), PB1-F2 [46] and PB1-N40 [47]. In segment 3, three other protein isoforms can be synthesized: PA-X, obtained by a second ORF accessed via ribosomal frameshifting [48], and two N-terminally truncated forms of PA, namely PA-N155 and PA-N182 [49]. Segment 7 also translates for M42 (functionally equal as M2), when an alternative initiation codon is read [47]. Segment 8 encodes for NS3, which corresponds to the NS1 isoform with an internal deletion of a motif consolidated by three antiparallel β-strands spanning codons 126 to 168 [50] (*Figure 1-1*). Some of these proteins are implicated in IAVs pathogenicity (see *1.2.6.1.*; "*Virulence determinants*").

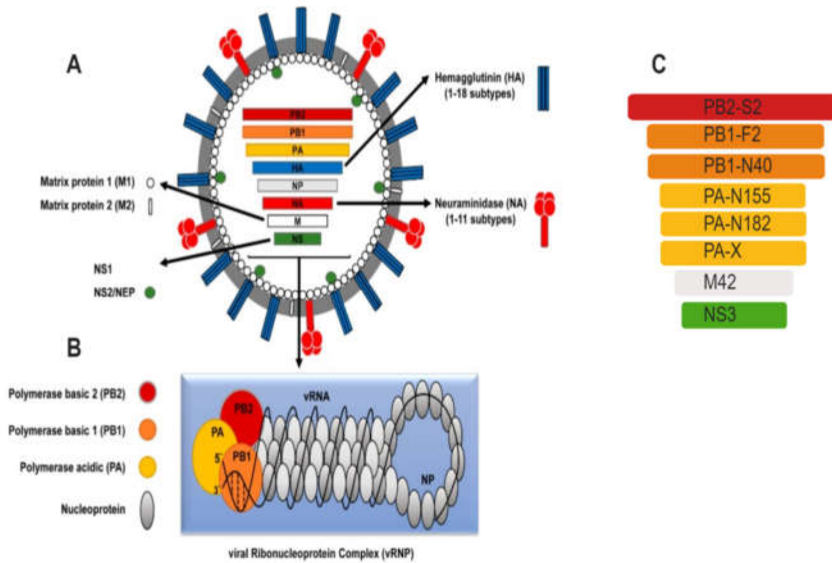

Figure 1-1. (A) Schematic structure of an IAV with eight negative-strand RNA segments, (B) an illustration of a vRNP structure, and (C) accessory proteins derived from corresponding (color) virus RNA segment. Image modified from [42].

1.2.3. Replication cycle

IAVs infection initiates with the recognition of the HA protein to terminal N-acetylneuraminic sialic acid (SA), monosaccharide usually encountered in glycoconjugates present on the surface of the host airway epithelial cells [51–53]. Once attached, the virus can internalize into target cells by receptor-mediated endocytosis [54–56] or, alternatively, by macropinocytosis [57,58]. After internalization, the virus moves to late endosomes and undergoes a conformational change in the HA protein owing to the low pH in the endosomal environment. Specifically, host cell proteases cleave HA into two subunits (HA1 and HA2), enabling the exposure of the fusion peptide [59–61].

Subsequently, the fusion peptide binds to endosomal membranes permitting the fusion of virus and endosome [62,63]. Moreover, the acidity of endosomes triggers the M2 ion channel acidifying the virus by pumping protons as well as K^+ ions into the virus [41,64,65]. The acidification promotes M1 depolymerization resulting in the capsid uncoating [66] and the disruption of protein-protein interactions making possible the dissociation of the viral ribonucleoproteins (vRNPs) [67].

vRNPs are released through the fusion pore into the cell cytosol and translocated to the host cell nucleus with the help of nuclear localization signals (NLSs). Transcription and replication take place in the nucleus. Viral negative ss-genome serves as a template for RdRp to synthesize two positive-RNA species: mRNAs for viral protein synthesis and complementary RNA (cRNA) further used for genomic vRNA replication [68,69]. After polyadenylation and 5'-capping, M1 and NEP/NS2 proteins help vRNAs to be transported into the cytoplasm via nucleoporins [70]. Envelope proteins (HA, NA and M2) are translated on membrane-bound ribosomes into the endoplasmic reticulum and non-envelope proteins (PB1, PB2, PA, NP, NS2, NS2 and M1) on cytosolic ribosomes. Post-translational modifications are held in the Golgi apparatus.

Sorting signals allow all proteins to be directed to the host cell membrane regions termed "lipid rafts" [71]. To ensure the new virions assembly, both glycoproteins (HA and NA) are essential to initiate the budding by deforming the plasma membrane [72–74]. Eventually, the role of M2 in membrane scission [75] and of NA in cleaving terminal sialic residues is important. In particular, the NA enzymatic activity,

which function prevents aggregation of the nascent virions at the cell surface, is essential for release of virus progeny [76–78]. Finally, the new virus generation leads to subsequent infection of the host's cells and virus spread (*Figure 1-2*).

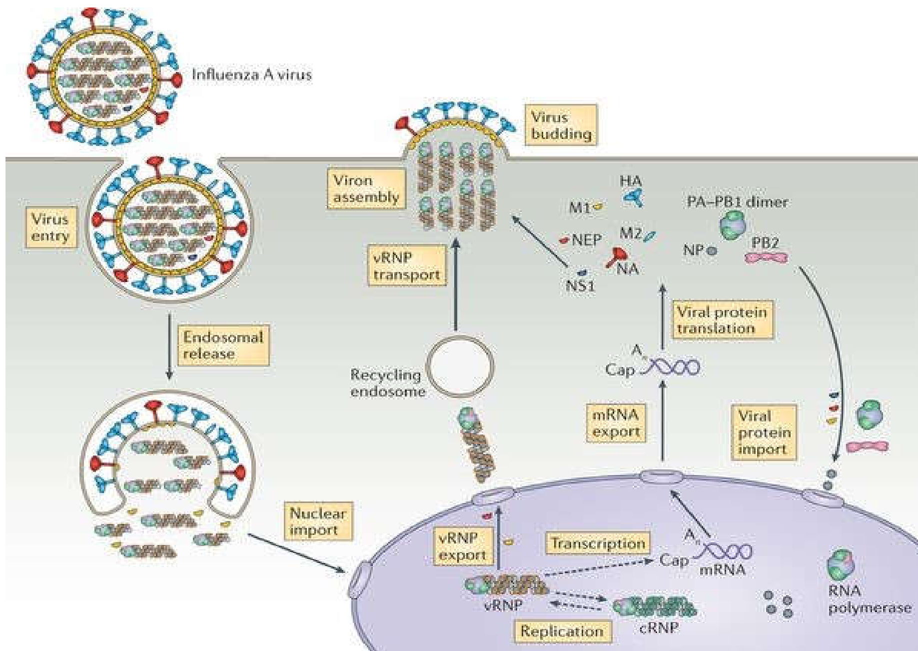

Figure 1-2. Illustration of the IAVs replication cycle. Image taken from [79].

Other cell types such as airway macrophages and dendritic cells (DCs) are also susceptible for infection, although no infectious progeny is released in these cases [80].

1.2.4. Host range

IAVs have global presence and their host range is wide as illustrated in *Figure 1-3*. They have been isolated from humans, birds (including ostriches), horses, marine mammals, camels, ferrets, minks, pigs, dogs,

cats and bats. In few occasions, IAVs have also been detected in tigers and leopards as well [81,82]. Furthermore, the interspecies transmission of IAVs is commonly occurring between humans, poultry and pigs. In particular, pigs may represent an intermediate host for the generation of new IAVs able to infect humans.

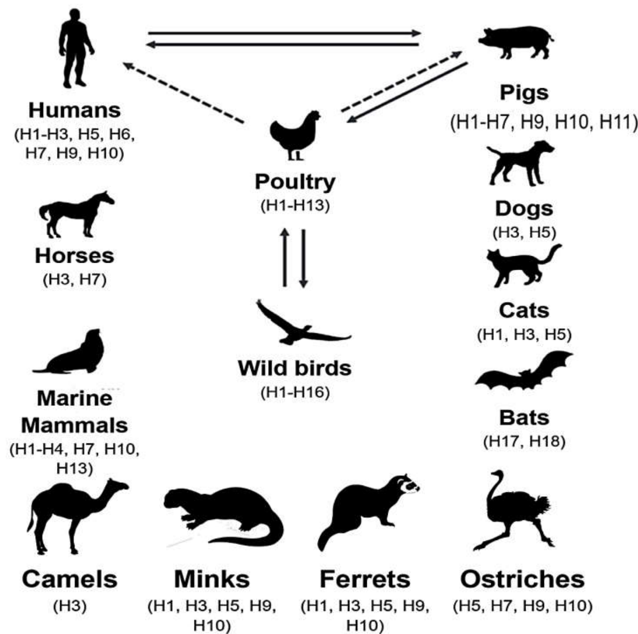

Figure 1-3. Host range of IAVs. The HA subtypes which have been isolated from the represented hosts are specified in parenthesis. The significant interspecies IAVs transmission is shown by solid (common) and dashed (sporadic) arrows (modified from [83]).

Poultry and swine are major sources of human infections with IAVs:

Avian influenza viruses (AIVs): As mentioned in section *1.1.*, waterfowl are the main reservoirs of IAVs, being the hosts of H1-H16 subtypes and mainly causing asymptomatic infections. Globally migrating wild birds, potential IAV carriers, may introduce IAVs to

poultry (e.g. chicken, turkeys, quails), which are susceptible to natural infections promoted by avian IAVs [84].

Isolates of AIVs have been typed either of "highly pathogenic avian influenza viruses" (HPAIVs) or "low pathogenic avian influenza viruses" (LPAIVs) (see *1.2.6.; "Pathogenesis and transmission"* and *1.2.6.1.;* "*Virulence determinants*"). HPAIVs are defined as these isolates causing>75% of mortality in a period of 10 days or that induce an "intravenous pathogenicity index" (IVPI) higher than 1.2. in a defined chicken population a mortality rate [85].

HPAIV subtypes are more restricted than LPAIVs. The first comprise isolates from H5Nx, H7Nx and H9N2 carrying a multibasic cleavage site in their HA protein [42,86]. Until mid-1950s, all HPAIVs were from the H7 subtype. In 1959, an H5N1 HPAIV was reported in chickens [87]. In 2002, a fatal outbreak of H5N3 HPAIV in wild birds was reported for the first time [88]. Since then, many others HPAIV outbreaks have been detected in wild birds [89–92].

In 1997 the first fatal outbreak of a HPAIV H5N1 was reported in humans [93]. Since then, AIVs from subtypes H5, H7 and H9 have been involved in human infections and to a minor extent, H6 and H10 isolates have also been detected [42] causing mild-to-fatal disease to their hosts. Acquiring gene segments from other co-circulating AIVs has expanded the host range frequently breaking the host barrier [94]. Human-to-human transmission of HPAIV strains, fortunately, has not been frequently reported [42,81].

Swine influenza viruses (SIVs): Direct introduction from distinct AIV subtypes, the reintroduction from human-adapted viruses and reassortment among human and avian viruses [95] are factors that have contributed to the establishment of IAVs in the swine herds, leading to the emergence of SIVs.

Although being infected by several IAV subtypes [96–98], only three subtypes have been established among swine herds. For decades, H1N1, H1N2 and H3N2 subtypes have been enzootic in pigs [6,99–101]. Other species such as wild boar [102] and turkeys [103] have also been infected by SIVs. Nevertheless, novel reassortments between enzootic SIVs or between SIVs and seasonal human IVs have been reported [104–106]. Human infections with SIVs have also occurred and in a few isolated cases were even lethal [107,108]. For example, in 2009 a reassortant IAV previously circulating in pigs emerged (H1N1pdm) infecting huge numbers of humans with fatal cases in thousands (See *1.3.2.*; "*Influenza A virus pandemics*").

1.2.4.1. Host receptors

The receptor preferences of distinct IAVs vary depending on the linkages to galactose present in the SA. NA on the surface of IAVs scans and searches the specific IAV receptors on the host cell depending on the IAV [109]. In 1990s, it was shown that while most avian influenza viruses (AIVs) preferentially attached to α-2,3-linked SAs linearly presented [110,111]; the human IAVs preferred the α-2,6 linkage, exposed in a "bent" presentation [112,113].

AIVs replicate efficiently in the avian gastrointestinal epithelium mainly expressing α-2,3 receptors. In humans, IAVs replicate in the upper respiratory tract (URT) where α-2,6 receptors are more prevalent. The lower respiratory tract (LRT), however, expresses more α-2,3 receptors where IAVs may attach. Consequently, the SA linkage receptor preferences of the IAVs correlated with the distribution of the SA linkages expressed on cells/tissues in their respective hosts [61]. Hence, it was determined that those variations in the SA receptors could define the IAVs host range [114].

Research in this field has established that virus tropism though not critical for infection, is a determinant for virus transmission [115–118]. It has also been shown that some strains of IAVs causing human pandemics bore mutations in the HAs from the former avian IAVs altering this way their SA receptor preferences (See *1.2.6.1.*; "*Virulence determinants*").

Owing to the presence of both the α-2,3 and α-2,6 receptors in the respiratory tract of pigs [119,120], pigs may act as "mixing vessels" allowing infection of both avian and human IAVs. This "mixing vessel" concept is narrower than originally thought. Current research indicates that the respiratory tract of the pigs resembles to that observed in humans [121] and points at the opportunity (changes of contact between infected birds and swine, sheer numbers and dense housing) rather than the physiology as the major cause of their interspecies transmission role [95].

1.2.5. Evolution

Two major mechanisms are described determining the evolution of IAVs. These mechanisms can enable IAVs to expand the host range and allow replication in other hosts as well:

i. **Antigenic drift** is due to the lack of proof-reading function of the RdRp, which leads to the gradual accumulation of point mutations in the IVs genome, thereby creating new IVs variants. This phenomenon is the main cause of the annually repeated seasonal epidemics.

ii. **Antigenic shift** is due to genetic segment reassortment, which can occur in cells infected by different (two or more) IVs. Resulting reassortment of IV genomes can enable the formation of viruses containing novel antigenic proteins, for which the hosts have no preexisting immunity, thereby leading to new pandemics. The resulting progeny also must have an efficient replication-competent set of internal genes and be able to spread from human to human.

Influenza vaccines must provide strain-specific protection against the circulating strains during specific seasons. Changes in antigenic drift or in antigenic shift may influence the efficacy of the seasonal flu vaccine. Thus, for humans, their composition is annually updated and immunogenically evaluated. In addition, if antigenic shift occurs that may result in pandemics, there is the need to manufacture and rapidly distribute a vaccine which protects and provides a long-lasting protection.

1.2.6. Pathogenesis and transmission

AIVs: While wild birds may carry LPAIVs without showing severe clinical signs and disease after transmission to domestic poultry, LPAIVs cause subclinical or mild illnesses (weight loss, ruffled feathers and/or a reduction in their egg production) [122]. On the contrary, HPAIVs cause fatal outbreaks in wild and domestic birds. Mortality can reach up to 90%-100%, often within 48 hours. In some instances, no clinical signs are recorded but sudden death occurs. In other circumstances, the disease is characterized by respiratory, nervous, reproductive and gastrointestinal signs [123]. Exceptionally, ducks do not exhibit any, or only very mild, clinical signs [84].

Transmission of AIVs mainly occurs through the fecal-oral route. Contaminated feeding grounds and surface waters, where AIVs can be retained are the major source of AIVs transmission [124]. Infected birds generally excrete AIVs via oropharyngeal and cloacal routes [125]. Airborne transmission of IAVs although, may occur but at a lower frequency [126,127].

Human IVs: IVs cause recurrent epidemics to humans and even evoke pandemics (See *1.3.*; *"Social and economic impact, epidemics and pandemics"*). In humans IVs infection cause pneumonia and acute respiratory failure, frequently complicated by bacterial coinfection [128]. Even though IVs may affect at all ages, the most vulnerable are the young children, the elderly and immunocompromised individuals.

Person to person transmission of human IVs is mediated by the air through droplets excreted by infected individuals via cough or sneeze. The rapid spread of the disease is accelerated particularly in crowded atmospheres. Infection frequency is higher in the winter in temperate climates since IVs survive longer when the weather is cold and dry [129].

Zoonotic IAV infections have been more frequently detected in people working in pig and poultry farms [130–132]. People in contact with wild birds (e.g. hunters) [133] and exposure to live poultry markets [134] are also a potential target of AIVs infections. The most common transmission route is through the contact with the eye conjunctiva and mucosal membranes (e.g. inhalation of dust, droplets) or swimming or bathing in contaminated water (e.g. household ponds) [135–138].

SIVs: Onset of clinical signs and nasal shedding begins after 24 hours of SIVs infection. Though some infections remain subclinical, others are clinical (25%-30%) [139]. Gross lesions are characterized by broncho-interstitial pneumonia (BIP) and there is no extra-respiratory evidence of infection.

SIV transmission initiates via the nasopharyngeal route (nose-to-nose contact or direct contact of mucus) through the contact between infected and susceptible pigs. SIVs are present in the excretions from infected pigs through coughing, sneezing and nasal discharges that also facilitate spread and airborne transmission of SIVs [140].

1.2.6.1. Virulence determinants

Some mutations in specific gene sequences have influenced virus virulence in one particular host. Nevertheless, the same mutation may not affect the IAVs virulence in other hosts [46]. Some virulence determinants of IAVs are described below:

I. HA:

HA affinity for receptors: SAs expressed on the host cells are described as receptors for HA. The affinity of the virus HA for specific SA may promote the adaptation of IAVs to specific hosts. Targeting α-2,6 SA receptors is a requirement for AIVs to enable efficient human-to-human transmission. Pandemic viruses acquired the ability to bind to α-2,6 SA receptors present on human cells [113,141]. Although some amino acid changes found in H5N1 HPAIVs receptor binding site enhanced the affinity to α-2,6 SA [142–146], HPAIVs H5N1 are not able to exclusively attach to specific α-2,6 SA. This fact reinforces why H5N1 did not show sustained transmission among humans [147].

HA cleavability: As explained in *section 1.2.3.*, the HA cleavage is a prerequisite for viral infectivity [148] and in AIVs, the cleavage susceptibility of uncleaved precursor haemagglutinin (HA0) correlates with the virulence of HPAIVs. As demonstrated, a mutant of an H5N1 HPAIV harboring the HA cleavage of a LPAIV becomes attenuated in mice [149]. This is related to the fact that while LPAIVs only possess a conserved arginine residue targeted by host trypsin-like proteases, HPAIVs possess multiple basic amino acids targeted by subtilisin-like proteases. Trypsin-like proteases are found extracellularly, secreted by

cells from the respiratory and intestinal tract, thus restricting infection in those tissues. On the other hand, subtilisin-like proteases are intracellular and ubiquitous, favoring systemic infections [150].

II. NS1:

This non-structural protein permits efficient viral replication since it suppresses the host innate immune response by influencing on distinct mechanisms, mainly counteracting with the cellular antiviral type I interferon (IFN) pathway. NS1 can interfere with the activation of the retinoic acid induced gene protein-I (RIG-1) innate pathway and thus, to IFN responses [151–153] (*See 1.4.1.; "Innate immunity triggered against IAVs infection"*). NS1 can also inhibit transcription factors, which will further impair IFN production (interferon regulatory factor-3 (IRF-3), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the activator protein-1 (AP-1) [154–156]) and interferon-stimulated genes (ISGs), which have antiviral effects (protein kinase R (PKR) and 2'-5'-oligoadenylate synthetase (OAS)-RNaseL) [157,158]. Furthermore, NS1 can also inhibit the nucleotide binding oligomerization domain (NOD)-like receptor family (NLRs) pyrin domain-containing 3 (NLRP3) inflammasome activation, interfering with the cleavage of pro-interleukin (IL)-1β and pro-IL-18 into mature forms (*See 1.4.1.; "Innate immunity triggered against IAVs infection"*) [159,160]. Moreover, NS1 blocks the proper processing of host cellular pre-mRNAs, by binding either to cleavage and polyadenylation specificity factor (CPSF30) [161,162] or to the poly(A)-binding protein II (PABPII) [163]. When attached to CPSF30, interfering with the cleavage of pre-mRNAs and when attached to PABPII, interfering with synthesis of the poly(A) tails.

III. NS3:

Although being identified so far in 33 strains, NS3 isoform seems to be aiding in viral transmission between hosts [44,50]. Several examples of avian viruses being transmitted to human (1977 H5N1, 1999 H9N2), swine (1979 H1N1) or canine [50,164] have supported this hypothesis.

IV. PA:

PA has been reported to play an important role in inhibiting host type I IFN signaling. A single mutation has been determined (K351E) to contribute to the mentioned role [165]. Also, in vitro, PA inhibits NF-κB transcription of H5N1 HPAIVs [166].

V. PA-X:

PA-X protein induces the shutoff of host protein expression, counteracting the antiviral responses in the host. PA-X widely degrades host RNA polymerase II (Pol II)-transcribed mRNAs and non-coding RNAs in the nucleus of infected cells [48] by means of its interaction with the host's 5'->3' Xrn1 exonuclease activity. However, it has been reported that the effects of PA-X on pathogenesis are host- and strain-specific.

VI. PB1-F2:

PB1-F2 protein disrupts mitochondrial antiviral signaling protein (MAVS) signaling and promotes inflammatory responses and apoptosis in immune cells [46,167,168]. During IAVs infection, PB1-F2 may facilitate secondary bacterial infections [169–171]. In vitro, it enhances polymerase activity by binding to the PB1 protein [172]. Its contribution

in the pathogenicity of the 1918, 1957 and 1968 pandemic viruses as well as H5N1 HPAIVs have also been demonstrated [169,173,174]. Specifically, N66S mutation in these pandemic virus (1918 H1N1 and H5N1) enhanced the pro-inflammatory cytokine release and the viral replication at lung level [173,175]. PB1-F2 mainly contributes to virulence of pandemic strains when it is recently derived from an avian reservoir [169], primordially, since PB1-F2 becomes truncated following introduction into humans or pigs [176].

VII. PB2:

PB2 enhances IAVs pathogenicity and can alter host range, most likely by controlling levels of vRNA replication. Punctual mutations in specific aa positions have further supported the role of PB2 in pathogenicity of IAVs. For instance, a replacement of lysine (K) at position 627 instead of a glutamic acid (E) enables IAVs to replicate in the URT of human [149]. This mutation seems to be influencing the temperature sensitivity of the PB2 allowing AIVs (usually replicating at nearly 41ºC) to replicate at a lower temperature of 33ºC [177]. Another mutation in PB2, PB2 D701N, is also known that triggers pathogenicity in mammals and is related to enhanced levels of virus replication, transmissibility and nuclear localization of PB2 [178]. In addition, PB2 interferes with MAVS signaling [179] or inhibits the transcription of the IFN-β gene induced upon an IV infection [180].

1.3. Social and economic impact of IVs

IVs cause seasonal epidemics, occasional pandemics and lethal zoonotic outbreaks universally. Estimations made by the World Health Organization (WHO) conclude that IVs are the global causative agents of a billion cases annually, being 290,000 to 650,000 among them lethal. Apart from the burden of IVs in the human population, IV epidemics promote a significant toll economically in productivity and financial resources. In industrialized countries, losses also involve the health care costs, worker absenteeism and workforce productivity losses [181]. Moreover, the threat of a future flu pandemic could emerge anywhere and spread universally.

Economic consequences of AIVs and SIVs are also severe not only for the potential occurring zoonosis concerning the public health but also for their impact on animal health.

AIVs can produce disease in chickens, turkeys and other birds of economic importance. Mild illnesses can influence the egg quality and final number of birds. Furthermore, H5 and H7 HPAIVs have mortality rates over 30% in humans [182]. Hence, the World Organization for Animal Health (OIE) makes mandatory to notify any HPAIVs and H5/H7 LPAIVs. The H5/H7 LPAIVs need to be reported due to their capability to acquire mutations leading to HPAIVs [183]. AIVs are one of the OIE priority topics; OIE in cooperation with the WHO and the Food and Agriculture Organization (FAO) exchange follow-up information of zoonotic AIVs. When any noticeable outbreak occurs, strict control strategies described in the *OIE Terrestrial Animal Health Code* must be implemented to eradicate disease. Disinfection, isolation

and culling are the main measures practiced to prevent its spread. Overall, AIVs cause enormous losses due to death and slaughtering of infected birds as part of control policies.

SIVs have also an enormous financial impact on the pork industry. A total of 100% of morbidity is reported, though mortality is rather low (1-4%) [139,140]. Losses are coming mainly through retarded growth of infected pigs leading to an increase in their finishing time [184]. A greater predisposition of pigs to secondary bacterial infections is also affecting the economy [140,184]. Another impact is the possibility of participating in the generation of a mutant/reassortant isolate that may spread between people initiating a pandemic as observed in the 2009 pandemic (See *1.3.2.*; "*Influenza A virus pandemics*"). During the pandemic, false perceptions on the safety of pork meat cause to the United States (US) pork industry estimated losses over $ 1 billion [185].

1.3.1. Epidemics

Evolution of seasonal IVs due to novel antigenic variants results in recurrent annual epidemics of disease. Seasonal influenza virus burden is associated with IAVs from subtypes H1N1, H3N2 and two IBVs of two distinct lineages (B/Victoria and B/Yamagata). Recurring seasonal influenza epidemics, that may last for 6-12 weeks, have infection rates of 10-30% in adults and 20-50% in children [186].

