UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

IMUNOGENICIDADE DE UMA VACINA NANOTECNOLÓGICA VIROSSOMAL CONTRA O VÍRUS INFLUENZA A EM SUÍNOS

VANESSA HAACH

PORTO ALEGRE
2023

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Tese apresentada como requisito parcial para obtenção do grau de Doutora em Ciências Veterinárias, nas áreas de Imunologia e Microbiologia – Virologia.

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2023

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

CIP - Catalogação na Publicação

Haach, Vanessa
Imunogenicidade de uma vacina nanotecnológica virossomal contra o vírus influenza A em suínos / Vanessa Haach. -- 2023.
112 f.
Orientadora: Ana Cláudia Franco.

Coorientadora: Rejane Schaefer.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Veterinária, Programa de Pós-Graduação em Ciências Veterinárias, Porto Alegre, BR-RS, 2023.

1. Vírus influenza A. 2. Vacina virossomal. 3. Imunidade humoral. 4. Imunidade celular. 5. Suíno. I. Franco, Ana Cláudia, orient. II. Schaefer, Rejane, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Vanessa Haach

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Aprovada em 28 de fevereiro de 2023

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Dedico este trabalho ao meu irmão Vanderlei Haach (*in memorian*), que sempre me apoiou e torceu por mim.

AGRADECIMENTOS

Agradeço à minha orientadora Ana Cláudia Franco pela oportunidade, confiança em mim depositada e aprendizados.

À minha coorientadora Rejane Schaefer por toda ajuda, dedicação, apoio, experiência, ensinamentos compartilhados, por sempre estar disposta a contribuir comigo e pela amizade.

À Ana Paula Bastos pela amizade, por toda ajuda, experiência, conhecimento compartilhado e por me proporcionar aprendizados no "mundo da imunologia".

À Danielle Gava pela ajuda incansável, experiência, conhecimento repassado, incentivo, apoio, pela parceria e amizade, e pelas palavras de conforto nos momentos que sempre precisei.

Ao Arlei Coldebella, Francisco Noé da Fonseca e Marcos Mores pelo conhecimento repassado, ajuda e contribuição com este trabalho.

À Franciana Volpato, Franciele Ianiski, Marisete Schiochet e Neide Simon pela ajuda, experiência compartilhada e amizade.

À Adriana Ibelli, Alexandre Tessmann, Jane Peixoto e Mônica Ledur pela colaboração neste projeto.

Às designers Marina Schmitt e Vivian Fracasso pelo auxílio neste trabalho.

Aos membros da banca por aceitarem o convite e contribuirem neste processo.

Aos meus pais, meu irmão (*in memorian*) e meu namorado por todo o apoio, incentivo e compreensão pela minha ausência.

Aos meus amigos e colegas do Laboratório de Sanidade e Genética Animal da Embrapa Suínos e Aves, e do Laboratório de Virologia do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul pela amizade e convivência.

A Embrapa Suínos e Aves pela estrutura, bem como os recursos financeiros para este trabalho ser realizado.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado.

A todos que contribuíram de alguma forma para a realização deste estudo. Muito obrigada!

RESUMO

O vírus influenza A (IAV) é um dos principais patógenos respiratórios em suínos, causando prejuízos econômicos à suinocultura e preocupações na saúde pública, devido a possibilidade de ocorrência de epidemias e pandemias. O IAV é endêmico em suínos e linhagens de vírus geneticamente e antigenicamente distintas, dos subtipos H1N1, H1N2 e H3N2, circulam em diferentes regiões geográficas. A cocirculação de linhagens distintas do IAV associadas à rápida evolução viral desafiam o desenvolvimento de vacinas eficazes. Os virossomas são partículas semelhantes a vírus que simulam uma infecção viral, todavia sem conter material genético, estimulando desta forma a indução de uma resposta imune antiviral, tanto humoral quanto celular. Assim, esse estudo teve como objetivo avaliar a imunogenicidade de uma vacina para influenza baseada em virossoma contendo subunidades proteicas (hemaglutinina – HA e neuraminidase – NA) dos subtipos H1N1 pandêmico, H1N2 e H3N2 isolados de suínos. Para isso, foram formados dois grupos de suínos com 40 dias de idade: G1 – não vacinados (n=10), e G2 – vacinados (n=33). Os suínos do grupo G2 foram vacinados com 1 mL da formulação vacinal (128 µg das proteínas HAs + adjuvante) nos dias 0 e 14 por via intramuscular. Os suínos dos grupos G1 e G2 foram avaliados por 28 dias, sendo que três suínos do grupo G2 permaneceram por 90 dias para avaliação da imunidade de memória. Resposta imune humoral e celular robusta contra os três subtipos de IAV foi observada nos suínos após as duas doses vacinais. Houve indução de títulos elevados de anticorpos específicos para HA e atividade de neutralização viral. Além disso, houve maturação significativa de macrófagos, e proliferação significativa de linfócitos B, células T $CD4^+$ e $CD8^+$ efetoras e de memória central, e linfócitos T $CD8^+$ produtores de interferon-γ. A vacina desenvolvida também demonstrou potencial para induzir resposta imune celular e humoral duradoura para os três subtipos virais até a idade de comercialização dos suínos (130 dias). Ademais, a vacina provou ser segura e não citotóxica para suínos. A utilização de vacinais virossomais permite flexibilidade para atualização dos antígenos vacinais a fim de refletir a diversidade genética e antigênica dos IAVs circulantes em suínos no Brasil. A vacinação de suínos pode ser altamente eficaz para reduzir o impacto da doença na produção de suínos e o risco de emergencia de vírus com potencial pandêmico.

Palavras-chave: Virus influenza A. Vacina virossomal. Imunidade humoral. Imunidade celular. Suíno.

ABSTRACT

Influenza A virus (IAV) is one of the main respiratory pathogens in swine, causing economic losses to the swine industry and public health concerns due to the possibility of epidemics and pandemics. The IAV is endemic in pigs and genetically and antigenically distinct virus lineages of subtypes H1N1, H1N2 and H3N2, circulate in different geographic regions. The cocirculation of distinct IAV lineages associated with rapid viral evolution is a challenge to the development of effective vaccines. Virosomes are virus-like particles that mimic a virus infection, however without genetic material, which stimulates both humoral and cellular antiviral immune response. This study aimed to evaluate the immunogenicity of a virosome-based influenza vaccine containing protein subunits (hemagglutinin – HA and neuraminidase – NA) of the subtypes H1N1 pandemic, H1N2 and H3N2 isolated from swine. For this, 40-day-old pigs were divided in two groups: G1 - non-vaccinated (n=10), and G2 - vaccinated (n=33). Pigs from G2 group were vaccinated with 1 mL of the vaccine formulation (128 µg of HAs proteins + adjuvant) on days 0 and 14 by intramuscular route. Pigs from G1 and G2 groups were evaluated for 28 days, in which three pigs from G2 group remained up to 90 days for evaluation of memory immunity. A robust humoral and cellular immune response against the three IAV subtypes was observed in pigs after two vaccine doses. There was induction of high titers of HA-specific antibodies and virus-neutralizing activity. In addition, there was significant maturation of macrophages, and significant proliferation of B lymphocytes, effector and central memory $CD4^+$ and $CD8^+$ T cells, and $CD8^+$ T lymphocytes producing interferon-γ. The developed vaccine also demonstrated the potential to induce a long-lasting cellular and humoral immune response to the three virus subtypes until the market age of pigs (130 days). Furthermore, the vaccine proved to be safe and non-cytotoxic to pigs. The use of virosomal vaccines allows flexibility for updating vaccine antigens in order to reflect the genetic and antigenic diversity of circulating IAVs in swine in Brazil. The vaccination of pigs can be highly effective in reducing the impact of the disease for the swine production and the risk of emerging viruses with pandemic potential.

Keywords: Influenza A virus. Virosomal vaccine. Humoral immunity. Cellular immunity. Swine.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

7-AAD	7-Aminoactinomicina D
ADCC	Citotoxicidade mediada por células dependente de anticorpos
AEC	3-amino-9-etilcarbazol
APC	Célula apresentadora de antígenos / Aloficocianina
BALF	Fluido de lavagem broncoalveolar
BD	Becton Dickinson
BRMSA	Brasil Microrganismo Suínos e Aves
BSA	Albumina sérica bovina
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CD	*Cluster of differentiation*
CD40L	Ligante CD40
CEUA	Comissão de Ética no Uso de Animais
CFSE	Éster succinimidílico de carboxifluoresceína
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Dióxido de carbono
CS&T	*Cytometer Setup and Tracking*
CSF1R	*Colony stimulating factor 1 receptor*
CTL	Linfócito T citotóxico
D	Dia
DC	Célula dendrítica
DCPC	1,2-dicaproil-sn-glicero-3-fosfocolina
DNA	Ácido desoxirribonucleico
EDTA	Ácido etilenodiamino tetra-cético
ELISA	*Enzyme-Linked Immunosorbent Assay*
FBS	Soro fetal bovino
Fc	Região do fragmento cristalizável
FITC	Isotiocianato de fluoresceína
FMO	*Fluorescence minus one control*
FSC	*Forward scatter*
FSC-A	*Forward scatter area*
FSC-H	*Forward scatter height*
H1N1pdm	H1N1 pandêmico

HA	Proteína hemaglutinina / Teste de hemaglutinação
H&E	Hematoxilina e eosina
HI	Inibição da hemaglutinação
IAV	Vírus influenza A
ICQ	Imunocitoquímica
IFN	Interferon
Ig	Imunoglobulina
IHQ	Imuno-histoquímica
IL	Interleucina
IM	Intramuscular
IN	Intranasal
IRF	Fator regulador de interferon
LAIV	Vacina viva atenuada para influenza
M	Proteína matriz
M2e	Domínio extracelular da proteína M2
mAbs	Anticorpos monoclonais
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
MDA5	Gene 5 associado à diferenciação de melanoma
MDCK	*Madin-Darby Canine Kidney*
MHC	Complexo principal de histocompatibilidade
NA	Proteína neuraminidase
NaCl	Cloreto de sódio
NCBI	*National Center for Biotechnology Information*
NF-κB	Fator nuclear κB
NK	Célula *natural killer*
NP	Nucleoproteína
NS	Proteína não estrutural
PA	Proteína polimerase ácida
PAMP	Padrão molecular associado a patógenos
PB1	Proteína polimerase básica 1
PB2	Proteína polimerase básica 2
PBMCs	Células mononucleares do sangue periférico
PBS	Solução salina fosfatada tamponada
PCV2	Circovírus suíno tipo 2

PerCP-Cy5.5	Proteína de clorofila peridinina-Cianina 5.5
pH	Potencial hidrogeniônico
PRR	Receptor de reconhecimento de padrões
PRRSV	Vírus da síndrome reprodutiva e respiratória dos suínos
qPCR	Reação em cadeia da polimerase em tempo real quantitativa
RIG-I	Gene I induzido por ácido retinoico
RNA	Ácido ribonucleico
RPE	R-Ficoeritrina
RPE-Cy7	R-Ficoeritrina-Cianina 7
RPMI	*Roswell Park Memorial Institute*
RT-PCR	Reação de transcrição reversa seguido da reação em cadeia da polimerase
RT-qPCR	Reação de transcrição reversa seguido da reação em cadeia da polimerase em tempo real quantitativa
SAS	*Statistical Analysis System*
SDS-PAGE	Eletroforese em gel de poliacrilamida de dodecil sulfato de sódio
SLA	*Swine leukocyte antigen*
SN	Soroneutralização
SPF	Livre de patógenos específicos
SSC	*Side scatter*
SVN	*Serum virus neutralization*
SWC	*Swine workshop cluster*
swIAV	Vírus influenza A suíno
$TCID_{50}$	Dose infectante para 50% da cultura de tecidos
Tfh	Célula T auxiliar folicular
Th	Célula T auxiliar
TLR	Receptor do tipo Toll
TNE	Tris-NaCl-EDTA
TNF	Fator de necrose tumoral
Tris	Tris (hidroximetil) aminometano
TUNEL	*Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling*
VAERD	Doença respiratória exacerbada associada à vacina
VLP	Partícula semelhante a vírus
WIV	Vírus inteiro inativado

LISTA DE SÍMBOLOS

α	Alfa
β	Beta
γ	Gama
^{o}C	Grau Celsius
=	Igual
κ	Kappa
λ	Lambda
≤	Menor ou igual a
µg	Micrograma
µm	Micrômetro
μm^2	Micrômetro quadrado
µM	Micromolar
mL	Mililitro
mM	Milimolar
M	Molar
-	Negativo
w/v	Peso/volume
%	Porcentagem
+	Positivo
n	Tamanho amostral
U	Unidade
x	Vezes
v/v	Volume/volume

SUMÁRIO

1 INTRODUÇÃO

O vírus influenza A (IAV) ocasiona infecção em aves e mamíferos, incluindo suínos e humanos, e representa risco para a saúde pública devido ao seu potencial de ocasionar pandemias (VINCENT; ANDERSON; LAGER, 2020). Em suínos, o IAV pode gerar prejuízos econômicos significativos, em consequência da alta morbidade, ocasionando redução na ingestão de ração e assim redução do ganho de peso, bem como custos com antimicrobianos para controlar as infecções bacterianas secundárias (RAJÃO et al., 2014). A doença em suínos apresenta muitas semelhanças clínicas e patológicas com a influenza em humanos, e os animais infectados manifestam sinais clínicos de tosse, dispneia e hipertermia (VAN REETH; VINCENT, 2019). O suíno é um hospedeiro importante na dinâmica e epidemiologia da infecção pelo IAV por expressar, nas células epiteliais do trato respiratório, receptores que permitem a ligação de vírus de origem aviária e humana, podendo, desta forma, sofrer infecção por IAVs oriundos de diferentes espécies, como a humana e a aviária, com possibilidade de sofrerem rearranjo gênico (MA; KAHN; RICHT, 2009).

Embora o Brasil possua uma das maiores populações de suínos do mundo, com mais de 42 milhões de cabeças (ABPA, 2022), antes de 2009 havia poucas evidências da circulação do IAV em rebanhos brasileiros. Após a emergencia em suínos do vírus influenza pandêmico H1N1 (H1N1pdm), transmitido por humanos a partir de 2009 (SCHAEFER et al., 2011), a vigilância e as pesquisas envolvendo o IAV em suínos foram intensificadas no mundo inteiro. Assim, outros subtipos virais, como o H1N2 e H3N2, e também os seus rearranjos começaram a ser detectados em suínos no Brasil (NELSON et al., 2015a). Nos últimos anos, como consequência de eventos de rearranjo gênico entre os vírus que circulam em suínos, além das mutações pontuais nos genes que codificam as glicoproteínas virais, houve um aumento expressivo da diversidade genética dos IAVs isolados de suínos (ANDERSON et al., 2021). Também tem sido observado que os IAVs detectados em suínos no Brasil são geneticamente distintos dos vírus que circulam em suínos em outros países (NELSON et al., 2015a; TOCHETTO et al., 2023). Esta rápida evolução viral, juntamente com a cocirculação de múltiplas linhagens distintas dos IAVs, são desafios para o controle da doença em suínos, bem como para o desenvolvimento de vacinas eficazes que induzam proteção cruzada entre os diferentes subtipos virais.

No Brasil, desde 2014, é comercializada uma vacina inativada para o vírus H1N1pdm, porém esta vacina não induz proteção cruzada para os subtipos H3N2 e H1N2, os quais são

antigenicamente distintos do vírus H1N1pdm (SCHAEFER et al., 2020). Desde 2017 começaram a ser comercializadas no país vacinas autógenas, as quais são produzidas a partir do próprio isolado do vírus que circula na granja ou em propriedades adjacentes. Entretanto, caso haja introdução de um vírus heterólogo, muito provavelmente uma nova vacina deverá ser produzida, uma vez que a imunidade cruzada entre subtipos virais é limitada, ou até ausente, dependendo da linhagem viral circulante na granja. Sendo assim, devido a alta variabilidade genética e antigênica dos IAVs circulantes em suínos no Brasil (JUNQUEIRA et al., 2023; NELSON et al., 2015a; SCHAEFER et al., 2020; TOCHETTO et al., 2023), a utilização de uma vacina polivalente para o IAV, contendo antígenos imunogênicos dos subtipos/linhagens virais predominantes em suínos (H1N1, H1N2 e H3N2) é extremamente importante, pois a utilização da vacinação é uma das medidas de controle e prevenção da influenza mais eficientes. O uso de plataformas vacinais distintas das tradicionais é interessante para o desenvolvimento de imunidade em suínos, como os virossomas, que são partículas semelhantes ao vírus, tanto no tamanho como na composição do envelope viral, na qual são acopladas proteínas virais imunogênicas, mas sem a presença de material genético viral. Os virossomas podem interagir com o sistema imunológico através de vias semelhantes a dos IAVs, e apresentar elevada imunogenicidade. O uso de vacinas eficazes, produzidas com antígenos virais que representem a atual diversidade genética e antigênica dos IAVs que circulam em suínos no país auxiliará no controle da doença em rebanhos suínos, reduzindo os custos da produção, bem como o risco de transmissão dos IAVs suínos para humanos.

1.1 **Objetivos**

1.1.1 Objetivo geral

Avaliar a imunogenicidade em suínos de uma vacina virossomal (previamente desenvolvida pela Embrapa Suínos e Aves) contendo subunidades proteicas (hemaglutinina – HA e neuraminidase – NA) dos vírus influenza A H1N1pdm, H1N2 e H3N2.

1.1.2 Objetivos específicos

- Avaliar a citotoxicidade da vacina virossomal para suínos.

- Avaliar a resposta imune humoral dos suínos vacinados, pela produção de anticorpos específicos para os três antígenos presentes na vacina virossomal.
- Caracterizar a resposta imune celular dos suínos imunizados com a vacina virossomal por imunofenotipagem das populações celulares.
- Analisar a participação dos linfócitos T e B na manutenção da imunidade de longa duração.