1.3.2. Influenza A virus pandemics

Pandemic IAVs were generated by antigenic shift due to the segmented genome of IVs.

I. **1918 Spanish influenza (H1N1):** In 1918-1919, the first IV pandemic is associated with the major number of deaths worldwide (50-100 million) [187]. The causative agent of this pandemic was characterized as H1N1 from an avian-origin virus [188,189]. Its reconstruction by means of reverse genetics allowed unraveling mechanisms of its high virulence phenotype and host susceptibility [190]. HA and PB2 were defined as key determinants in transmission of this pandemic H1N1 IV [191].

II. **1957 Asian flu (H2N2):** In 1957, a novel IAV emerged within humans in southern China that was associated with one million fatal outcomes [192]. It was characterized to be a reassortant of the 1918/H1N1 and an avian IAV virus, since it harbored H2, N2 and PB1 segments from an avian-derived virus [193–195].

III. **1968 Hong Kong flu (H3N2):** In 1968, a reassortant of the H2N2 virus with another avian IV emerged in Hong Kong. The 1968 pandemic virus carried H3 instead of H2 and a PB1 segment from avian origin [193,195]. One million of fatalities were reported during the pandemics [84].

IV. **1977 Russian influenza (H1N1):** An H1N1 IV re-emerged in China causing a pandemic in 1977, leading to severe disease and fatalities primarily in the young population (greater than 50%) [84,147]. High similarity was encountered within the

re-emerged virus and the H1N1 from 1950s [196]. The re-emergence was presumably caused by a laboratory stock accidental release.

V. **2009 H1N1pdm09**: Last reported flu pandemic was in 2009, with more than 18,000 deaths associated with the infection [197]. Triple reassortants H1N2 and H3N2 first arose in the late 1990s harboring:

i. the PB2/PA segments of North-American avian IVs,
ii. the PB1 segment from a human H3N2 IVs,
iii. the HA/NP/NS segments from human H1N1 IVs.

The triple assortants of H1N2and/or H3N2 reassorted with a Eurasian H1N1 avian-like swine virus (segments NA/M), finally resulting in the emergence of H1N1pdm09 [198,199] (*Figure 1-4*). Prior to its human spread, H1N1pdm09 seemed to be circulating asymptomatically in the swine population [200]. Likewise, the Russian flu, H1N1pdm09 caused a severe disease with a high incidence in the younger population, pregnant women and population with chronic diseases. The youngers were also more affected, assumedly linked to the lack of cross-reactive antibodies to the H1N1-typed virus circulating between 1918 and 1943 [201].

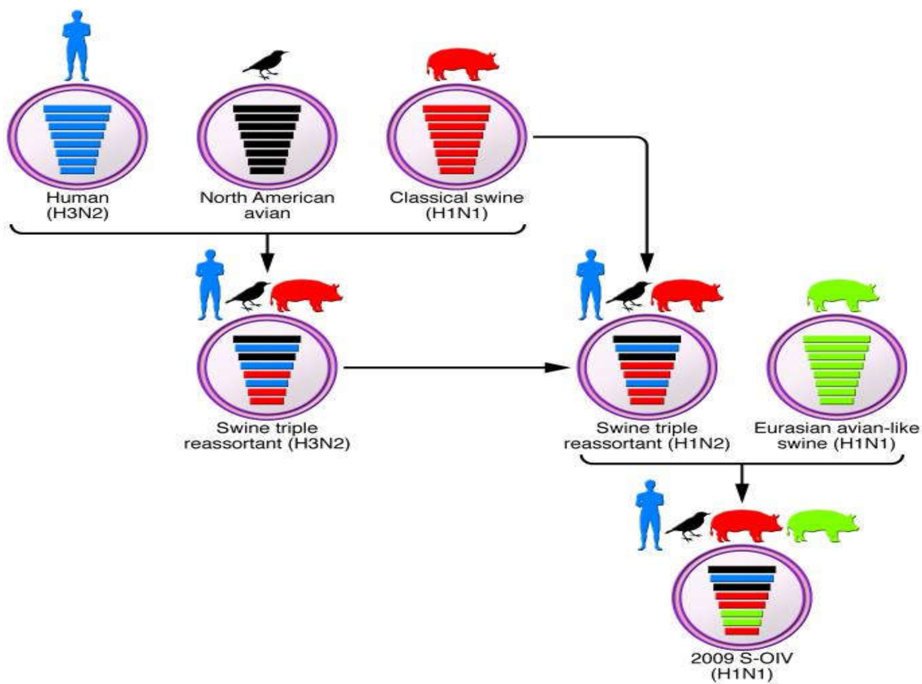

Figure 1-4. Origin of H1N1pdm09. Gene segments of H1N1pdm09 are colored depending on their host origin: avian, human or swine. Image taken from [147].

1.4. Immune responses to IAVs infection

Understanding the host immunity response to IAVs infection is crucial to interfere with the severity of the disease and to design vaccine candidates. The host immune response to IAVs infection triggers both the innate and the adaptive immune responses. Innate response is quicker and comprises a number of factors that can interfere with IAVs infection and viral replication. However, adaptive immunity owns antigen specificity and memory, being effective to combat recurrent IAV infections. Nevertheless, in many cases IAVs still can still evade host immunity by distinct mechanisms and establish a proper infection.

1.4.1. Innate immune response to IAVs infection

Albeit nonspecific, innate immunity serves to initiate the first line of host defense against IV and triggers proinflammatory responses. Several components like physical barriers (mucus and collectins), host pathogen recognition receptors (PRRs) compounds and distinct host cells (target epithelial cells and immune effector cells) consolidate the host innate immunity against IAVs.

Whilst mucus and collectins aim to prevent infection of the IAVs to target cells, several PRRs can be induced upon IAVs infection implying subsequent expression of type I and III IFNs (*Figure 1-5*).

PRRs are receptors that distinguish non-self conserved structures, referred to as pathogen associated molecular patterns (PAMPs), in the IAV infected cells. To date, the PRRs described to sense IAVs include the RIG-I, the melanoma differentiation associated gene 5 (MDA-5), the toll-like receptors (TLR3, TLR7 and TLR8) and the NLRs, namely the NRLRP3 and NRLRP5.

RIG-I and MDA-5 are cytosolic receptors which can identify viral single stranded RNAs and transcriptional IAV intermediates in IAV infected cells. Upon the recognition, both of them associate with MAVS. This interaction triggers a downstream cascade signaling at the outer mitochondrial membrane [202], which ends up with the activation of transcriptional factors such as IRF3, IFR7 and NF-κB [203,204]. Subsequently, IRF3 and IRF7 promote the production of type I and III IFNs and NF-κB, the expression of pro-inflammatory cytokines such as IL-6, tumor necrosis alpha (TNF-α) and IL-1β.

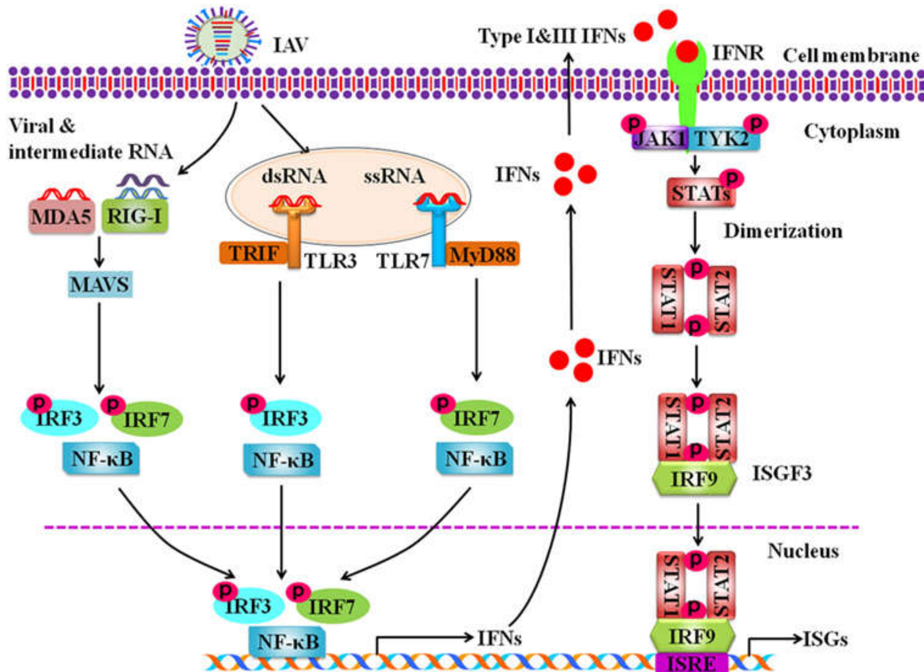

Figure 1-5. Representation of the induced host PRRs of the innate immune response orchestrated upon an IAV infection and subsequent expression of type I and III IFNs. Image taken from [205].

Toll-like receptors, are PRRs that recognize PAMPs at cell surface or internally at endosomes or lysosomes [206]. In particular, TLR3, TLR7 and TLR8: can sense IAV compounds during virus replication. All of the three are localized at the surface of the endosomes and lysosomes. While TLR3 recognizes dsRNA structures in endosomes [207] or other unidentified RNA structures in macrophages and DCs [208], TLR7 recognizes ssRNAs exposed by viral capsid degradation in the acidified endosomes in plasmacytoid dendritic cells (pDCs) [209]. Upon recognition, both TLR3 and TLR7 mediate a downstream signaling that activates either IRF3, IRF7 and NF-κB; leading to the production of type I IFN and pro-inflammatory cytokines, as RIG-I and MDA-5 pathways

[209]. On the other hand, little is known about the role of TLR8 in sensing IAVs, except their capability in human to recognize ssRNA and their subsequent IL-12 production by monocytes and macrophages [210].

As described, triggering PRRs leads to type I IFNs (IFN-α/β) and type III IFNs (IFN-λ) production [211]. Type I IFNs interact with IFN receptor (IFNAR) and activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling. This reaction ends up in an antiviral state in the cell caused by the expression of numerous IFN-stimulated genes (ISGs) that target different steps of IAVs cycle [212]. Type III IFN (IFN-λ) activates the same cascade but is mainly produced by pDCs [213] and interacts with IFNL receptors in the lung. Thus, IFN-λ controls locally the IAVs infection in the lung [205,214].

The airway epithelial cells upon being infected by IAVs can release antiviral or chemotactic molecules (such as TNF-α and IL-1), which can lead to the recruitment/migration of the innate effector cells to the infection site. These immune effector cells can play quite diverse roles to clear the virus.

Monocytes and alveolar macrophages release pro-inflammatory cytokines such as TNF-α and IL-6 but also can limit the spread of IAVs by opsono-phagocytosing IAV particles and/or phagocytosing apoptotic infected cells [215,216]. In contrast, natural killer (NK) cells, cytotoxic lymphocytes, can mediate target and lysis of IAV-infected cells [217,218].

In addition, upon an IAV infection, dendritic cells (DCs), can acquire viral antigens and degrade them into small peptides by means of proteasomes present in the cytosol. Then, they present them to naïve

and memory T lymphocytes. Depending on how the antigen uptake takes place, two mechanisms can be triggered. When DCs present the virus-derived antigens by major histocompatibility complex (MHC) class I molecules, this results in $CD4^+$ T helper (Th) cells activation. On contrary, when the molecules used are MHC-class II, the viral antigens are recognized by specific $CD8^+$cytotoxic T cells (CTLs).

1.4.2. Adaptive immune responseto IAVs infection

Adaptive immune responses are specific to pathogen and consists humoral (virus-specific antibodies) and cellular (T cells) immunity.

1.4.2.1. Humoral immunity to IAVs

Infection of IAVs leads to the production of virus antigen-specific antibodies. In particular, antigens expressed on the surface of the virus like HA, NA and M2 are targets of humoral immune mechanism.

IAVs infection induces production of HA-specific antibodies that are directed to the globular and stem part of the HA. As mentioned in section *1.2.3.*, HA protein is important and crucial in initiating the IAVs infection cycle. Antibodies produced against the HA-globular part can bind to the HA receptor-binding site (RBS) and prevent the IAVs infection (by blocking the RBS). Besides, HA specific antibodies also facilitate antibody-dependent cellular cytotoxicity (ADCC). Nevertheless, these antibodies are ineffective when IAVs acquire mutations in their head region [219].

The antibodies induced against HA stem part can be neutralizing the IAVs and/or non-neutralizing (e.g. ADCC antibodies) [220]. Owing to the highly conserved epitopes consolidating the HA-stem part and because they are not under immense immune pressure [221], HA-stem epitopes are suggested as promising universal vaccine candidates (*See 1.7.; "Universal vaccines"*). Conserved regions of the HA-stem part however, bear particular features which divide them into two phylogenetically different groups: Group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 subtypes) and Group 2 (H3, H4, H7, H10, H14 and H15), which afford a non-equivalent protective efficacy. Besides, their immune-subdominance is another of their drawbacks since HA-globular head masks the stem of the IAV virion [222].

Published reports also suggest that specific antibodies against virus surface protein NA facilitate ADCC and prevent virus spread since they inhibit the NA enzymatic activity [223,224]. In addition, M2 and NP specific antibodies can also be produced and, although non-neutralizing, may promote protection against distinct IAVs [225–227] eliminating the virus by activating complement cascade ending up with cytolysis [228].

IAVs target the respiratory epithelial cells encountered in the mucosal tissues of the respiratory tract. To limit IAVs infection, presence of IgA, IgM and IgG antibodies in the mucosa is fundamental. Mucosal or secretory IgAs are locally produced and can afford local protection of airway epithelial by neutralizing HA and NA viral proteins [229,230].

A high IgM level, hallmark of primary infection, it is associated with a quicker clearance of IAVs. Likewise, it is also correlated with an elevated IgG antibody titer thatis dominant during secondary responses occurring

in the periphery [231–233] subjected to the interplay between innate early IgM response and subsequent IgG production [234]. Although some of those antibodies are short-living others can even last for a lifetime being produced by Ab-secreting cells (ASCs).

1.4.2.2. Adaptive cellular immunity to IAVs

IAVs infection activates both the helper $CD4^+$ and cytotoxic $CD8^+$ T lymphocytes.

I. Helper $CD4^+$T cells

Naïve helper $CD4^+$ T cells differentiate into Th1 or Th2 phenotypes after activation [235–237]. Th1 or Th2 cell types depends on their stimulators including antigen, co-stimulatory molecules, cytokines secreted by DCs, epithelial cells, and inflammatory cells [238–240]. Th1 cell subset produce mainly IFN-γ and IL-2 and are involved in cell mediated immunity and may further promote the differentiation of $CD8^+$T cells into CTLs [241,242]. On the other hand, Th2 type cells release IL-4 and IL-13, which predominant role is stimulating B cell responses [233,243] that significantly leads to class-switching of antibody, affinity maturation and generation of long-lived plasma cells.

II. Cytotoxic $CD8^+$ T cells

Recognition of IAV epitopes with MHC class I molecules on antigen-presenting-cells (APCs), promote the differentiation of naïve $CD8^+$ T cells, draining in the lymph nodes into cytotoxic cells [232,233,244].

Immunomodulators like type I IFNs, IFN-γ, IL-2 and IL-12 are described to help $CD8^+$ T cell differentiation process [245,246]. When activated upon detecting IAV infected cells, $CD8^+$ T cells migrate to the infection site and kill infected cells by secreting cytotoxic granules containing perforin and granzymes (e.g. GrA and GrB) [247,248]. These specific CTL cells can be reactivated upon secondary IAVs infection and it has been revealed that their specificity is mainly directed to conserved IAV epitopes. Hence, their response is characterized for being of a heterosubtypic nature [249].

III. Th17, T regs and T follicular helper (Tfh) cells

Other subsets that can be activated against IAVs are the Th17 and the T regs, whose role is to control/regulate the cellular immune response [232]. Th17 cells promote T helper responses by secreting IL-6. This cytokine hampers the T regs to suppress the T helper responses [250,251]. The Th17 cytokine, IL-17, has a controversial role in terms of being or not being indispensable in an effective influenza vaccination [252,253]. Albeit it has been reported a role in recruiting B cells to the pulmonary infection site upon H5N1 infection [254].

T regs contribute in controlling lung inflammation during an IAV infection by dampening both exaggerated innate and adaptive immune responses against IAVs [255,256]. Furthermore, it is described that T regs release IL-35 that is upregulated during secondary infections and suppresses the inflammatory responses [257].

Moreover, in terms of effector activity (humoral memory and vaccination), other subset named as T follicular helper (Tfh) cells are an area of extreme interest [258]. Tfh are believed to carry out effector $CD4^+$ T cell Th2 responses (antibody switch, affinity maturation and long lived plasma cell generation) in secondary lymphoid tissues [259].

1.4.3. Immune response in swine

Immunity to SIVs infection in pigs is relatively less studied in comparison to humans and rodents. Pig innate immune system shares many features with that of humans, albeit there is alarge contribution of the γδ-T-lymphocytes [260]. These cells can contribute to kill some IAV-infected cells [261] and can be activated by either the PRRs and/or cytokines receptors (innate mechanisms) [262] or via T cell receptor (TCR) (adaptive mechanisms) [263].

Adaptive immune response in pigs against SIVs, in particular the humoral responses, are similar to mice [264]. IgG and IgA are the main isotypes elicited against SIVs infection and are mainly involved in protection. Furthermore, maternal derived antibodies (MDA) are essential to protect piglets in the first weeks of live [265].

Cell-mediated immunity (CMI) to SIVs helps reducing the viral titer in lungs similar to humans [266]. Increased numbers of $CD4^+$ and $CD4^+CD8^+$ cells in bronchoalveolar lavage fluid (BALF) during SIVs infection in pigs suggest an important role of this cell population during infection [267]. Indeed, at least some of these double positive cells subset

may be effector memory T cells (T_{EM}) [268]. Similar as in humans, the increase in the numbers of $CD8^{+}$T cells migrating to lungs and BALF peak at 7 days pots infection [269].

1.4.4. Immune response in avian species

Avian species differ in many aspects of immunity from mammals, in particular regarding organs and cells, functional mechanisms, genes and secreted molecules. One example is their primary lymphoid organs. T-cell development usually occurs in the thymus like the mammals, while B lymphopoiesis develops in a unique organ existing in birds, the bursa of Fabricius. Avian species also lack eosinophils and instead of neutrophils they carry heterophils. Furthermore, birds lack organized lymph nodes [270], although not all of them [271].

Other secondary organs involved in the immunity of birds are: the spleen, bone marrow, Harderian gland and lymphoid tissues such as bronchial-associated lymphoid tissue (BALT), conjunctiva-associated lymphoid tissue (CALT) and gut-associated lymphoid tissue (GALT). GALT also involve the tonsils and the Peyer's Patches in the intestines.

In chicken, B cells when differentiated to plasma cells secrete IgM, IgA and IgY antibodies [272,273]. The same antibodies are present in other birds such as pheasants, turkeys, quails [274] and ducks [275,276]. While IgM and IgA share features from mammalian counterparts, IgY equivalent with IgG in mammals, differs in the number of C_H domains of the heavy chains [273,277]. Moreover, in the avian Harderian gland

IgA-typed antibodies predominate. No equivalents of IgD or IgE are described in any avian specie [277].

Immunity in avian species consists of both the innate and adaptive immune responses. Innate immunity includes TLRs, NOD-receptors and RIG-I/MDA5 pathways. Albeit it seems RIG-I to be inexistent in the chicken specie [278]. Reports indicate that adaptive immunity in avian species involves the paradigm of $CD4^{+}$ helper T cells with its two subsets and the $CD8^{+}$ response is also described [279].

1.4.5. Immune correlates of protection for influenza

The European Committee for Medicinal products for Human Use (CHMP) is in charge of licensing human medicinal products, including vaccines. The criteria and correlates of protection followed when evaluating IV vaccines are described in *Table 1-1*. While seasonal vaccines need to accomplish one or more criteria, pandemic vaccines need to meet all three criteria.

As mentioned in the table below, an influenza vaccine is considered protective when the hemagglutination inhibition (HI) titer is ≥ 40. In conditions of pre-exposure to the virus, geometric mean titers (GMT) and seroconversion parameters are more representative to better distinguish the response mediated by the vaccine.

Nonetheless, the appropriateness of this protection correlate has been widely questioned [280–282]. Principally due to its low sensitivity to avian and IBV strains [283,284] but also since these established

parameters only target healthy adults, not including population at risk such as elderly, children and immunocompromised population [285–287].

For instance, some studies suggest CMI as correlate of protection in the elderly population while other demand higher HI titers, particularly in children, to achieve protection.

Alternative assays are required to overcome such limitations but also to assess other immune markers such as anti-NA, anti-M2, anti-NP, $CD4^+$ and $CD8^+$ T cells and mucosal response that can be included in the next generation vaccines as immune correlates.

Table 1-1. European CHMP criteria for evaluation of influenza vaccine immunogenicity (copied from [288]**).**

	Adults	Older adults (>60 years)
GMT increase	2-5	2
Seroconversion/significant increase*	70%	30%
Seroprotection*	40%	60%

Abbreviations: GMT: Geometric mean titer. *In HI tests, seroconversion corresponds to: negative pre-vaccination serum (HI < 1:10), post-vaccination serum HI ≥ 1:40; pre-vaccination serum >1:10, significant increase: at least a four-fold increase in titer. Seroprotection corresponds to the percentage with serum HI ≥ 1:40.

Swine influenza vaccines: According to the OIE guidelines (Chapter 3.8.7.;-Influenza A virus of swine, OIE) [289], vaccines against swine

influenza must induce protection against both the subtypes H1 and H3. Moreover, statistically significant reduction of the virus in the respiratory tract (both in terms of titers and duration of shedding) and minimizing the clinical signs and lung lesions in the vaccinated pigs are the criteria of efficacy in a vaccine against SIVs. These vaccines need to be pure, safe and potent.

Avian influenza vaccines: Survival and a reduction of the viral shedding are the efficacy requirements that need to be demonstrated for vaccine licensing against AIVs, according to OIE guidelines (Chapter 3.3.4.; Avian influenza (infection with avian influenza viruses, OIE) [139]. A minimum of 80% of the vaccinated and challenged birds (with a dose of 10^6) need to survive and must reduce stastistically both the titer and the number of birds shedding the virus from oropharynx and/or cloaca.

In both AIVs and SIVs the vaccine efficacy focuses on reducing viral shedding, because this way the transmission is also reduced and, at the same time, the risk of zoonosis to humans. Survival, clinical signs and lung lesions will reduce the economic impact of the disease and will contribute to animal welfare.

1.5. Vaccines

Prophylactic vaccines are a forefront method to prevent or, in the most optimal cases, to eradicate, various types of infectious diseases [290]. For influenza, vaccination is the best primary method of infection prevention and control and, if effective, they contribute to mitigate the harm and to combat the huge economical losses caused by the virus at both human and animal health stages.

1.5.1. Current vaccines in humans

Two types of vaccines have been developed to protect individuals against IVs:

i. Seasonal influenza vaccines

In 1945, the first inactivated seasonal vaccine against IVs was approved [291]. Since then, several types of vaccines have been licensed. *Table 1-2* summarizes the licensed influenza vaccines in Europe for the 2017-2018 season. Overall, influenza vaccines are divided into trivalent influenza vaccines (TIVs) or quadrivalent influenza vaccines (QIVs). Whilst, TIVs target two IAV strains (subtypes H1N1 and H3N2) and one IBV strain, tetravalent vaccines target also an additional B strain (containing Victoria and Yamagata lineages). As seen in *Table 1-2*, inactivated-type vaccines predominated (inactivated whole, inactivated split or subunit split vaccines). However, one live attenuated influenza vaccine (LAIV) has also been licensed. Split inactivated vaccines consist on ether and/or detergent disrupted IVs while subunit split vaccines only harbor purified and enriched HA and NA viral components. These vaccines are generally produced using egg-based or cell-based technology [292].

The strain vaccine composition is adapted, manufactured and delivered annually prior to the flu season. They must comply with the recommendations of the continuous surveillance by WHO Global Influenza Surveillance and Response System (GISRS).

Moreover, several measures have been implicated to improve the efficiency of these seasonal vaccines, like increasing antigen dose [293,294], intradermal administration route [295–300] and including adjuvants such as MF59® [301,302] and AS03 [303] adapted for high risk population.

Lately, another type of vaccine has been licensed in the United States (US), FluBlock®, produced in a baculovirus expression system. The first licensed human influenza vaccine employing recombinant DNA technology [304,305].

ii. Pandemic mock-up vaccines

For pandemic preparedness, the WHO coordinates the development of influenza candidate vaccine viruses (CVVs). Upon a pandemic IAV emergence, the particular CVV could be rapidly licensed speeding up the regulatory approval process. Particularly, developing vaccines against HPAIVs H5N1 and H7N9 subtypes are in focus. In addition, CVVs against H9N2 and virus variants of H1 and H3N2 subtypes are also being tailored [306].

Table1-2: Vaccines licensed and commercialized in Europe for the last season 2017/2018.

Product name	Vaccine type	Adjuvant	Administration route	Produced in egg/cell	Age recommended	Available for use in the following countries
Trivalent vaccines						
Influvac®	Inact/sub unit	None	IM/Sc	Egg	> 6 months	All EU/EEA countries
Imuvac®	Inac/subu nitt	None	IM/Sc	Egg	> 6 months	UK
Fluarix Alpahrix Influsplit	Inact/split	None	IM/Sc	Egg	> 6 months	All EU/EEA countries
Agrippal	Inact/sub unit	None	IM	Egg	> 6 months	All EU/EEA countries
Fluad	Inact/sub unit	Squalene (MF59)	IM	Egg	> 65 years	Austria, Germany, Italy, Spain, UK
Fluval AB	Inact	Aluminum phospahte gel (AS03)	IM	Cell	> 6 months	Hungary
Afluria* Enzira**	Inact	None	IM	Egg	> 5 years	Belgium, Czech republic, Denmark, Finland, Germany, Greece, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Romania, Spain, Sweden, United Kingdom
Vaxigrip**	Inact	None	IM/Sc	Egg	> 6 months	All EU/EEA countries
Istivac	Inact	None	IM/Sc	Egg	> 6 months	Portugal/Spain
Mutagrip	Inact	None	IM/Sc	Egg	> 6 months	Italy/Spain
Intanza	Inact	None	Id	Egg	> 18 years	Austria/UK
Quadrivalent vaccines						
Vaxigrip Tetra	Inact	None	IM/Sc	Egg	> 6 months	Austria, Belgium, Bulgaria, Croatia, Czech republic, Estonia, Finland, Germany, Greece, Hungary, Italy, Ireland, Latvia, Lithuania, Malta, Poland, Romania, Slovenia, Sweden, UK
Fluarix Tetra**	Inact/Spli t	None	IM	Egg	> 6 months	Belgium, France, Germany, Italy, Spain, Switzerland, UK
Fluenz Tetra/ Flumist Quadrivalent *	LAIV	None	In	Egg	> 24 months to 17 years	Austria, Finland, Germany, Norway, Sweden, UK

Abbreviations: Inact: Inactivated; IM: intramuscular; Sc: subcutaneous; Id: intradermal; In: intranasal; EU: European Union; EEA: European Economic Area

1.5.2. Current vaccines in swine

Current vaccines against SIVs are whole or split inactivated, non-replicating alphavirus RNA particles, LAIV or autogenous. The commercial SIVs vaccines more commonly used in Europe and US are depicted in *Table 1-3*. They all contain (whole, split or HA) from H1 and H3 IAV subtypes. In US, novel vaccines harbor 4-5 strains in response to the antigenically distinct clusters circulating in herds. All inactivated-type SIVs vaccines are propagated in egg-based or cell-based technologies and formulated with oil-in-water adjuvants. A viral vector vaccine consisting alphavirus-like replicon particles (RP) (Swine Influenza Vaccine, RNA; Harrisvaccines, Ames, IA, USA), in which replication is restricted since it does not contain all the genes for packaging, is also currently on market in US [307]. Additionally, an attenuated-type vaccine was also authorized recently against SIVs by United States Department of Agriculture (USDA).

Despite the predominance of the inactivated-typed vaccines, they are limited to confer protection against different IV subtypes (heterovariant or heterosubtypic viruses) and currently ongoing research focuses to overcome this inconvenience. For instance, the development of autogenous/custom inactivated vaccines by individual farms is allowed in US and Canada. However, such vaccines are only used when the circulating strain in the herd does not match with commercial vaccines. MDA are another obstacle for the inactivated vaccine, since they may interfere with the vaccine response to post-weaning vaccination.

Table 1-3. Current commercial vaccines against SIVs licensed in the US and Europe.

Manufacturer	Commercial vaccine	Strains (Subtype)	Formulation	Adjuvant	Where
IDT Biologika GmbH	Respiporc Flu 3	Bakum/IDT1769/2003 (H3N2) Bakum/1832/2000 (H1N2) Haselünne/IDT2617/2003 (H1N1)	Whole/Inactivated	Carbomer 971 P NF	Europe
IDT Biologika GmbH	RESPIPORC FLUpan H1N1	A/Jena/VI5258/2009(H1N1)pdm09	Whole/Inactivated	Carbomer 971 P NF	
Zoetis	FluSure XP®	A/Sw/Oklahoma/0726H/2008 (H1N2): cluster delta 1- H1N2 A/Sw/Iowa/110600/2000 (H1N1): cluster gamma H1N1 A/Sw/North Carolina/394/2012 (H3N2): cluster IV-A H3N2 A/Sw/Minnesota/872/2012 (H3N2): cluster IV-B H3N2	Whole/Inactivated	Amphigen®	US
Zoetis	FluSure® Pandemic	A/California/04/2009 (H1N1): pandemic H1N1	Whole/Inactivated	Amphigen®	
Harrisvaccines	Swine Influenza Vaccine, RNA	Cluster IV H3N2	Whole/Inactivated	None	
Boehringer Ingelheim	Ingelvac Provenza™	α-cluster H1 Cluster IV H3N2 A/swine/Nebraska/97901-10/2008	LAIV	None	

Abbreviations: LAIV: Live attenuated influenza vaccine; US: United States.

Progress towards new more effective inactivated vaccines is rather slow. Currently, LAIV and alphavirus vaccine, despite their safety concerns, are commercially available in some parts of the world and induce cellular/mucosal immunity and reduce viral shedding [308–310].

1.5.3. Current influenza vaccine in avian

Until the 1990s, the stamping out policy (euthanasia of infected and contact flocks) was the main countermeasure to control and eradicate HPAIV outbreaks in poultry. The widespread of HPAIVs outbreaks and their endemic situation in several countries (China, Hong Kong, Indonesia and Vietnam) made vaccination a common control measure [311]. For endemic LPAIV of H5N2 and H9N2 [312,313], vaccines are also utilized as a control strategy. These vaccines are tailored as 'autogenous' vaccines containing the strain detected in the field. For AIVs, distinct vaccine types are licensed and can be categorized as;

i. Inactivated AIV vaccines

Inactivated AIV vaccines are adjuvanted, parentally injected and grown in embryonated chicken eggs. Protection in poultry is achieved by inducing solid systemic humoral response when matched to the challenge strain. However, biosafety level 3 (BSL-3) facilities are required to develop HPAIVs vaccines. Due to the fact that HPAIVs mutate from LPAIVs, matching LPAIV strains are searched to be manufactured. Another approach is the reverse genetics (RG) technology. RG alters the HA cleavage site and inserts the *HA* in a vaccine virus backbone, permitting their production in BSL2-facilities [314]. Some RG vaccines have been licensed in China, Egypt, US and Mexico [315,316].

Nonetheless, differentiating infected from vaccinated animals (DIVA) strategy is an inconvenience when using this vaccination type.

ii. Subunit vaccines

***In vitro* expressed HA protein:** Large quantities of HA can be expressed in an *in vitro* system and can be used for vaccines either as crude extract or purified HA. This approach is more convenient since it does not require safety concerns and yet provides comparable immune response to IAVs like the inactivated vaccines. For example,*Volvac® B.E.S.T. AI + ND.*A vaccine produced in baculovirus expression system technology (BEST) against H5 subtype has been recently licensed in Egypt [317].

***In vivo* expressed HA (NA also in some cases) protein:** Live administered viral vectors with inserts of the *HA* gene as either cDNA or RNA are used as AIVs vaccines. Examples of such vaccines are fowlpoxvirus [315,318–320], Newcastle disease virus (NDV) [315,321,322], herpesvirus of turkeys (HVT) [323], alphavirus [324] and duck enteritis virus (DEV) [324] vaccines. Viral vectors generally induce cellular immunity promoting protection but with low HI titers. Alphaviruses, apart from inducing humoral immune response, also induce cellular immune responses [325,326]. Likewise, subunit vaccines also allow the use of the DIVA concept, which is not generally possible when conventional inactivated vaccines are used. Depending on the viral vector type, the vaccines can be host-specific [327] or can be used in a mass-vaccination approach by water or aerosol application [324]. However, the use of these vaccines is more restricted since their production costs are more elevated than the ones of the non replicating vaccines [324].

1.6. Universal vaccines

Developing a universal flu vaccine inducing a broad and a long-lasting immune response, potentially defeating virus antigenic variation has been widely pursued by vaccine virologists. The vaccine targets for universal IV vaccines are conserved sites on influenza proteins [328]. The most common targets include the type of epitopes summarized here:

**Epitopes from the HA globular head*: They inhibit hemagglutination although they are susceptible to antigenic variations. Conserved epitopes close to the RBS are investigated [329–331]. Other approaches are the search of antibodies which could mimic the conserved residues within the HA RBS [332–336].

**Epitopes from the HA-stem part:* Due to their immune-subdominant property (See *1.4.2.1.*; "*Humoral immunity to IAVs*"), headless HA proteins have been constructed [337]. However, not all the stem could be introduced into the construct. Thus, serially immunizing with chimeric HA molecules with distinct globular parts have been engineered [338]. This way, each time, a primary response will be induced by the head domain whilst the stem part will be boosted [339].

**Epitopes from the M2:* M2 has an extracellular domain highly conserved among human IAVs [340,341]. However, this domain is weakly immunogenic. Exploring new avenues to enhance its immunity, some candidates already used in clinical trials have been developed (M2e-HBc (hepatitis B virus core) fusion protein, M2e-flagellin fusion vaccine) [342–344].

**Epitopes from NP and M1:* As internal proteins, they induce T-cell responses and, thus, facilitating cross-reactive protection. An approach consisting in Vaccinia virus Ankara (MVA) expressing NP and M1 is undergoing clinical phases [345,346].

**Epitopes from NA:* conserved epitopes near their enzymatic site could be of great interest [347]. Moreover, a potential universal NA-inhibiting antibody has been determined [348].

Moreover, as aforementioned, innovative delivery systems are widely tested with those conserved sequences:

- Adjuvants: to elicit robust immune responses either by triggering innate immunity or by facilitating antigen delivery. Nonetheless, they use to require of extensive safety measures.
- Fusing the peptides with carrier proteins: For effective presentation of antigens and enhancing their immunogenicity. Example: M2e fused with bacterial flagellin [343,344].
- Expressed in viral vectors: this strategy mimics the viral infection inducing the cellular immune response as well [349,350].
- Virus like particles (VLPs): VLPs can present multimeric peptide antigens, as being explored for the M2e domain [351–353].
- Phage-based nanoparticles: Easy production at large scale, low cost and immunogenic [354–356].
- Prime/boost with DNA prime and recombinant adenovirus or protein vaccine boost [357].

1.7. Informational Spectrum Methodology (ISM)

The emergence of the bioinformatics, *in silico* tools have been a catalyst in drug and peptide-based vaccine designing [358]. This is because a computational virtual screening is more effective in terms of time and cost in comparison to experimental conventional screening [359–361]. Specifically, the informational spectrum methodology (ISM) is an example of one of these *in silico* tools and encompasses three stages. The first step is the transformation of the amino acid (aa) sequences into numerical sequences. This is possible because an electron-ion interaction potential (EIIP) is assigned to each aa. By using the discrete Fourier transformation (a mathematical approach), the numerical sequence can be transformed into a frequency domain; this creates an informational spectrum (IS). When finding common informational characteristics of sequences, these are determined by cross-spectrum or consensus informational spectrum (CIS). In the last step, the antigens which sharing a common frequency components in their IS are considered immunologically cross-reactive [362,363].

Some studies have used this *in silico* approach to identify important informational characteristics of protein-protein interactions, protein-DNA interactions and structure/function of proteins [363]. Moreover, ISM has also promoted the *de novo* design of biologically active peptides [364] as well as assessment of the possible effects created by mutations/substitutions of aa [365] and predict novel interactions [366]. A plausible example is the well-characterized information of the conserved glycoprotein gp120 regions of the human immunodeficiency virus type 1 (HIV-1), revealed by means of ISM, prompting the

interaction between CD4, CCR5 and CXCR4 receptors [366–368].

Our group has used several ISM predicted epitopes supposed to be immunogenic and conserved as potential vaccines against IAVs. One of them (NF-34) elicited a strong cellular response, with a notable cross-reactive effect with different IAV subtypes [331]. In the present PhD thesis, all three studies employed predicted ISM sequences.

CHAPTER II

Hypothesis and Objectives

2. Hypothesis and objectives

2.1. Hypothesis:

One of the surface proteins of IVs, the hemagglutinin (HA), plays a key function in recognition and entry of the viral genome into the target host cells. Similarly, targeting HA is highly desired in achieving protection against IAVs infection. Conserved HA peptides (HA-epitopes) capable of eliciting antibodies that inhibit virus attachment to target cells are attractive immunogens to include in vaccine formulations against influenza virus infection. Using ISM technology, potential antigenic epitopes from HA, that share common immunogenically cross-reactive informational characteristics among different IAV subtypes, were selected and used as subunit vaccine against IAV infection. Subunit vaccines nonetheless, are often poor immunogens that require a potent adjuvant to be more efficacious. Bacterial components like flagellin as well as some costimulatory molecules, for example CTLA4, on the other hand, are powerful tools that can modulate both the innate and/or adaptive immune response and may serve as effective adjuvants. Including such adjuvants along with conserved HA epitopes in vaccine formulations may provide a broad and efficacious cross-protective immune response against heterovariant/heterosubtypic influenza virus infections.

2.2. General and specific objectives:

The general objective of the present thesis is to improve IAV vaccine efficacy. To this end, various vaccine formulations, based on *in silico*

predicted HA-epitopes, combining with different adjuvants co-administered either in plasmid or soluble form were developed. The vaccine formulations were further evaluated in different animal infection models to validate the efficacy of vaccine to induce effective immune response and protection against IAV infections.

I. To evaluate DNA influenza vaccine, encoding one HA-epitope (NG-34) fused with cytotoxic T lymphocyte-associated antigen (CTLA4), against a heterologous (SwH3N2) IV challenge in pig model. The vaccine efficacy was also evaluated in pigs presenting MDA *(Chapter III).*

II. To evaluate DNA influenza vaccine, encoding a combination of conserved immunogenic HA-epitopes along with flagellin (VC4-flagellin), in SIV-seronegative and SIV-seropositive pigs (*Chapter IV*) against IV challenge.

III. To test the vaccine efficacy of soluble larva extracts expressing flagellin-NG34/CS17 (two HA-epitopes fused with flagellin) against a heterologous (H7N1 HPAIV) IV challenge in chickens *(Chapter V).*

PART II:

Studies

"Somewhere, something incredible

is waiting to be known"

Carl Sagan

CHAPTER III

Study I: Conserved HA-peptide NG-34 formulated in pCMV-CTLA4-Ig reduces viral shedding in pigs after a heterosubtypic influenza virus SwH3N2 challenge.

3. Study I

“Conserved HA-peptide NG34 formulated in pCMV-CTLA4-Ig reduces viral shedding in pigs after a heterosubtypic influenza virus SwH3N2 challenge”

3.1. Abstract

Previously, our group has reported the use of conserved hemagglutinin peptides (HA-peptides) derived from H1-influenza virus as a potential multivalent vaccine candidate. Immunization of swine with these HA-peptides elicited antibodies that recognized and neutralized heterologous influenza viruses *in vitro* and demonstrated strong hemagglutination-inhibiting activity. In the present work, we cloned one HA-peptide (named NG34) into a plasmid fused with CTLA4 which is a molecule that modifies T cell activation and with an adjuvant activity interfering with the adaptive immune response. The resulting plasmid, named pCMV-CTLA4-Ig-NG34, was administered twice to animals employing a needle-free delivery approach. Two studies were carried out to test the efficacy of pCMV-CTLA4-Ig-NG34 as a potential swine influenza vaccine, one in seronegative and another in seropositive pigs against SIV. The second one was aimed to evaluate whether pCMV-CTLA4-Ig-NG34 vaccination would overcome MDA. After immunization, all animals were intranasally challenged with an H3N2 influenza strain. A complete elimination or significant reduction in the viral shedding was observed within the first week after the challenge in the vaccinated animals from both studies. In addition, no challenged heterologous virus load was detected in the airways of vaccinated pigs. Overall, it is suggested that the pCMV-CTLA4-Ig-NG34 vaccine formulation could potentially be used as a multivalent vaccine against influenza viruses.

3.2. Introduction

Influenza-like disease in pigs started occurring in both US and Europe in connection with the human influenza pandemic in 1918. Proof of this is the close relationship between the early H1N1 swine viruses with the human influenza virus of 1918, as determined by genetic analyses [184].

SIVs co-circulating in European swine are from the subtypes H1N1 (SwH1N1), H3N2 (SwH3N2) and H1N2 (SwH1N2) [100,101] that are also equivalent to the more prevalent subtypes in North America [369]. Thus, in line with the circulating subtypes in the swine population [100,101,369], current commercial vaccines available for pigs consist of two or three inactivated SIV strains belonging to the aforementioned subtypes. Vaccines are commonly administered in pregnant sows to stimulate passive antibody transfer via colostrum. Regrettably, protection with these commercial vaccines is only achieved when the strain either closely or completely matches with the challenged virus [8,370–373]. The reported lack of protection against divergent strains is thought to be associated with the poor stimulation of the mucosal and cellular immunity provided by inactivated-typed vaccines [8,374,375]. It is thus fundamental to seek new vaccine strategies eliciting robust immune response and protection against drifted or emerging strains occurring from antigenic drift or antiviral shift, respectively.

The new emergent vaccine formulas must take MDA interference into account since the presence of MDA inhibit/neutralize current vaccines [376,377]. Even though optimal vaccination of animals should begin at the time of disappearance of maternal antibodies, this approach is often unfeasible due to a high degree of variability in antibody titers between individuals [378]. Similarly, inactivated-type vaccines may pose drawbacks in piglets with MDA by suppression of the antibody responses [379–381], and because they may enhance respiratory diseases upon influenza infection [382,383] or cause an aggravated pneumonia called vaccine associated enhanced respiratory disease (VAERD) [384]. Thus, an additional aim of this work was to evaluate the capacity of the vaccine to provide protection against a heterologous influenza virus strain to individuals with MDA.

Our group has previously reported the use of an HA1-based peptide (NF-34) in pigs as potential vaccine [331]. Partial virus clearance after an intranasal challenge with the homologous pH1N1 (pandemic swine-origin A/Catalonia/63/2009 H1N1 IV) influenza virus was observed along with a strong humoral and T-cell response in animals vaccinated with the NF-34 HA-peptide. Although NF-34 HA showed concomitant detection of antibody response it did not totally correlate with neutralizing activity [331]. In the present study, NF-34 peptide has been modified (named NG-34) and employed in a DNA vaccine approach (pCMV-CTLA4-Ig-NG34). In addition, to ameliorate safety and large-scale vaccination approaches, a needle-free intradermal administration technique intra dermal application of liquids (IDAL® device) was applied.

The research objectives were two-fold in two different swine experiments depending on the initial presence/absence of MDA. Firstly, to assess vaccine efficacy against a heterologous SIV isolate in seronegative animals (SwH3N2) and, secondly, to investigate whether farm animals could clear a heterologous virus challenge, thus overcoming MDA obstacles.

In the present work, we demonstrated that immunization of seronegative pigs with pCMV-CTLA4-Ig-NG34 antigen formulation induced neutralizing antibodies that inhibited hemagglutination of a heterologous SIV. Moreover, immunized conventional farm pigs (both the influenza virus seropositive and seronegative) were fully or partially protected against a heterologous influenza virus challenge as they either completely eliminated or significantly reduced virus secretion and cleared the virus from the respiratory airways.

3.3. Materials and methods

3.3.1. Ethics statement

All animal studies presented in this work were approved by IRTA's Ethics Committee for Animal Experimentation and the Animal Experimentation Commission from the Catalonia Government (Spain) in compliance with the Directive, EU 63/2010, the Spanish Legislation (RD 53/2013) and the Catalan Law 5/1995 and Decree 214/1997. Treatment with anesthetics or analgesics was not considered because

animals did not suffer from the disease and/or experimental manipulation.

3.3.2. Experimental design

The results presented in this chapter are representative of two almost identical experimental studies performed in swine, whose outlines are described in *Figure 3-1*. In both studies, animals were observed daily during the course of the experiments by monitoring for flu-like clinical signs and rectal temperature profiles; and the severity of clinical signs was assessed from 0 to 3 according to a previously described scoring [385]. Animals received water and food *ad libitum.*

Clinically healthy pigs were selected and tested for presence of specific antibodies in sera against the influenza NP using the ID Screen® Influenza A Antibody Competition ELISA (ID VET, France) kit. SIV-seronegative animals were selected for study I. For MDA positive pig studies, piglets were obtained from vaccinated sows and were controlled for having NP antibodies by ELISA. Moreover, in both cases, RT-qPCR (see *3.3.7.*; *"Quantitative real time RT-PCR (RT-qPCR)")* was also determined to ensure animals were not exposed to IAVs.

In a first study, 10 five-to-six week-old, influenza A virus seronegative, Yorkshire x Landrace pigs were used. Animals were housed, vaccinated and challenged in the animal BSL-3 facility at CReSA (Barcelona, Spain). Animals were randomly distributed into two groups of 5 animals each (Group A = non-vaccinated pigs, animals 1-5; Group B = vaccinated pigs, animals 6-10). After an acclimatization period of 5 days, five pigs were vaccinated twice with an interval of 3 weeks by injecting the final

plasmid formulation using a needle-free device (IDAL®, MSD Animal Health, Salamanca, Spain), on the dorsal side of the back of each animal as previously described [386].

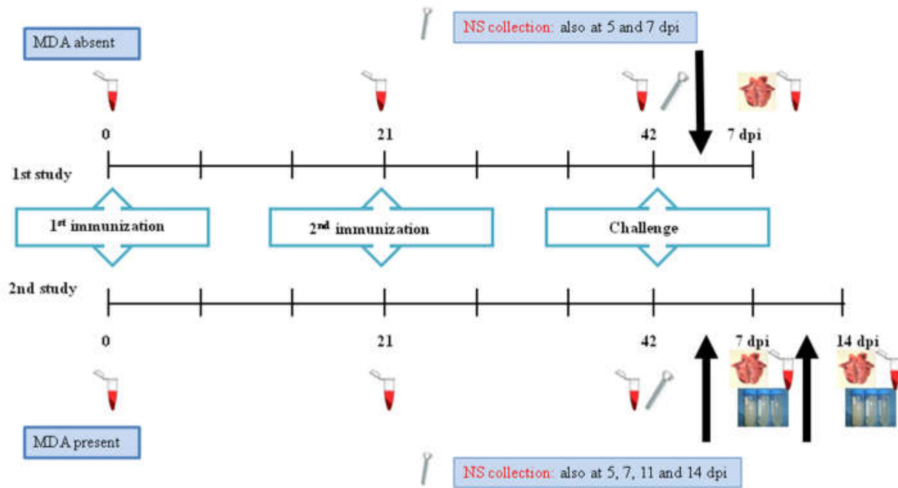

Figure 3-1. Experimental outline of two studies. (A) 1st study outline: seronegative pigs against SIV were vaccinated at 0 and 21 days, and challenged at 42 days. Sera were collected each pre-vaccination, pre-challenge and at necropsy day (7 dpi), as indicated. Lung tissues at 7 dpi. Nasal swabs collection took place at challenge day and at all days indicated in the figure. (B) 2nd study outline: seropositive pigs against SIV, vaccinated at 0 and 21 days, and challenged at 42 days. Sera were collected each pre-vaccination, pre-challenge and necropsy days (7 and 14 dpi). Lung tissues were obtained at 7 and 14 dpi. In this case, BALFs were also collected at 7 and 14 dpi. Nasal swabs collection took place at challenge day and at all days indicated in the figure. Dpi, days post-inoculation; NS, nasal swabs.

The final plasmid formulation used for immunization consisted of 600 µg DNA construct pCMV-CTLA4-Ig-NG34 (applied in 3 shots/vaccination) mixed with Diluvac Forte® from MSD Animal Health (1:1 v/v). The remaining five pigs were sham-vaccinated (control group). Two weeks after the second vaccination, both groups received an intranasal challenge of a SIV H3N2 isolate at a concentration of 10^6 $TCID_{50}$/mL in 3 mL saline solution into each animal (1.5 mL/nostril) by using the MAD® device (Intranasal Mucosal Atomizing Device, Teleflex® Inc. NC, USA). Euthanasia and necropsies were carried out 7 days post-inoculation (dpi). Sera from all individuals were collected previous to each immunization, before the challenge, and at 7 dpi. Nasal swabs were collected before challenge and at 5 and 7 dpi. Eventually, lung tissues were obtained and fixed by immersion in 10% neutral buffer formalin to perform the histopathological analysis.

The second study was performed applying identical conditions as those of the first study, albeit with influenza A virus seropositive animals. The goal of this second study was to simulate conditions generally occurring under usual conventional farm conditions. For that reason, animals were vaccinated in the farm and were transported to BSL-3 facilities for challenge. Each group consisted of six animals (Group A = non-vaccinated pigs, animals 1-6; Group B = vaccinated group, animals 7-14), and three pigs were euthanized at either 7 or 14 dpi. Sample collection was similar in both studies until day 7, albeit at different times. In this study, nasal swabs were collected before challenge and at 5, 7, 11 and 14

dpi. Moreover, BALFs were collected from the right lung of each pig at the time of euthanasia (7 and 14 dpi).

A complementary study with three SIV-seronegative pigs was performed in order to evaluate the immunogenic effect of the plasmid without the NG-34 sequence (empty vector). The same experimental design as the described for the study 1 was carried out (*Figure 3-1*), but animals were vaccinated with 600 µg DNA construct pCMV-CTLA4-Ig. Here, BALFs were also collected at 7 dpi.