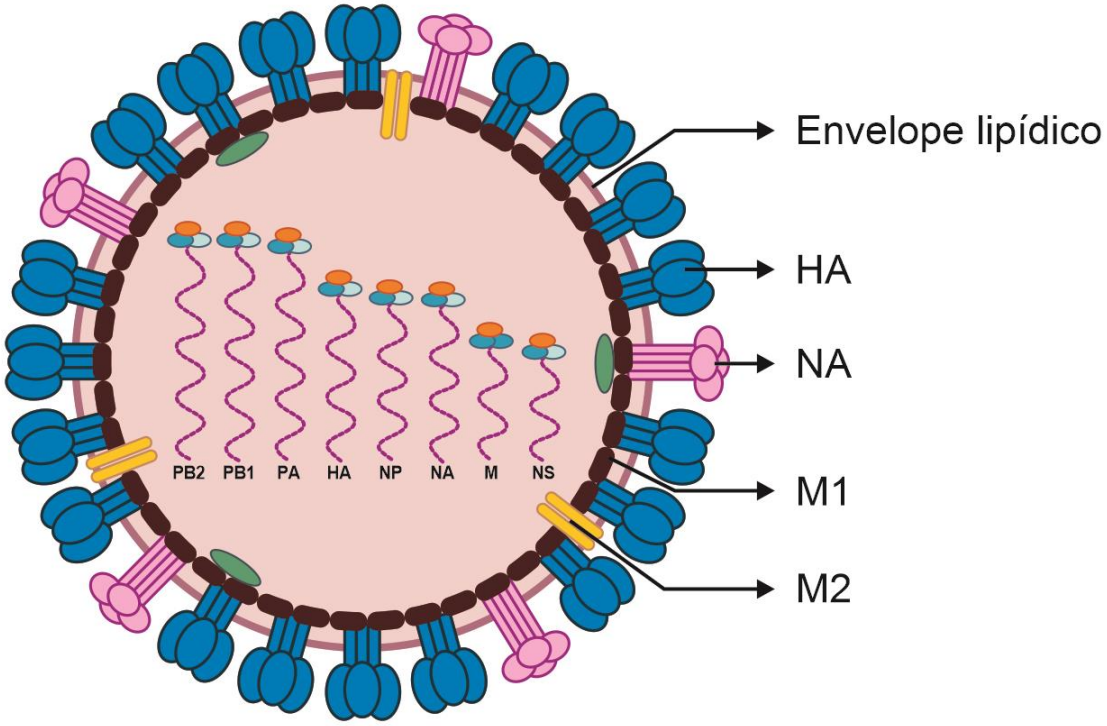
2 REVISÃO DE LITERATURA

2.1 Vírus influenza

Os vírus influenza pertencem a família *Orthomyxoviridae*, que é uma família de vírus RNA de sentido negativo composta por nove gêneros: *Alphainfluenzavirus*, *Betainfluenzavirus*, *Deltainfluenzavirus*, *Gammainfluenzavirus*, *Isavirus*, *Mykissvirus*, *Quaranjavirus*, *Sardinovirus* e *Thogotovirus* (ICTV, 2023). Os vírus pertencentes ao gênero *Alphainfluenzavirus* (*Influenzavirus A*) são classificados em subtipos e os demais gêneros apenas em tipos virais. A nomenclatura segue um padrão universal com as seguintes informações: tipo do vírus, espécie hospedeira de origem (exceto vírus de origem humana), região geográfica de origem, número da amostra, ano de isolamento, subtipo da amostra (entre parênteses); como por exemplo, A/*swine*/*Brazil*/07/2011(H1N1). Em suínos, o vírus influenza A (IAV) é o único tipo viral que apresenta relevância clínica (VAN REETH; VINCENT, 2019).

Os IAVs são pleomórficos, com 80 a 120 nanômetros de diâmetro, possuem capsídeo helicoidal e um envelope lipídico que os tornam altamente suscetíveis a solventes e detergentes, além de serem sensíveis a temperaturas elevadas (VAN REETH; VINCENT, 2019). O genoma viral é segmentado, composto por oito segmentos lineares de RNA de fita simples com polaridade negativa, denominados como segue: polimerase básica 2 (PB2), polimerase básica 1 (PB1), polimerase ácida (PA), hemaglutinina (HA), nucleoproteína (NP), neuraminidase (NA), matriz (M), e não estrutural (NS) (GONEAU et al., 2018) (Figura 1). Os segmentos gênicos codificam 12 a 13 proteínas, sendo que o segmento PB1 codifica as proteínas PB1, PB1-F2 e PB1-N40, o segmento PA codifica as proteínas PA e PA-X, o segmento M codifica as proteínas M1 e M2, o segmento NS codifica as proteínas NS1 e NS2/NEP. Os outros segmentos codificam apenas uma proteína cada (VINCENT et al., 2017; ZHANG; GAUGER; HARMON, 2016). Os genes M e NP codificam proteínas internas e, por conseguinte, são mais conservados. Os genes HA e NA codificam as glicoproteínas de superfície, as quais são alvo do sistema imune do hospedeiro, e consequentemente são mais propensas a sofrerem mutações (GAYMARD et al., 2016). A glicoproteína HA é formada por uma região globular (HA1), mais variável, e uma região da haste (HA2), mais conservada (KRAMMER, 2019) (Figura 2).

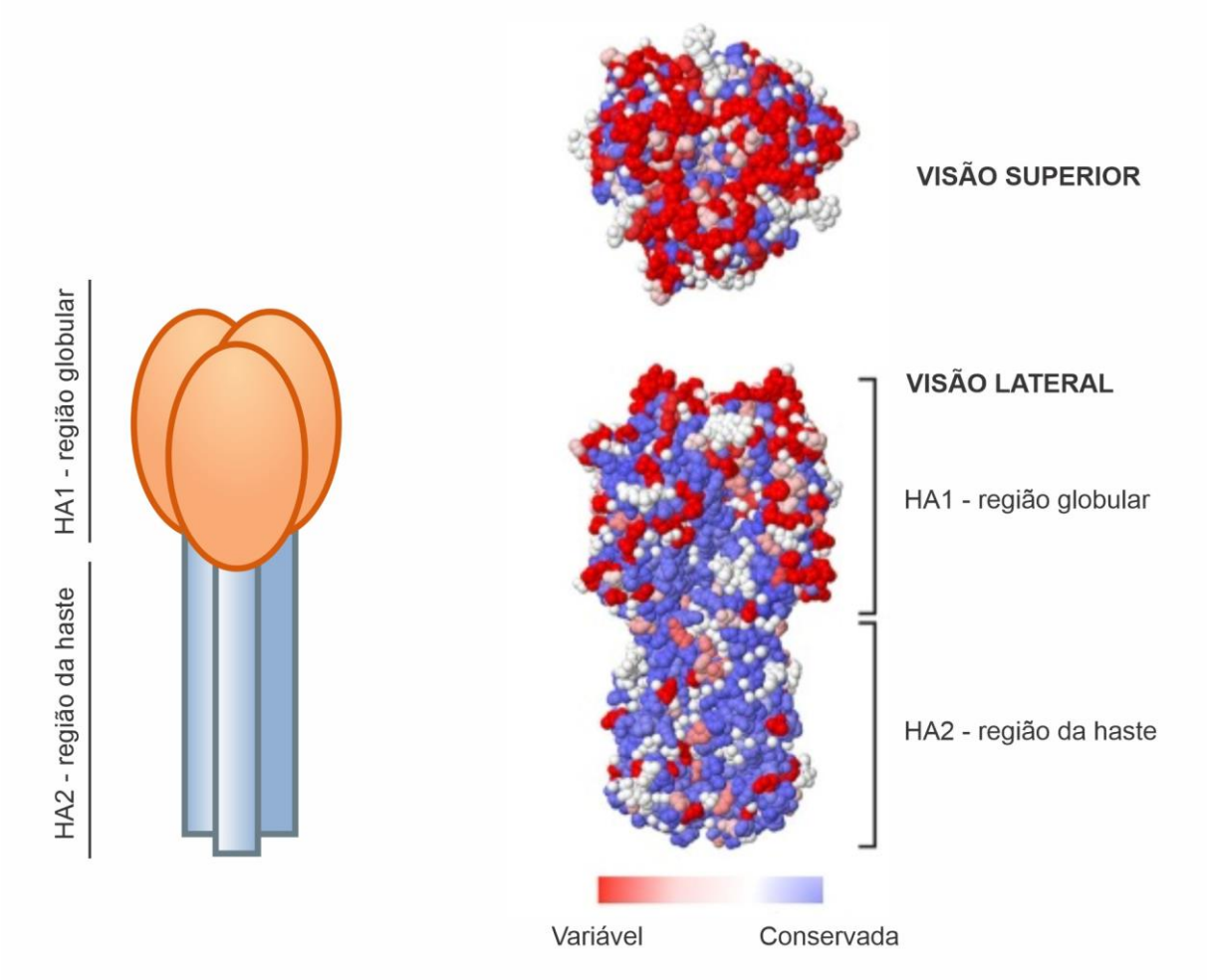
Figura 1 – Diagrama do vírus influenza A mostrando os oito segmentos gênicos virais, destancando as glicoproteínas de superfície e o envelope viral.



Legenda: Exterior: HA = hemaglutinina codificada pelo gene HA; M1 = matriz 1 codificada pelo gene M; M2 = matriz 2 codificada pelo gene M; NA = neuraminidase codificada pelo gene NA. Interior: HA = segmento hemaglutinina; M = segmento matriz; NA = segmento neuraminidase; NP = segmento nucleoproteína; NS = segmento não estrutural; PA = segmento polimerase ácida; PB1 = segmento polimerase básica 1; PB2 = segmento polimerase básica 2.

Fonte: Adaptado de RAJÃO; PÉREZ, 2018.

Figura 2 – Glicoproteína de superfície hemaglutinina do vírus influenza A, mostrando a região globular (HA1) e a região da haste (HA2).



Fonte: Adaptado de KREIJTZ; FOUCHIER; RIMMELZWAAN, 2011; RAJÃO; PÉREZ, 2018.

A glicoproteína de superfície mais abundante, a HA, possui capacidade de se ligar à célula do hospedeiro, ocasionando a fusão celular e a entrada viral (ZHANG et al., 2013). A HA contém epítopos essenciais para ativar o sistema imune, os quais são determinantes dos mecanismos de mutação e troca de segmentos gênicos (WANG et al., 2016). Logo, a alta variabilidade da HA permite que os IAVs escapem da ação do sistema imunológico do hospedeiro. A proteína NA tem como função a clivagem de porções de ácido siálico, promovendo a liberação dos vírions produzidos na célula hospedeira, facilitando a dispersão do IAV (WANG et al., 2012).

As aves silvestres aquáticas são os reservatórios dos IAVs na natureza. Nestes animais já foram identificados dezesseis tipos diferentes de HA (H1-H16) e nove tipos de NA (N1-N9) (GAMBLIN; SKEHEL, 2010; NEUMANN; KAWAOKA, 2015) sendo que a determinação dos subtipos virais tem como base a antigenicidade das glicoproteínas de superfície (HA e NA)

(NELSON; VINCENT, 2015; SHORT et al., 2015). Em 2009 e 2010, foram identificados em morcegos na Guatemala e no Peru os subtipos H17N10 e H18N11, porém não há registros de que estes novos vírus possam infectar outras espécies animais além dos próprios morcegos (TONG et al., 2012, 2013; WU et al., 2014b). Além disso, no Brasil, detectou-se em morcegos o subtipo H18N11 (CAMPOS et al., 2019). Os IAVs infectam uma ampla variedade de espécies, aves (selvagens e domésticas), humanos, suínos, equinos, caninos, felinos e mamíferos marinhos (BROWN, 2000; SHORT et al., 2015). Em suínos, os subtipos considerados endêmicos na maioria dos países produtores de suínos são H1N1, H1N2 e H3N2 (BAUDON et al., 2017; CIACCI-ZANELLA et al., 2015; LEWIS et al., 2016; NELSON et al., 2015b).

Existem dois mecanismos responsáveis pela grande variabilidade genética dos IAVs: *antigenic drift* e *antigenic shift*. O *antigenic drift* é ocasionado por uma alta frequência de erros introduzidos pela enzima RNA polimerase do vírus durante o processo de cópia do genoma viral durante a replicação, devido à ausência de um sistema de correção de erros ou *proofreading*, resultando em mutações pontuais nos diferentes genes (HAMPSON, 2002; ZHANG; GAUGER; HARMON, 2016). Nos genes que codificam as proteínas HA e NA, mutações pontuais ocorrem com maior frequência devido à pressão de seleção induzida pelos mecanismos de defesa do hospedeiro (CORREIA; ABECASIS; REBELO-DE-ANDRADE, 2018; DE, 2018; KLEIN et al., 2014). O *antigenic shift* ou *reassortment* ocorre quando dois ou mais vírus infectam uma mesma célula do hospedeiro e trocam segmentos gênicos, podendo surgir um vírus novo distinto dos vírus parentais (LOWEN, 2018; ZHANG; GAUGER; HARMON, 2016). O novo vírus que sofreu rearranjo gênico pode apresentar propriedades antigênicas e patogênicas distintas dos vírus parentais, podendo também ser capaz de infectar outras espécies (GARTEN et al., 2009). Os erros induzidos pela polimerase viral durante a replicação e o genoma segmentado dos vírus influenza possibilitam uma rápida evolução dos IAVs, e assim, uma alta variabilidade nas glicoproteínas de superfície, o que contribui para que os vírus não sejam neutralizados pela imunidade prévia do hospedeiro. Mutações pontuais, rearranjos gênicos e transmissão interespécies, com introdução e manutenção em suínos de genes oriundos de aves e humanos, favorecem o aumento da diversidade genética dos vírus circulantes, contribuindo para a perpetuação destes vírus na natureza (VINCENT et al., 2017).

2.2 **Epidemiologia e dinâmica da infecção por influenza**

O vírus influenza é reconhecido no mundo inteiro pela sua capacidade em gerar ondas epidêmicas e pandêmicas em humanos, como ocorreu em 1918 com um vírus do subtipo H1N1, de origem aviária, pandemia que ficou conhecida como gripe espanhola (TRILLA; TRILLA; DAER, 2008). Nesta ocasião, a infecção de suínos foi concomitante com a infecção de humanos, e os primeiros relatos da influenza em suínos ocorreram nos Estados Unidos e na Europa (JOHNSON; MUELLER, 2002; TUMPEY et al., 2005a). Posteriormente, em 1957 ocorreu mundialmente a emergencia do vírus H2N2, pandemia que ficou conhecida como gripe asiática, mas que não afetou suínos e, em 1968 ocorreu a pandemia pelo vírus H3N2, isolado pela primeira vez em Hong Kong (COX; SUBBARAO, 2000). A última pandemia de influenza em humanos iniciou no México e Estados Unidos no ano de 2009, e foi causada pelo vírus influenza A/H1N1 de origem suína (H1N1pdm), que logo se espalhou para outros países (MEDINA; GARCÍA-SASTRE, 2011; SMITH et al., 2009). O H1N1pdm surgiu como resultado de eventos de rearranjo gênico entre linhagens de influenza detectadas em suínos na América do Norte e Eurásia (GARTEN et al., 2009; SMITH et al., 2009). Logo após a emergencia do H1N1pdm em humanos, o vírus foi rapidamente transmitido para suínos em vários países (VINCENT et al., 2014). No Brasil, o primeiro surto de influenza causado pelo vírus H1N1pdm em suínos foi descrito em 2010 (SCHAEFER et al., 2011), mas o vírus já estava presente em suínos desde 2009 (RAJÃO et al., 2013a).

Quando o IAV é introduzido pela primeira vez em uma granja, ocorre a forma epidêmica da doença, podendo haver acometimento de até 100% dos animais de diferentes faixas etárias. Uma vez que a doença torna-se estabelecida em um rebanho, a mesma ocorre na forma endêmica, comumente surgindo na fase de creche, quando há diminuição da imunidade passiva (VAN REETH; VINCENT, 2019). Em situações de endemia, a influenza pode acometer suínos durante todo o ano, embora a infecção ocorra com mais frequência nos períodos mais frios do ano.

A transmissão do IAV entre humanos e suínos é bidirecional, entretanto os eventos de transmissão humano-suíno são mais frequentes do que o inverso, sendo considerada a maior zoonose reversa de doenças infecciosas com ocorrência global documentada até o momento (NELSON; VINCENT, 2015). Estes eventos de transmissão viral de humanos para suínos contribuem para o aumento da diversidade genética dos vírus que circulam em populações de suínos mundialmente (RAJÃO; VINCENT; PEREZ, 2019).

Os IAVs apresentam uma restrição parcial em relação à infecção de novos hospedeiros, apesar da ocorrência de transmissão viral interespécies. Na maioria dos casos de transmissão interespécies, os vírus dificilmente são mantidos com sucesso na nova espécie hospedeira, permanecendo preferencialmente em seus hospedeiros originais, o que limita a propagação do vírus em outras espécies (LANDOLT; OLSEN, 2007; WEBSTER et al., 1992). Provavelmente, a capacidade de "quebrar" a barreira interespécies varia entre os subtipos virais, e existem evidências de que os oito segmentos gênicos são importantes na determinação da espécie hospedeira suscetível (LANDOLT; OLSEN, 2007; NEUMANN; KAWAOKA, 2006). Porém, vários estudos mostram que a proteína HA é um dos principais determinantes da especificidade dos IAVs por ser responsável pela ligação do vírus ao receptor de ácido siálico. Além disso, a existência de variações nos receptores de ácido siálico também podem diferir entre as espécies hospedeiras do IAV (ITO, 2000; MATROSOVICH et al., 2000; NICHOLLS et al., 2008).

Para que ocorra a infecção viral, a proteína HA precisa ligar-se aos receptores de ácido siálico presentes nas células do hospedeiro (VAN REETH; VINCENT, 2019). Existem várias formas quimicamente distintas dos ácidos siálicos, e diferentes vírus influenza apresentam distintos níveis de afinidade a estes. As diferenças de afinidade podem determinar qual a espécie animal pode ser infectada por determinado vírus. Sendo assim, em nível molecular, os ácidos siálicos são ligados ao açúcar galactose por meio de ligações α2,3 ou α2,6. Os sítios de glicosilação em receptores celulares do tipo α2,3 são os preferenciais para ligação de vírus aviários e os do tipo α2,6 são os preferenciais para vírus de mamíferos (IMAI; KAWAOKA, 2012). As células epiteliais do trato respiratório de suínos expressam ambos os receptores (α2,3 e α2,6) (ITO et al., 1998; VAN REETH; VINCENT, 2019). Entretanto, os suínos expressam em maior abundância os receptores do tipo humano (α2,6) comparado aos receptores do tipo aviário (α2,3), e a distribuição destes receptores no epitélio respiratório de suínos varia de acordo com a porção do trato respiratório (NELLI et al., 2010; VAN POUCKE et al., 2010). Por esta razão, os suínos podem ser infectados por vírus de origem aviária e humana e, desta forma, servirem como um "vaso de mistura" ou "*mixing vessel*" para a emergencia de novos vírus (BROWN, 2000; JANKE, 2014; NELSON et al., 2014; RAJÃO; VINCENT; PEREZ, 2019). Por conseguinte, no epitélio respiratório do suíno, o vírus influenza A pode adaptar-se, replicar, tornando-se infeccioso para outras espécies, e ainda, pode sofrer rearranjos gênicos que facilitem a transmissão interespécies (CRISCI et al., 2013).

Os IAVs estão distribuídos mundialmente em rebanhos suínos, porém os subtipos e linhagens virais presentes em cada continente diferem significativamente (VAN REETH; VINCENT, 2019). As características genéticas e antigênicas dos IAVs estão relacionadas com

as regiões geográficas. Desta forma, os vírus isolados na América do Norte diferem dos vírus isolados na Europa, e estes, diferem dos vírus isolados na Ásia (ANDERSON et al., 2021; KUNTZ-SIMON; MADEC, 2009; VINCENT et al., 2014). Isto ocorre devido a introduções virais independentes em suínos e manutenção de linhagens do vírus influenza de origem aviária, humana e suína (BROWN, 2000). Nelson et al. (2015a) relataram que os IAVs detectados em suínos no Brasil são geneticamente distintos dos vírus que circulam em suínos em outros países. Situação similar foi encontrada através da análise genética dos IAVs detectados em suínos em outros países da América Latina, como a Argentina, Chile e Colômbia (BRAVO-VASQUEZ et al., 2017; CAPPUCCIO et al., 2011; NELSON et al., 2015c; PEREDA et al., 2011). Os três subtipos (H1N1, H1N2 e H3N2) circulantes em suínos são classificados globalmente em linhagens da HA: H1 1A (H1 clássico e H1 pandêmico), H1 1B (H1 de origem humana), H1 1C (H1 de origem aviária) e H3 (H3 classificado pela década de introdução do vírus ancestral) (ANDERSON et al., 2021). No Brasil circulam sete linhages de vírus H1 e H3: H1 1A (H1 pandêmico), H1 1B (1B.2.3, 1B.2.4 e 1B.2.6) e H3 (1990.5.1, 1990.5.2 e 1990.5.3) (JUNQUEIRA et al., 2023; TOCHETTO et al., 2023).

2.3 **Patogenia, sinais clínicos e lesões histológicas causadas pela infecção por influenza**

O IAV é um dos principais patógenos causadores de infecção respiratória aguda em suínos (VINCENT; ANDERSON; LAGER, 2020). A doença é caracterizada por apresentar alta morbidade (até 100%) e baixa mortalidade (geralmente menos de 1%) (VAN REETH; VINCENT, 2019; VINCENT; ANDERSON; LAGER, 2020). A elevada morbidade nos rebanhos afetados pode ocasionar a perda de produtividade devido à redução na ingestão de ração, e consequentemente, redução do ganho de peso (SCHMIDT et al., 2016). Além disso, as infecções bacterianas secundárias concomitantes fazem com que haja aumento de custos com o uso de antimicrobianos. Assim, a doença em suínos ocasiona uma perda econômica significativa para a suinocultura em vários países (RAJÃO et al., 2014).

A principal via de transmissão viral é através do contato direto entre os suínos, por meio das secreções nasofaríngeas de animais infectados pelo vírus. Também ocorre transmissão do vírus por aerossóis eliminados durante a tosse e espirros, bem como por meio de fômites e pela introdução de animais infectados no rebanho (ROMAGOSA et al., 2011; TORREMORELL et al., 2012). A infecção viral é limitada às células epiteliais do trato respiratório superior e inferior, podendo haver replicação viral na mucosa nasal, tonsilas, traqueia, linfonodos

traqueobronquiais e pulmões, e raramente é detectada viremia (RAJÃO et al., 2014; VAN REETH; VINCENT, 2019). A excreção do vírus ocorre durante a fase aguda da doença (a partir de 24 horas pós-infecção), quando ocorre eliminação de altas concentrações de vírus nas secreções respiratórias dos suínos (JANKE, 2013). Na forma epidêmica, a doença aparece de forma abrupta, acometendo todo ou grande parte do rebanho após um período de incubação de um a três dias, com os sinais clínicos regredindo de forma rápida entre cinco e sete dias pós-infecção, desde que não ocorram infecções bacterianas secundárias (RAJÃO et al., 2014; VINCENT; ANDERSON; LAGER, 2020).

Os sinais clínicos frequentemente observados em suínos infectados pelo IAV são hipertermia, prostração, anorexia, dispneia, taquipneia, hesitação em movimentar-se e tosse (LANGE et al., 2009; VINCENT et al., 2010a; VINCENT; ANDERSON; LAGER, 2020). Também podem ocorrer infecções subclínicas, caracterizadas pela ausência de doença clínica respiratória significativa. Geralmente infecções restritas ao trato respiratório superior resultam em infecção subclínica ou com sinais clínicos brandos, quando comparado às infecções do trato respiratório inferior (VAN REETH; VINCENT, 2019).

Em casos de infecção pelos subtipos H1N1, H1N2 e H3N2 os sinais clínicos observados são semelhantes, não sendo observados em estudos experimentais diferenças na patogenicidade ou virulência entre os subtipos (LANDOLT et al., 2003; RICHT et al., 2003; SRETA et al., 2009; VINCENT et al., 2006, 2009). Entretanto, a gravidade da doença pode ser determinada por fatores como idade do animal, condição imunológica, pressão de infecção, condições climáticas, manejo e infecções concomitantes (VAN REETH; VINCENT, 2019). Usualmente, o IAV é encontrado associado a outros patógenos, e infecções secundárias causadas pelo *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Mycoplasma hyopneumoniae*, *Glaesserella parasuis*, *Streptococcus suis* tipo 2, circovírus suíno tipo 2, vírus da síndrome reprodutiva e respiratória dos suínos e coronavírus respiratório suíno podem aumentar a severidade do quadro clínico (BROCKMEIER; HALBUR; THACKER, 2002; JUNG; HA; CHAE, 2005; RECH et al., 2018; THACKER; THACKER; JANKE, 2001).

Comumente, as lesões macroscópicas observadas em pulmões de suínos infectados pelo IAV são caracterizadas como áreas de consolidação pulmonar, com coloração vermelho-púrpura afetando, principalmente, os lobos cranioventrais, podendo se estender aos lobos caudais e acessório. A porcentagem do tecido pulmonar com consolidação varia, podendo o pulmão ser afetado em mais de 50%. Também em vários casos de infecção aguda severa pode-se observar congestão das mucosas, vias aéreas preenchidas por exsudato fibrinoso, edema pulmonar, bem como linfonodos brônquicos e mediastínicos aumentados (GAUGER et al.,

2012; JANKE, 2014; KHATRI et al., 2010; LÓPEZ, 2007; VAN REETH; VINCENT, 2019). Devido às infecções secundárias, especialmente bacterianas, as lesões podem ser exacerbadas (VAN REETH; VINCENT, 2019). As lesões ocasionadas pelo IAV podem ser observadas por até duas semanas após a infecção, desaparecendo após três semanas (JANKE, 2000). Microscopicamente, as lesões observadas são bronquite e bronquiolite necrosante com infiltrado linfocítico peribronquiolar leve, pneumonia broncointersticial, necrose multifocal e acúmulo de células inflamatórias, principalmente neutrófilos e células epiteliais descamadas no lúmen, podendo levar a bronquiolite obliterante (GAUGER et al., 2012; KHATRI et al., 2010; LÓPEZ, 2007; VAN REETH; VINCENT, 2019; VINCENT et al., 2010a; WATANABE et al., 2012).

2.4 **Diagnóstico de influenza A**

Diferentes métodos de diagnóstico de influenza A em suínos têm sido utilizados pelos laboratórios. Entretanto, nos últimos anos o diagnóstico tornou-se mais complexo devido a grande diversidade genética e rápida evolução dos IAVs cocirculantes em suínos (DETMER et al., 2013). O sucesso da detecção do IAV em suínos depende de alguns fatores, como escolha do animal para a amostragem, a correta colheita das amostras, armazenamento adequado e envio rápido ao laboratório de diagnóstico (SCHAEFER et al., 2013). Os métodos de diagnóstico podem ser aplicados para a detecção do antígeno ou ácido nucleico viral; ou para identificação de anticorpos específicos contra o vírus, ou seja, detectam a resposta do hospedeiro à infecção (DETMER et al., 2013). Para a detecção de IAV podem ser colhidas amostras de tecido pulmonar, lavado broncoalveolar, traqueia, secreção nasal, fluido oral, obtidos preferencialmente de suínos durante a fase aguda da infecção, e para a detecção de anticorpos devem ser utilizadas amostras de soro, preferencialmente pareadas (CULHANE; GARRIDO-MANTILLA; TORREMORELL, 2020).

Para a detecção de anticorpos em soros de suínos podem ser utilizados os ensaios de inibição da hemaglutinação (HI) (KITIKOON; GAUGER; VINCENT, 2014), soroneutralização (SN) (GAUGER; VINCENT, 2020a) e ELISA (*Enzyme-Linked Immunosorbent Assay*) (GAUGER; VINCENT, 2020b; GOODELL et al., 2016). Os ensaios de HI e SN são mais trabalhosos do que os ensaios comerciais de ELISA, porém fornecem mais informações sobre os anticorpos detectados. O teste de SN detecta anticorpos neutralizantes, sendo mais sensível do que o teste de HI, apesar deste último ser considerado o “padrão-ouro”

para detectar e quantificar anticorpos contra influenza correlacionados com proteção (GAUGER; VINCENT, 2020a; KITIKOON; GAUGER; VINCENT, 2014). A presença de anticorpos contra o IAV indica contato prévio com o agente, mas não indica ocorrência de infecção. Além disso, a implementação da vacinação contra o IAV em muitos rebanhos dificulta a interpretação dos resultados sorológicos, uma vez que os mesmos não diferenciam anticorpos induzidos pela vacinação dos anticorpos produzidos após infecção natural (JANKE, 2014).

A análise histopatológica é realizada para a identificação de lesões microscópicas sugestivas de infecção pelo IAV nos pulmões de suínos, porém não são confirmatórias. Assim, os tecidos fixados devem ser analisados pela técnica de imuno-histoquímica (IHQ) para detecção do antígeno viral associado às lesões histológicas observadas, com a utilização de anticorpos específicos marcados com corantes ou enzimas (PANTIN-JACKWOOD, 2020).

Comumente, a detecção do antígeno viral é realizada a partir do isolamento do IAV em ovos embrionados de galinhas livres de patógenos específicos (SPF; *specific pathogen-free*) e/ou em cultivo celular, principalmente com o uso da linhagem de células MDCK (*Madin-Darby Canine Kidney*) (ZHANG; GAUGER, 2020), seguido do teste de hemaglutinação (HA). Para confirmar o isolamento viral é realizado o teste de imunocitoquímica (ICQ), imunofluorescência direta ou transcrição reversa seguida de reação em cadeia da polimerase (RT-PCR) / transcrição reversa seguida de reação em cadeia da polimerase em tempo real quantitativa (RT-qPCR). O isolamento viral é considerado o método "padrão-ouro" no diagnóstico de influenza, e a partir da sua propagação é possível realizar estudos de caracterização genética e antigênica, patogenia e produção de antígenos vacinais. Embora o isolamento viral seja tradicionalmente utilizado no diagnóstico, esta técnica é bastante laboriosa (resultado obtido em uma ou duas semanas) e apresenta limitações, uma vez que depende da viabilidade viral (JANKE, 2000). Isto pode comprometer o diagnóstico da doença, levando a detecção de amostras falso-negativas, caso o diagnóstico for baseado apenas em isolamento viral (RICHT et al., 2004). Além disso, o laboratório precisa possuir estrutura física adequada para a realização do cultivo celular ou ter disponível ovos de galinhas SPF.

Para o diagnóstico virológico são utilizadas técnicas moleculares que detectam o RNA viral, como RT-PCR e RT-qPCR (ZHANG; HARMON, 2020). Comparadas a outros métodos, as técnicas moleculares são menos afetadas pela qualidade da amostra, e são mais sensíveis e específicas, permitindo a detecção de ácido nucleico viral mesmo em amostras com baixa carga viral (GUNSON; COLLINS; CARMAN, 2006). Também são testes mais rápidos, usados para avaliar um grande número de amostras em um curto período de tempo (DETMER et al., 2013; ZHANG; GAUGER; HARMON, 2016). Além disso, o uso de RT-qPCR possibilita a

quantificação da carga viral presente nas amostras biológicas. Para o diagnóstico do IAV são utilizados pares de iniciadores para amplificação do gene M (gene mais conservado geneticamente). No entanto, os ensaios baseados no gene M identificam apenas o tipo viral e não o subtipo (DETMER et al., 2013; ZHANG; GAUGER; HARMON, 2016; ZHANG; HARMON, 2020). Para subtipagem do IAV por RT-PCR e RT-qPCR, são utilizados pares de iniciadores e sondas específicas para os genes HA e NA, o que permite a distinção entre os subtipos virais circulantes em suínos, além de possibilitar a detecção de infecções mistas em um único animal (DETMER et al., 2013; HAACH et al., 2019; RICHT et al., 2004). Contudo, a alta taxa de mutação observada nos segmentos HA e NA (VAN REETH; VINCENT, 2019) evidencia a necessidade de uma atualização periódica dos pares de iniciadores e sondas empregadas nas técnicas de RT-PCR e RT-qPCR, a fim de detectar novos vírus emergentes (DETMER et al., 2013; ZHANG; HARMON, 2020).

Nos últimos anos, frequentemente realiza-se o sequenciamento genético seguido de análises comparativas de sequências e análises filogenéticas para caracterização molecular dos IAVs e para melhorar a compreensão sobre a epidemiologia e as relações evolutivas dos vírus (ZHANG; GAUGER; HARMON, 2016). Para determinar as sequências genômicas dos IAVs, muitos laboratórios ainda utilizam o método tradicional de Sanger, porém vários laboratórios empregam o uso das tecnologias de sequenciamento de nova geração (LEE, 2020).

2.5 **Imunidade contra influenza**

2.5.1 Imunidade passiva

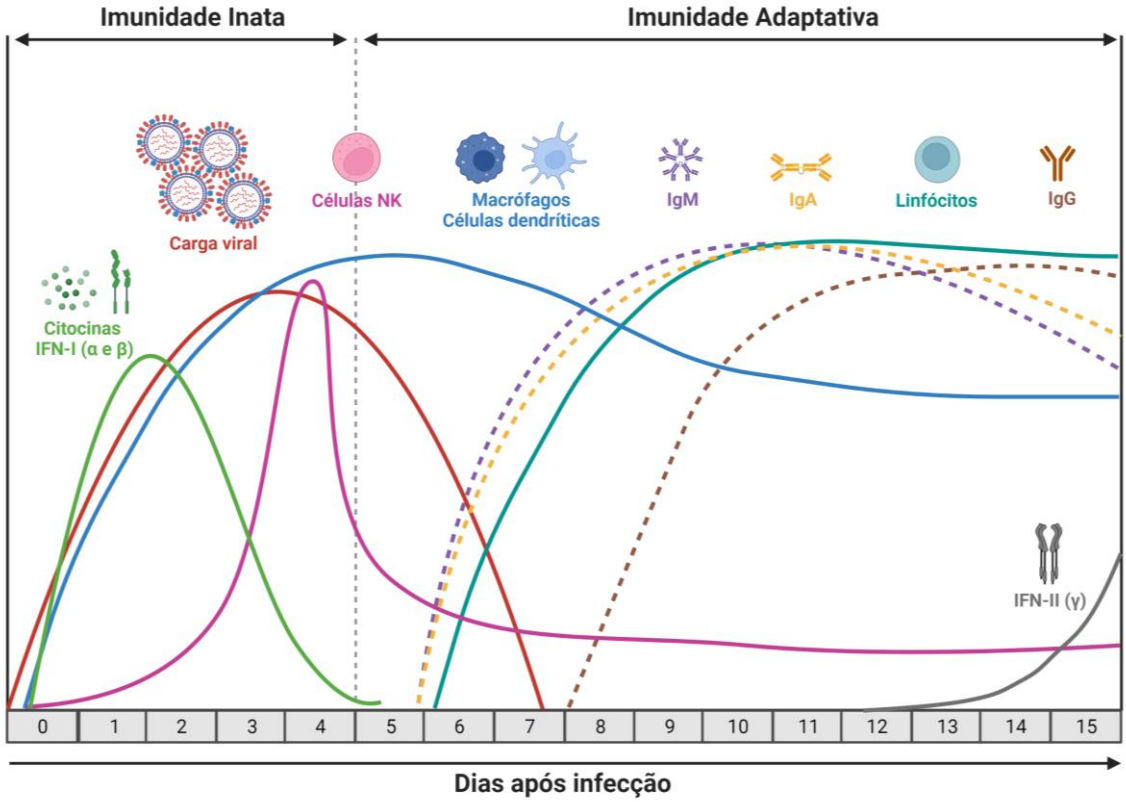
Em suínos lactentes, a imunidade passiva é adquirida através do colostro que contêm anticorpos e células do sitema imune materno, e pode controlar a doença clínica nos leitões, mas não impede a infecção e a excreção viral (ALLERSON et al., 2013; LOEFFEN et al., 2003). A presença de anticorpos maternos para o IAV protege os leitões no período inicial de vida contra vírus antigenicamente relacionados, porém, interferem no desenvolvimento da resposta imune ativa, por vacinação ou infecção (KITIKOON et al., 2006). Isso acontece devido aos anticorpos maternos se ligarem ao antígeno vacinal e ao receptor Fc, formando uma ligação cruzada entre o receptor de célula B e o receptor Fc, inibindo assim a produção de anticorpos em resposta ao reconhecimento do antígeno (ALBRECHT; ARCK, 2020; VAZQUEZ-PAGAN; SCHULTZ-CHERRY, 2021). Os níveis de anticorpos maternos em leitões são

dependentes da concentração inicial de anticorpos da fêmea, e declinam no soro de leitões até a sexta semana de vida em rebanhos não vacinados para a influenza (JANKE, 2000).