3.3.3. Cells, virus and antigens

Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. The cell cultures were kept at 37°C with 5% CO_2 atmosphere in a humidified incubator.

SwH3N2 (A/swine/Spain/003/2010 H3N2 IV) [GenBank JQ319724 and JQ319726] influenza virus was used in this study for the intranasal challenge. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated in MDCK cells according to Reed and Muench method [387].

The sequence identity between antigen of interest (NG-34) and challenge virus, SwH3N2, used in this study is of 26% (*Table 3-1*).

Table 3-1. Amino acid sequence of NG-34 (peptide of interest) and the HA from the challenged virus (A/swine/Spain/001/2010 (H3N2).

Antigen	Aminoacid sequence
NG-34	NSDNGT**CYP**G**D**FID**Y**EE**LR**EQLS**S**VSSF**E**RFEIF
HA from A/swine/Spain/001/2010 (H3N2)	KA - FSN**CYP**Y**D**VPE**Y**TS**LR**SLIAS**S**GTL**E**FTNED

The amino acid identity between sequences is depicted in bold.

Purified hemaggluinins of A/California/04/09(H1N1)pdm09 and A/Aichi/2/1968 (H3) were purchased from SinoBiological (Sino Biological, cat. no. 40340-V08B and 11707-V08H; respectively) and were used as antigens.

3.3.4. Sample collection

Nasal swabs collected at predetermined time points were placed in 500 µL of PBS with antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). Serum samples were obtained from the jugular vein. After necropsy, BALFs from animals from the second study were also obtained by introducing 150 mL of PBS into the right lung of each pig, massaged gently and recollected into 50 mL falcon tubes [388]. BALFs were further centrifuged to remove cells. All supernatant samples were stored at -80°C until analysis.

3.3.5. Pathological assessment

Complete necropsies were performed, with special emphasis on macroscopic examination of lung parenchyma. Moreover, samples from apical, medial and cranial part of diaphragmatic pulmonary lobes were taken and fixed by immersion in 10% buffered formalin. Lung tissues were then embedded in paraffin, cut in 5 µm sections, stained in hematoxylin and eosin, and the severity degree of BIP was scored in a blinded fashion by a single pathologist using established criteria [389].

3.3.6. Plasmid synthesis

Two amino acids were replaced from the original HA1-based peptide (NF-34) to design the NG-34 peptide (*Table 3-2*). NG-34 peptide derives from the HA1 protein of the pH1N1 A/Catalonia/63/2009 strain [GenBank: ACS36215] and was theoretically predicted by the ISM, and mapped within the flanking region of the HA1. In order to enhance immunogenicity, the NG-34 peptide sequence was reverse-translated and cloned into an expression vector encoding human IgG fused with the extracellular domain of CTLA4. EndoFree plasmid gigakit (Qiagen, Barcelona, Spain) was used for purification and large-scale plasmid production. The plasmid was resuspended in sterile saline solution and stored at -20°C until use.

Table 3-2. Amino acid modifications in peptide NF-34 to obtain the NG-34 peptide.

Peptide	Aminoacid sequence
NF-34	NS**E**NGTCYPGDFIDYEELREQLSSVSSFE**K**FEIF
NG-34	NS**D**NGTCYPGDFIDYEELREQLSSVSSFE**R**FEIF

The amino acids differences between sequences are depicted in bold type.

3.3.7. Quantitative real time RT-PCR (RT-qPCR)

A TaqMan RT-qPCR was carried out to determine and quantify viral RNA in nasal swabs and BALFs collected at different time-points during the study. Extraction of RNA was carried out using NucleoSpin RNA isolation kit (Macherey-Nagel GmbH&CoKG, Düren, Germany) according to the manufacturer's instructions. Primers and probes used in this study, one-step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA, USA) and amplification conditions ran in a Fast7500 equipment (Applied Biosystems) to amplify the conserved fragment of the matrix (M) gene of influenza viruses are described elsewhere [390]. Samples in which fluorescence was undetectable were considered negative.

3.3.8. Subtypic quantitative real time RT-PCR (RT-qPCR)

Viral RNA extracted with NucleoSpin RNA isolation kit (Macherey-Nagel GmbH&CoKG, Düren, Germany) was tested to amplify a 95 base-pairs (bp) fragment of the *HA* gene of the challenged strain A/swine/Spain/003/2010 H3N2 IV. Based on a previous work, specific primers for the subtype H3 were designed [391]: forward 5'-TCCTTTGCCATATCATGCTTTTTG-3, and reverse 5'-ATGCAAATGTTGCACCTAATGTTG-3'. Specificity of primers was checked utilizing BlastN [392] through the Influenza Research Database (https://www.fludb.org/brc/blast.spg?method=ShowCleanInputPage&decorator=influenza) [393]. Real time amplification was performed employing the Power SYBR Green RNA-to-CT 1-Step Kit (Applied Biosystems, P/N #4389986) following manufacturer's indications and using 2 µL of eluted RNA in a total volume of 20 µL. In brief, for RNA-to-C_T 1-Step, the real-time PCR was performed using a Fast7500 equipment (Applied Biosystems, Foster City, CA, USA) and following cycles: 48ºC for 30 min (for cDNA synthesis), 95ºC for 10 min (transcriptase inactivation), followed by 95ºC for 15 s and 60ºC for 1 min for 40 cycles. Dissociation curve (melting curve) analyses were performed employing the parameters of a hot start at 60ºC for 15 s and measuring the fluorescence every 0.5ºC until 95ºC to confirm specific amplification.

Prior to the setting up of the RT-qPCR, standards were constructed. The amplification conditions for 95 bp of the HA fragment were: a reverse transcription at 50ºC for 30 min, an initial denaturation reaction at

95ºC for 15 min and 40 PCR-cycles of 94ºC 30 s, 55ºC 1 min and 72ºC 1 min. The obtained *HA* gene fragment amplicon was cloned into pGEM-T vector (Promega Madison, WI, USA) and transformed by heat shock in *Escherichia coli* TOP10 competent cells (Invitrogen, Paisley, UK). The recombinant plasmid was purified using the QIAprep Spin kit (Qiagen) and quantified by using BioDrop*µLITE* Spectrophotometer (BioDrop Ltd, Cambridge, UK). The copy number of recombinant plasmid was calculated as described elsewhere [394] by following the formula: N (molecules per µL) = (C (DNA) µg/µL/K (fragment size in bp)) × 182.5 × 10^{23} (factor derived from the molecular mass per the Avogadro constant). Serial 10-fold dilutions of known concentration were made and the standard curves were generated using copies of the recombinant plasmid harbouring the *HA* gene fragment from the SwH3N2 isolate.

3.3.9. Immunoassays

Virus-antigen specific serum antibodies were detected by enzyme-linked immunosorbent assays (ELISA). The influenza virus proteins used to detect specific antibodies were hemagglutinins (HA) from A/California/04/09(H1N1)pdm09 and A/Aichi/2/1968(H3N2). Briefly, ELISA plates (Costar, Corning Incorporated) were coated overnight with 2 µg/mL recombinant influenza hemagglutinin protein antigen in sodium bicarbonate (50 mM) buffer at 4ºC. Blocking was performed using 3%BSA/PBS for 1 hour at room temperature following washes with 1% Triton X-100/PBS. Sera were diluted 1:100 in the

blocking buffer and added to the 96 well plates during an incubation period of 1 hour at room temperature. Then, plates were washed four times and incubated during 30 minutes at 37°C with an anti-pig IgG (whole molecule)-Peroxidase (Sigma) diluted 1:10000 with the washing buffer. Plates were again washed four times with 1%Triton X-100 PBS and 50 µL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added during 8 to 10 minutes for the enzymatic reaction. Finally, the reaction was stopped by adding 50 µL of 1 N H_2SO_4. All samples were analyzed in triplicates.

3.3.10. Inhibition of the hemagglutination assay (IHA)

IHA was performed following the standard procedures described previously [331]. Positive and negative sera against H3N2 were purchased from GD Animal Health, Deventer, The Netherlands. All sera were analyzed in duplicate.

3.3.11. Statistical analysis

Mean and standard deviations were calculated with Excel 2007 (Microsoft Office) and statistical differences between the groups were calculated. Briefly, all data obtained were first normalized by Shapiro-Wilk test and later the groups were compared using either the t-test (in case of normally distributed data), or the Wilcoxon test (in case of non-normally distributed data). All calculations were carried out using R statistical software (http://cran.r-project.org/).

3.4. Results

3.4.1. Clinical and pathological evaluation

In the first study, one animal from unvaccinated group (animal 5) manifested fever at 4 and 7 dpi. From the vaccinated group, fever could be detected in four out of five pigs; three of them (animals 6, 7 and 8) had fever during days 2, 3 and 4 post-inoculation and one of them (animal 9) on days 3 and 4. No other clinical signs could be observed, except one vaccinated animal (animal 8), which was coughing at 4 and 5 dpi. Likewise, four out of six animals had fever in the unvaccinated group (animals 1, 2, 5 and 6) and three out of six from the vaccinated group (animals 7, 8 and 11) from the second study. Also, one animal from each group was coughing (animal 1 and 14, both at 3 dpi), and only one vaccinated pig (animal 7) showed apathy and a loss of weight after the challenge.

In the first study, no differences in the histological lesions in the lung tissues were found at 7 dpi between groups. No differences in the severity of the histological lesions in the lung tissues were detected at 7 or 14 dpi in the second study (*Table 3-3*). *Figure 3-2* shows different sections of histological lung tissues illustrating the different scoring values (0, 1, 2 or 3). Additionally, besides the BIP scoring, other pathological findings were also recorded. In the first study, animal 8 had bronchiolitis fibrosa obliterans. In the second study, suppurative bronchopneumonia was present in one animal from the unvaccinated group (animal 1) and in 3 out of 6 animals from the vaccinated group (animals 7, 9 and 10), pig 8 had fibrous pleuritis.

Table 3-3. Scoring and observations given to each hematoxylin-eosin stained preparation for every single pig of 2nd study.

Vaccine applied	Animal identification	Necropsy date	Broncho-interstitial pneumonia (BIP)
Unvaccinated	1	7 dpi	2
	2	7 dpi	2
	5	7 dpi	3
	3	14 dpi	0.5
	4	14 dpi	1.5
	6	14 dpi	0.5
Vaccinated	7	7 dpi	3
	9	7 dpi	3
	12	7 dpi	2
	8	14 dpi	2
	10	14 dpi	0.5
	11	14 dpi	1

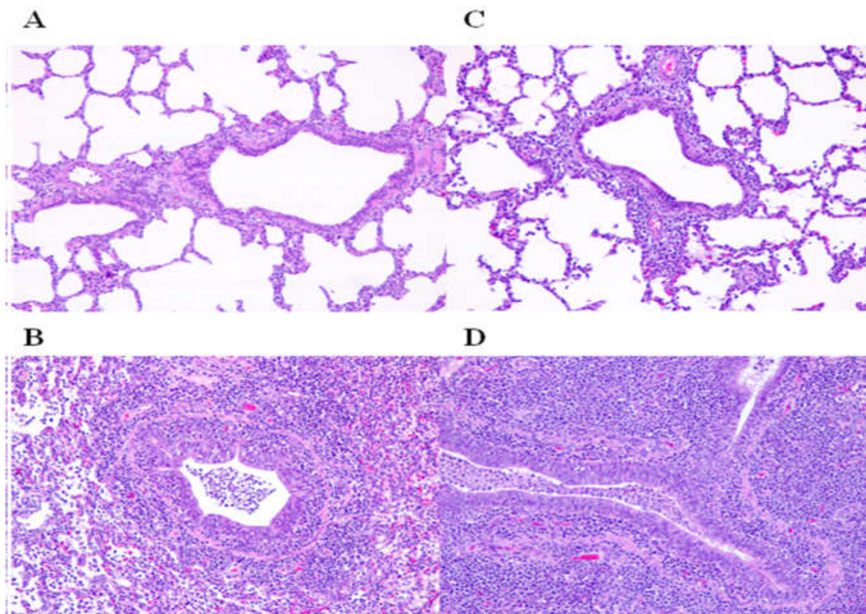

Figure 3-2. Representative sections from lung samples fixed with formalin and embedded in paraffin, stained with hematoxylin and eosin and microscopically evaluated following the scoring system [389] **(magnification 100x):** (A) scoring 0, (B) scoring 1, (C) scoring 2, and (D) scoring of 3.

3.4.2. Immunization with pCMV-CTLA4-Ig-NG34 eliminates or significantly reduces viral shedding

In comparison to the control group, pigs immunized twice with pCMV-CTLA4-Ig-NG34 showed reduced viral shedding within the first week after challenge in both studies (*Figure 3-3*). The mean of genomic equivalent copies (GEC) per mL of the vaccinated pigs was inferior to the mean of the unvaccinated group at 5 and 7 dpi (*Figure 3-3A*). Remarkably, in the first study (seronegative pigs) from the vaccinated group, in three out of five animals no viral RNA was detected at 7 dpi. In contrast, viral RNA could be detected in all the five pigs from the unvaccinated group ($P<0.01$) (*Figure 3-3A*). Moreover, one animal from the unvaccinated group (animal 3) died, most likely due to a secondary bacterial infection. A reduction in the subtypic RNA shedding was also observed at 5 dpi in pCMV-CTLA4-Ig-NG34 vaccinated seronegative animals; however, no virus was detectable in the vaccinated and non-vaccinated animals on day 7 post-inoculation (*Figure 3-3B*).

In animals with MDA (Study II; seropositive pigs), the mean of GEC per mL was lower at 5 and 7 dpi than those observed in the unvaccinated group. Noteworthy, at 7 dpi, in two out of the six animals (animals 8 and 9) we could not detect viral RNA ($P<0.05$) (*Figure 3-3C*). Regarding subtypic viral RNA in MDA positive animals, at 5 and 7 dpi, the mean of GEC per mL, was lower than those of the unvaccinated group. Furthermore, at 7 dpi viral RNA could not be detected in two vaccinated animals (*Figure 3-3D*). The unvaccinated-infected pigs continued shedding influenza virus up to 14 dpi (*Figure 3–3C*) whereas IV virus was

not detected in vaccinated group at 11 dpi, and only in one vaccinated pig at 14 dpi (*Figure 3-3C*). Results from the vaccinated pigs with pCMV-CTLA4-Ig (empty plasmid) showed that none of the three pigs were reducing the viral shedding at 7 dpi (*S1 Table*).

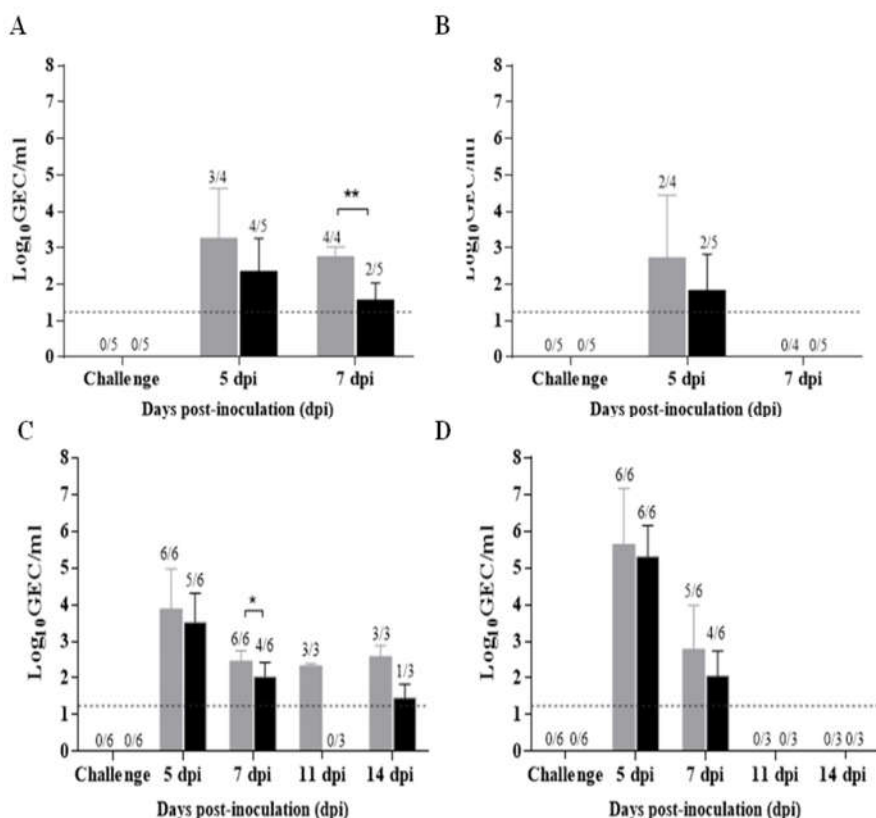

Figure 3-3. Viral detection in nasal swabs samples by RT-qPCR. (A) Mean values of **GEC per mL** obtained from nasal swabs samples (1st study) collected at 0, 5 and 7 dpi, from seronegative animals and (B) subtypic RT-qPCR results from the 1st study (C) Mean values of GEC per mL obtained from nasal swabs samples (2nd study) collected at 0, 5, 7, 11 and 14 dpi from seropositive animals and (D) subtypic RT-qPCR from the 2nd study. Grey bars correspond to Group A (unvaccinated group) and black bars to Group B (pCMV-CTLA4-Ig-NG34 vaccinated group). Dpi, days post-inoculation. Dashed lines indicate the detection limit of the assays: 1.24 log_{10}GEC/mL. Error bars indicate the mean ± SEM.

3.4.3. Humoral response to a heterologous influenza virus after pCMV-CTLA4-Ig-NG34 vaccination

To determine whether the NG-34 peptide of H1N1 origin could confer protection against a heterosubtypic circulating influenza strain, pigs were challenged with the H3N2 influenza virus subtype. In addition, attempting to potentially improve the vaccine efficiency, we decided to deliver the antigen in a DNA vaccination approach instead of peptide. Immunogenicity of the vaccine was examined by the presence of specific antibodies, raised against H1 and H3, and their ability to inhibit the hemagglutination against the challenged heterologous virus in the sera collected from vaccinated and non-vaccinated animals. Specific immune response in the BALFs collected from MDA seropositive animals (from the 2nd study) was also examined by testing for specific H1 and H3 antibodies.

pCMV-CTLA4-Ig-NG34 immunization in pigs elicited antibodies that were recognizing both the H1 and H3 hemagglutinin subtypes (*Figures 3-4A and B*). Increased antibody levels were observed at 35 post-vaccination days (PVD, pre-challenge) in the vaccinated group (*Figures 3-4A and B*). The levels of antibodies were higher against H1 (*Figure 3-4A*), since NG-34 belongs to H1 subtype. Furthermore, sera from all vaccinated animals, collected 7 days after the intranasal challenge with SwH3N2 influenza virus, manifested a potent boost in H3 subtype-specific antibodies in comparison to non-vaccinated SwH3N2 influenza virus infected control group (*Figure 3-4B*).

Additionally, we evaluated the H1 and H3 subtypes HA-specific IgG titers in BALFs samples. While not statistically significant, vaccinated animals achieved a greater antibody titer at the two necropsy points of the 2nd study, with increased values at 14 dpi (*Figures 3-4C and D*). The difference encountered was greater for antibodies against the H3 subtype among groups and, in a higher rate, likely due to the fact that an H3 virus was used for the challenge.

None of the pigs vaccinated with pCMV-CTLA4-Ig (empty plasmid) raised antibodies against H3 subtype before or after the challenge against SwH3N2 (*S1 Table*).

To further evaluate whether antibodies obtained from seronegative pigs (Study I) could inhibit the attachment of the virus to the chicken red blood cells (cRBCs), an IHA against the challenged virus was carried out. Albeit only detecting HI activity at 7 dpi, results displayed in *Figure 3-5* suggest that all swine from the vaccinated group had significantly higher detectable HI titers than the non-vaccinated pigs ($P<0.05$). None of the pigs vaccinated with pCMV-CTLA4-Ig (empty plasmid) could inhibit the hemagglutination of SwH3N2 (*S1 Table*).

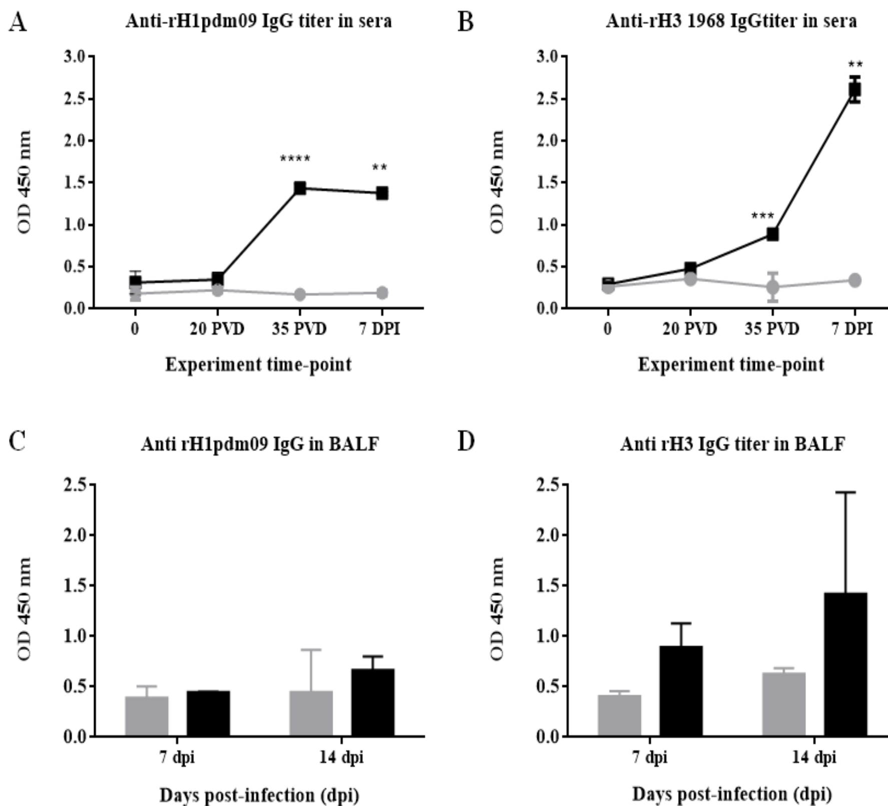

Figure 3-4. Serum antibody HA-specific IgG titers detected in sera and BALFs samples by ELISA test. Mean of serum antibody levels detected in all individuals at time-points 0, 20 PVD and 35 PVD, and 7 dpi of Groups A and B (A) against HA from A/California/04/09(H1N1)pdm09, and (B) against HA from A/Aichi/2/1968(H3N2) are shown. Mean of BALFs antibody levels detected in pigs necropsied at 7 and 14 dpi of Groups A and B (C) against HA from A/California/04/09(H1N1)pdm09, and (D) against HA from A/Aichi/2/1968(H3N2). Grey circles/bars designate group A (unvaccinated group), and black squares/bars designate group B (pCMV-CTLA4-Ig-NG34 vaccinated group). OD, optical density. PVD, post-vaccination days and dpi, days post-inoculation. Error bars indicate the mean ± SEM. Statistically significant differences between vaccine treatment groups (*P* value <0.05) are marked with **: *P*<0.01, ***: *P*<0.001, ****:P<0.0001.

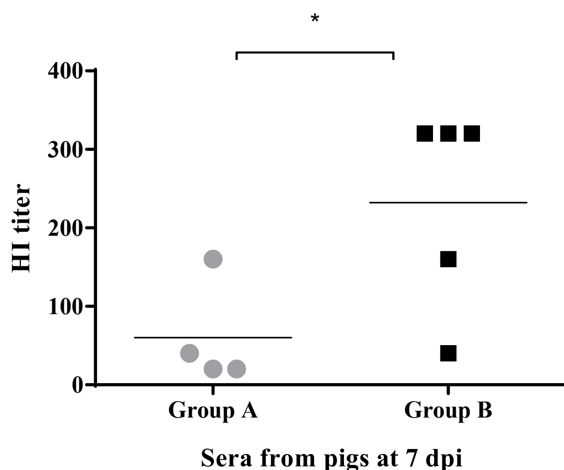

Figure 3-5. IHA activity at 7 dpi against SwH3N2 in sera from seronegative pigs (Study I). HI titer obtained with sera from unvaccinated (Group A) and vaccinated (Group B) pigs, at 7 dpi against the SwH3N2. Grey circles designate group A (unvaccinated group), and black squares designate group B (pCMV-CTLA4-Ig-NG34 vaccinated group). HI: Hemagglutination inhibition. Error bars indicate the mean ± SEM and statistically significant differences between vaccine treatment groups (*P* value <0.05) are marked with *:*P*<0.05.

3.4.4. Virus detection in BALFs

BALFs collected in the second study were also used to assess whether at 7 dpi and 14 dpi SIV RNA could be detected in the respiratory tract of the lungs. While no viral RNA could be detected at 14 dpi in any of the vaccinated pigs, differences between groups were evident at 7 dpi. Influenza virus RNA could not be detected in any of the BALFs collected from the vaccinated pigs. In contrast, BALFs from two out of the three non-vaccinated pigs were positive for SIV RNA (*Figure 3-6*),

demonstrating a clearance of viral RNA in the respiratory airways from the pCMV-CTLA4-Ig-NG34 vaccinated pigs. Data gathered from the complementary study showed that none of the three pigs vaccinated with pCMV-CTLA4-Ig (empty plasmid) cleared the infection in the lung (*S1 Table*).