2.5.2 Resposta imune inata

A resposta imune inata é uma resposta rápida e é a primeira linha de defesa do organismo, incluindo respostas químicas, físicas e celulares, desencadeando respostas pró-inflamatórias. Durante uma infecção pelo IAV, o sistema imune inato reconhece componentes virais conservados chamados de padrões moleculares associados a patógenos (PAMPs), através de receptores de reconhecimento de padrões (PRRs), como o receptor do tipo Toll (TLR) 3, TLR7, TLR8, gene I induzido por ácido retinoico (RIG-I) e gene 5 associado à diferenciação de melanoma (MDA5) (CAO, 2016; OUYANG et al., 2014). Estes PRRs ativam fatores de transcrição, incluindo o fator regulador de interferon (IRF) 3, IRF7 e fator nuclear κB (NF-κB), causando a expressão de citocinas e interferons (IFNs) (HISCOTT et al., 2006; KUMAR; KAWAI; AKIRA, 2011) (Figura 3). Os PRRs são capazes de distinguir as moléculas "próprias" das "não próprias", como RNAs de origem viral, presentes no interior das células infectadas (CHEN et al., 2018). A infecção por IAV induz a expressão de IFNs do tipo I (IFN-α e IFN-β) e do tipo III, também conhecidos como IFN-λ, os quais apresentam propriedades antivirais que inibem a replicação do IAV (VINCENT et al., 2008a; WANG et al., 2009).

Figura 3 – Curva da resposta imunológica inata e adaptativa do vírus influenza A em suínos. Cinética geral da carga viral e diferentes componentes do sistema imunológico durante uma infecção pelo vírus influenza A em suínos. A magnitude das respostas está relacionada com os dias após a infecção (0-15).



Legenda: IFN = interferon; Ig = imunoglobulina; NK = *natural killer*.

Fonte: Criado com BioRender.com.

O primeiro alvo dos IAVs são as células epiteliais das vias aéreas, as quais produzem moléculas antivirais e quimiotáticas que iniciam a resposta imunológica pelo recrutamento rápido de células efetoras, como as células *natural killer* (NK), monócitos e neutrófilos (CHEN et al., 2018). A fim de limitar a replicação viral e preparar as células para a imunidade adaptativa, todos os tipos celulares possuem mecanismos próprios para interagir com as células infectadas pelo vírus (CHEN et al., 2018). O fator de necrose tumoral (TNF) α e a interleucina (IL) 1 induzem moléculas de adesão endotelial, que desencadeiam a migração de células imunes inatas, como macrófagos, células dendríticas (DCs) e células NK para o local da infecção (CHEN et al., 2018) (Figura 3). Os macrófagos ativados fagocitam as células infectadas pelo IAV, limitando a disseminação viral e regulando a resposta imune adaptativa (TUMPEY et al.,

2005b). As células NK provocam a lise das células infectadas, auxiliando na eliminação da infecção pelo IAV (GUO; KUMAR; MALARKANNAN, 2011).

Quando os linfócitos T imaturos e de memória reconhecem antígenos virais que foram apresentados pelas DCs, a resposta imunológica adaptativa é iniciada. As DCs imaturas sob o trato respiratório monitoram a presença de patógenos invasores (BAHADORAN et al., 2016; HOLT et al., 2008). As DCs convencionais migram dos pulmões para os linfonodos, após a infecção pelo IAV, onde apresentam os antígenos virais para os linfócitos T (HEER et al., 2008; HINTZEN et al., 2006). Os epítopos degradados no citosol são exportados para o retículo endoplasmático, ligando-se às moléculas do complexo principal de histocompatibilidade (MHC) de classe I, sendo transportados para a membrana celular através do complexo de Golgi para o reconhecimento por células T $CD8^+$. Os epítopos degradados em endossomos/lisossomos ligam-se às moléculas do MHC de classe II, sendo transportados para a membrana celular para o reconhecimento pelas células T $CD4^+$. Este processo pode levar à proliferação de células B e à maturação dos plasmócitos secretores de anticorpos (VAN DE SANDT; KREIJTZ; RIMMELZWAAN, 2012).

2.5.3 Resposta imune adaptativa

A imunidade adaptativa é importante para a eliminação viral, recuperação e proteção contra a reinfecção. Desta forma, as células T e B apresentam um papel importante na imunidade adaptativa. O sistema imune responde rapidamente às infecções pelo IAV, resultando na eliminação do vírus do trato respiratório em até uma semana após a infecção. Com o passar do tempo, os títulos de anticorpos e a produção das células imunológicas diminuem, mas as populações de células T e B de memória são mantidas nas vias aéreas e nos tecidos linfóides. Em uma nova infecção pelo IAV, estas células irão desencadear uma resposta imune amplificada e acelerada (VAN REETH; MA, 2013).

Após infecção primária pelo IAV, é induzida proteção contra a reinfecção pelo mesmo vírus ou por vírus muito semelhante antigenicamente (DE VLEESCHAUWER et al., 2011; VAN REETH et al., 2003). Provavelmente, os principais mediadores desta imunidade homóloga sejam os anticorpos vírus-neutralizantes específicos para a HA. Devido à circulação simultânea de diferentes subtipos e linhagens do vírus influenza, os suínos normalmente são expostos a IAVs antigenicamente distintos durante sua vida (VAN REETH; VINCENT, 2019). Pode haver proteção cruzada completa ou parcial entre as linhagens H1 e entre as linhagens H3

(BUSQUETS et al., 2010; DE VLEESCHAUWER et al., 2011; QIU; DE HERT; VAN REETH, 2015; VAN REETH et al., 2003). No entanto, a proteção cruzada entre vírus H1 e H3 é muito restrita ou nula (KAPPES et al., 2012; QIU; DE HERT; VAN REETH, 2015).

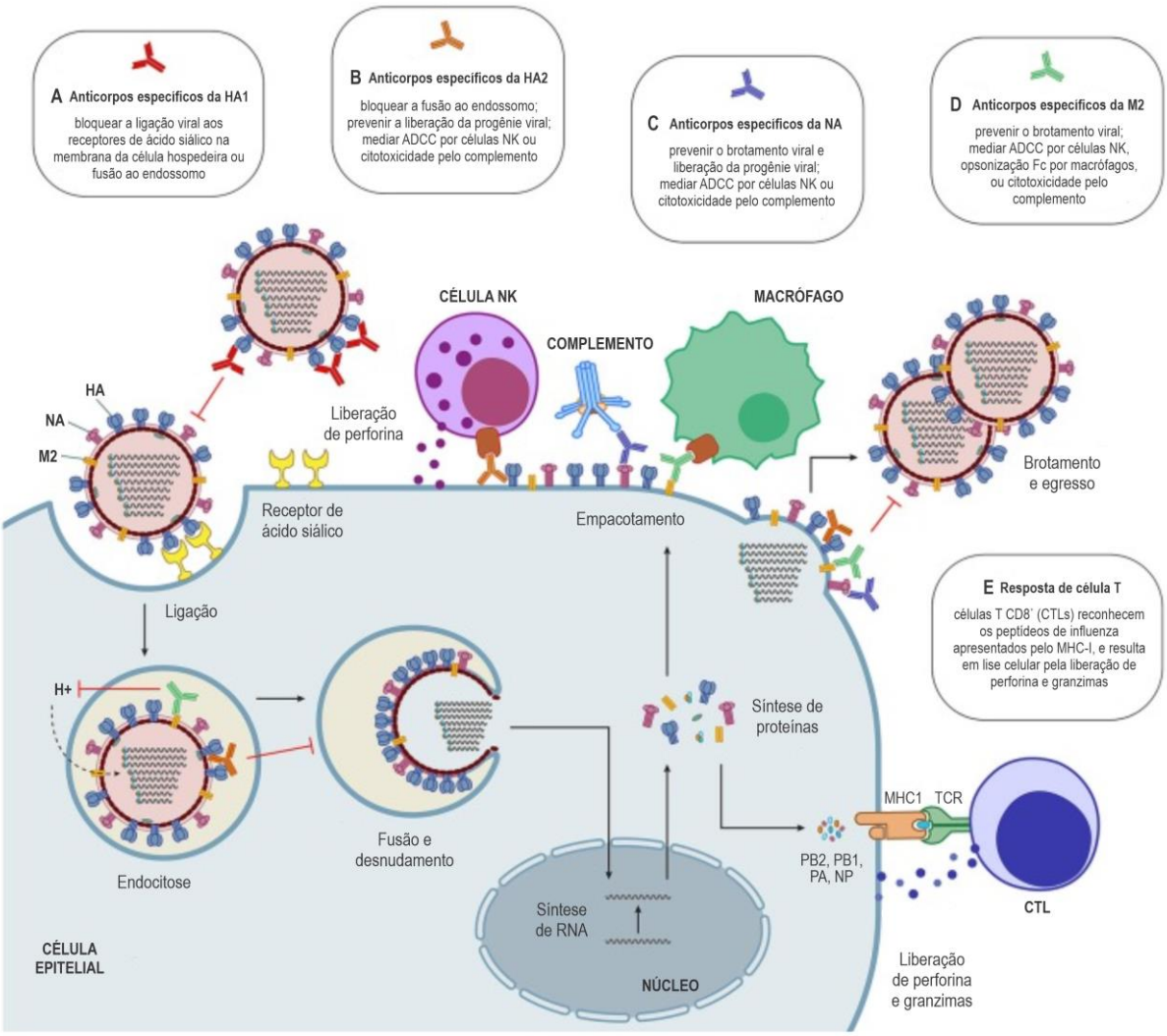
2.5.3.1 Imunidade humoral

Durante a infecção pelo IAV, o antígeno viral é apresentado e as células B imaturas localizadas nos linfonodos mediastínicos diferenciam-se em células produtoras de anticorpos (JUNG; LEE, 2020). Além disso, os anticorpos não neutralizantes produzidos pelos plasmócitos, colaboram para acelerar a expansão dos linfócitos T $CD8^+$ de memória e facilitar a eliminação viral (RANGEL-MORENO et al., 2008). A imunoglobulina (Ig) do isotipo IgG é o anticorpo dominante neste processo, e o isotipo IgA é importante para proteção no trato respiratório (CHEN et al., 2018). As células B iniciam a resposta contra o IAV aproximadamente três dias após a infecção, e os plasmócitos começam a secretar IgG no sétimo dia pós-infecção (WAFFARN; BAUMGARTH, 2011). Nas vias aéreas do trato respiratório, o isotipo IgM possui atividade neutralizante, mediando as respostas imunes iniciais (CHOI; BAUMGARTH, 2008). No soro suíno, as imunoglobulinas dos isotipos IgM e IgG são dominantes, sendo a IgM detectada no início da infecção, com pico sete dias após a infecção, e posteriormente é detectada a IgG, que atinge o pico de produção 25 dias após a infecção ou mais tarde (Figura 3). Os títulos permanecem elevados durante várias semanas e começam a declinar cerca de oito a dez semanas após a infecção. No trato respiratório superior, células secretoras de anticorpos no tecido da mucosa nasal produzem anticorpos do isotipo IgA, que são detectados quatro dias após a infecção (HEINEN et al., 2000; LARSEN et al., 2000; LEE et al., 1995; VAN REETH; LABARQUE; PENSAERT, 2006). No trato respiratório inferior, em amostras de lavado broncoalveolar de suínos infectados com IAV, são detectados os isotipos IgG e IgA (HEINEN et al., 2000; KITIKOON et al., 2006; LARSEN et al., 2000), atingindo o pico em torno de dez dias após a infecção (GEURTS VAN KESSEL et al., 2008) (Figura 3).

A resposta por anticorpos é dirigida principalmente para as proteínas HA, NA, M e NP (SHAW; PALESE, 2013). Os anticorpos contra a proteína HA são neutralizantes e a principal resposta protetora contra o IAV, sendo predominantemente dirigidos para a região globular da HA (HA1) (RAJÃO; PÉREZ, 2018). Porém, a região HA1 é altamente variável, abrigando mutações que podem levar à variação antigênica (Figura 2). Assim, a maioria dos anticorpos específicos para a região HA1 são apenas protetores contra vírus antigenicamente relacionados.

Os anticorpos produzidos contra a região HA1 bloqueiam a ligação do vírus aos receptores das células do hospedeiro e neutralizam a infectividade viral (Figura 4). Já a região da haste da HA (HA2) é mais conservada, desta forma os anticorpos contra a região HA2 apresentam reação cruzada, podendo se ligar a diferentes subtipos, porém usualmente estão presentes em baixos níveis (EKIERT et al., 2009; KRAMMER, 2019) (Figuras 2 e 4). A liberação dos vírus das células infectadas é limitada pelos anticorpos produzidos contra a proteína NA. Por sua vez, os anticorpos contra as proteínas NA e M2 do IAV restringem a replicação viral, e desencadeiam a morte das células infectadas por mecanismos dependentes de anticorpos (RAJÃO; PÉREZ, 2018) (Figura 4).

Figura 4 – Resposta imunológica induzida por diferentes proteínas do vírus influenza A.



Fonte: Adaptado de RAJÃO; PÉREZ, 2018.

2.5.3.2 Imunidade celular

As respostas de células T são detectadas a partir de sete dias após a infecção (HEINEN; DE BOER-LUIJTZE; BIANCHI, 2001; KHATRI et al., 2010), e são amplamente direcionadas contra epítopos presentes em todas as proteínas virais. Os linfócitos T consistem em populações funcionalmente distintas, sendo as células T $CD4^+$ e células T $CD8^+$ as mais bem definidas. Os linfócitos T $CD4^+$ auxiliam as respostas de imunidade celular e humoral. Os linfócitos T $CD8^+$ se diferenciam em linfócitos T citotóxicos (CTLs), produzindo citocinas e moléculas efetoras para restringir a replicação viral e matar as células-alvo infectadas pelo IAV (VAN REETH; VINCENT, 2019) (Figura 3). Os CTLs são específicos para epítopos de proteínas conservadas do IAV, principalmente NP e M1, conferindo resposta imunológica de reação cruzada, ou seja, heterossubtípica (GRANT et al., 2016; SRIDHAR, 2016). Além disso, os suínos também possuem uma população de linfócitos T $CD4^+CD8^+$ duplamente positivos, que possuem propriedades de células T auxiliares, citolíticas e memória, importantes para "depuração viral" (DE BRUIN et al., 2000; ZUCKERMANN, 1999).

As células T $CD8^+$ reconhecem o IAV apresentado pelas células apresentadoras de antígenos (APCs) associadas às proteínas do MHC de classe I (JUNG; LEE, 2020). As DCs que migraram dos pulmões para os linfonodos ativam a proliferação e diferenciação das células T $CD8^+$ imaturas em CTLs (HO et al., 2011; KREIJTZ; FOUCHIER; RIMMELZWAAN, 2011). Também contribuem para esta diferenciação, IFNs do tipo I, IFN-γ, IL-2 e IL-12 (PIPKIN et al., 2010; WHITMIRE; TAN; WHITTON, 2005). Dentro do citoplasma dos CTLs existem numerosos grânulos que contêm moléculas, incluindo perforinas e granzimas. As perforinas se agrupam na superfície da membrana plasmática das células-alvo que expressam o antígeno associado ao MHC de classe I formando poros, consequentemente, promove a difusão passiva das granzimas para induzir apoptose (VOSKOBOINIK; WHISSTOCK; TRAPANI, 2015) (Figura 4). Além disso, a replicação viral pode ser restringida pela clivagem de proteínas virais e de células hospedeiras envolvidas na replicação e defesa viral, devido a ação das granzimas (ANDRADE, 2010; VAN DOMSELAAR; BOVENSCHEN, 2011). Os CTLs também podem expressar citocinas que interagem com receptores das células infectadas com IAV, promovendo apoptose (ALLIE; RANDALL, 2017). Ademais, as células T $CD8^+$ produzem IFN-γ e TNF-α, que contribuem para os mecanismos de defesa viral (SCHMIDT; VARGA, 2018). As células T $CD8^+$ também controlam a inflamação pulmonar durante uma infecção aguda do IAV pela produção de IL-10 (SUN et al., 2009).

Após a infecção pelo IAV, os CTLs e DCs que tornaram-se específicos circulam no sangue, órgãos linfóides e no local da infecção (CHEN et al., 2018). Em uma infecção secundária por IAV, os CTLs respondem rapidamente, e a eficiência dos CTLs durante a infecção secundária é afetada pelo processo de ativação e diferenciação recebido na infecção primária (VAN GISBERGEN et al., 2011). Com o passar do tempo, há redução na citotoxicidade das células T $CD8^+$ de memória relacionada a diminuição da expressão das moléculas citolíticas e da capacidade de matar as células-alvo (GRANT et al., 2016). A disseminação do IAV das vias respiratórias superiores para o pulmão é impedido pelos linfócitos T $CD8^+$ de memória do epitélio nasal, na qual bloqueiam o desenvolvimento de doença pulmonar (PIZZOLLA et al., 2017). Para restringir a replicação viral e facilitar a eliminação viral, as células T $CD8^+$ de memória dos pulmões podem contribuir para a defesa de infecções de IAVs heterólogos (VAN BRAECKEL-BUDIMIR; HARTY, 2017). Além disso, durante uma infecção por IAV, os monócitos presentes nos pulmões contribuem para o estabelecimento de linfócitos T $CD8^+$ neste tecido (DUNBAR et al., 2017).

As células T $CD4^+$ reconhecem o IAV associado às proteínas do MHC de classe II nas APCs, como DCs, macrófagos e células B (JUNG; LEE, 2020). Ademais, os linfócitos T $CD4^+$ expressam ligantes coestimuladores que contribuem para a ativação das células B, e assim, a produção de anticorpos, como o ligante CD40 (CD40L) (SWAIN; MCKINSTRY; STRUTT, 2012). Durante a infecção pelo IAV, as DCs migram dos pulmões para os gânglios linfáticos ativando as células T $CD4^+$ (LUKENS et al., 2009). Antígenos, moléculas coestimuladoras e citocinas secretadas pelas DCs, células epiteliais e células inflamatórias estimulam a diferenciação dos linfócitos T $CD4^+$ em linfócitos T auxiliares (Th) do tipo 1 para responder em uma infecção do IAV (CHEN et al., 2018). As células Th1 ativam macrófagos alveolares e expressam citocinas antivirais, como IFN-γ, TNF-α e IL-2. A diferenciação dos linfócitos T $CD8^+$ para eliminar a infecção viral é regulada pela IL-2 e IFN-γ que são expressos pelas células Th1 (CHEN et al., 2018). Além das células Th1, as células T $CD4^+$ também podem diferenciar-se em linfócitos Th2, linfócitos Th17, células T reguladoras, e células T auxiliares foliculares (LUCKHEERAM et al., 2012; ZHU; YAMANE; PAUL, 2010). As células Th2 ligam-se a antígenos associados ao MHC de classe II e produzem IL-4 e IL-13 para promover respostas de linfócitos B (LAMB et al., 1982). As células T reguladoras por sua vez, contribuem para reparação e manutenção dos tecidos, bem como na supressão das respostas imunológicas e inflamação (ARPAIA et al., 2015; BETTS et al., 2012).

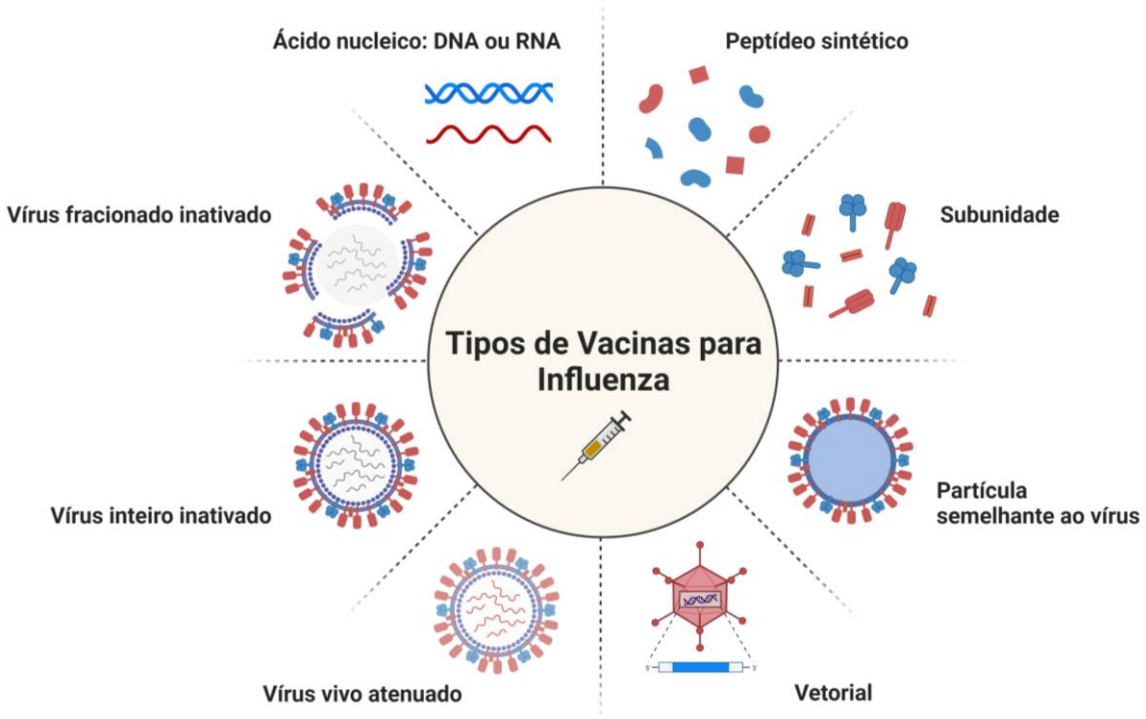
2.6 **Medidas de prevenção e controle para influenza**

Entre as principais medidas de controle para a prevenção da introdução do IAV em rebanhos suínos, destacam-se: a implementação de medidas de biosseguridade de rebanhos, como por exemplo, manter os animais em local limpo e seco, observar as boas práticas de produção, como boa higiene, ventilação das instalações, limpeza e desinfecção das instalações entre lotes e implementação de vazio sanitário, monitorar e testar os animais que entram no rebanho (por exemplo, fêmeas de reposição), evitar transportar os suínos durante a fase aguda da infecção, evitar o contato dos suínos com outras espécies animais e vacinar anualmente contra o vírus influenza todas as pessoas que entram em contato com os suínos (SCHAEFER et al., 2013; TORREMORELL et al., 2009, 2012; WHITE; TORREMORELL; CRAFT, 2017). Caso o vírus seja introduzido no rebanho, e na ocorrência de doença clínica mais severa, pode ser indicado o uso de antitérmicos, expectorantes e antimicrobianos para combater infecções bacterianas secundárias (TORREMORELL et al., 2009, 2012). As medidas de biosseguridade são importantes para o controle de doenças nos rebanhos, porém a principal medida de controle da influenza é a vacinação.

2.6.1 Vacinas para influenza

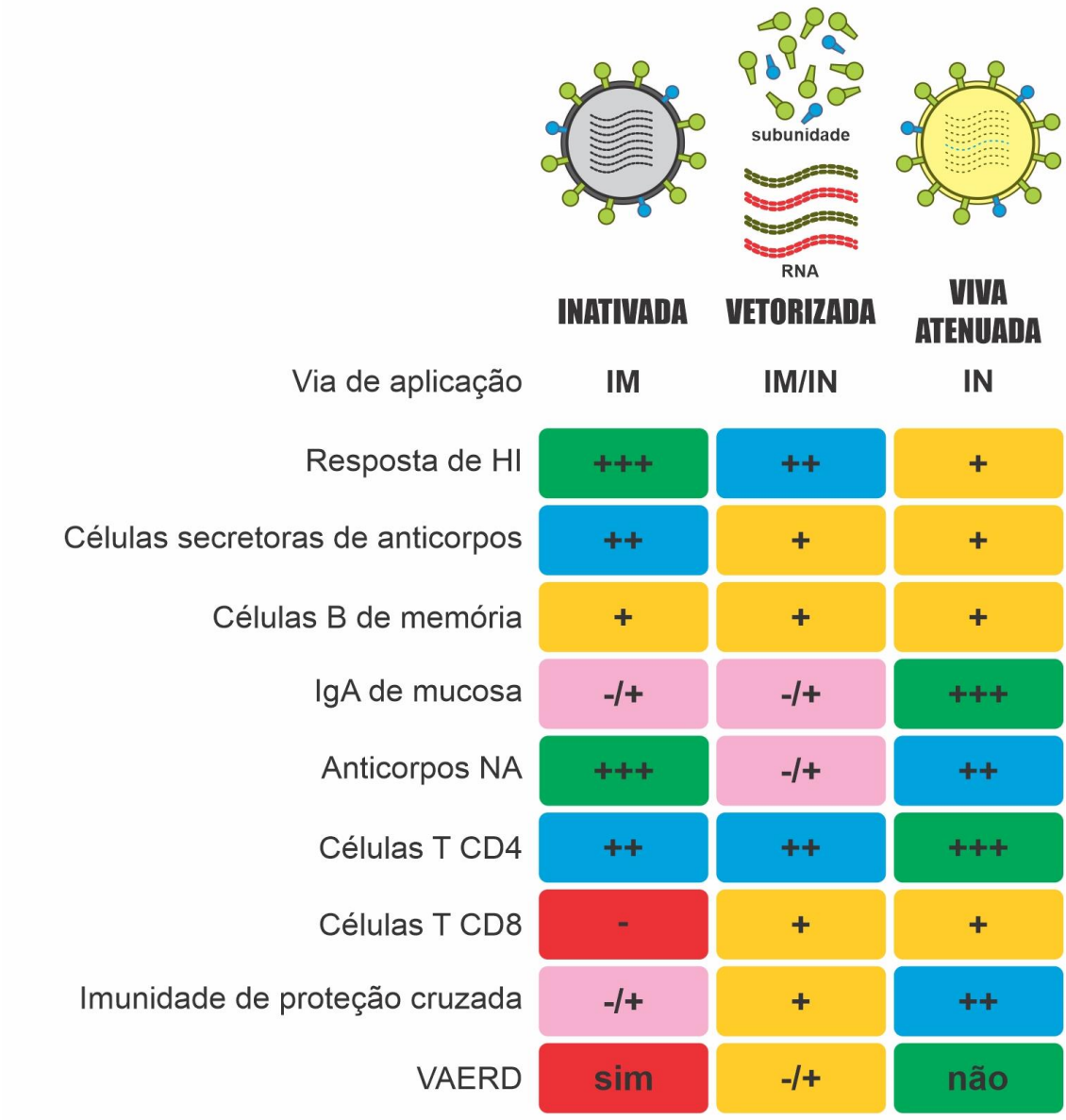
A utilização da vacina para o controle do vírus influenza tem sido uma das medidas profiláticas adotadas em rebanhos suínos em vários países. A vacinação tem por objetivo induzir uma resposta imune protetora, reduzindo a excreção viral, as lesões pulmonares, e a doença clínica. A resposta de memória, idealmente, deve proteger contra infecções futuras por IAV antigenicamente semelhante ao antígeno da vacina. Ademais, vários fatores influenciam a resposta imune do hospedeiro após a vacinação, como por exemplo: a plataforma ou tipo de vacina (Figura 5), a via de administração da vacina, e a apresentação dos antígenos ao sistema imunológico (VINCENT et al., 2017) (Figura 6).

Figura 5 – Diferentes tipos de vacinas para o vírus influenza.



Fonte: Criado com BioRender.com.

Figura 6 – Diferentes plataformas vacinais e vias de administração da vacina para influenza em suínos, e resposta imunológica resultante.



Legenda: IM = intramuscular; IN = intranasal; HI = inibição da hemaglutinação; NA = neuraminidase; VAERD = doença respiratória exacerbada associada à vacina (*vaccine-associated enhanced respiratory disease*).
Fonte: Adaptado de VINCENT et al., 2017.

A proximidade genética e antigênica entre a vacina e os vírus circulantes em suínos, imunogenicidade dos vírus vacinais, carga antigênica e adjuvante são determinantes para a potência da vacina e reatividade cruzada (VAN REETH; VINCENT, 2019). A imunogenicidade das vacinas é aumentada pelo uso dos adjuvantes, pela indução de um melhor processamento e entrega do antígeno às APCs, estímulo à produção de citocinas

imunomoduladoras específicas, ativação da resposta imune inata (DE VEER; MEEUSEN, 2011) e também pela produção de anticorpos com maior reação cruzada (VINCENT et al., 2017). Em suínos, a maioria das vacinas contra o IAV são acrescidas de adjuvantes a base de óleo, como por exemplo, Emulsigen-D e Montanide (RAJÃO; PÉREZ, 2018; SOUZA et al., 2018).

As vacinas mais utilizadas contra o IAV são as produzidas com vírus inteiro inativado (comerciais e autógenas). A vacinação para o IAV é rotineiramente realizada pelos produtores de suínos na América do Norte e Europa (MA; RICHT, 2010; RAJÃO et al., 2013b). Entretanto, esta não é uma prática de rotina nas granjas brasileiras. Desde 2014 é comercializada no Brasil uma vacina produzida com uma amostra do vírus H1N1pdm (A/California/04/2009; FluSure, Zoetis). Já em 2017, vacinas autógenas, produzidas a partir de amostras virais isoladas no próprio rebanho, começaram a ser utilizadas no país. De um modo geral, a eficácia das vacinas pode ser prejudicada pela presença de anticorpos maternos no momento da vacinação dos leitões (KITIKOON et al., 2006), bem como pela rápida evolução viral e aumento da diversidade genética e antigênica do vírus. Desta forma, isto traz desafios para o desenvolvimento de vacinas que induzam proteção cruzada para todas as linhagens virais circulantes (RAJÃO et al., 2014).

Para as fêmeas primíparas (leitoas) recomenda-se vacinar duas vezes antes do parto e para as porcas recomenda-se vacinação de reforço semestral, que geralmente é realizada de três a seis semanas antes do parto (VAN REETH; MA, 2013; VAN REETH; VINCENT, 2019). Em rebanhos vacinados, os anticorpos maternos persistem até cerca de 14 semanas de idade, protegendo os leitões durante a fase de creche (MARKOWSKA-DANIEL; POMORSKA-MÓL; PEJSAK, 2011). A combinação da vacinação de leitoas/porcas com a vacinação de leitões pode ser difícil, pois a imunidade passiva prolongada pode interferir no desenvolvimento da imunidade pós-vacinação dos leitões (VAN REETH; VINCENT, 2019). Para evitar a interferência dos anticorpos maternos em rebanhos com porcas com altos níveis de anticorpos por vacinação ou infecção, a vacinação dos leitões deve ser adiada até a idade de 12 a 16 semanas (VAN REETH; MA, 2013).

2.6.1.1 Vacinas inativadas

Comumente, utilizam-se as vacinas contendo vírus inativado para o controle de influenza, por apresentarem custo de produção relativamente baixo, amplo histórico de práticas

de fabricação, segurança e eficácia. Dentre as vacinas inativadas, encontram-se as vacinas com vírus inteiro, vacinas com vírus fracionado (*split-virus*) e vacinas de subunidades (YAMAYOSHI; KAWAOKA, 2019). Estas diferem na organização estrutural e componentes antigênicos, tendo impacto na imunogenicidade da vacina (SOEMA et al., 2015a). As vacinas contendo vírus inteiro são inativadas quimicamente com formaldeído, etilenoimina binária ou β-propiolactona, concentradas e purificadas. Na produção de vacinas com vírus fracionado, ocorre tratamento do antígeno viral com éter dietílico ou detergente, que rompe o envelope viral expondo todas as proteínas (NEURATH et al., 1971). Nas vacinas de subunidades há a separação do nucleocapsídeo e lipídios das glicoproteínas de superfície por etapas adicionais de purificação (LAVER; WEBSTER, 1976).

A maioria das vacinas disponíveis mundialmente para suínos são compostas por vírus inteiro e inativadas, acrescidas de adjuvante de emulsão, óleo em água, e administradas pela via intramuscular (SANDBULTE et al., 2015). Comumente, para suínos é necessário aplicação de duas doses de vacinas, com duas a três semanas de intervalo (RAJÃO et al., 2014). A proteção induzida pelas vacinas inativadas é dirigida contra vírus antigenicamente similares, estimulando altos níveis de anticorpos séricos contra a HA, todavia a produção de anticorpos de mucosa é limitada (HOLZER et al., 2019; RAJÃO et al., 2014). Uma limitação deste tipo de vacina é que sua capacidade de induzir proteção é afetada pela presença de alterações antigênicas nos vírus de campo, bem como pela redução de reação cruzada no ensaio de HI (RAJÃO; PÉREZ, 2018). Além disso, quando os suínos vacinados são desafiados com um vírus homossubtípico, mas antigenicamente distinto, pode haver intensificação dos sinais clínicos da doença nos animais vacinados. Este fenômeno é conhecido como doença respiratória exacerbada associada à vacina (VAERD; *vaccine-associated enhanced respiratory disease*) (GAUGER et al., 2011; RAJÃO et al., 2014), caracterizada por pneumonia broncointersticial severa com bronquiolite necrótica e hiperplasia (GAUGER et al., 2011, 2012).

As vacinas inativadas tradicionais induzem uma resposta imune celular muito limitada, ou até mesmo ausente, sendo menos eficazes em infecções causadas por vírus heterólogo. Além disto, estas vacinas são fracas indutoras de imunidade inata (RAJÃO; PÉREZ, 2018). As vacinas baseadas em vírus inteiro inativado protegem os suínos contra a doença clínica, e dependendo do título de anticorpos vírus-específico obtidos pelos ensaios de HI ou SN, a replicação viral pode ser completamente bloqueada ou reduzida (VAN REETH; VINCENT; LAGER, 2016). Estudos prévios demonstraram maior proteção, com redução de doença clínica e excreção viral em suínos vacinados com vacinas comerciais contendo vírus inteiro inativado, ao serem desafiados com vírus semelhante (KITIKOON et al., 2006, 2013; LEE; GRAMER;

JOO, 2007; LOVING et al., 2013), do que com vírus antigenicamente distintos (VAN REETH; VAN GUCHT; PENSAERT, 2003; VINCENT et al., 2008b, 2010b). Parys et al. (2022) demonstraram que alternar três diferentes vacinas de IAV inativadas em suínos pode resultar em resposta mais ampla de anticorpos e em proteção, do que o procedimento de vacinação tradicional de reforço com a mesma vacina.

2.6.1.1.1 Vacinas autógenas

As vacinas autógenas são vacinas inativadas personalizadas para cada granja ou propriedades adjacentes, usando os seus isolados recentes, fornecendo desta forma proteção contra vírus homólogo. A diversidade de IAVs circulantes em suínos juntamente com a limitação de IAVs presentes em vacinas comerciais, provavelmente é o principal motivo para o uso de vacinas autógenas nos rebanhos suínos (VINCENT et al., 2008a). A produção destas vacinas envolve as mesmas etapas exigidas para vacinas comerciais, compreendidas por isolamento viral, purificação, concentração do antígeno, inativação, acréscimo de adjuvante, e registro junto ao Ministério da Agricultura, Pecuária e Abastecimento (MAPA). As vacinas autógenas também devem atender aos requisitos gerais para a produção de vacinas virais, garantindo a inativação viral completa, e testadas quanto à segurança e à pureza. Geralmente, testes de potência e eficácia não precisam ser realizados pelos fabricantes de vacinas autógenas (MAPA, 2019; SANDBULTE et al., 2015).

Este tipo de vacina pode ser produzida mais rapidamente do que uma vacina comercial atualizada. Porém, o período para isolar, caracterizar o vírus e produzir a vacina é maior em comparação a realização de um pedido de vacina comercial já disponível (SANDBULTE et al., 2015). Além disto, pode haver variação nos produtos produzidos por diferentes empresas, devendo-se levar isto em consideração ao escolher este tipo de vacina. Recomenda-se uma avaliação completa da vacina de escolha para determinar a eficiência no rebanho, bem como uma avaliação sorológica dos suínos após a vacinação. Provavelmente, há distinção na eficácia de vacinas autógenas e comerciais entre rebanhos, devido aos vírus específicos que circulam em cada granja (SANDBULTE et al., 2015).

2.6.1.2 Vacinas vivas atenuadas

Existem diferentes métodos de atenuação viral para a produção de vacinas. As mutações que geram atenuação viral podem ser inseridas em um ou mais segmentos gênicos. As abordagens de atenuação podem, por exemplo, tornar o vírus adaptado ao frio e sensível à temperaturas mais altas (PENA et al., 2012), prejudicar a capacidade do vírus de resistir ao interferon do tipo I da célula hospedeira, com o truncamento da proteína NS1 (SOLÓRZANO et al., 2005), ou pela modificação do local de clivagem da HA para requerer a enzima elastase (MASIC; BABIUK; ZHOU, 2009).