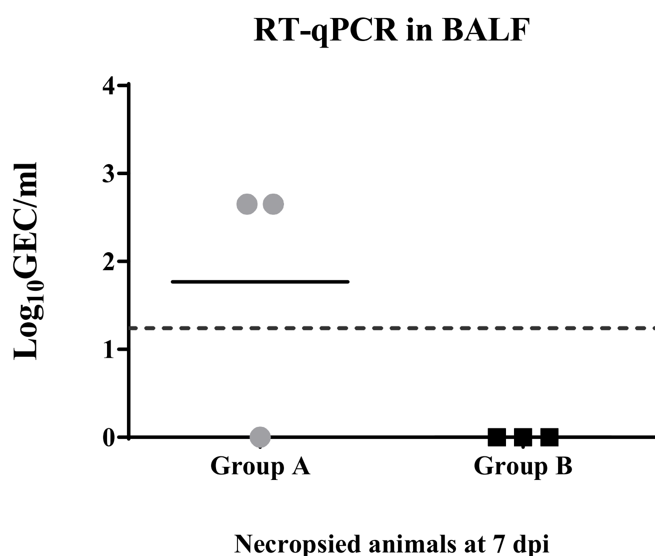

Figure 3-6. Influenza viral RNA detection in BALFs performed by RT-qPCR. GEC per mL values obtained in BALFs samples, obtained from MDA positive animals (Study 2), from unvaccinated (Group A) or vaccinated (Group B) pigs at 7 dpi, corresponding to necropsy day. Grey circles designate group A (unvaccinated group), and black squares designate group B (pCMV-CTLA4-Ig-NG34 vaccinated group). Dashed lines indicate the detection limit of the assay: 1.24 log_{10}GEC/mL.

3.5. Discussion

Conserved peptides are highly desirable vaccine antigens/candidates for various reasons [395], particularly with regard to safety and ease of production. NG-34, the peptide antigen used in this study, is relatively conserved and, as we reported previously [331], it consists of both a B and T cell epitope. For this dual role of the peptide fomenting both humoral and cellular responses in conventional pigs and the demonstrated *in vitro* cross-protective immune reaction [331], we are seeking new strategies to present the antigen in a formulation that can potentiate the immune response and confer protection against influenza virus infection. For this purpose, NF-34 was modified to NG-34. Immunization of mice with this modified HA-peptide (NG-34) elicited sustained antibodies with strong neutralizing capacity [396]. Moreover, by cloning NG-34 into the pCMV-CTLA4-Ig plasmid, our intention was to target the APCs with the objective to induce an enhanced immune response. CTLA4 was chosen due to its described adjuvant like role at low doses as it delivers fused antigens to APCs [397]. Data reported in mice [397] indicated that targeting antigens to APCs by means of CTLA4 increased both the humoral and the cellular responses. The role of CTLA4-Ig as an adjuvant has also been reported in other studies including a model of asthma [398]. Although it has been described that IgG2a production is predominant after DNA immunization [399], mice immunized with DNA-CTLA4-Ig generated enhanced levels of distinct IgG subclasses (IgG1, IgG2a, IgG2b), with a predominance of the IgG1 subtype [397]. This suggests that CTLA4 might have caused a non-

specific change in the immune response, possibly by a direct stimulation of APCs.

An intradermal delivery approach was chosen for vaccine prototype delivery mainly owing to a large number of studies [400–402] warranting higher antibody titers by this method in comparison to gene injection into skeletal muscles. Furthermore, using this approach we facilitated DNA uptake by skin-associated-lymphoid tissue that may play a role in inducing cytotoxic T cells against viruses or intracellular pathogens [401]. We used the DNA vaccine delivery approach first described by [403] which was previously applied also using another influenza DNA vaccine in pigs with challenge [404]. Optimal influenza DNA plasmid doses (moles) were identified and suggested using this delivery method in pigs [386]. Using the same delivery method in pigs but another multivalent influenza DNA vaccine we also were able to break MDA and protect pigs from influenza challenge. Since the DNA vaccine and the challenge strain (H1N1psm2009) were different, we cannot compare. However, the influenza DNA studies both suggest that naked DNA vaccine seems all very powerful in protecting pigs from heterologous influenza strains of both H1N1 and H3N2. Similar results have been consistently reported by others using different influenza DNA and delivery methods [405,406].

Introduction of the NG-34 peptide sequence into plasmid together with CTLA4 further improved the immunogenicity and protective potential of the peptide-based vaccine previously reported by our group [331].

Moreover, seeing the complementary study data using pCMV-CTA4-Ig it is evident that the combination of CTLA4 with NG-34 is fundamental. An additional DNA adjuvant effect may be obtained using the Diluvac diluent containing tocopherol [407]. Vaccinated animals completely eliminated virus from the lung within 7 days after challenge as demonstrated from BALFs samples collected from seropositive pigs. Additionally, in seronegative vaccinated animals, viral shedding was also reduced to basic levels within 5 to 7 days after infection, suggesting that transmission of the virus could greatly be reduced with the vaccine approach used. Interestingly, vaccinated MDA positive could also reduce virus replication and shedding, suggesting that pCMV-CTLA4-Ig-NG34 vaccine could overcome a possible inhibition/delay in inducing an active antibody and/or cellular immune response [379,386]. We occasionally see virus secretion from the vaccinated pigs at 14 dpi, but we assume this might be by contact (residual) coming from the non-vaccinated animals. The virus clearing effects could apparently be linked to CTLA4-Ig vaccination that is involved in IgG1 activity promoting Th2 response, in a possible transportation of the antigens in lymphoid organs [408] and in an increase of the B cell and T cell response [397]. In addition, elevated levels of anti-HA specific antibodies at 35 PVD and 7days after a H3N2 inoculation in the vaccinated pigs might have played a role in the elimination of the heterologous challenged virus. Additionally, these antibodies potently inhibited the hemagglutination activity of the challenged virus at 7 dpi. Moreover and as reported in [331] vaccination induced and maintained antibody cross-reactive response against H3N2 and H1N1 subtype. Furthermore, a tendency of a higher IgG titer in

BALFs against H3N2 and H1N1 subtypes was observed in the vaccinated MDA animals compared to the non-vaccinated ones.

Clinical signs and lung lesions were similar between groups. However, in the first study, more animals from the vaccinated groups had fever than the animals from the unvaccinated group. Likewise, there was one seropositive pig from the unvaccinated group (2nd study, animal 11) that cleared the virus at 7 dpi, at least based on viral RNA presence in BALFs samples. This animal coincided to have had the highest MDA levels of the group at the onset of the experiment. Besides, due to the small number of samples studied for SIV RNA and antibody titers in BALFs, no statistical analyses were performed.

In summary, intradermal application of pCMV-CTLA4-Ig-NG34 DNA vaccine might represent a potential alternative to combat SIVs and could overcome MDA-associated blockage of the virus secretion. We anticipate that reducing/blocking/eliminating the influenza virus shedding after infection is crucial for concomitant transmission to indirect naïve contact pigs. Nonetheless, more studies are indispensable (with larger groups) and might be mainly addressed to examine whether the presented formulation is also capable of promoting a solid response against other widely circulating swine influenza subtypes.

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CHAPTER IV

Study II: DNA vaccine based on conserved HA-peptides induces strong immune response and rapidly clears influenza virus infection from vaccinated pigs.

4. Study II

"DNA vaccine based on conserved HA-peptides induces strong immune response and rapidly clears influenza virus infection from vaccinated pigs"

4.1. Abstract

The present work aimed to evaluate a new DNA influenza vaccine based on distinct conserved HA-peptides fused with flagellin and applied together with Diluvac Forte® as adjuvant. Two experimental pig studies were performed to test DNA-vaccine efficacy against SIVs in pigs vaccinated with a needle-free device (IDAL®). The first one was performed with SIV-seronegative pigs intranasally challenged with a pH1N1. In the second study, SIV-seropositive animals were intranasally challenged with an H3N2 SIV-isolate. Both experiments demonstrated a complete elimination or significantly reduced viral shedding within the first week after challenge, suggesting vaccine efficacy against both the H1 and H3 influenza virus subtypes. In addition, the results proved that MDA did not constitute an obstacle to the vaccine approach employed. Moreover, elevated titers in antibodies both against H1 and H3 proteins in serum and in BALFs was detected in the vaccinated animals along with a markedly increased mucosal IgA response. Additionally, vaccinated animals developed stronger neutralizing antibodies in BALFs and higher inhibiting hemagglutination titers in sera against both the pH1N1 and H3N2 influenza viruses compared to unvaccinated, challenged-pigs. It is proposed that the described DNA-vaccine formulation could potentially be used as a multivalent vaccine against SIV infections.

4.2. Introduction

SIVs are common throughout pig populations worldwide and they generally cause coughing, sneezing, nasal discharges, fever, conjunctivitis, respiratory difficulties, lethargy, decreased food intake [140,405,409,410] and, in some instances, abortions in pregnant sows due to fever [289,411].

In spite of the reduction in clinical signs and high antibody titers induced both in serum and alveolus, commercial vaccines have some weaknesses [369,412]. Apart from not being sufficiently protective when the strain does not closely match with the ones included in the vaccine product, they do not confer protection when facing against heterovariant or heterosubtypic challenges [8,371–373]. Some research studies have hypothesized that this matter could be related to the lack of cell-mediated and/or mucosal responses provided by the inactivated-type vaccines [8,374,375]. Moreover, it is also evidenced that MDA may interfere in the development of immunity provided by vaccination [377,381]. Fundamentally, piglets with MDA at vaccination showed prolonged flu-like clinical signs, more severe SIV-pneumonia and suppression of both humoral and cellular responses in comparison to vaccinated MDA-seronegative piglets [377].

For these abovementioned reasons, many efforts have been directed to design a universal vaccine that should cover all relevant subtypes of influenza, including varying field strains, and able to avoid the likelihood

of emergence of forthcoming pandemic strains. The ideal vaccine should also overcome MDA interference. Currently, conserved areas of the virus proteins are targeted for the design of such vaccine [331,413–416]. In fact, those designs are based on combining different well-conserved epitopes to improve their protection and strain coverage.

Our group has defined new vaccine strategies utilizing conserved epitopes of the IAVs, specifically from the HA protein [331]. In the present work, with the aim to improve our vaccine prototype, a DNA vaccine encoding a combination of HA-conserved immunogenic epitopes along with flagellin (VC4-flagellin) was designed. Selection of these HA peptide epitopes (from H7, H5N1 or pH1N1) was based on the encoded informational spectrum frequencies that are common for the IVs judged by ISM. Previously, it has been shown that antigens which share a common frequency component in their informational spectra are immunologically cross-reactive [417].

Instead of immunizing animals with this new construct via intramuscular, a needle-free approach (IDAL® device) was used. This administration route is safer because of the needle-free system and easy to be used in large-scale vaccination programs [403,404]. Moreover, to test the broad-based immunity and the protective efficacy of the vaccine, both MDA-seropositive and MDA-seronegative animals were used in this study. Further, animals were challenged with either pH1N1 or SwH3N2 to assess the cross-protective effect of the vaccine.

Herewith, we demonstrated that vaccination with VC4-flagellin DNA induced high titers of seroprotective/neutralizing antibodies and contributed in reducing the viral shedding of the vaccinated pigs in presence and absence of MDA.

4.3. Materials and methods

4.3.1. Immunogen and expression vector construction

Four conserved HA-peptides were predicted in silico by the ISM [362,418] and expressed along with a flagellin-derived construct, which was also designed by the ISM bioinformatics platform [419]. The predicted peptides along with the flagellin were organized in tandem to construct the multipeptide: PQRERRRKKRGLFGAIAGVEVVNATETVERTNIPRICSKGKRTVD LGQCGLLTIQVGANDGETIDIDLKQINSQTLSSSGSSGSSGSSIDAA LAQVDALRSDLGAVQNRFNSAGVEVVNATETVERTNIPRICSKGK RTVDLGQCGLSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNV, designated hereafter as VC-4-flagellin. This sequence was reverse-translated with codon optimization for swine expression and cloned into the pCDNA3.1(+) plasmid (GenScript, NJ, USA). *Table 4-1* describes each of the four predicted HA epitopes. Expression of the construct was controlled by vitro transfection (data not shown). Purification of the plasmid at large-scale production was performed with the EndoFree plasmid Gigakit (Qiagen, Barcelona, Spain). Purified plasmid DNA was quantified by using Biodrop μLITE Spectrophotometer (BioDrop Ltd,

Cambridge, UK), resuspended in sterile saline solution and kept at -20ºC until used.

Table 4-1. Amino acid sequences from the HA-epitopes used in the VC4-flagellin construct, aa positions from their respective consensus IVs are also indicated.

HA-epitopes	Aa positions*	Consensus virus subtype	GenBank Id
PQRERRRKKRGLFGAIA	337-357	H5N1	AAC32098.1
GVEVVNATETVERTNIPRICSKGKRTVDLGQCGLLTI	41-77	H7N1	AGT40751.1
	37-71	H7N7	ACN80240.1
	33-67/ 37-71	H7N8	AFP99768.1
	41-75	H7N9	ASV61404.1
GVEVVNATETVERTNIPRICSKGKRTVDLGQCGL	41-74	H7N3	APD70004.1
		H7N6	ANK78016.1
		H7N7	ANC28237.1
		H7N9	AJU15322.1
SLPFQNIHPITIGKCPKYVKSTKLRLATGLRNV	168-200	H1N1	ALN12227.1

*the most common aa positions of HA-epitopes, though they also could be encountered in other aa positions. The aa positions shown are according to the reference cited from the GenBank database. Abbreviations: aa= amino acid; HA= hemagglutinin; Id=identification.

4.3.2. Cells and antigens

Madin-Darby Canine Kidney (MDCK, ATCC CCL-34) cells were used to prepare viral stocks and to perform the seroneutralization assays on

BALFs. MDCKs were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine.

Hemagglutinins of A/California/04/09(H1N1)pdm09 and A/Aichi/2/1968 (H3N2) were acquired from SinoBiological (Cat no. 40340-V08B and 11707-V08H; respectively, SinoBiological Inc., PA, USA) and were used as purified antigens for in-house ELISA test developments.

4.3.3. Ethics statement

Experiments with SIVs were performed at the BSL-3 facilities at IRTA-CReSA (Barcelona, Spain). The experiment protocols were supervised and approved by the Ethical and Animal Welfare Committee of IRTA and the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia.

In addition, both conducted research studies followed the Directive UE 63/2010, the Spanish Legislation, RD 53/2013, the Catalan Law 5/1995 and Decree 214/1997.

Animals from Study II were housed in a conventional farm during the immunization, and transferred to BSL-3 facilities one week prior to challenge (adaptation period).

4.3.4. Animal experimental design

Two experiments were carried out to assess the DNA-vaccine efficacy *in vivo* (*Table 4-2*). Clinically healthy pigs were selected and tested for presence of specific antibodies in sera against the influenza NP using the ID Screen® Influenza A Antibody Competition ELISA (ID VET, France) kit. SIV-seronegative animals were selected for Study I. For MDA positive pig studies, piglets were obtained from vaccinated sows and were controlled for having NP antibodies by ELISA. Moreover, in both cases, RT-qPCR (see *4.3.8.*; *"Quantitative real time RT-PCR (RT-qPCR)")* was also determined to ensure animals were not exposed to IAVs.

Table 4-2. Schematic outline of Studies I and II.

Study	Groups	Nº animals (n)	MDA	Challenged virus
Study I	Group A: unvaccinated	5	Absence	pH1N1
	Group B: pCDNA3.1(+)-VC4-flagellin	5		
Study II	Group A: unvaccinated	6	Presence	SwH3N2
	Group B: pCDNA3.1(+)-VC4-flagellin	6		

4.3.4.1. Study I (SIV-seronegative pigs/challenged with pH1N1)

Ten 5-to-6-week-old male pigs seronegative against SIV were randomly divided into two groups: animals 1-5 (Group A, n=5) and animals 6-10 (Group B, n=5). Animals from group B were immunized twice with a 21-day interval period. The immunizations consisted of 600 µg (3 IDAL® shots/200 µg/100 µL animal) of the VC4-flagellin DNA construct mixed

in a ratio of 1:1 (v/v) with Diluvac Forte® adjuvant (MSD Animal Health, Salamanca, Spain) applied with the IDAL® device (MSD Animal Health) on the dorsal side of the back of each pig [404]. Animals from A group were sham-vaccinated by administration of 2 mL/animal of PBS+Diluvac Forte®. Two weeks after booster immunization, all pigs were intranasally challenged with pH1N1. All animals were euthanized seven days dpi with an overdose of pentobarbital followed by exsanguination.

4.3.4.2. Study II (SIV-seropositive pigs/challenged with SwH3N2)

Twelve 4-week-old SIV-seropositive male or female pigs were separated into two groups: animals 1-6 (Group A, n=6) and animals 7-12 (Group B, n=6). Animals from groups A (sham-vaccinated) and B (VC4-flagellin DNA-vaccinated) were immunized as described in Study I. Two weeks after the second immunization, animals were intranasally challenged with SwH3N2. Animals were euthanized with an overdose of pentobarbital followed by exsanguination either at 7 or 14 dpi.

4.3.5. Sampling and clinical records

Flu-like clinical signs were monitored during all the experiment. Fever was considered when rectal temperatures reached values above 40ºC [385]. The sampling schedule for both experiments is represented in *Table 4-3*. Briefly, nasal swabs were collected to determine the presence of

viral RNA and sera were collected to analyse the humoral immune response at different time-points.

Table 4-3. Sampling schedule for studies I and II.

Sample	Study	0 PVD	21 PVD	35 PVD	5 dpi	7 dpi	11 dpi	14 dpi
Sera	Study I	✓	✓	✓		✓		
	Study II	✓	✓	✓		✓		✓
Nasal swabs	Study I	✓		✓	✓	✓		
	Study II	✓		✓	✓	✓	✓	✓
BALF	Study I							
	Study II					✓		✓
Lung tissues	Study I					✓		
	Study II					✓		✓

Abbreviations: BALF= bronchoalveolar lavage fluid; dpi= days post-inoculation; PVD= post-vaccination days.

Complete necropsies were performed at the indicated times after infection (7dpi, Study I; 7 or 14 dpi, Study II). Gross pictures were taken from both sides of the lung to assess the macroscopiclung lesion score. Subsequently, three lung samples were collected (apical, middle and diaphragmatic lobes) from the left lung and fixed by immersion in 10% neutral buffered formalin to perform histopathological analysis. BALFs were also collected immediately from the right lung after post-mortem examination [388]. The BALF supernatants obtained were stored at -80ºC to investigate antibody response (IgG and IgA) and to assess seroneutralizing titers against the challenged virus.

4.3.6. Pathological procedures

Only in study II, the macroscopically affected lung area (%) from each individual was quantified by image analysis (IA) (ImageJ® online free software) as previously described [420]. Formalin fixed tissues of animal from both studies were dehydrated and embedded in paraffin wax, sectioned at 3-5 μm and stained with hematoxylin-eosin (HE) for examination under light microscopy. In all lung samples, a semi-quantitative scoring method was determined as previously described [389].

4.3.7. SIVs and inoculum preparation

The viruses used for inoculation were the pH1N1 virus (A/Catalonia/63/2009 H1N1 IV) [GenBank GQ464405-GQ464411 and GQ168897] and the SwH3N2 (A/swine/Spain/003/2010 H3N2 IV) [GenBank JQ319724 and JQ319726]. The infectious virus titres were determined by following the Reed and Muench methodology [387]. All pigs were intranasally inoculated with a total dose of $10^6 TCID_{50}$/mL diluted in 3 mL saline solution and delivering a final volume of 1.5 mL/nostril using a mucosal atomisation device (MAD® Nasal; Teleflex® Inc. NC, USA) to mimic aerogenous infection.

4.3.8. Quantitative real-time RT-PCR (RT-qPCR)

Viral RNA quantification was performed in nasal swab samples using the NucleoSpin RNA isolation kit (Macherey-Nagel GmbH&CoKG, Düren, Germany) following the manufacturer's instructions. Subsequently, a TaqMan RT-qPCR designed to detect influenza viruses (IVs) using the PCR primers and hydrolysis probe already described [390] was run in a Fast7500 equipment (Applied Biosystems, Foster City, CA, USA) with the conditions already set and described [421].

4.3.9. IgGs and IgAs ELISAs

To assess IgG antibody responses against the purified antigens from H1N1 and H3N2 in sera and BALFs samples, specific ELISA tests were developed. Briefly, 96 well plates were coated with 2 µg/mL of each HA antigen diluted in 50 mM sodium bicarbonate buffer and incubated overnight at 4ºC. After blocking with 3%BSA/PBS (100µL/well) for 1 hour at room temperature (RT) either serum from individuals diluted at 1:100 or neat BALFs samples were added (50µl/well) to the coated plate, followed by 1 hour incubation at RT. Plates were washed three times with 1% Triton X-100/PBS, and anti-pig IgG (whole molecule)-Peroxidase (Sigma-Aldrich, MO, USA) diluted 1:10,000 was added to wells followed by 30 minutes incubation at 37ºC. After washing the plates four times (1% Triton X-100/PBS), 50 µL of TMB substrate solution was added to the wells and allowed to develop protected from light exposure for 10 minutes. Reaction was stopped by adding 50 µL of

1 N H_2SO_4 and the optical density (OD) was measured at 450 nm. Each sample was run in triplicates.

An in-house ELISA to detect mucosal response (IgA) against hemagglutinins from H1 and H3 subtypes was run in BALF samples. For that purpose, a previous protocol was followed with few modifications and by means of using IgA antibody (Cat no. AA140p; AbD Serotec, Oxford, UK) [422]. Briefly, the high-binding 96-well plates (Costar, Corning Incorpororated, NY, USA) were coated with 2 µg/mL of each HA in 50 mM sodium bicarbonate buffer and incubated overnight at 4ºC. Samples were diluted 1:1 with blocking buffer.

4.3.10. Inhibition of the hemagglutination assay (IHA)

HI titers were obtained following the standard protocol instructions out of the OIE (2012) [289] using cRBCs and 4 hemagglutination units of either pH1N1 IV or SwH3N2 IV. All sera were analyzed in duplicates. Positive and negative reference sera (purchased at the GD Animal Health, Deventer, The Netherlands) were used to validate the technique. “Seroprotective” titer (HI≥40) has been used as a criteria of immunogenicity in a vaccine and standard for licensure [288,423–425].

4.3.11 Serum neutralization test (SNT)

MDCK cells were seeded into 96- well tissue culture plates to achieve confluence the following day. After 24 hours, BALFs samples were inactivated at 56ºC for 30 minutes and serially diluted two-fold up to 1: 2,560 dilution using DMEM supplemented with 1% penicillin/streptomycin and 1% L-glutamine. In parallel, to promote a proper cleavage of the hemagglutinin protein, the H3N2 virus was trypsinized using porcine-trypsin (Sigma-Aldrich, MO, USA) for 30 minutes at 37ºC. After this step, the virus was added to the diluted BALFs to yield final concentrations of 100 $TCID_{50}$/well. Serum-virus mixtures were incubated at 37ºC temperature for 2 hours and were added to PBS 1X washed MDCK cells. Media controls (no virus) and virus controls (no serum) were included on each plate. Reference positive and negative sera against H3N2 (GD Animal Health, Deventer, The Netherlands) were also incorporated. Each sample dilution was plated in duplicates. After an incubation period of 7 days, the plates were read. SNT titers were calculated as 50% endpoints for the greatest serum dilution giving complete inhibition of the virus growth [387].

4.3.12. Flow cytometry

In order to identify the phenotype of T cells, peripheral blood mononuclear cells (PBMCs) were isolated before the challenge from whole blood by density centrifugation using Histopaque®-1077 gradient (Sigma-Aldrich, MO, USA) and performed the flow cytometry using monoclonal antibodies (mAbs). Cell numbers were calculated using a

dye solution and the cell concentration was adjusted to 10^6 cells/well, and single- or double-stained with surface antibodies diluted in PBS 1% anti-CD4 (clone 74-12-4, IgG2b) Alexa Fluor® 647-labelled (BD Pharmingen™, CA, USA) and anti-CD8 (clone 76-2-11, IgG2a) fluorescein isothiocyanate (FITC)-labelled (BD Pharmingen™, CA, USA). Cells were acquired by means of FACSCalibur (Becton Dickinson FACSAria I) (Becton Dickinson, CA, USA), and the positive frequencies analyzed by FACSDiva software, version 8.01. Gated images of different cell populations are shown in *S1 Figure*.

4.3.13. Statistical analyses

Mean and standard deviations of studied parameters were calculated with Excel 2007 (Microsoft Office). All data obtained were first normalized by the Shapiro-Wilk test and the t-test (in case of normally distributed data) or the Wilcoxon test (in case of non-normally distributed data) and were subsequently used to compare A and B groups within each study. Statistical analyses were performed using the R statistical software (http://cran.r-project.org/) and the significance was depicted depending on the significance threshold obtained: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****).

4.4. Results

4.4.1. Study I (SIV-seronegative pigs/challenged with pH1N1)

4.4.1.1. Clinical and pathological evaluation

Previous to the challenge, all animals were clinically healthy. Upon challenge, one animal out of five (pig 1) from the unvaccinated group had fever at 6 dpi and also one animal (pig 8) from the VC4-flagellin vaccinated group had fever but only at 2 dpi. Also, one animal (pig 2) from the unvaccinated group displayed loose feces at 7 dpi. No other clinical signs were recorded.