As vacinas contendo vírus vivo atenuado mimetizam a rota natural de infecção se forem administradas por via intranasal, resultando em uma resposta imunológica sistêmica e de mucosa (BARRÍA et al., 2013), além de uma menor taxa de reações adversas. Diferentemente das vacinas de vírus inteiro inativado, as vacinas vivas atenuadas induzem resposta imune celular e humoral, podendo induzir IgA de mucosa no trato respiratório superior se for administrada via intranasal, fornecendo uma resposta imunológica mais duradoura e com reatividade cruzada abrangente (HOFT et al., 2017; LOVING et al., 2012). No entanto, as vacinas vivas atenuadas geralmente induzem títulos de anticorpos mais baixos do que uma vacina de vírus inteiro inativado (BASHA et al., 2011). As vacinas vivas atenuadas induzem a produção de células T $CD4^+$ e $CD8^+$ específicas, fornecendo uma resposta imunológica mais ampla, com sensibilidade menor à correspondência antigênica entre a vacina e os IAVs circulantes (CHENG et al., 2013). Além disso, vacinas vivas atenuadas podem induzir resposta imunológica pulmonar e células de memória residentes em tecidos, que podem proporcionar maior proteção do que as células T $CD8^+$ de memória circulantes, bem como, auxiliar na proteção contra IAVs heterólogos (WU et al., 2014a; ZENS; CHEN; FARBER, 2016). Ainda, durante a replicação viral intracelular as vacinas vivas atenuadas ativam TLR3 e TLR7, levando a regulação de citocinas inflamatórias (DIEBOLD, 2008).

Gauger et al. (2014) demonstraram que suínos vacinados com uma vacina viva atenuada e posteriormente desafiados com um vírus heterólogo, não apresentaram VAERD. Outros estudos em suínos vacinados com vacina viva atenuada também resultaram em proteção superior contra infecções heterólogas (KAPPES et al., 2012; LOVING et al., 2012; VINCENT et al., 2007). Todavia, o uso de vacinas vivas atenuadas apresentam o risco de reversão do vírus vacinal, e a possibilidade da ocorrência de rearranjo gênico entre o vírus vacinal e os vírus endêmicos em suínos (SHARMA et al., 2020).

2.6.1.3 Vacinas vetoriais

As vacinas vetoriais utilizam como vetor um vírus já bem caracterizado, como adenovírus, alfavírus, poxvírus, mas com replicação limitada e que apresentam ao sistema imune do hospedeiro uma proteína de um vírus-alvo contra a qual se quer uma resposta imunológica (RAJÃO; PÉREZ, 2018). No vetor viral são removidos alguns genes não essenciais, fazendo com que a replicação do vetor viral seja deficiente. No local da deleção são inseridos os genes de interesse, como por exemplo o gene HA do vírus influenza. O vetor produzido com o gene de interesse terá a capacidade de ligar-se aos receptores celulares do hospedeiro e direcionar a expressão do gene inserido, contudo, não será capaz de espalhar-se além das células iniciais infectadas (VANDER VEEN; HARRIS; KAMRUD, 2012).

A tecnologia de vacinas vetoriais permite uma rápida atualização do gene de interesse, por meio de clonagem (do amplicon ou do gene sintetizado) no vetor viral, possibilitando o rápido estabelecimento de replicons para a produção de vacinas. Este sistema possui a capacidade de gerar vacinas personalizadas, como as autógenas, com vírus circulantes em cada rebanho (RAJÃO; PÉREZ, 2018; SANDBULTE et al., 2015). No entanto, um dos riscos existentes em vacinas baseadas em vetores virais é a possibilidade de imunidade anti-vetor, podendo induzir tolerância à vacina (SOEMA et al., 2015a). A imunidade pré-existente ao vetor utilizado tende a reduzir as respostas imunológicas pela vacina vetorial (ERTL, 2016).

As vacinas vetoriais induzem resposta imune humoral e celular, bem como imunidade de mucosa se administradas por via intranasal (TUTYKHINA et al., 2011), além dos antígenos de interesse serem expressos em sua conformação nativa, o que resulta em maior especificidade dos anticorpos (RAJÃO; PÉREZ, 2018). Estas vacinas podem estimular o sistema imune inato, bem como induzir respostas de células T $CD4^+$ e $CD8^+$, e células B (ERTL, 2016; EWER et al., 2016; HUMPHREYS; SEBASTIAN, 2018). Em suínos, demonstrou-se que vacinas vetoriais induziram proteção contra o desafio com vírus homólogo (BOSWORTH et al., 2010; WESLEY; TANG; LAGER, 2004). Além disso, outros estudos também mostraram proteção parcial contra vírus heterólogo (ABENTE et al., 2019; BRAUCHER et al., 2012; VANDER VEEN et al., 2013), e indução de anticorpos IgA em suínos vacinados por via intranasal (BRAUCHER et al., 2012). Ainda, relatou-se resposta robusta de anticorpos neutralizantes, bem como resposta de células T $CD8^+$ específicas (JOSHI et al., 2021).

2.6.1.4 Vacinas de ácido nucleico

As vacinas baseadas em ácido nucleico (DNA e RNA) possibilitam a indução da expressão do antígeno de interesse nas células do hospedeiro, induzindo imunidade celular e humoral, resultando em uma proteção ampla, sem expor o hospedeiro ao vírus viável (KIM; JACOB, 2009; MA; RICHT, 2010). Estas vacinas de ácido nucleico podem estimular o sistema imune inato, e também permitem que os antígenos expressos sejam apresentados pelo MHC de classe I e II, estimulando células T $CD4^+$ e $CD8^+$ (LI; PETROVSKY, 2016).

Esta tecnologia é segura, rápida, estável, escalável e não há necessidade de multiplicar o vírus em ovos embrionados, como na produção tradicional de vacinas contra influenza (KUTZLER; WEINER, 2008). Além disso, permite a combinação de diferentes IAVs e a substituição de um IAV por outro (SANDBULTE et al., 2015). Produz-se uma cópia de DNA do segmento de RNA viral que codifica a proteína do vírus de interesse, liga-se a um plasmídeo de expressão, o DNA plasmidial é inserido em bactérias, e os plasmídeos são posteriormente purificados. Os plasmídeos purificados são administrados ao hospedeiro permitindo a produção do antígeno de interesse nas células, resultando em uma resposta imune contra este antígeno (SOEMA et al., 2015a).

Geralmente, as vacinas de DNA contra influenza são baseadas na expressão da HA. Ao comparar estas vacinas com as vacinas tradicionais contra a influenza foi observada imunidade humoral subótima em humanos e animais (RAJÃO; PÉREZ, 2018). Para aumentar a imunogenicidade, é necessária otimizar a eficiência da vacina com o uso de adjuvantes, diferentes métodos e doses de administração, doses maiores de vacina. Entretanto, estas otimizações podem aumentar o custo da vacina e não ser economicamente viável, em comparação com as outras plataformas de vacinas (SANDBULTE et al., 2015). Fotouhi et al. (2017) observaram que camundongos vacinados com uma vacina de DNA acrescida de adjuvante apresentaram aumento significativo nos títulos de IgG e IFN-γ, em comparação com os camundongos que receberam a vacina de DNA sem adjuvante. Além disso, estudos em suínos demonstraram resposta imune mediada por células e por anticorpos (BORGGREN et al., 2016), e forte indução de resposta humoral que resultou na redução da carga viral nos pulmões dos suínos vacinados (GORRES et al., 2011; LARSEN; OLSEN, 2002).

Um dos riscos em relação a segurança das vacinas de DNA é que anticorpos produzidos contra o vetor plasmidial podem tornar a vacina ineficaz. A produção contínua de antígenos de influenza no hospedeiro pode alterar o sistema imunológico ou induzir tolerância contra estes antígenos (SOEMA et al., 2015a).

As vacinas de RNA mensageiro permitem a produção de antígenos nas células hospedeiras, porém são mais seguras do que as vacinas de DNA, pois abrigam apenas os componentes necessários para a expressão da proteína, sendo rapidamente degradadas (RAJÃO; PÉREZ, 2018). As vacinas de RNA podem estimular o sistema imune inato através do TLR3, TLR7, TLR8, RIG-I e MDA5, induzindo IFN do tipo I e citocinas inflamatórias (ZHANG et al., 2019). Estas vacinas também podem promover resposta robusta de células T $CD4^+$ e $CD8^+$, além de respostas de anticorpos neutralizantes (PARDI et al., 2018). Uma vacina de RNA mensageiro protegeu tanto camundongos jovens quanto mais velhos contra desafio com diferentes IAVs, além de induzir proteção clínica em suínos, reduzindo a excreção viral após o desafio com vírus homólogo (PETSCH et al., 2012).

2.6.1.5 Vacinas recombinantes, com base em proteínas e partículas semelhantes a vírus (VLPs; *virus-like particles*)

A tecnologia do DNA recombinante possibilita a produção de antígenos proteicos do IAV que podem induzir diferentes respostas imunológicas. Além de promover respostas de anticorpos contra a proteína HA, também podem desenvolver resposta imune contra a região da haste da HA (HA2) e proteína M2, potencializando a reação cruzada (SOEMA et al., 2015a). As vacinas produzidas usando o sistema de expressão de baculovírus para proteínas virais permitem a expressão *in vitro* de grandes quantidades das proteínas HA e NA do IAV em um processo de fabricação relativamente rápido (RAJÃO; PÉREZ, 2018). As partículas semelhantes a vírus (VLPs; *virus-like particles*) são partículas de vírus recombinante que não possuem nenhum componente genômico, sendo formadas apenas por proteínas estruturais. Ademais, a produção das VLPs pode utilizar diferentes sistemas de expressão, como baculovírus, células de insetos, bactérias e plantas (LI et al., 2017; LOW et al., 2014; PILLET et al., 2016; VALERO-PACHECO et al., 2016). As VLPs não são replicantes, possuem a morfologia semelhante aos vírus, e assim, a antigenicidade, podendo ativar o sistema imune inato (RAJÃO; PÉREZ, 2018). As VLPs podem estimular as APCs, especialmente as DCs, ocorrendo a apresentação de antígenos por moléculas do MHC de classe I e II para ativação de células T $CD4^+$ e $CD8^+$ (KESHAVARZ et al., 2019; MOHSEN et al., 2017). Além disso, a ativação dos linfócitos B resulta em uma resposta imune humoral robusta (BRAUN et al., 2012; CIMICA; GALARZA, 2017).

Estudos com vacinas VLPs relataram indução de títulos robustos de anticorpos, bem como redução das lesões pulmonares e da excreção viral após o desafio em suínos (HERNANDEZ; MILLER; VAUGHN, 2016; PYO et al., 2012). Também observou-se redução nos sinais clínicos respiratórios (KITIKOON et al., 2009) e da replicação viral nos suínos vacinados e desafiados (LOEFFEN et al., 2011).

2.6.1.5.1 Vacinas virossomais

Os virossomas são partículas semelhantes ao vírus, tanto em tamanho como na composição do envelope viral, contudo não possuem material genético viral (HUCKRIEDE et al., 2005; WILSCHUT, 2009). Os virossomas podem interagir com o sistema imune através de vias semelhantes a dos vírus, e apresentar elevada imunogenicidade e antigenicidade específica (HUCKRIEDE et al., 2005; WILSCHUT, 2009). Nas vacinas virossomais, as proteínas HA e NA são integradas em lipossomas de bicamada de fosfatidilcolina, formando virossomas unilamelares com diâmetro de 150 nanômetros aproximadamente, que posteriormente irão se fusionar com as células-alvo, mas sem resultar em infecção das células, uma vez que os virossomas não possuem RNA viral (HERZOG et al., 2009). Além dos virossomas mimetizarem o vírus, a sua capacidade de se ligar e fusionar às células do hospedeiro, como faz o vírus nativo, também pode contribuir para uma imunidade robusta (HUCKRIEDE et al., 2005; WILSCHUT, 2009).

Os virossomas possuem a capacidade de estimular a expressão de MHC de classe I e II, e moléculas coestimulatórias, induzindo a maturação de DCs. As glicoproteínas do envelope do IAV na superfície virossomal facilitam a apresentação de antígenos pelas APCs, como as DCs, para os linfócitos T, provocando uma resposta imunológica robusta, estimulando resposta de Th1 e Th2, e induzindo resposta de CTLs (HUCKRIEDE et al., 2005; WILSCHUT, 2009; ZURBRIGGEN, 2003). Ademais, as vacinas virossomais levam a ativação de células B, resultando na produção de anticorpos. Além da resposta sistêmica de anticorpos IgG, também podem induzir resposta imune local de anticorpos IgA pela administração da vacina por via intranasal (BERNASCONI et al., 2016).

Os peptídeos podem ser utilizados para induzir resposta imunológica específica de células B e T contra epítopos conservados. Os virossomas também são usados como sistemas de entrega de antígenos peptídicos (SOEMA et al., 2015a). A imunização intramuscular de camundongos com virossomas contendo peptídeo NP do IAV induziu resposta de CTLs

específicos que foram capazes de lisar as células-alvo infectadas com o IAV (ARKEMA et al., 2000). Virossomas com epítopo para células T, acrescido de adjuvante, aumentou a resposta de células T $CD8^+$ específicas, resultando em recuperação dos camundongos desafiados com IAV heterólogo (SOEMA et al., 2015b). Ainda, Kammer et al. (2007) demonstraram que uma vacina virossomal induziu resposta imune humoral e celular robusta em camundongos, com indução de células T $CD8^+$.

Na Europa, há uma vacina virossomal trivalente licenciada para uso em humanos que se mostrou segura e com boa imunogenicidade (HERZOG et al., 2009; HERZOG; METCALFE; SCHAAD, 2002). Abdoli et al. (2014) desenvolveram uma vacina virossomal para H1N1 e H3N2, e observaram que os camundongos vacinados com os virossomas tiveram uma perda de peso menor e carga viral reduzida nos pulmões em comparação aos camundongos controles não vacinados após o desafio, além de desenvolverem uma resposta robusta de anticorpos. Além disso, galinhas vacinadas com virossomas apresentaram altos títulos de anticorpos por HI, e de anticorpos IgG e IgA no soro, proliferação de IFN-γ, bem como redução da excreção viral (MALLICK et al., 2011, 2012).

2.6.1.6 Vacinas universais

As vacinas universais têm como princípio tentar superar as desvantagens da natureza altamente mutável dos IAVs, baseando-se no desenvolvimento de respostas imunológicas contra epítopos conservados. Estas vacinas tem por objetivo estimular a resposta humoral e celular, induzindo imunidade neutralizante de ampla proteção cruzada, além de evitar as atualizações, prolongando as respostas imunológicas (RAJÃO; PÉREZ, 2018). No entanto, as proteínas conservadas utilizadas normalmente, são também proteínas pouco imunogênicas, requerendo estratégias para melhorar a imunogenicidade (RAJÃO; PÉREZ, 2018). As vacinas universais têm como alvo os epítopos conservados da HA, NA ou domínio extracelular da proteína M2 (M2e) para induzir anticorpos de reação cruzada, e proteínas internas NP ou M1 para induzir respostas de células T (RAJÃO; PÉREZ, 2018).

A região da haste da HA (HA2), é muito mais conservada entre os subtipos virais, diferentemente da região globular da HA (HA1). Os anticorpos contra a HA1 neutralizam a infecção, prevenindo a ligação do vírus aos receptores celulares ou a fusão da membrana. Os anticorpos contra a HA2 normalmente não neutralizam o vírus, mas protegem através da inibição de entrada, inibição da liberação da progênie viral, indução de citotoxicidade mediada

por células dependente de anticorpos (ADCC) ou da citotoxicidade pelo complemento (RAJÃO; PÉREZ, 2018). Em uma infecção pelo IAV, os anticorpos são produzidos principalmente contra a região HA1 e em baixos níveis contra a região HA2 (CORTI et al., 2011). Além disso, há regiões específicas na HA1 que são alvos para as vacinas universais, como o local de ligação ao receptor na HA1 (RAJÃO; PÉREZ, 2018). Estudos demonstraram que vacinas que possuem HA2 como antígeno, protegeram camundongos contra o desafio homólogo (CORTI et al., 2011) e heterossubtípico (STEEL et al., 2010), e uma vacina com antígeno HA2 e diferentes HA1s protegeu contra o desafio heterólogo (KRAMMER et al., 2013).

A proteína NA é mais conservada do que a proteína HA do IAV. Os anticorpos contra a NA interferem na liberação dos vírus na superfície celular, reduzindo a quantidade de progênie viral produzida, e também podem mediar ADCC (RAJÃO; PÉREZ, 2018). Em camundongos vacinados com antígeno NA, observou-se imunidade contra vírus homólogo e proteção parcial contra infecção heteróloga (WOHLBOLD et al., 2015). Suínos vacinados com uma vacina inativada contendo as proteínas HA e NA desenvolveram VAERD ao serem desafiados com um vírus antigenicamente distinto para ambas as proteínas, entretanto, o fenômeno VAERD não foi observado no desafio com um vírus com HA não homóloga mas com NA homóloga (RAJÃO et al., 2016).

Os anticorpos contra a M2e não neutralizam o vírus, mas evitam o brotamento viral, medeiam a morte de células infectadas por células NK ou macrófagos através de ADCC, opsonização Fc ou ativação do complemento (EL BAKKOURI et al., 2011). A proteína M2e é pouco imunogênica, pois está presente em pequenas quantidades no IAV (WU et al., 2007). Sendo assim, são utilizadas estratégias para melhorar a imunogenicidade, como adjuvantes, formas multiméricas da M2e, co-imunização com outras vacinas de IAV, fusão com proteínas transportadoras (KIM et al., 2013; LEE et al., 2015; TANG et al., 2017).

Os CTLs são direcionados principalmente para as proteínas internas do IAV, como NP e M1, gerando respostas imunológicas de reação cruzada (HILLAIRE et al., 2013). As proteínas NP e M possuem epítopos conservados que são compartilhados por vários subtipos do IAV (LEE et al., 2008). Vacinas compostas pelas proteínas internas induziram resposta de células T, bem como reduziram a excreção viral pós-desafio em humanos (LILLIE et al., 2012) e em suínos (MORGAN et al., 2016). As vacinas indutoras de CTLs auxiliam na redução da gravidade da doença após uma infecção heteróloga, contribuindo para a eliminação viral e restringindo a progressão da doença, uma vez que estas respostas imunes mediadas por células

não evitam a infecção. Desta forma, seriam mais eficientes se utilizadas em complemento às tecnologias que estimulam produção de anticorpos (RAJÃO; PÉREZ, 2018).

3 **ARTIGO CIENTÍFICO 1**

Os materiais e métodos, resultados e discussão que compõem esta tese são apresentados a seguir no formato de artigo científico. A imunogenicidade da vacina virossomal polivalente para influenza A em suínos é apresentada a seguir no formato de artigo científico. Este artigo científico foi publicado no periódico *Virology Journal* (20:181, 2023, DOI 10.1186/s12985-023-02153-5) e é intitulado "*A polyvalent virosomal influenza vaccine induces broad cellular and humoral immunity in pigs*".

Virology Journal

RESEARCH

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A polyvalent virosomal influenza vaccine induces broad cellular and humoral immunity in pigs

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Abstract

Background Influenza A virus (IAV) is endemic in pigs globally and co-circulation of genetically and antigenically diverse virus lineages of subtypes H1N1, H1N2 and H3N2 is a challenge for the development of effective vaccines. Virosomes are virus-like particles that mimic virus infection and have proven to be a successful vaccine platform against several animal and human viruses.

Methods This study evaluated the immunogenicity of a virosome-based influenza vaccine containing the surface glycoproteins of H1N1 pandemic, H1N2 and H3N2 in pigs.

Results A robust humoral and cellular immune response was induced against the three IAV subtypes in pigs after two vaccine doses. The influenza virosome vaccine elicited hemagglutinin-specific antibodies and virus-neutralizing activity. Furthermore, it induced a significant maturation of macrophages, and proliferation of B lymphocytes, effector and central memory $CD4^+$ and $CD8^+$ T cells, and $CD8^+$ T lymphocytes producing interferon-γ. Also, the vaccine demonstrated potential to confer long-lasting immunity until the market age of pigs and proved to be safe and non-cytotoxic to pigs.

Conclusions This virosome platform allows flexibility to adjust the vaccine content to reflect the diversity of circulating IAVs in swine in Brazil. The vaccination of pigs may reduce the impact of the disease on swine production and the risk of swine-to-human transmission.

Keywords Influenza A virus, Virosomal vaccine, Humoral immunity, Cellular immunity, Swine

Background

Influenza A virus (IAV) subtypes H1N1, H1N2 and H3N2 are endemic in swine herds globally, causing economic losses for the swine industry and public health concerns. The control of influenza in pigs in Brazil is a challenge due to co-circulation of multiple genetically distinct viruses of subtypes H1N1, H1N2 and H3N2. Human seasonal IAVs of subtypes H1N1 and H1N2 were introduced in swine in the middle of 1980s, and in the early 2000s, respectively, and formed three Brazilian genetic clades within the lineage H1-1B (1B.2.3, 1B.2.4 and 1B.2.6) [1–3]. In the middle 1990s, a human seasonal H3N2 IAV was

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introduced in swine and diversified into three genetic clades (1990.5.1, 1990.5.2 and 1990.5.3) [3]. Regarding to H1N1 pandemic (pdm) IAV, several human-to-swine spillover events have occurred since 2009, however, only four of these introductions resulted in sustained onward transmission in swine, giving rise to four distinct genetic clusters within the lineage 1A.3.3.2 [4]. A substantial antigenic diversity between distinct subtypes and lineages of Brazilian swine IAVs (swIAVs) has been found, and may impact vaccination [5]. In recent years, the genetic and antigenic diversity of swIAVs has expanded through reassortment events among viruses co-circulating in pigs and accumulation of amino acid changes in genes encoding the viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1, 6]. The HA binds to cell receptors and mediates virus entry into cells, and is the main antigen against which neutralizing antibodies are induced during infection or vaccination [7]. The HA gene is highly variable, harboring mutations that can lead to antigenic variation and, consequently an antigenic mismatch between the vaccine and infection strain that can lead to vaccine failure, and thus immune escape [8].

Vaccination is the most effective measure to mitigate and control influenza-associated morbidity and mortality in swine populations. Additionally, IAV vaccination in pigs contributes to human health by reducing zoonotic transmission and the appearance of "variants" in humans, as well as the emergence of pandemics. However, the rapid viral evolution and co-circulation of multiple distinct IAV lineages pose a challenge for the development of cross-protective vaccines [9]. Whole inactivated influenza virus (WIV) vaccines are commercially available in many countries around the world [10] and induce humoral immune response and protection in pigs against challenge with homologous virus [11]. Live-attenuated influenza vaccine (LAIV) induces a cell-mediated immune response and improved protection against challenge with heterologous virus but may reassort with wild-type IAV or revert virulence [12, 13]. Other vaccine platforms using viral vectors, nucleic acid-based particles and virus-like particles have also been tested in pigs [14–17]. These vaccines stimulate antibody and cell-mediated responses, are safe and can be used to construct polyvalent vaccines that can be updated [18]. In Brazil, a WIV containing the H1N1pdm virus as well as autogenous vaccines have been commercialized since 2014 and 2017, respectively. However, IAV vaccination in pigs is not a common practice in Brazilian farms.

Therefore, novel vaccine strategies that induce wide cross protection, are safe and can be rapidly updated are required. Virosomes consist of reconstituted viral envelopes, but without virus genetic material [19, 20]. Their use is an alternative for the control of influenza in pigs since they mimic virus infection, eliciting a broad immune response [20]. Influenza virosomes preserve the receptor-binding and membrane fusion activities of the HA, allowing the presentation to the major histocompatibility complex (MHC) class I and II, interacting with the immune system through pathways similar to IAVs, and resulting in high immunogenicity [19]. Virosomal influenza vaccines have already been developed for humans and poultry [21, 22]. However, its use in pigs has been poorly studied.

The aim of this study was to assess the immune response kinetics of a virosomal-based influenza vaccine containing the viral envelope proteins of H1N1pdm, H1N2, and H3N2 IAVs in pigs.

Methods

Viruses and vaccine

A/swine/Brazil/025-15/2015 1A.3.3.2 (H1N1pdm; NCBI GenBank Accession HA = MH559931 and NA = MH559933; BRMSA 1710), A/swine/Brazil/223-15-1/2015 1B.2.4 (H1N2; NCBI GenBank Accession HA = MH560035 and NA = MH560037; BRMSA 1698) and A/swine/Brazil/028-15-8/2015 1990.5.2 (H3N2; NCBI GenBank Accession HA = MH559963 and NA = MH559965; BRMSA 1697) were the viruses used in this study. H1N1pdm and H1N2 viruses were propagated in specific pathogen-free (SPF) embryonated chicken eggs and H3N2 virus was propagated in Madin–Darby canine kidney (MDCK) cells [23]. The three viruses were individually concentrated by tangential ultrafiltration, followed by ultracentrifugation, and the pellets were resuspended in TNE buffer (10 mM Tris, 100 mM NaCl and 1 mM EDTA, pH 7.4).

The virosomal influenza vaccine was prepared as previously described by Fonseca et al. [24]. Briefly, the three concentrated viruses were mixed with 200 mM of 1,2-dicaproyl-sn-glycero-3-phosphocholine (DCPC), then diluted 1:2 (v/v) in TNE buffer, and the final mixture was incubated in an ice bath for 30 min to ensure viral dissolution. Viral nucleocapsids were removed by ultracentrifugation (100,000 × g for 30 min at 4 °C). The supernatant was extensively dialyzed against TNE buffer for 48 h at 4 °C to remove the DCPC, which led to the self-assembling of virosomes. The virosomal particle was characterized previously by Fonseca et al. [24]. The influenza vaccine contained 128 µg of total HA and 20% (v/v) of Emulsigen®-D (MVP Laboratories) per mL. The HA concentration corresponded to 6, 21 and 73% for H1N1, H1N2 and H3N2, respectively, as evaluated by SDS-PAGE [24].

Animal study design

Forty-three (43) four-week-old pigs were obtained from a SPF herd composed of crossbreed pigs (MS 115–composite terminal sire developed by Embrapa Swine and Poultry with Landrace × Large White sows). All pigs were previously tested negative for IAV antibodies (Multispecies Influenza A Antibody Test kit, BioChek) and IAV RNA [25]. All pigs were transferred to the biosafety level 1 (BSL1) experimental facility one week before the beginning of the experiment and cared for in compliance with the Animal Use Ethics Committee of Embrapa Swine and Poultry (protocol number 001/2017). The pigs were randomly divided into two groups: G1: 10 non-vaccinated pigs; and G2: 33 vaccinated pigs. Pigs from G2 were vaccinated on days (D) 0 and 14 with 1 mL of the adjuvanted virosomal IAV vaccine, by the intramuscular route in the neck. Pigs were daily monitored for clinical signs, behavior, appetite and temperature or any adverse effects related to vaccination. Blood and nasal swab samples were collected from all pigs on D0, D14 and D28. Three pigs from the G2 group were kept in the experimental facility until D90 to assess the long-term immunity induced by vaccination, and additional blood and nasal swab samples were collected on D60 and D90. The pigs were anesthetized with 6 mg/kg of Zoletil® (Zolazepam + Tiletamine; 100 mg/mL, Virbac) by the intramuscular route and euthanized with one step electrocution followed by bleeding. Necropsy was performed on D28 for the G1 and G2 groups, and on D90 for the three remaining pigs from the G2 group. Spleen from all pigs were excised and kept in RPMI 1640 medium supplemented with 1 × penicillin, streptomycin and fungizone for the in vitro cell proliferation assay. Bronchoalveolar lavage fluid (BALF) [26] and blood with anticoagulant (BD Vacutainer® EDTA K2) were collected for cell profile analysis by flow cytometry. Lung, mediastinal lymph node, spleen, liver and kidney fragments were collected and preserved in 4% paraformaldehyde for histopathological assessment (Fig. 1A, B).

Diagnostic microbiology

The presence of other respiratory pathogens was investigated as follows: RNA was extracted from nasal swab samples using MagMAX™ Viral RNA Isolation kit (Thermo Fisher Scientific) and tested by RT-qPCR targeting IAV/matrix gene [25]. Serum and BALF samples were submitted for DNA extraction using the DNeasy® Blood & Tissue kit (Qiagen). Serum samples were evaluated by qPCR for detection of porcine circovirus type 2 (PCV2) [27]. BALF samples were evaluated by qPCRs for detection of *Actinobacillus pleuropneumoniae* [28], *Glaesserella parasuis* [29], *Mycoplasma hyopneumoniae* [30] and *Pasteurella multocida* [31]. Samples were not tested for porcine reproductive and respiratory syndrome virus (PRRSV), since Brazil is considered free of PRRS [32].

Histopathological evaluation and immunohistochemistry

Lung, mediastinal lymph node, spleen, liver and kidney tissue samples fixed in 4% paraformaldehyde, were routinely processed and stained with hematoxylin and eosin (H&E) [33]. The mediastinal lymph node samples were evaluated for PCV2 detection by immunohistochemistry [34]. Additionally, to confirm that the vaccine was not cytotoxic to the pigs, the qualitative and quantitative analysis of apoptotic cells was performed in lung, mediastinal lymph node, spleen, liver and kidney tissues by using the DeadEnd™ Colorimetric TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) System kit (Promega) in accordance with the manufacturer's recommendations but using the 3-amino-9-ethylcarbazole (AEC) chromogen. Microscopic analysis was carried out using optical microscopy (Axio Scope.A1, Zeiss) at 400 × magnification and consisted of blind quantification of positive and total cells in ten different fields, each field measuring 37,000 μm^2.

Serological analysis

Serum samples were evaluated for HA-specific and virus-neutralizing antibodies. For this, the serum samples were treated and submitted to the hemagglutination inhibition (HI) [35] and serum virus neutralization (SVN) [36] assays. The same IAV strains composed the virosomal vaccine in this study were used as antigens in the HI and SVN assays: A/swine/Brazil/025-15/2015(H1N1), A/swine/Brazil/223–15-1/2015(H1N2) and A/swine/Brazil/028-15-8/2015(H3N2). The results were reported as geometric mean antibody titers.

Determination of cellular profile

The cellular profile from the blood and BALF collected from the pigs was assessed by flow cytometry. Heparinized whole blood samples were diluted 1:3 (v/v) in PBS and the peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll-Paque™ PLUS, GE Healthcare), following the manufacturer's recommendations. PBMCs were resuspended in flow cytometry buffer (PBS supplemented with fetal bovine serum (FBS, 2% v/v, Gibco), bovine serum albumin (BSA, 2% w/v, Sigma-Aldrich) and sodium azide (0.01% w/v, Sigma-Aldrich)). BALF samples were centrifuged at 800 × g for 10 min at 4 °C and the pellet was resuspended in flow cytometry buffer. The PBMC and BALF cell concentration was adjusted and distributed to

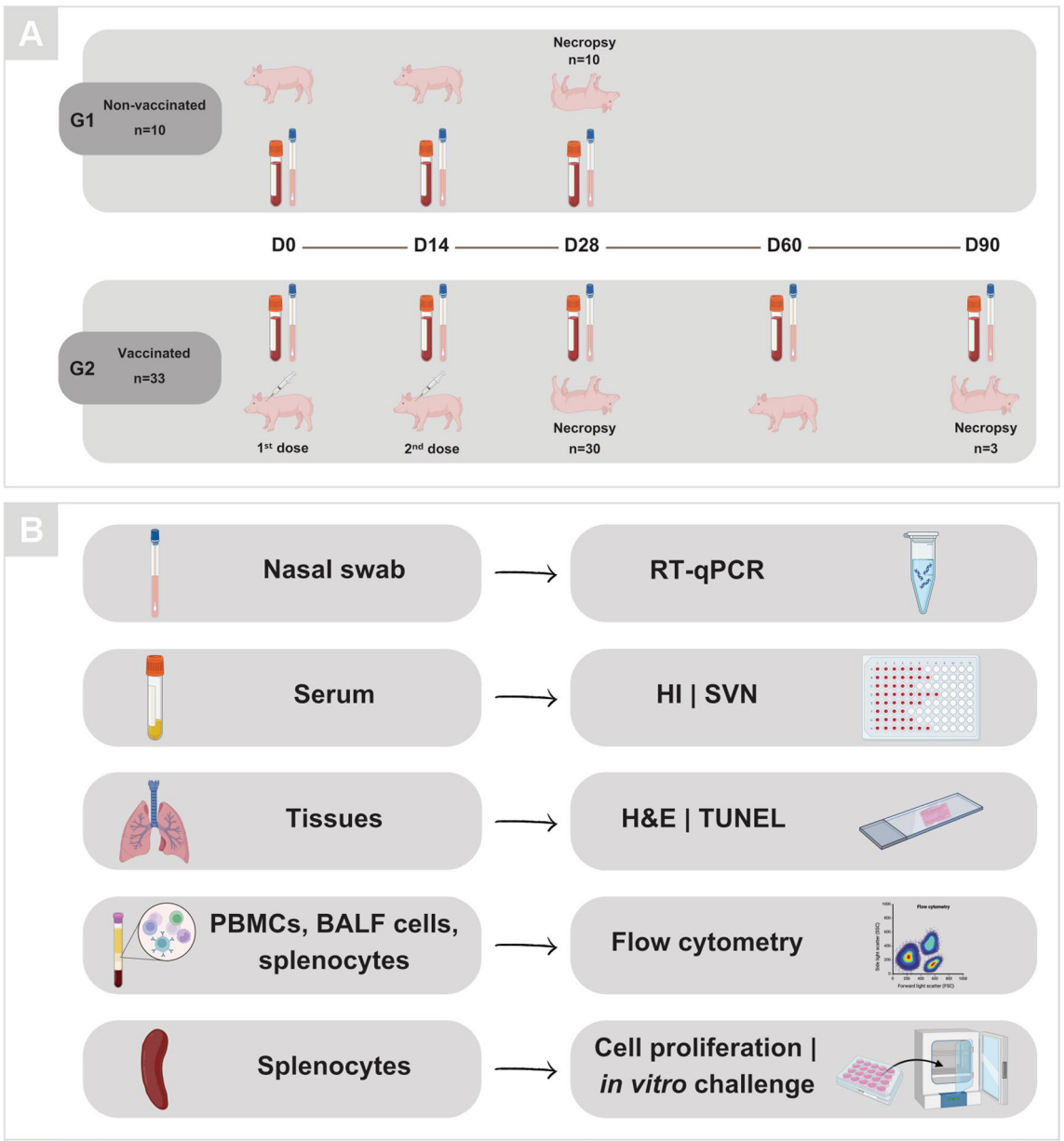

Fig. 1 Experimental design. **A** Timeline of G1 (non-vaccinated) and G2 (vaccinated) groups, highlighting the time points (D0, D14, D28, D60 and D90) of blood and nasal swab collection, administration of virosomal influenza vaccine (first and second dose) and necropsy of pigs. **B** Main laboratory assays carried out with biological samples collected from pigs of G1 and G2 groups. BALF = Bronchoalveolar lavage fluid; D = day; H&E = hematoxylin and eosin; HI = hemagglutination inhibition; PBMCs = peripheral blood mononuclear cells; SVN = serum virus neutralization. Illustration created with BioRender.com

approximately 1×10^6 cells/well (96-well plate). The cells were stained with specific monoclonal antibodies (mAbs) [37] to assess major lymphocyte, monocyte/macrophage, granulocyte and natural killer (NK) cell populations by four-color flow cytometry (Table 1). The PBMCs and BALF cells resuspended in flow cytometry buffer were incubated for 30 min at room temperature with a cocktail of specific mAbs. The fluorochrome-labeled mAbs used for the PBMCs and BALF cells are described in Table 2. For intracellular staining, the cells were treated with the Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD), according to the manufacturer's instructions, and then the cells were stained with CD79a (the epitope recognized by the mAb is located in the cytoplasmic domain) and CD3 (the PPT3 clone recognizes an extracellular and intracellular epitope on CD3) mAbs. All antibodies used

Table 1 Antibodies used in flow cytometry

Antibody	Species	Clone	Isotype	Fluorochrome	Dilution
7-AAD	NA	NA	NA	7-AAD	1:400
Granulocytes	Mouse anti-pig	6D10	IgG2a	FITC	Neat
Macrophages	Mouse anti-pig	BA4D5	IgG2b	RPE[a]	Neat
CSF1R	Mouse anti-pig	ROS8G11	IgG2a	APC	Neat
SLA Class II	Mouse anti-pig	2E9/13	IgG2b	FITC	Neat
IFN-γ	Mouse anti-pig	P2G10	IgG1	PerCP-Cy5.5	Neat
CD3e	Mouse anti-pig	PPT3	IgG1	FITC	1:10
				RPE-Cy7[b]	
				APC[c]	
CD4α	Mouse anti-pig	MIL17	IgG2b	RPE	1:100
CD5	Mouse anti-pig	1H6/8	IgG2a	FITC	Neat
CD8α	Mouse anti-pig	MIL12	IgG2a	FITC	1:100
				RPE	
CD14	Mouse anti-pig	MIL2	IgG2b	FITC	1:10
CD16	Mouse anti-pig	G7	IgG1	RPE	1:10
SWC7 or CD19	Mouse anti-bovine	CC55	IgG1	RPE-Cy7[b]	Neat
CD25	Mouse anti-pig	K231.3B2	IgG1	RPE-Cy7[b]	Neat
CD27 or SWC2	Mouse anti-pig	B30C7	IgG1	APC	1:10
CD45RA	Mouse anti-pig	MIL13	IgG1	FITC	Neat
CD79a	Mouse anti-human	HM57	IgG1	RPE	1:10
CD335	Mouse anti-pig	VIV-KM1	IgG1	APC	1:100
IgG1 isotype control	Mouse	NA	IgG1	FITC	1:10
				RPE	
				APC	
	Mouse anti-pig			RPE-Cy7[b]	Neat
				PerCP-Cy5.5[d]	Neat
IgG2a isotype control	Mouse	NA	IgG2a	FITC	1:10
				RPE	
				APC	
IgG2b isotype control	Mouse	NA	IgG2b	FITC	1:10
				RPE	

[a] Monoclonal antibody dilution following RPE labeling (Serotec)

[b] Monoclonal antibody dilution following RPE-Cy7 labeling (Serotec)

[c] Monoclonal antibody dilution following APC labeling (Serotec)

[d] Monoclonal antibody dilution following PerCP-Cy5.5 labeling (Serotec)

NA = Not applicable

in the staining were previously titrated for their optimum concentrations. To evaluate fluorochrome unspecific staining, respective isotype controls for anti-IgG1, anti-IgG2a and anti-IgG2b were analyzed in the preliminary procedure to set up technical parameters.