Challenge with pH1N1 caused subclinical infection in all pigs and minor histopathological changes observed at the necropsy. No differences in the severity of microscopic lung lesions between vaccinated and unvaccinated animals were recorded. Apart from the lung scorings based on BIP, other pathological findings were documented. Multiple abscesses with bacterial colonies were visualized in animal 7; animal 8 had fibrous pleuritis and animal 10 showed pulmonary congestion and edema. All three animals belonged to the VC4-flagellin vaccinated group.

4.4.1.2. Vaccination using VC4-flagellin limited or reduced pH1N1 viral load

A reduced mean of GEC per mL was observed at 5 and 7 dpi in the vaccinated group compared to the unvaccinated group. Furthermore, two out of five animals cleared the virus at 5 dpi and a total of three out of five animals at 7 dpi. All pigs from the unvaccinated group showed viral RNA until the end of the experiment (*Figure 4-1A*).

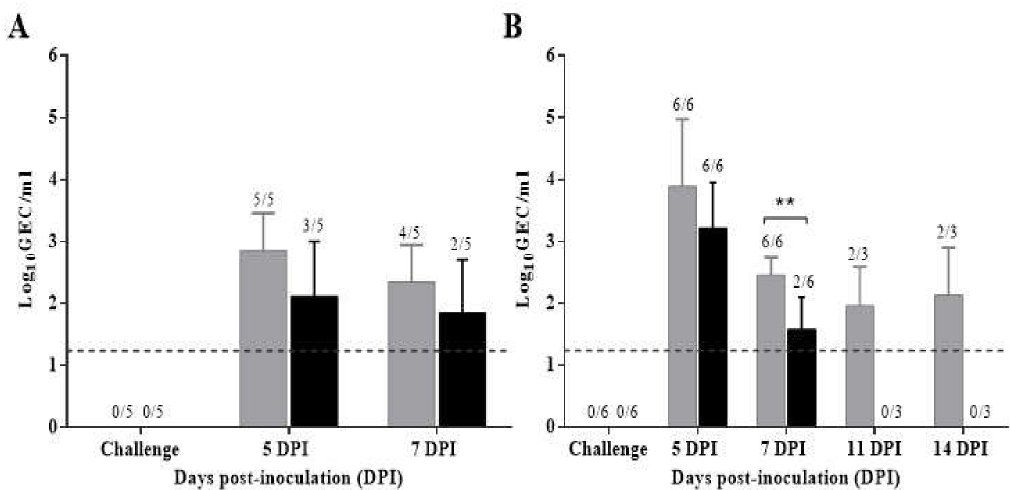

Figure 4-1. Viral RNA load in nasal swabs by RT-qPCR. (A) Mean of GEC per mL obtained from nasal swabs from seronegative pigs (Study I) collected at 0, 5 and 7 dpi (B) and from nasal swabs from seropositive animals (Study II) collected at 0, 5, 7, 11 and 14 dpi. Group A (unvaccinated animals) is represented grey bars and Group B (pCDNA3.1(+)-VC-4-flagellin vaccinated group) by black bars. Dpi, days post-inoculation. Dashed lines indicate the detection limit of the assays: 1.24 log_{10}GEC/mL. Error bars indicate the mean ± SEM.

4.4.1.3. Vaccination using VC4-flagellin induced higher IgG titers in sera against both the H1 and H3 subtypes

Pigs immunized with the VC4-flagellin vaccine manifested a boost in IgG antibodies against H1 and H3 subtypes in sera compared to unvaccinated groups (*Figure 4-2A and B*), being the peak at 35 PVD (*Figure 4-2A and B*). The increased antibody level was significant ($P<0.01$) for H1 subtype. In comparison to the H3 subtype, the increment in the antibody levels at 35 PVD was also significant, but with a higher *P* value ($P<0.05$). This difference could probably be attributed to one particular animal in this group (pig 10: vaccinated challenged with pH1N1) that, unlike the four other animals from the VC4-flagellin group, did not show seroconversion against H3 neither upon vaccination with VC4-flagellin nor after challenge with pH1N1 IV.

4.4.1.4. Vaccination using VC-4 flagellin promoted higher HI titers in sera against pH1N1

Likewise, to discriminate whether the antibodies obtained in sera could also block viral entry, we carried out an IHA against the pH1N1. Two of the five VC4-flagellin vaccinated pigs showed values ≥40 before challenge and, unexpectedly, one pig from the unvaccinated group (*Figure 4-3A*). Nevertheless, the differences among groups were more illustrative at 7 dpi when all pigs from the VC4-flagellin vaccinated displayed HI titers ≥40. By contrast, only two unvaccinated pigs (animal 11 and 12) obtained seroprotective titers (animal 1, 1:40; animal 12; 1:160) at 7 dpi (*Figure 4-3A*).

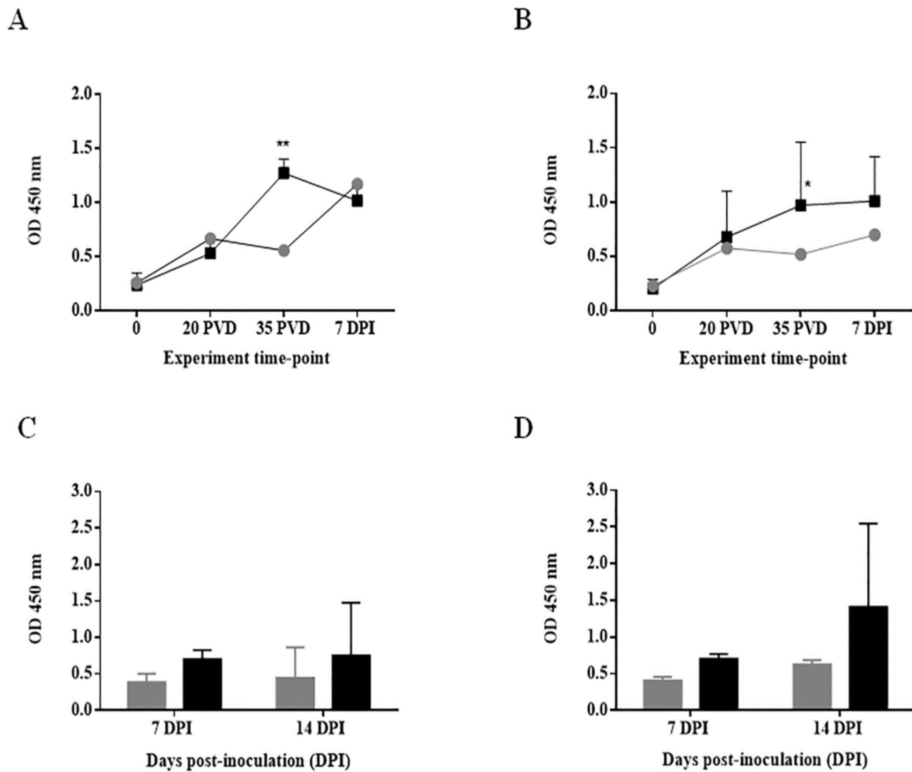

Figure 4-2. Serum antibody HA-specific IgG titers detected in sera and BALFs samples by ELISA test. Mean of serum IgG antibody levels detected at 0, 20 PVD and 35 PVD, and 7 DPI of Groups A and B (A) against HA from A/California/04/09(H1N1)pdm09, and (B) against HA from A/Aichi/2/1968(H3N2) are represented. Mean of BALFs IgG antibody levels detected in pigs sacrificed at 7 and 14 dpi of Groups A and B (C) against HA from A/California/04/09(H1N1)pdm09, and (D) against HA from A/Aichi/2/1968(H3N2). Grey circles/bars refer to group A (unvaccinated group), and black squares/bars refer to group B (pCDNA3.1(+)-VC4-flagellin vaccinated group). OD, optical density. PVD, post-vaccination days and dpi, days post-inoculation. Error bars indicate the mean ± SEM. Statistically significant differences between vaccine treatment groups (*P* value <0.05) are marked with *: *P*<0.05, **: *P*<0.01.

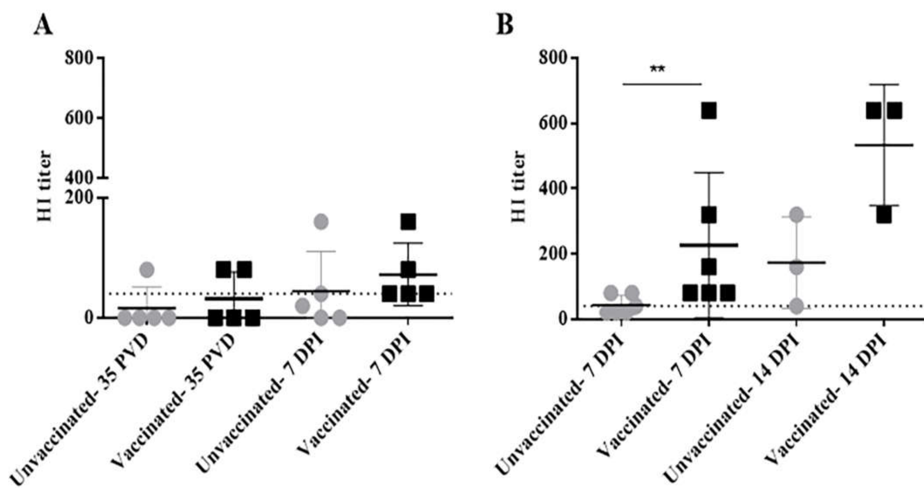

Figure 4-3. HI activity against pH1N1 from seronegative pigs (Study I) and against H3N2 from seropositive pigs (Study II). HI titers obtained with sera from unvaccinated (Group A) and vaccinated (Group B) pigs against (A) the pH1N1 from SIV-seronegative pigs (Study I) and (B) the SwH3N2 from SIV-seropositive pigs (Study II). Grey circles refer to group A (unvaccinated group) and black squares depict group B (pCDNA3.1(+)-VC-4-flagellin vaccinated group). HI, hemagglutination inhibition. DPI, days post-inoculation. Error bars indicate the mean ± SEM and statistically significant differences between vaccine treatment groups are marked with **: $P<0.01$.

4.4.2. Study II (SIV-seropositive pigs/challenged with SwH3N2)

4.4.2.1. Clinical and pathological evaluation

Clinical examination revealed that four animals of each group (unvaccinated and VC4-flagellin vaccinated) exhibited fever. These animals corresponded to pig number 1 (fever at 6 and 7 dpi), 2 (fever at the challenge day), 5 (fever at 3, 4 dpi) and 6 (fever from 3 to 7 dpi) from

the unvaccinated group. From the VC4-flagellin group, the pigs with fever were the number 7 (fever at 4 dpi), 10 (fever at 3 and 7 dpi), 11 (fever at 2, 5 and 7 dpi) and 12 (fever from 2 to 4 dpi). Referring to clinical signs, one unvaccinated pig (pig 1) was coughing at 3 dpi. Three pigs vaccinated with VC4-flagellin were also coughing: pig number 7 and 8 (both coughing at 3 dpi) and pig 11 (at 4 dpi).

Using ImageJ® analysis tools, the percentage of affected lung area of pigs were examined. Results revealed that two out of the three unvaccinated pigs (pig 1 and 5) had multifocal pulmonary cranio-ventral consolidation lesions: 4.11% and 2.93%, respectively, observed on the dorsal side of the lung. From the VC4-flagellin vaccinated group only one pig (pig 9) had dorsally (3.04%) and ventrally (2.61%) visible macroscopic lesions. Lungs collected at 14 dpi did not have lesions.

Intranasal inoculation with SwH3N2 caused a mild infection in all pigs and minor histopathological changes observable at necropsy. No differences in the severity of microscopic lung lesions of vaccinated and unvaccinated animals were recorded. Representatively, observations detected at 7 and 14 dpi from the second study are depicted in *Table 4-4*. Other pathological findings documented were one unvaccinated pig (animal 1) and two vaccinated pigs (animal 7 and 9) with suppurative bronchopneumonia. In addition, one immunized pig (animal 8) had fibrous pleuritis.

Table 4-4. Pathological microscopic score for all the animals from Study II based on BIP-compatible lesions. BIP was assessed by a semi-quantitative scoring (0-3, indicating lack of, mild, moderate or severe pneumonia lesions, respectively).

Group	Animal Id	Dpi	BIP scoring
A: Unvaccinated group	1	7	2
	2		2
	5		0.5
	3	14	1.5
	4		3
	6		0.5
B: pCDNA3.1(+)-VC4-flagellin vaccinated group	7	7	3
	9		2
	12		3
	8	14	0.5
	10		1
	11		2

Abbreviations: BIP= broncho-interstitial pneumonia; dpi= days post-inoculation; Id=identification.

4.4.2.2. Vaccination using VC4-flagellin limited or reduced SwH3N2 viral load

The mean of GEC from the VC4-flagellin vaccinated group was lower than the unvaccinated group at 4 and 7 dpi. Notably, four out of six VC4-flagellin vaccinated pigs cleared the virus at 7 dpi. Conversely, none of the unvaccinated group was able to clear the virus at 7 dpi ($P<0.01$) (*Figure 4-1B*). The unvaccinated-infected pigs continued shedding influenza virus up to 14 dpi (*Figure 4-1B*) whereas IV virus was not detected in VC4-flagellin vaccinated group (*Figure 4-1B*).

4.4.2.3. Vaccination using VC4-flagellin induced superior IgG titers in BALFs against both the H1 and H3 subtypes

The presence of specific antibodies against H1 and H3 was also examined in the BALF samples from seropositive animals. The average of IgG antibody OD values at 7 and 14 dpi of the H1 and H3 subtypes were higher in the vaccinated group (*Figure 4-2*C and *D*). Considering that the challenged virus in study II belonged to H3 subtype, enhanced antibody values against H3 were expected (*Figure 4-2D*) in comparison to H1 subtype. At 7 dpi, all H3 antibody OD values from the VC4-flagellin vaccinated group were higher than the ones of the unvaccinated group (*Figure 4-2D*).

4.4.2.4. Vaccination using VC4-flagellin promoted higher HI titers in sera against SwH3N2

HI results against the SwH3N2 evidenced that the VC4-flagellin vaccinated pigs had higher HI titers at 7 and 14 dpi than the unvaccinated animals. At 7 dpi, all pigs from the VC4-flagellin vaccinated group exhibited a positive HI titer (≥40) ($P<0.01$). Contrarily, only three out of six animals of the unvaccinated group remained with seroprotective titers ≥40. In addition, at 14 dpi, the inhibiting capacity of the three remaining VC4-flagellin vaccinated animals (animal 7, 1:640; animal 11, 1:320; animal 12, 1:640) was higher than the remaining three unvaccinated (animal 3, 1:40; animal 4, 1:320; animal 5, 1:160) (*Figure 4-3B*).

4.4.2.5. Vaccination using VC4-flagellin induced stronger IgA responses in BALF samples

The mucosal antibody response was investigated in the BALF samples. Against H1 subtype, vaccinated pigs elicited an increase in the IgA response in comparison to unvaccinated pigs at 7 dpi (*Figure 4-4A*). At 14 dpi, the same tendency was observed although the OD values of antibodies were less than at 7 dpi. At 7 and 14 dpi, the VC4-flagellin vaccinated pigs exhibited elevated IgA values compared to the unvaccinated pigs when analyzing IgAs against H3 subtype (*Figure 4-4B*).

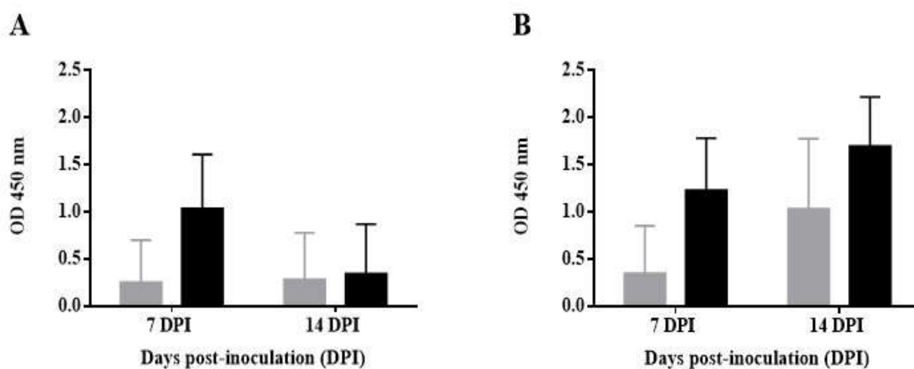

Figure 4-4. Antibody HA-specific IgA titers detected in BALFs samples from Study II. Mean of BALFs IgA antibody levels detected in pigs sacrificed at 7 and 14 dpi of Groups A and B (A) against HA from A/California/04/09(H1N1)pdm09, and (B) against HA from A/Aichi/2/1968(H3N2). Grey bars refer to group A (unvaccinated group), and black bars refer to group B (pCDNA3.1(+)-VC4-flagellin vaccinated group). OD, optical density. PVD, post-vaccination days and DPI, days post-inoculation. Error bars indicate the mean ± SEM.

4.4.2.6. Vaccination using VC4-flagellin promoted higher SNT titers in BALFs

After determining that the VC4-flagellin vaccinated group displayed higher antibody titers in the BALFs compared to the unvaccinated pigs, we were intrigued to find wether the elicited antibodies could neutralize the virus. VC4-flagellin vaccinated pigs showed higher mean values of seroneutralizing antibody titers in BALFs than the unvaccinated pigs at 7 and 14 dpi (*Figure 4-5*). Moreover, at 7 dpi, all animals from the vaccinated group manifested seroneutralizing titers (animal 6, value 1:20; animal 8, value 1:20; animal 9, 1:60). None of the unvaccinated animals developed seroneutralizing antibodies. At 14 dpi, two out of three unvaccinated pigs achieved seroneutralizing titers (animal 3, 1:60; animal 4, 1:320), but to lesser extent than immunized pigs (animal 8, value 1:320; animal 11, value 1:1280; animal 12, value 1:120).

4.4.2.7. Vaccination using VC4-flagellin promoted higher percentage of double-positive T-cells CD4-CD8

A numeric increment of phenotypic population of T-cells CD4 SP (single positive) and T-cells CD8 SP was observed in the vaccinated group in comparison to the unvaccinated group prior to challenge and such increase was significant for CD4-CD8 DP (double positive) cells ($P<0.001$) (*Figure 4-6*).

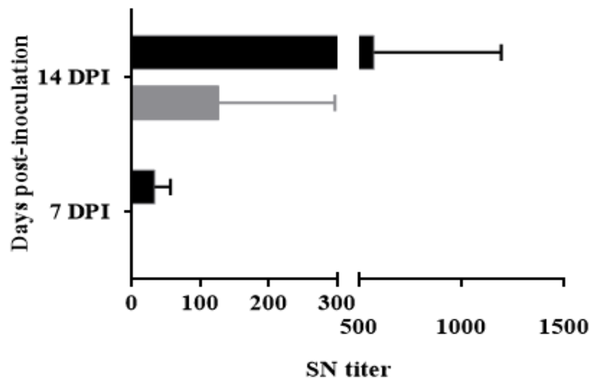

Figure 4-5. Seroneutralization (SN) titers detected in BALF samples from Study II by seroneutralization assay. Mean of seroneutralization titers detected at 7 and 14 dpi of Groups A and B against the A/swine/Spain/003/2010 H3N2 IV challenged virus. Grey bars refer to group A (unvaccinated group) and black bars depict group B (pCDNA3.1(+)-VC-4-flagellin vaccinated group). SN, seroneutralization. DPI, days post-inoculation. Error bars indicate the mean ± SEM.

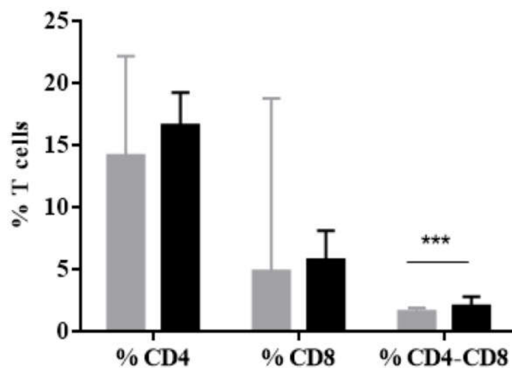

Figure 4-6. Flow cytometry from PBMCs isolated at 35 PVD (Study II). Mean of the percentages of T cells CD4, CD8 and CD4-CD8 DP from unvaccinated (Group A) and vaccinated (Group B). Grey bars refer to group A (unvaccinated group) and black bars depict group B (pCDNA3.1(+)-VC-4-flagellin vaccinated group). Error bars indicate the mean ± SEM and statistically significant differences between vaccine treatment groups are marked with ***: $P<0.001$.

4.5. Discussion

Each year there is a must to identify the strains of influenza A and B viruses that will be circulating in the next season, in order to manufacture the best option for seasonal influenza vaccines. Consequently, universal vaccines against influenza virus making use of highly conserved epitopes or proteins have been investigated during years. Thus, the present studies describe how the combination of several conserved HA-peptides in a DNA approach constitutes a potential influenza vaccine for use in conventional pigs.

To generate further information of this novel universal vaccine prototype, two experiments were designed. Study I was developed under a more favorable scenario: SIV-seronegative pigs challenged with a homologous virus (pH1N1) for the HA-peptide: SLPFQNIHPITIGKCPKYVKSTKLRLATGLRNV. By contrast, Study II was designed to evaluate vaccine efficacy when the MDA were present and a heterologous virus was challenged (SwH3N2), representing a more unfavorable scenario. Subtypes H1 and H3 were the ones chosen to be inoculated in view of being the most prevalent subtypes circulating in swine herds.

All HA-peptides included in the DNA approach presented were selected by means of the ISM platform, which is based on virtual spectroscopy [362,418]. HA-peptides were selected since well-matched antibodies to the HA can block an influenza virus infection and also contribute to the clearance of the virus from the lungs [426,427]. Furthermore, to obtain an improved presentation of the antigens and to enhance

immunogenicity, flagellin was fused to the conserved HA-peptides. Such approach should result more potent and efficacious since incorporates TLR-ligands (such as flagellin) [428]. The flagellin ligand fused to an antigen of interest has been shown to yield vaccines able to induce higher IgG responses by means of improving APCs functions [428–432]. Moreover, flagellin induces TLR5 signaling and this pathway triggers the recruitment of granulocytes and macrophages/monocytes in the respiratory airways. Subsequently, the production of cytokines and chemokines required to initiate strong humoral and cellular responses is primed [419]. This characteristic is coherently related to the flagellin stimulation of monocytes to produce IL-10 and TNF-α cytokines [433], of the NK cells to deliver IFN-γ and α-defensins and of the T cells to proliferate and produce cytokines and chemokines (e.g. IL-10, IL-8 and IFN-γ) [434]. Furthermore, it is a usual practice to include flagellin (FliC) as an adjuvant in novel universal vaccine approaches to face influenza viruses [428,435–439]. It is reported in those studies that conserved influenza epitopes linked to the flagellin either at the N or C terminus, or inclusive in its hypervariable region, did not impair the proper binding of the flagellin ligand to the TLR5.

Due to the final length of the construct and since DNA vaccines can provide the activation of both humoral and cellular responses, the construct VC4-flagellin was reverse-translated into a pCDNA3.1(+) plasmid. Moreover, DNA-based vaccines may cross-protect when facing heterologous swine influenza viruses without being as hazardous as the attenuated-type [440]. Besides, a suitable delivery platform of the vaccine was sought. At the very end, an intradermal delivery approach seems to

promote higher antibody titers than the intramusucular route [400–402]. The overall approach used was also selected because the optimal doses of DNA plasmid (moles) to be used were already described [386].

Thus, in this work, the VC-4-flagellin construct administered intradermally mixed with Diluvac Forte® adjuvant was tested as a vaccine candidate in pigs with or without MDA. Diluvac Forte® was mixed with the vaccine formulation but also was administered to unvaccinated pigs. Intriguingly, the VC4-flagellin vaccinated pigs demonstrated a reduction/clearance of the viral shedding in days 5 and 7 in Study I (seronegative animals, pH1N1 challenged) and in Study II (seropositive animals, H3N2 challenged). Therefore, we anticipate that MDA antibodies were not an apparent obstacle for the vaccine to reduce viral shedding and, eventually, to potentially block the viral transmission. Surprisingly, unlike unvaccinated pigs, seropositive vaccinated pigs did not shed the challenge H3N2 virus at 11 or 14 dpi although they were constantly in contact with unvaccinated infected animals. This fact indicated that vaccination with VC4-flagellin not only limited the virus shedding from vaccinated pigs but, most possibly, also prevented re-infection in a contaminated environment. Further studies are needed to prove this assumption using contact infection experiments in larger groups.

Previous to challenge, the vaccinated animals of Study I could recognize both the H1 and H3 IV-subtypes. In consequence, seroconversion and a cross-protecting effect against the two IV-subtypes were demonstrated. Apart from the post-vaccination seroconversion, the HI titers of ≥40 are

considered to constitute a marker that correlates in vitro with protection [288,423–425]. Analyzing HI titers against pH1N1 (Study I, SIV-seronegative pigs/challenged with pH1N1) seroprotective antibodies could be found in 2 out of 5 pigs prior to the challenge. Noticeably, all the five vaccinated pigs manifested seroprotective titers at 7 dpi. Moreover, seropositive vaccinated pigs elicited higher HI values against SwH3N2 at 7 and 14 dpi than the unvaccinated group, thus confirming that MDA were not interfering with the vaccine effect.

Nevertheless, the vaccine failed to reduce the influenza clinical signs and lung lesions. Indeed, no relevant differences were found between groups. Also, it is vital to take into consideration that the clinical picture and disease caused by the pH1N1 in pigs it is generally mild and subclinical [441,442]. In fact, in Study I very little number of pigs manifested fever or any clinical sign. Also, we assume that pig 7 was suffering from a pathological disorder before being transported to the experimental unit, since had fever before challenge and bacterial colonies were present in its lungs.

IgG and IgA antibody titers against H1 and H3 subtypes and their seroneutralizing effect against the challenge virus were analyzed in the BALFs of Study II. Overall, IgG antibodies were to a higher rate against both the H1 and H3 subtypes in DNA-vaccinated animal group than the unvaccinated group at 7 and 14 dpi. The major difference among groups was observed at 14 dpi against the H3 subtype. Assuming that the challenged virus in Study II was an H3N2 virus, elevated H3 antibodies were expected. Furthermore, a stronger seroneutralizing effect could be

observed in the BALF samples obtained from the vaccinated pigs than in the samples from the unvaccinated ones. IgA antibodies were also to a higher rate against both the H1 and H3 subtypes in the vaccinated group than the unvaccinated one at 7 days pot-infection. A comparable tendency of IgA antibodies was observed in BALFs collected at 14 dpi against H3 subtype, indicating an enhanced mucosal immune response induced after VC-4-flagellin vaccination in pigs. This is in line with studies claiming that mucosal immune response is necessary for the design of an universal influenza vaccine as it is the first line of defense against IVs [443,444]. We consider that mucosal immune response (IgA) elicited after VC-4-flagellin vaccination in pigs might have contributed in limiting virus shedding and cross-protection, as reported previously also by others [445,446].

VC-4-flagellin vaccination in pigs, interestingly, also showed an increase in the frequency of the CD4-CD8 DP T cells subset. In fact, results from an earlier report [268] evidenced that some CD4-CD8 DP T cell subset likely belong to effect or memory T cells (T_{EM}). This data was only analyzed at pre-challenge time point and not followed after the challenge. Further investigation would be necessary to ultimately define the role of the CD4-CD8 DP T cell subset in protection and clearance of IV after VC-4-flagellin vaccination in pigs.

Our results strongly indicate that HA specific immune response effectively contributed to control influenza infections after VC-4 flagellin vaccination without MDA apparent interference. Promoting a solid systemic mucosal response and blocking viral transmission by reducing

earlier the viral shedding were the key outcomes in the VC4-flagellin vaccination approach. Therefore, VC4-flagellin as such maybe an interesting vaccine candidate against H1 and H3 subtypes. However, more studies are crucial in order to vaccinate with VC4-flagellin and mitigate clinical manifestations and lung pathology.

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CHAPTER V

Study III: Conserved HA-peptides expressed along with flagellin in *Trichoplusia ni* larvae protects chicken against intranasal H7N1 HPAIV challenge.

5. Study III

"Conserved HA-peptides expressed along with flagellin in *Trichoplusia ni* larvae protects chicken against intranasal H7N1 HPAIV challenge"

5.1. Abstract

Given the outbreaks of IVs infection reported in the human population, H5 and H7 IV subtypes have been regarded as potential candidates for an upcoming IV pandemic. The immunization of poultry where such IVs are endemic is crucial to prevent possible zoonoses. Up to date, inactivated, live-attenuated and recombinant vaccine types have been licensed for poultry. Recombinant vaccines provide several advantages over conventional vaccines used. Our group has been focused on conserved HA-epitopes as potential vaccine candidates to obtain multivalent and broad immune responses against IV subtypes infection. In this study, two conserved epitopes (NG-34 and CS-17) fused to increase the immune response with the TLR5 ligand, the flagellin, were produced in a Baculovirus platform based on *Trichoplusia ni* larvae as living biofactories. The efficacy of the experimental vaccine formulation was evaluated against a highly pathogenic heterologous H7N1 IV subtype influenza virus challenge in chickens. The vaccine consisted insoluble extracts obtained from larvae expressing the construct, designated as flagellin-NG34/CS17. The flagellin-NG-34/CS17 vaccine was able to protect the vaccinated chickens and was effective in blocking viral shedding orally and cloacally. A single fatal outcome and virus shedding in 2 out of 12 of the vaccinated chicken were observed. Furthermore, no apparent clinical signs were monitored in 10 out of 12 vaccinated and virus challenged individuals. The mechanism of protection conferred by flagellin-NG34/CS17 vaccine against an H7N1 HPAIV infection in chicken is currently under investigation.

5.2. Introduction

H7 subtype has become highly prevalent in avian species with some strains considered of having pandemic potential due to several human outbreaks caused in the past years. Though being the causative agents of severe poultry outbreaks [447–449], H7 barely infected humans [450]. In 2003, a H7N7 IV subtype emerged in the Netherlands infecting at least 89 humans. One of those ended up with a fatal case due to severe pneumonia [448,451]. Similarly, in 2013, an outbreak in China with the H7N9 subtype produced high morbidity and mortality in the population. The H7N9 subtype infected over 1625 humans with a fatality rate of almost 40% [452]. Genetic changes observed in the strain such as the specific lysine at the 627 position of the polymerase basic protein 2 (PB2), suggest that adaptation to mammals could have occurred [453]. Moreover, other subtypes such as H7N2, H7N3, H7N4 have also been detected and related to sporadic human infections [81,454–456].

Controlled vaccination campaigns in some endemic countries may prevent possible outbreaks caused by AIVs in the poultry. At present, most of the poultry influenza vaccines are inactivated-typed, many of them using reverse genetics to alter the cleavage site of HPAIV strains [314]. Nevertheless, alternatives have been sought to tackle the shortcomings of such vaccine for example failure to induce cellular/mucosal immune response, their egg-based manufacture which takes a minimum of 3-6 months time [457–460] and their lack of protection across subtypes. Furthermore, with conventional vaccines it is

hard differentiating infected from vaccinated animals [461]. To overcome these drawbacks, many current AIV vaccines are based on LAIVs [324] or have used recombinant IV proteins [462–465].

LAIVs against AIVs have been widely licensed using distinct viral vectors however, some hurdles such as potential reassortment of the vaccine strains with the circulating strains or the demand to be cultured in egg-based technologies have limited their advance [466]. In contrast, recombinant IV vaccines do not possess such limitations. Concretely, the Baculovirus are non-pathogenic insect viruses which do not replicate and do not harm mammalian cells [467,468]. Thus facilitating their production without requiring high-level biosafety facilities [469]. Other advantages they procure are the high expression yields of recombinant proteins and their sustainment of the native conformation since they can be post-tranlationally modified [470]. On the whole, their safety and their cost-effectiveness to scale-up, reducing development time, have contributed as attractive and demanding IV vaccine vehicles [467,468,471].

In the present study, Baculovirus were selected as suitable influenza vaccine technology platform. As biofactories, insect larvae from *Trichoplusia ni* (*T.ni*), the natural host for the commonly used *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) vector, was used as an excellent host insect for recombinant production [472]. Besides, a novel baculovirus vector expression cassette, TopBac®, was used to

optimize the baculovirus expression vector system (BEVS) system by conferring enhanced viability, integrity of the recombinant proteins and up to 4-fold increase in recombinant protein production [473,474].

Hemaglutinin-based vaccines using the baculovirus approach have been widely investigated [469,475–478]. Safety, immunogenicity and efficacy were demonstrated with those approaches in human [304,477]. Hemagglutinin is the mostly selected recombinant protein from IVs due to its potent antigenic properties favoring antibodies which can prevent IV infection [426,427]. However, the antigenic changes that the HAs constantly undergo make probable that the IV can eventually evade the host immune response [219]. Such inconvenient occurs with the current seasonal inactivated influenza vaccines and required to be annually updated. By selecting conserved viral targets as immunogens, it is sought to obtain universal vaccines which confer broad protection against several subtypes [331,414–416]. Those vaccines should confer protection against epidemic viruses and even ever-changing viruses.

Our research group has been dedicated to use conserved-HA epitopes as vaccine candidates [331]. Thus, two *in silico* predicted conserved epitopes of the HA were selected as vaccine candidates. One of them (NF-34) already used in pig model, was used but modified in two aminoacid positions (NG-34) (See *Table 3.2.*; *"Chapter III, Study I*). An H5 conserved epitope corresponding to the cleavage site of HPAIVs viruses (CS-17) was additionally linked to NG-34. Both epitopes were fused to phase 2

flagellin from *Salmonella typhymurium* to generate a more potent vaccine [428,430,479]. The final formulation (flagellin-NG34/CS17) was produced in the Baculovirus system and the soluble extracts obtained with the homogenization of larvae expressing flagellin-NG34/CS17 were used as a vaccine against IV infection in chicken.

The final goal of this work was to study the vaccine efficacy of soluble extracts expressing flagellin-NG34/CS17 construct against a heterologous virus challenge in chicken.

Our results indicate that chickens immunized twice with flagellin-NG34/CS17 were protected against a lethal challenge with a heterologous influenza A virus (H7 subtype). Immunized animals showed no clinical signs of infection and did not shed virus. An increased IgM/IgY was also evident in some of the vaccinated chicken.

5.3. Materials and methods

5.3.1. Immunogen and plasmid construction

The prediction of the peptides belonging to the HA protein (HA-epitopes) was developed by means of ISM approach [362,418]. *Table 5-1* depicts the aminoacidic sequence of the two conserved HA-peptides (NG-34 and CS-17) used in the vaccine approach, which are connected

by the linker sequence: SSGSSGSSGSS. Moreover, at the N-terminal site of that sequence, the complete sequence of *Salmonella typhimurium* phase 2 flagellin (GenBank: AAC94993.1) was inserted as an adjuvant. The whole *cDNA* was chemically synthesized (GenScript, Piscataway, NJ, USA) following various requirements. Firstly, the complete sequence was introduced in between the *BamH*I and *Xba*I sites of the pcDNA3.1(+) vector with the flagellin sequence sited at the N-terminal site of the NG34/CS17 epitopes and delimited by *Xho*I and *Cla*I restriction sites. Moreover, a 6-His tag was added at the C-terminal site of the NG34/CS17 sequence delimited by *EcoR*I and *Xba*I sites for the later protein purification (*Figure 5-1*). Finally, the codon usage from *Bombyx mori* was utilized to optimize the sequence in order to improve the epitope expression in the *Trichoplusia ni* larvae by means of: (http://www.ebi.ac.uk/Tools/st/emboss_backtranseq/). The whole sequence of interest was excised from the pCDNA3.1(+) vector with *BamH*I and *Xba*I enzymes and inserted in the same restriction sites of the TopBac3.2® vector (Algenex, Madrid, Spain), the donor plasmid with an expression cassette characterized for being formed by baculovirus-derived regulatory elements which work in cascade [362,418].

Table 5-1. Amino acid sequences from the HA-epitopes: NG-34 and CS-17, used in the vaccine approach.

HA-epitopes	Aa positions	Consensus virus subtype	GenBank Id
NSDNGTCYPGDFIDYEELREQLSSVSSFERFEIF (NG-34)	59-92	pH1N1	ACS36215
PQRERRRKKRGLFGAIA (CS-17)	337-357	H5N1	AAC32098.1

Aa positions referenced are in accordance with the reference cited from the GenBank database. Abbreviations: aa= amino acid; HA= hemagglutinin; Id=identification

Figure 5-1. Illustration of the sequence of interest and restriction sites inserted in the pCDNA3.1(+) vector.

5.3.2. Construction of the recombinant baculovirus

The resulting donor plasmid, named as pTB3.2 flagellin-NG34/CS17, was used to generate the corresponding recombinant baculovirus (rBac) by using the Bac-to-Bac® Baculovirus Expression System (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions. The expression cassette was integrated into the baculovirus genome (bacmid) contained into the DH10Bac™ chemically competent *E.coli* cells (Invitrogen, San Diego, CA, USA) through Tn7 transposition functions. The recombinant bacmid was transfected into Sf21 cells using

CellfectinII® Reagent (Invitrogen, USA) and following the manufacturer's instructions. After 96 hours post-transfection the culture medium containing the rBac was harvested. To reach high-titers of the baculovirus stock, the rBac obtained was propagated and amplified again in Sf21 insect cells for 96 hours. Baculovirus from the second passage were titrated in duplicate by a standard plaque protocol in 6-well plates containing Sf21 cells according to the protocol described at the Bac-to-Bac® Baculovirus Expression System user guide (Invitrogen, No. MAN0000414). Virus titer was determined as plaque-forming units (PFU). Eventually, baculovirus stocks were stored at 4°C for daily use and -80°C for long-term storage.

5.3.3. Infection of insect larvae

Trichoplusia ni (*T. ni*, Cabbage looper) larvae were reared following a previously described methodology [480–482]. *T. ni* larvae in stage of fifth-instar larvae (last instar larvae before pupation) were sedated by incubation at 4°C for 15 minutes and then were injected near the proleg (forward of the body cavity) with 5 µL of the recombinant baculovirus diluted with *T. ni* medium formulation Hink (TNM-FH) medium (PAN Biotech GmbH, Germany) to reach a dose of 5.5×10^6 pfu/mL. Inoculated larvae were kept at 28°C for 72 hours in growth chambers and then frozen at -20°C until processed.

5.3.4. Protein extraction, determination and quantification

Total soluble protein (TSP) extract from the frozen *T. ni* larvae infected with the rBac was obtained by mixing 16 mL of a extraction buffer (PBS 1X, Brij®35 0.1%: Scharlau, Barcelona, Spain; BR00171000, phenylmethylsulfonyl fluoride (PMSF) 1mM and 1,4-dithiothreitol (DTT) 5 mM per gram of larvae biomass and homogenizing twice using a Bag Mixer blender (www.interscience.fr) for 2 min (total protein extract, TP). Later, a step of sonication (Bandelin Sonopuls 3200HD, tip TT13, amplitude 50%) with three cycles of 15 seconds and a break in between in ice of 45 seconds was carried out followed by a centrifugation step at 2000 g for 30 minutes at 4ºC. Supernatant was then filtered with a 22 μm Miracloth filter paper (Calbiochem®, Merck, UK) (total soluble protein extract, TSP). Samples were resolved in 12% SDS-polyacrilamide gels both for Coomassie blue staining and transference onto a nitrocellulose membrane (Bio-Rad, USA) to perform a Western blot analysis. Transferred membranes were blocked in PBS-0.1% Tween 20 (PBST)-4% skim milk overnight at 4ºC. They were then incubated at room temperature (RT) for 1 h with an anti-His monoclonal antibody (mAb) (Clontech) diluted 1:2000 in PBST. After two washes of 15 min with PBST, a second incubation was made using an anti-mouse IgG horseradish peroxidase (HRP)-labeled conjugate (GE Healthcare, USA) diluted 1:2000 in PBST for 1 h. All membranes were developed using enhance chemiluminescence (ECL) reagent. Images were captured by ChemiDoc™ XRS Gel Imaging System (Bio-Rad, USA) and analyzed using the ImageLab™ Software (Bio-Rad, version 6).

Total protein concentration of the extract was determined by the Bradford method using a protein assay kit (Bio-Rad, USA) [483] and the quantification of the flagellin-NG34/CS17 expressed in *T. ni* larvae was carried out by measurement of band densitometry in Western Blot images with the Image Lab ™ Software (Bio-Rad, version 6) using a standard curve with a known quantified 6xHis-tagged protein (ASFV p54 protein).

5.3.5. Cells, virus and antigen

*Spodoptera frugiperda Sf*21 cell line (Invitrogen, San Diego, CA, USA) was cultured at 27 °C in TNM-FH medium (PAN Biotech GmbH, Germany) supplemented with 10% heat-inactivated fetal bovine serum (PAN Biotech GmbH) and gentamicin (50 µg/ml) (PAN Biotech GmbH) as antibiotic. Cell viability was calculated by means of trypan blue staining and determined with the frequency (%) of living cells with respect to the total number of cells.

MDCK, ATCC CCL-34 cells were used for seroneutralization assays. Cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-glutamine was required for the MDCK proper culture.

The H7N1 HPAI virus strain used in this study was kindly provided by Dr. Moreno from the Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER) and corresponds to a fifth passage A/chicken/Italy/5093/99. It was propagated in 11-day-old

embryonated specific pathogen free (SPF) chicken eggs, collected their allantoic fluid and 0.45 µM filtered. Virus was then diluted tenfold in phosphate buffer saline (PBS) for titration in SPF eggs according to the method of Reed and Muench [387] and, later on, diluted in PBS to obtain a dose of $10^{4.5}$ embryo lethal dose 50% (ELD_{50}) in 0.1 mL (100 µL).

The H7 protein used was acquired from Sino Biological (cat no.40169-V08H1), which corresponds to the strain: A/turkey/Italy/4602/99 (H7N1).

5.3.6. Ethics statement

All chickens were retained and handled according to procedures reviewed and approved by the IRTA's Ethics Committee for Animal Experimentation and the Animal Experimentation Commission from the Autonomous Community of Catalonia Government. Additionally, the study was in accordance with the Directive, UE 63/2010; the Spanish Legislation, RD 53/2013; the Catalan Law 5/1995 and Decree 214/1997.

5.3.7. Experimental design

Thirty-four SPF chickens (VALO BioMedia, Salamanca, Spain) were hatched and randomly divided in three different negative isolators with HEPA-filtered air under BSL-3 containment conditions at IRTA-CReSA. Throughout the experiment, chickens were feed with food and water *ad libitum*. One isolator (group A= animals 1-12) was used as negative control and individuals were subcutaneously sham-vaccinated with PBS mixed 70:30 with Montanide™ 71 VG ISA. Chickens in the second isolator (group B= 13-24) were vaccinated at 10 days of age with flagellin-NG34/CS17 and after 21 days of interval a boost vaccination followed. In parallel, in the third isolator (group C=25-34) chickens were immunized only once with flagellin-NG34/CS17 (without receiving boost vaccination). The animals from Group B and C were subcutaneously vaccinated with 0.250 mL/animal of soluble larvae extracts containing 15 µg/mL of flagellin-NG34/CS17 and mixed in a ratio of 70:30 with Montanide™ 71 VG ISA. Two weeks later, all chickens (group A-C) were intranasally inoculated with 100 µL diluted infectious allantoic fluid containing $10^{4.5}$ ELD_{50} H7N1 HPAIV. Two days pre-challenge onwards all the chickens were monitored daily for flu-like clinical signs. The experiment terminated at 10 days post-infection, when all the remaining chickens were euthanized. Oropharyngeal (OS) and cloacal swabs (CS) samples were collected at 1, 2, 3, 5, 7 and 9 dpi and, in addition, blood sampling to collect sera in tubes without anticoagulant was undertaken previous to the challenge (33 PVD) and at 10 dpi. All the samples gathered were stored at -80ºC until its use.

5.3.8. Pathological assessment

Daily monitoring for clinical signs was carried out according to OIE guidelines [139] and semi-quantitative scoring: healthy (0), sick (1), severely sick (2), moribund or dead (3) was established. Chickens presenting one of the potential flu-clinical signs were classified as sick (1) chickens manifested two or more of the potential flu-clinical signs were classified as severely sick (2) and were euthanized by inoculating intravenously sodium pentobarbital in a dose of 100 mg/kg (Dolethal®, Vétoquiunol, France). The potential flu-clinical signs were the following ones: respiratory involvement, depression, diarrhea, edema of the face and/or head, cyanosis of the exposed skin or wattles and nervous signs.

5.3.9. Quantitative real time (RT-qPCR)

All swab samples (OS and CS) were examined for viral RNA quantification. Viral RNA was extracted using the kit NucleoSpin RNA isolation kit (Macherey-Nagel GmbH&CoKG, Düren, Germany) and a 99 bp fragment of the M gene amplified as already described [421] in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). The detection limit of the technique was 0.84 log_{10}GEC/mL viral RNA copies/sample.

5.3.10. H7-specific ELISAs

ELISA plates were coated with 2 µg/mL of H7 antigen diluted in 50 mM sodium bicarbonate buffer and incubated overnight at 4ºC. Then, plates

were blocked for 1 hour at 37ºC with 100μl/well 3%BSA/PBS1x. Diluted 1:100 chicken sera (50μl/well) were later incubated during 1 hour at 37ºC. Plates were then four times washed with 0.5%Tween20/PBS 1x and incubated 45 minutes at 37ºC with either diluted 1:50000 HRP-conjugated goat anti-chicken IgY (ab6877, Abcam) (50μl/well) or diluted 1:50000 HRP-conjugated goat anti-chicken IgM (ab112813, Abcam). After four more washing steps, 50 μl/well of TMB substrate solution was dispensed and, after 10 minutes time protected from light stopped with 1 N H_2SO_4. Plates were finally read at an OD of 450nm. Samples were evaluated in duplicates. Positive H7N1 and negative control anti-serums (GD Animal Health, Deventer, The Netherlands) were included in each plate.

5.3.11. Inhibiton of the hemaggluatination assay (IHA) and serum neutralization test (SNT)

HI tests were developed according standard protocols [139]. Briefly, sera were incubated with 4 HA units of the H7N1 challenged strain and the sera-virus mixture was further incubated with 1% cRBCs. Positive H7N1 and negative control anti-serums (GD-Animal Health, Deventer, The Netherlands) were included in each plate. All samples were evaluated in duplicates. HI titers were defined as the highest sera dilution where inhibition of the hemagglutination was detected.

For SNT, plates containing $5*10^4$cells/well were seeded and incubated overnight in order to achieve confluence. Chicken inactivated sera

samples (56ºC for 30 minutes) were serially diluted two-fold up to 1: 2,560 and incubated with the H7N1 virus (100 $TCID_{50}$/50 µL) during 2 hours at 37ºC and in a 5% CO_2 atmosphere. Serum-virus mixtures were then added to PBS 1X washed MDCK cells. Cytopathic effect (CPE) was monitored after 7 days. SNT titers were expressed at the highest dilution of serum showing CPE. Media controls (no virus) and virus controls (no serum) were included in each plate. Samples were evaluated in duplicates. Positive H7N1 and negative control anti-serums (GD Animal Health, Deventer, The Netherlands) were included in each plate.

5.3.12. Statistical analysis

The significance between the survival curves was determined by Kaplan-Meier survival analysis with log-rank (Mantel-Cox) test. Normality assessment was used in the rest of analysis, with a Shapiro-Wilk test followed by: Student's t-test (when data was normally distributed) or Wilcoxon test (when data was non-normally distributed). Differences among groups were considered statistically significant when the *P* values were less than 0.05 and are represented with: $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***) and $P<0.0001$ (****). R statistical software was used for developing all the statistical analysis (http://cran.r-project.org/).

5.4. Results

5.4.1. Production of recombinant flagellin-NG34/CS17 in *T. ni* larvae

Fifth instars *T. ni* larvae were inoculated with 27500 pfu of the TB3.2 flagellin-NG34/CS17 baculovirus and total soluble protein extract was obtained after 72 hour of infection and analyzed by SDS-PAGE and Western blot (*Figure 5-2*A). Flagellin-NG34/CS17 recombinant protein was detected by Western blot using an anti-6x His-tag monoclonal antibody as a major band of about 65 kDa. All the recombinant protein produced was solubilized with the conditions used for the extraction. For immunization experiments, the amount of flagellin-NG34/CS17 antigen in the soluble larvae extract was quantified by Western blot using a standard curve with a 6xHis-tagged protein (*Figure 5-2B*). Concentration was determined in 28.6 µg of flagellin-NG34/CS17 per ml of TSP larvae extract.

5.4.2. Immunization with flagellin-NG34/CS17 protected chickens against heterologous influenza virus challenge.

More than 91% (11/12) of the chickens vaccinated twice with flagellin-NG34/CS17 survived against a challenge with H7N1 influenza virus until the end of the experiment. In contrary, only two animals (16.6%) survived from the control group. Animals which received only one vaccination also succumbed to infection and, at the end of the experiment, only two animals (22.2%) remained alive (*Figure 5-3*).

Chickens vaccinated twice remained healthy and only one animal (number 15) died on day 6 after challenge. Chickens in the control group showed signs of disease and the majority of animals died between 5 and 8 dpi. Chickens which received only one vaccination died also between 5 and 8 dpi (*Figure 5-3*).

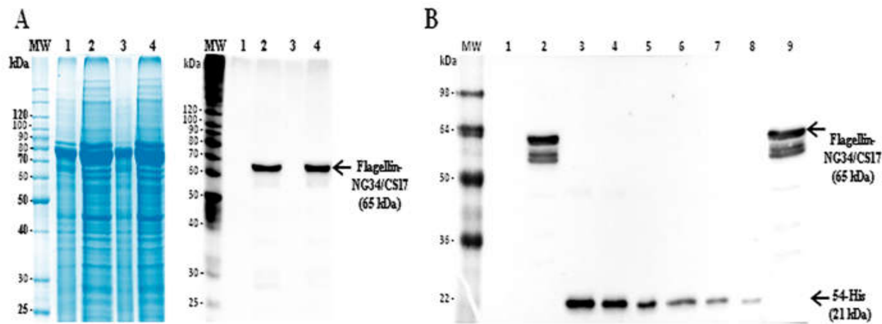

Figure 5-2. Production of recombinant flagellin-NG34/CS17 protein in *T. ni* larvae (A) Coomassie blue staining (left) and anti-6xHis-tag Western Blot (right) of protein extracts (10µl loaded/lane) obtained from empty rBac TB3.2-Ni (a recombinant TopBac baculovirus without a gene of interest) (lane 1: TP, lane 3: TSP) or rBac flagellin-NG34/CS17 (lane 2: TP, lane 4: TSP) infected *T. ni* larvae run in a 10% SDS-PAGE gel. Arrow indicates the band corresponding to flagellin-NG34/CS17 protein in the Western Blot image. MW: BenchMark™ Unstained Protein Ladder (Invitrogen). **(B)** Quantification by anti-6xHis-tag Western Blot of the amount of flagellin-NG34/CS17 protein contained in the soluble larvae extract. Lanes: (1) TSP extract from empty rBac TB3.2-Ni infected *T. ni* larvae (5µl loaded), (2 and 9) TSP extract from rBac TB3.2 flagellin-NG34/CS17 infected *T. ni* larvae (5µl loaded), (3 to 8) p54-His standard curve (150, 100, 50, 40, 20, 10 ng/lane, respectively). Arrows indicate the bands corresponding to flagellin-NG34/CS17 and p54-His proteins. MW: See Blue Plus2 Prestained Ladder (Invitrogen).

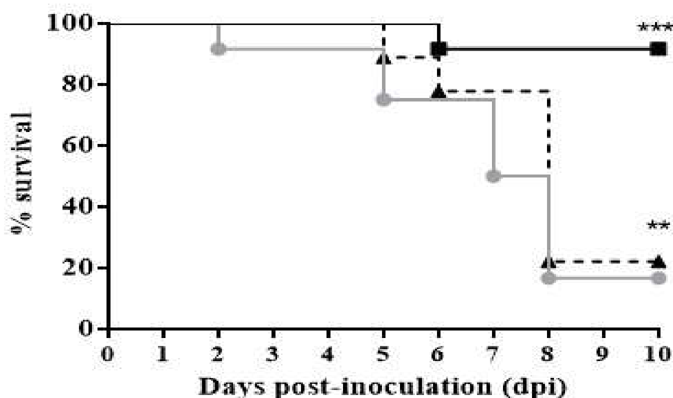

Figure 5-3. Survival curves of unvaccinated and once/twice flagellin-NG34/CS17 vaccinated chicken upon challenge with 0.1mL of $10^{4.5}$ELD$_{50}$ of a H7N1 HPAIV. Survival rates (%) of the three groups are depicted upon challenge with A/chicken/Italy/5093/99 until 10 dpi. Group A (unvaccinated animals) is depicted by grey circles, Group B (flagellin-NG34/CS17; twice vaccinated animals) by black squares and Group C (flagellin-NG34/CS17; once vaccinated animals) by black triangles. Statistically significant difference concerning survival among Group A and Group B ($P<0.001$) is depicted as ***: The significant difference ($P< 0.01$) among Group B and Group C is depicted as: **.

5.4.3. Flagellin-NG34/CS17 immunization saved chickens from flu-like clinical manifestations

No clinical signs of disease were observed in majority of chickens vaccinated twice with flagellin-NG34/CS17. Only one chicken showed flu like symptoms (apathy) and was euthanized at 6 dpi for ethical

reasons. One more chicken from the flagellin-NG34/CS17 vaccinated group also manifested signs of sickness at 8 dpi (ruffled feathers and mucus secretions) but recovered during the course of the experiment.

All animals from the control unvaccinated group were found sick with signs of apathy right from the first day of challenge. Apathy appeared more severe as one animal died at 2 dpi and other 2 animals at 5 dpi. Signs of flu-like sickness were more apparent and severe that obliged to sacrifice most of the unvaccinated chickens at 7 and 8 dpi. As described, euthanasia was solely applicable when two or more flu-like clinical signs were observed [139].

5.4.4. Flagellin-NG34/CS17 immunization limited oropharyngeal and cloacal H7N1 viral shedding

Significant differences were observed in the viral shedding both from oropharynx and cloaca of chickens vaccinated with flagellin-NG34/CS17 (*Figure 5-4*). While the chickens vaccinated with flagellin-NG34/CS17 had viral RNA detection values almost at the basal level, the unvaccinated group showed variations in the viral secretion that peaked at the seventh day post-inoculation (both orally and cloacally). Differences in the OS viral shedding among groups became more apparent between 5 and 9 dpi. Significant differences in the mean values of 2.73 log_{10} GEC/mL (unvaccinated group) and 1.01 log_{10} GEC/mL (vaccinated group) were noticed at 7 dpi ($P<0.001$). At 9 dpi, a

statistically significant difference among groups in the mean of shed virus: 3.23 $\log_{10}$ GEC/mL (unvaccinated group) and 0.93 $\log_{10}$ GEC/mL (vaccinated group) was also visualized (*P*<0.01).

Similarly, cloacal viral shedding was more apparent and evident among vaccinated and unvaccinated groups right from 2 dpi. Unvaccinated animals were secreting higher number of IVs than the flagellin-NG34/CS17 vaccinated animals. At 7 dpi, significant differences (*P*<0.001) in the mean values among groups: 3.47 $\log_{10}$ GEC/mL (unvaccinated) and 1.16 $\log_{10}$ GEC/mL (vaccinated) were detected.

It should be noted that only two chickens remained alive from unvaccinated challenged group at 9 dpi. Viral secretion in OS, as recorded by RT-qPCR, was observed in both of the chickens, while viral secretion from CS was detected only from one animal (chicken number 5).

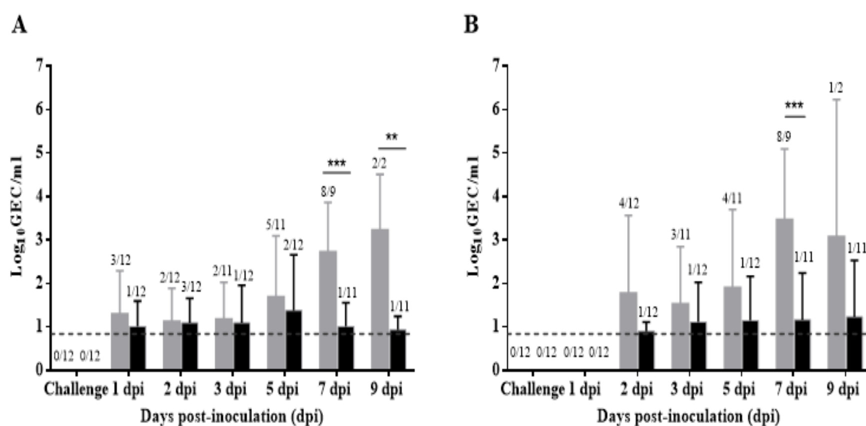

Figure 5-4. Viral RNA shedding detected in oral and cloacal swabs by RT-qPCR. (A) Mean of GEC per mL obtained from oral swabs from sampled at 0, 1, 2, 3, 5, 7 and 9 dpi (B) and from the cloacal swabs sampled at 0, 1, 2, 3, 5, 7 and 9 dpi. Group A (unvaccinated animals) is depicted by grey bars and Group B (flagellin-NG34/CS17; twice vaccinated animals) by black bars. Dpi, days post-inoculation. GEC, genomic equivalent copies. Rates above the bars indicate the relation between positive chickens and the total of chickens examined. Dashed line marks the detection limit of the technique: 0.84 log_{10}GEC/mL. Error bars represent the mean ± SEM. Statistically significant differences concerning shedding among groups (*P* value <0.05) are depicted as *** *P*<0.001, **:*P*<0.01.

5.4.5. Flagellin-NG34/CS17 immunization induced an increase in IgM and improved IgY response.

Flagellin-NG34/CS17 vaccinated chicken displayed a higher mean of IgM antibodies against H7 in sera at 33 PVD than the unvaccinated chickens (*P*<0.05) (*Figure 5-5*). This tendency was also observed at 10 dpi. IgY antibody levels, on the other hand, were accelerated slightly in the

flagellin-NG34/CS17 vaccinated chickens at 33 PVD though this tendency was not maintained at 10 dpi. In fact, higher mean of IgY antibodies at 10 dpi were observed in the unvaccinated chickens rather than in the chickens vaccinated with flagellin-NG34/CS17. This tendency may attribute to the fact that only two chickens remained alive on 9 dpi and, one of them (chicken 2) showed higher amount of antibodies.

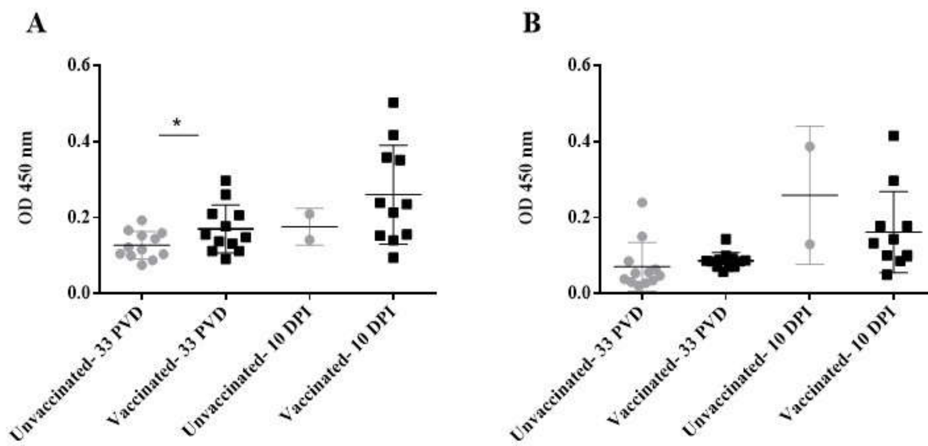

Figure 5-5. HA-specific IgM and IgY titers in sera obtained by ELISA test. (A) Mean of IgM HA-specific antibody levels detected at 33 PVD and 10 DPI of Groups A and B (B) Mean of IgY antibody levels detected at 33 PVD and 10 DPI of Groups A and B. Group A (unvaccinated animals) is depicted by grey circles and Group B (flagellin-NG34/CS17, twice vaccinated animals) is depicted by black squares. OD, optical density. PVD, post-vaccination days and DPI, days post-inoculation. Error bars indicate the mean ± SEM. Statistically significant difference concerning OD values among groups (P value <0.05) is marked as *: P<0.05

5.4.6. Flagellin-NG34/CS17 induced negative HI and SN titers against H7N1.

HI and SN titers were negative in both the flagellin-NG34/CS17 vaccinated and unvaccinated chickens. An HI titer of 1:80 was only observed in only one flagellin-NG34/CS17 vaccinated chicken that also showed flu-like clinical symptoms and recovered later (chicken 23).

5.5. Discussion

The monitoring and controlling the AIVs in the poultry is essential to further decrease their prevalence in avian species and the probable risk of human infections with HPAIV of zoonotic potential. Thus, immunization of poultry is vital as a prophylactic method when there is risk of IVs incursion and when the areas are endemic to such viruses. Moreover, considering the reported outbreaks in humans with H5 and H7 subtypes, WHO recommends not only vaccines against seasonal IVs, but also pandemic preparedness against potential pandemic strains [306].

In addition, the concept of developing a universal influenza vaccine using conserved IV epitopes is widely sought. In the present study, two conserved HA-epitopes (NG-34 and CS-17) selected by *in silico* prediction were linked and used as vaccine candidates. The NG-34 peptide belongs to a highly conserved region of the HA, the E domain [113]. The CS-17 peptide, corresponds to the cleavage site of the HA from H5 HPAIV. Previous immunization studies using polypeptide covering the cleavage site from IVs subytpes A and B have elicited neutralizing antibody

responses and conferred protection in mice [14,484]. Additionally, flagellin was fused to HA-epitopes (NG-34 and CS-17) in order to enhance immunogenicity of these peptides. It is known that flagellin induces TLR5 downsignaling pathway that ends up in the upregulation of immune cells, chemokines, and cytokines, promoting also the antigen uptake by the DCs to be further presented to the helper T cells [485,486]. Similar studies were also carried out previously with flagellin ligand in IV vaccines and effective adjuvants for poultry [487–489].

We have used baculovirus technology to express and produce flagellin-NG34/CS17 vaccine against IV infection. Previous reports [480] indicated that extracts from larvae infected with an empty recombinant baculovirus (BacNi, a recombinant baculovirus with no foreign gene) does not interfere with the immune reaction associated to the antigen. Similarly, prior to virus challenge, we have not observed any local immune reaction upon flagellin-NG34/CS17 vaccine application produced in Baculovirus system.

More than the 80% of chickens vaccinated twice survived the IV challenge reducing significantly the virus load in oropharyngeal and cloacal mucosal tissues. This is in accordance with the standard set by OIE [139]. We are aware that a higher viral dose must be used to achieve a 90% of the mortality in the control unvaccinated challenged animal group. However, it should be taken into account that one of the two survivors in the unvaccinated group was showing deteriorated signs of sickness and most probably needed euthanization adding up to increasing numbers of mortality in unvaccinated challenged group.

Protection against IVs is generally attributed to anti-HA antibodies, determined by ELISA, HI and SNT [490]. In this study, however, hemaglutination-inhibiting/neutralizing antibodies were either very low or below detection levels. Elevated IgM antibody levels in the flagellin-NG34/CS17 vaccinated group however were recorded. We assume that simple ELISA test detect also lower affinity antibodies that are missed in HI or neutralization assays [491]. There is however, no consensus of the ELISA titers required in order to achieve protection [492,493]. Protection without the need of high HI and/or neutralizing titers against H7 subtype IVs has been described previously [494,495]. Reports indicate that candidate inactivated vaccines against potential pandemic viruses (H5/H7) have induced low immunogenicity, required two doses as well as adjuvant in their composition. Possible explanations of these low inhibiting/neutralizing titers are focused on the levels of glycosylation of the HA protein [496,497].

Previous reports also suggest that non-neutralizing antibodies may confer protection against IVs [201,490,498]. These antibodies either interact with the complement [499,500] or recruit NK cells/monocytes leading to kill IV-infected cells by antibody-dependent cellular cytotoxicity (ADCC) [501,502]. IgM antibodies, on the other hand, could neutralize IVs as efficiently as IgY antibodies in the presence of complement [503]. Experiments with passive serum transfer from vaccinated to naïve chicken may unravel the mechanism of antibody induced protection. Possible involvement of cellular immune response in flagellin-NG34/CS17-associated protection in chicken against IV challenge is

under investigation. In our previous work we showed that NG-34 peptide included in the vaccine formulation induces strong T cell response [331].

In conclusion, flagellin-NG34/CS17 may serve as a potential vaccine to protect against heterologous IV infections in chicken. It further limits IV transmission by blocking virus shedding from vaccinated animals. Further experiments however are needed to understand completely the immune mechanisms associated to the protection conferred. Whether this vaccine would protect animals from other HPAIV infections is currently under investigation.

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PART III:

General Discussion and Conclusions

CHAPTER VI

General Discussion

Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.

Marie Curie

6. General discussion

IVs remain a major health threat although for many years efforts have been focused on exploring strategies to obtain a protective universal vaccine able to provide immunity against drifted and pandemic IVs. Their wide host range, antigenic drift and shift make IVs difficult to eradicate and combating influenza viruses is one of the major focus of the influenza research community.

The three studies embedded in this thesis (Chapter III-Chapter V) were designed to potentially induce a broad and a multivalent immune response against IVs by employing distinct vaccines approaches. The vaccine strategy implicated in these studies made use of *in silico* predicted HA-conserved epitopes sharing common informational characteristics that are immunogenically cross-reactive among different IV subtypes and may provide resistance to heterotypic influenza viruses (as described in [417]). In order to enhance immunogenicity, HA-peptides were linked with different biological adjuvants either in a DNA (plasmid) approach or as soluble antigen formulation. Mainly, since adjuvants are often characterized for not inducing robust responses, and thus, suitable vaccine delivery to protect peptides from degradation and regulating release of antigens to the immune cells summed with appropriate adjuvants are essential to increase its immunogenicity [395].

The first two studies were undertaken in pigs for many reasons (Chapter III--IV). Apart from all the economic losses in the pig industry caused by the IVs, pigs are also hosts that may lead to zoonoses in humans with the

risk of a pandemic (such as the 2009 pandemic). Moreover, results obtained in pigs hosts might be extrapolated to the humans as the distribution of SA among the respiratory tracts of pigs and humans are very similar [121].

An alternative to conventional inactivated vaccines that generally lack heterovariant and heterosubtypic protection, may enhance disease and experience interference by MDA [8] was taken into account in both the studies (Chapter III--IV). Thus, the capability of vaccine formulations and application approach to break the MDA was investigated in both cases (Chapter III--IV).

Results gathered from Study I (Chapter III) using pCMV-CTLA4-Ig-NG34 in a needle-free vaccine approach in SIV-seronegative and SIV-seropositive pigs showed that the vaccine formulation was effective in reducing viral secretion, in clearing the virus from the lungs, in inducing antibodies against H1 and H3 subtypes and in promoting HI neutralizing antibodies. Results obtained with the vaccine approach used in the study also indicate that the initial presence of MDA could be bypassed. We assume that the effects observed might be in part of Th2 type T cell response that results in the induction of IgG1 and further increase in humoral and cellular response. All these immune effects are valuable components in an efficacious SIV vaccine against influenza virus infection. Moreover, DNA vaccine used in this work, administered in a needle-free approach eases the mass-vaccination and quicker massive production in case of pandemic [504]. To note, the orchestrated immune response has been induced by a single HA-peptide from H1 influenza subtype combined with the CTLA4 molecule, unlike to all the

SIV vaccines on the market described in *section 1.5.2.*; "*Current vaccines in swine*".

Lots of current studies are using conserved peptides which are tested in pigs (in order to get T-cell cross-protective response) [505–507]. This is because both the $CD4^+$ T helper cells [508] and CTLs [509,510] can clear IAVs and/or help in inducing high titers of strain-specific antibodies [511]. Moreover, antigen-specific T cells are also associated with reductions in the IV shedding [512,513]. However, in those studies, other methodologies have been developed to present the antigen in order to augment their immunogenicity [505,506].

pCMV-CTLA4-Ig-NG34 vaccine did not mitigate pneumonic lung lesion and reduced pigs from flu-like clinical signs suggesting an improvement in the vaccine formulation. Possibly introducing HA-specific T cell epitopes that can induce CTLs could be an option as $CD8^+$ cytotoxic T cells are shown to be associated with reducing clinical severity in mice and humans [514,515].

In chapter IV, we took advantage of bacterial surface protein flagellin, known to induce/enhance innate immune response, fused together with HA-peptides. The resulting VC4-flagellin construct was used to immunize pigs and the vaccine efficacy assessment was evaluated. Both the homologous and heterologous virus (SwH3N2) challenges were used in these studies. Besides, efficacy of VC4-flagellin vaccine was evaluated in both the SIV-seronegative and SIV-seronegative (MDA positive) animals.

We believe that VC-4 flagellin vaccine could be used as a multivalent vaccine against SIV infection in seronegative and seropositive pigs. Vaccination with VC-4 flagellin reduced viral shedding and induced higher HI titers compared to unvaccinated pigs. Stronger IgG antibody responses against H1 and H3 subtypes as well as higher seroneutralizing titers, increased mucosal IgA response in BALFs and elevated amount of DP CD4 CD8 T-cells were induced.

Data collected in this work also points to a cross-reactive antibody effect and that MDA could be overcome by immunizing pigs with VC4-flagellin, a criterion that lacks in commercially available vaccines. Moreover, the VC-4 flagellin vaccination approach induced mucosal immune response crucial for a good protective universal influenza vaccine candidate as IgA from nasal and lung washes can neutralize homo- and heterologous influenza virus challenges [516]. This correlates with other studies [517] which have demonstrated that the humoral mucosal antibody response can be induced when bacterial cytosolic flagellin is recognized by the NLR family members: NAIP5/NLRC4 inflammasome [518]. Additionally, increase in DP CD4 and CD8 T cells that is correlated in other viral pig diseases with protective responses is also induced [519–522].

Wild birds are the natural reservoir of IAVs which can easily infect poultry. Occurring outbreaks in humans coming from HPAI AIVs in poultry (mainly H5 and H7 subtypes) make also crucial that universal

vaccine prototypes are sought and implemented in poultry to aid in animal health and combat zoonotic stages.

HA- peptides fused to flagellin were produced in the baculovirus platform and the soluble extracts of infected larvae expressing the construct used as vaccine prototype against H7N1 subtype. Chickens vaccinated twice with flagellin-NG34/CS17 were protected against H7N1 virus challenge, viral shedding was blocked and were free of any flu-like clinical symptom, criteria that are needed and mentioned in OIE standards for a suitable avian influenza vaccine [139]. Nevertheless, SNT and IHA results were negative making it difficult to state mechanism/s associated to observed protection and demand further research. Similarly, protection, without high HI titers against H7 subtypes has also been reported by others [494,495] suggesting the need of other/different immune correlates for protection against IVs infection.

In summary, we assume that ISM could be a good predictive tool to potentially select antigenic determinants/epitopes with cross-reactive immunological spectrum and may help in detection/protection against heterologous influenza virus challenge as seen in this work. Nevertheless, the approaches herein tested are initial step to the complicate issue of getting a universal IV vaccine and highlight the need of more studies (to further improve immune response, to test approaches in larger groups and against other IAVs, to understand protection mechanism, etc.) to finally develop an effective/efficient and suitable multivalent IAV vaccine.

CHAPTER VII
Conclusions

"Nobody said it was easy…

No one ever said it would be this hard".

Coldplay.

7. Conclusions

1) Cross-reactive antibodies (against both H1 and H3) and high HI titers were achieved in pigs vaccinated with pCMV-CTLA4-NG34 and challenged with a heterologous H3N2 IV. Furthermore, the levels of anti-H3 specific antibodies exponentially increased after SwH3N2 challenge in vaccinated pigs.

2) Increasing levels of anti-H3 antibodies in the vaccinated pigs correlated with the reduction of the viral shedding observed after H3N2 challenge. This reduction in the viral shedding was also observed in pCMV-CTLA4-NG34 vaccinated SIV-seropositive pigs, leading to the clearance of the H3N2 virus in their lungs.

3) pCMV-CTLA4-NG34 partially protects against heterologous H3N2 challenge in the presence of pre-existing antibodies as differences in the degree of pneumonic lesions or flu-like clinical signs were not observed.

4) DNA vaccine based on conserved HA-epitopes and flagellin (VC-4 flagellin) elicited cross-reactive antibodies against H1 and H3 subtypes. Moreover, higher HI titers in the VC-4 flagellin vaccinated pigs were observed after pH1N1 and SwH3N2 IV challenge.

5) The antibodies correlated with the reduction of the viral shedding observed in VC4-flagellin vaccinated SIV-seronegative and SIV-

seropostive pigs. In the BALFs, VC-4 flagellin vaccinated pigs elicited elevated mucosal immune response and higher seroneutralizing titers.

6) VC4-flagellin could be used as multivalent vaccine against SIV infections in both the seronegative and seropositve pigs. However, improvement is needed to mitigate clinical signs and lung lesions.

7) Flagellin-NG34/CS17 vaccination in chicken protected animals from H7 HPAIV strain and as such it represents a potential vaccine candidate against H7 HPAIV strains in the chickens.

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CHAPTER VIII

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APPENDIX

CHAPTER III:

S1 Table. Summary of results obtained in a preliminary trial performed in pigs vaccinated with pCMV-CTLA4-Ig (empty vector) and challenged with H3N2 SIV.

Assay	Mean	Mean SD
Virus quantification by RT-qPCR in NS	2,80[a]	0,526
Virus quantification by RT-qPCR in BALFs	4,01[b]	0,535
ELISA for H3-antibody detection	0,35[c]	0,079
HI test	0,00[d]	0,00

Abbreviations: BALFs, bronchoalveolar fluids; dpi, days post-inoculation; GEC, genomic equivalent copies; HA, hemagglutinin; HI,hemagglutination inhibition; IV, influenza virus; NS, nasal swabs; OD, optical density; PVD, post-vaccination day; SIV, swine influenza virus.

[a]Mean Log_{10} GEC/mL of NS collected from animals immunized with pCMV-CTLA4-Ig at 7 dpi with 106 TCID50/mL H3N2 SIV.

[b]Mean Log_{10} GEC/mL of the BALFs from animals immunized with pCMV-CTLA4-Ig at 7 dpi with 106 TCID50/mL H3N2 SIV.

[c]Mean of OD450 nm values against HA (A/Aichi/2/1968(H3N2)) from sera samples at 35 PVD obtained by ELISAs.

[d]Mean HI titers against A/swine/Spain/003/2010 H3N2 IV from sera samples at 7 dpi with 106TCID50/mL H3N2 SIV.

APPENDIX

CHAPTER IV:

S1 Figure. Plots for gating CD4, CD8 SP T-lymphocytes and CD4-CD8 DP lymphocytes in flow cytometry. A) lymphocytes B) CD4, CD8 T-lymphocytes and CD4-CD8 DP lymphocytes plot C) CD4 T-lymphocytes, D) CD8 T-lymphocytes.