The stained cells were acquired using an Accuri™ C6 Plus flow cytometer (BD). Fifty thousand events were analyzed based on forward scatter (FSC) and side scatter (SSC), using Accuri™ C6 Plus (BD) and FlowJo™ (Tree Star Inc.) software. Before sample analysis, flow cytometer settings were verified using Cytometer Setup and Tracking beads (CS&T beads, BD) as described in the manufacturer's instructions. Compensation beads were used with single stains of each antibody to establish the compensation settings. The SSC threshold was set at 8,000 units to eliminate debris. Gates considered to indicate positive and negative staining cells were set based on fluorescence minus one (FMO) tests of samples, and these gates were performed systematically on each sample, allowing minor adjustments for SSC variability. Dead cells were excluded by discrimination with 7-AAD dye, according to our protocol previously described [38].

Table 2 Panels of fluorochrome-labeled monoclonal antibodies

Panel	Fluorochrome-labeled monoclonal antibodies for PBMCs
A	7-AAD (BD Biosciences)
B	RPE-macrophages (clone BA4D5), FITC-SLAII (clone 2E9/13)
C	FITC-CD14 (clone MIL2), RPE-CD16 (clone G7)
D	FITC-CD3e (clone PPT3), RPE-CD4α (clone MIL17), RPE-Cy7-CD25 (clone K231.3B2)
E	RPE-Cy7-CD3e (clone PPT3), RPE-CD4α (clone MIL17), FITC-CD8α (clone MIL12), APC-CD335 (clone VIV-KM1)
F	RPE-Cy7-CD3e (clone PPT3), RPE-CD4α (clone MIL17), APC-CD27 (or SWC2, clone B30C7), FITC-CD45RA (clone MIL13)
G	RPE-CD79a (clone HM57), RPE-Cy7-SWC7 (or CD19, clone CC55), FITC-CD5 (clone 1H6/8)
Panel	Fluorochrome-labeled monoclonal antibodies for BALF cells
A	7-AAD (BD Biosciences)
B	FITC-granulocytes (clone 6D10)
C	APC-CSF1R (clone ROS8G11), FITC-SLAII (clone 2E9/13)
D	FITC-CD14 (clone MIL2), RPE-CD16 (clone G7)
E	RPE-Cy7-CD3e (clone PPT3), RPE-CD79a (clone HM57), FITC-CD5 (clone 1H6/8)
F	RPE-Cy7-CD3e (clone PPT3), RPE-CD4α (clone MIL17), FITC-CD8α (clone MIL12), APC-CD335 (clone VIV-KM1)
Panel	Fluorochrome-labeled monoclonal antibodies for in vitro cell proliferation assay
A	7-AAD (BD Biosciences), RPE-macrophages (clone BA4D5)
B	RPE-CD79a (clone HM57), RPE-Cy7-SWC7 (or CD19, clone CC55)
C	APC-CD3e (clone PPT3), RPE-CD4α (clone MIL17), RPE-Cy7-CD25 (clone K231.3B2)
D	APC-CD3e (clone PPT3), RPE-CD8α (clone MIL12), RPE-Cy7-CD25 (clone K231.3B2)
E	RPE-Cy7-CD3e (clone PPT3), RPE-CD4α (clone MIL17), APC-CD27 (or SWC2, clone B30C7)
F	RPE-Cy7-CD3e (clone PPT3), RPE-CD8α (clone MIL12), APC-CD27 (or SWC2, clone B30C7)
G	APC-CD3 (clone PPT3), RPE-CD8α (clone MIL12), PerCP-Cy5.5-IFN-γ (clone P2G10)

Monoclonal antibodies used to stain peripheral blood mononuclear cells (PBMCs), bronchoalveolar lavage fluid (BALF) cells and in vitro cell proliferation assay

In vitro cell proliferation assay

Due to the lack of an adequate biosafety structure for the challenge of pigs with influenza virus, the in vitro challenge by culturing swine splenocytes and stimulating them with the three vaccine virus strains was performed. For this, spleen fragments were mechanically dissociated under aseptic conditions and filtered through a 70 µm Nylon Cell Strainer (Corning). After this, red blood cells were depleted using Pharm Lyse™ Buffer (BD Biosciences) for five minutes at room temperature. The splenocytes were obtained after the addition of RPMI 1640 medium to stop the lysis reaction, followed by washing the cells twice with RPMI 1640 medium. Splenocytes were labeled with 2.5 µM carboxyfluorescein succinimidyl ester (CFSE) by applying the CellTrace™ CFSE Cell Proliferation kit (Invitrogen), for 15 min at 37 °C in the dark. The reaction was stopped by adding six volumes of RPMI 1640, supplemented with 10% FBS, followed by incubation for 5 min in an ice bath, in the dark. Finally, the cells were washed three times with RPMI 1640-10% FBS, and further resuspended at a concentration of 5×10^6 cells/mL in the same medium. Viable cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco), 1 mM GlutaMAX (Gibco), 25 mM HEPES (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 50 M 2-mercaptoethanol (Gibco) and 100 U/mL penicillin–streptomycin (Sigma-Aldrich) at 37 °C under 5% CO_2. During 96 h in the dark, cells were stimulated in vitro by adding 8000 $TCID_{50}$/mL of each of the three vaccine viruses separately (H1N1, H1N2 and H3N2). For the negative control, culture medium was added to one well of the microplate (non-virus-stimulated cells). For the positive control, two wells of the microplate were prepared, one well with 3 µg/mL of Concanavalin A from *Canavalia ensiformis* (Sigma-Aldrich) and another well with 10 µg/mL of lipopolysaccharide from *Escherichia coli* (Sigma-Aldrich).

CFSE in combination with mAbs, enabled the concomitant access to cell proliferation and activation status of cell subpopulations. Flow cytometry analysis was performed to identify and quantify lymphocyte subpopulations (CD3, CD4, CD8, CD79a mAbs), to measure the levels of cellular activation marker expression (CD25, CD19 mAbs), cellular memory marker expression (CD27 mAb), interferon-γ (IFN-γ) cytokine and also to quantify macrophages (BioRad Serotec) (Table 1). Proliferation was detected by loss of CFSE fluorescence [38].

Cells obtained after 96 h of stimulation were resuspended in flow cytometry buffer, and the cell concentration was adjusted and distributed to approximately

1×10^5 cells/well (96-well plate). To assess major lymphocyte and macrophage populations, cells were stained with the fluorochrome-labeled mAbs described in Table 2. For intracellular staining, the cells were treated with the Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD), according to the manufacturer's instructions, and stained with CD79a, CD3 and IFN-γ mAbs. The respective isotype controls and the acquiring stained cells were performed as described in the previous section.

The gate was performed as described in a previous study conducted by our research group [38]. Gates were set using the non-virus-stimulated sample for each individual pig. In summary, the gate was based on forward scatter (FSC) and side scatter (SSC) properties to estimate lymphocyte population and debris exclusion. The doublet cells were subjected to doublet plotting showing forward scatter height (FSC-H) against forward scatter area (FSC-A). Dead cells were excluded from the analysis using 7-AAD staining. Counterstaining with CD3e/CD79a allowed us to gate on T and B cells, respectively (Fig. 2). Among T lymphocytes, subsets of $CD4^+$ and $CD8^+$ T cells were captured using panels. The gate to identify monocyte/macrophage cell population was based on which T lymphocytes could be recovered from $CD4^{bright}$ cells. Among B-lymphocytes cells were defined as the sum of $CD79a^+$, the gate is similar to what has been described for T lymphocytes. The gate for positive cells to $CD79a^+$ was determined, which was differentiated in $SWC7^+$ (conventional B cells).

Statistical analysis

Differences between vaccinated (G2) and non-vaccinated (G1) groups were evaluated using the two-sided Student's t test in the statistical analysis system (SAS) [39]. *P* values ≤ 0.05 were considered statistically significant.

Results

Absence of clinical signs and respiratory pathogens

No respiratory clinical signs, changes in behavior, appetite or hyperthermia were observed in the pigs during the experiment. Also, no local inflammatory reactions or any adverse effects related to the vaccination were identified. During the experiment, two pigs died, one on D14 (from G2 group) and another on D22 (from G1 group). Both deaths were not related to the experiment. The pig from G2 group had unintended lesion on vagus nerve during blood collection on jugular groove which led to respiratory and cardiac depression followed by death. For the pig from G1 group, a necropsy was performed and no macroscopic or microscopic lesions were observed. Thus, the G1 and G2 groups remained with 9 pigs and 32 pigs, respectively.

All nasal swab samples collected on D0, D14, D28, D60 and D90 were negative for IAV by RT-qPCR. Moreover, serum samples collected on D0, D28 and D90 were negative for PCV2 and BALF samples were negative for *Actinobacillus pleuropneumoniae*, *Glaesserella parasuis*, *Mycoplasma hyopneumoniae* and *Pasteurella multocida* by qPCR.

Safety of the virosomal influenza vaccine in pigs

In the histopathological analysis, no significant lesions were detected in lung, spleen, liver and kidney tissue samples collected from all pigs. However, in mediastinal lymph node samples, follicular lymphoid hyperplasia was observed in 71.87% (23/32) of pigs from the G2 group, and mild histiocytic infiltration was observed in 11.11% (1/9) of pigs from the G1 group and 15.62% (5/32) from the G2 group. All mediastinal lymph node samples tested negative for PCV2 by immunohistochemistry.

In the TUNEL assay, there was no difference in the number of apoptotic cells observed in lung, mediastinal lymph node, spleen, liver and kidney tissues between non-vaccinated pigs (G1 group) and vaccinated pigs (G2 group) (Additional file 1: Fig. S1).

The polyvalent influenza virosome vaccine proved to be safe and non-cytotoxic to pigs, as demonstrated by the results obtained in the TUNEL assay and by the lack of adverse reactions after vaccination. The follicular lymphoid hyperplasia observed in the mediastinal lymph nodes from vaccinated pigs remains to be investigated, since immunohistochemistry of mediastinal lymph nodes and qPCR from serum samples were negative to PCV2.

Virosomal influenza vaccine elicited humoral immune response in pigs

All serum samples from the non-vaccinated pigs (G1 group) were negative for IAV by HI and SVN assays.

For the vaccinated pigs (G2 group), antibodies to H3N2 virus were detected in 18.75% (6/32) of the pigs (HI titers of 40–80) 14 days after the first vaccine dose. Antibodies to the three vaccine antigens were detected after the second vaccine dose (D28) as follows: for H1N1 virus, 18.75% (6/32) of pigs had HI titers of 40–160; for H1N2 virus, 46.88% (15/32) of pigs had HI titers of 40–160; and for H3N2 virus, 100% (32/32) of pigs had HI titers of 160–1280 (Fig. 3A). Antibodies to H1N2 (HI titer of 40) and H3N2 (HI titer of 160) were detected in one out of three pigs from the G2 group on D90 (Additional file 2: Fig. S2A).

Virus-neutralizing-antibodies for H1N2 were detected in 3.13% (1/32) of pigs (titer of 10) and for H3N2 in 100% (32/32) of pigs (titers of 10–160) 14 days after the first vaccine dose. On D28, virus-neutralizing antibodies were detected in all pigs from the G2 group as follows:

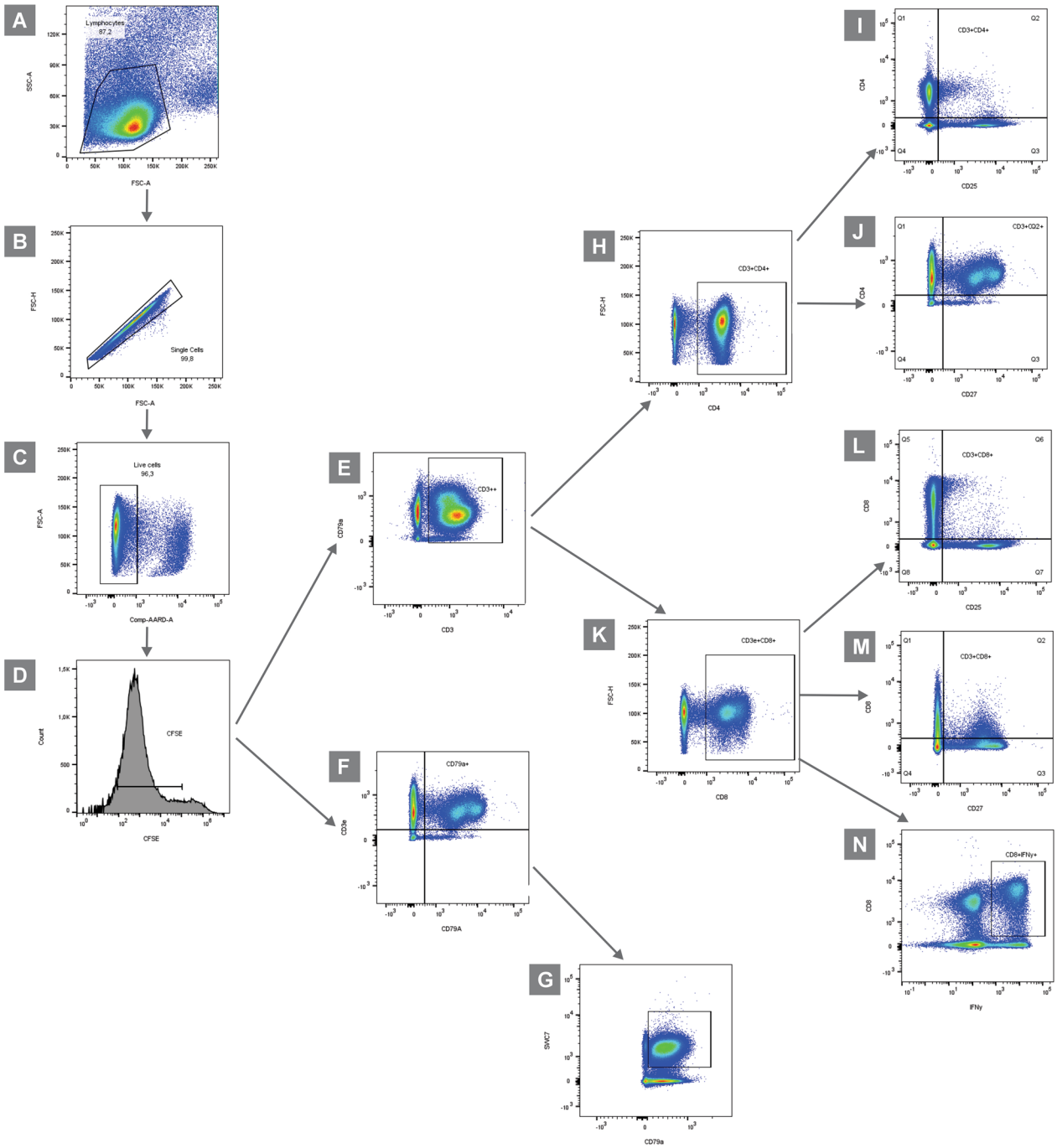

Fig. 2 Hierarchical gating strategy applied to samples from the experiment. **A** Flow cytometry dot plot gate shows lymphocytes (left). **B** The forward scatter (FSC) A × FSC-H dispersion was used to gate singlet cells. **C** Viable cells were defined by a gate encompassing the 7-AAD negative cells in a FSC-A vs. 7-AAD dot-plot. **D** Flow cytometry histograms gated into $CFSE^{+}$ events displaying the $CFSE^{low}$ region (the cell proliferation region). Results of cell proliferation with H1N1, H1N2 and H3N2 challenges. **E** From the viable cell gate (7-AAD negative), the CD3 vs. CD79a negative dot-plot was used to define T lymphocytes, and **F** CD79a positive vs. CD3 negative dot-plot was used to define B lymphocytes. **G** SWC7 vs. CD79a from a gate on cell population, the frequencies of conventional B cells. **H**, **K** From a gate on each cell population (exemplified here by $CD4^{+}$ T cells and $CD8^{+}$ T cells, respectively), the frequencies of **I** effector $CD4^{+}$ T cells ($CD4^{+}CD25^{+}$), **J** central memory $CD4^{+}$ T cells ($CD4^{+}CD27^{+}$), **L** effector $CD8^{+}$ T cells ($CD8\alpha^{+}CD25^{+}$), **M** central memory $CD8^{+}$ T cells ($CD8\alpha^{+}CD27^{+}$), and **N** cytotoxic $CD8^{+}$ T cells ($CD8\alpha^{+}IFN\gamma^{+}$) were determined in the respective dot-plots

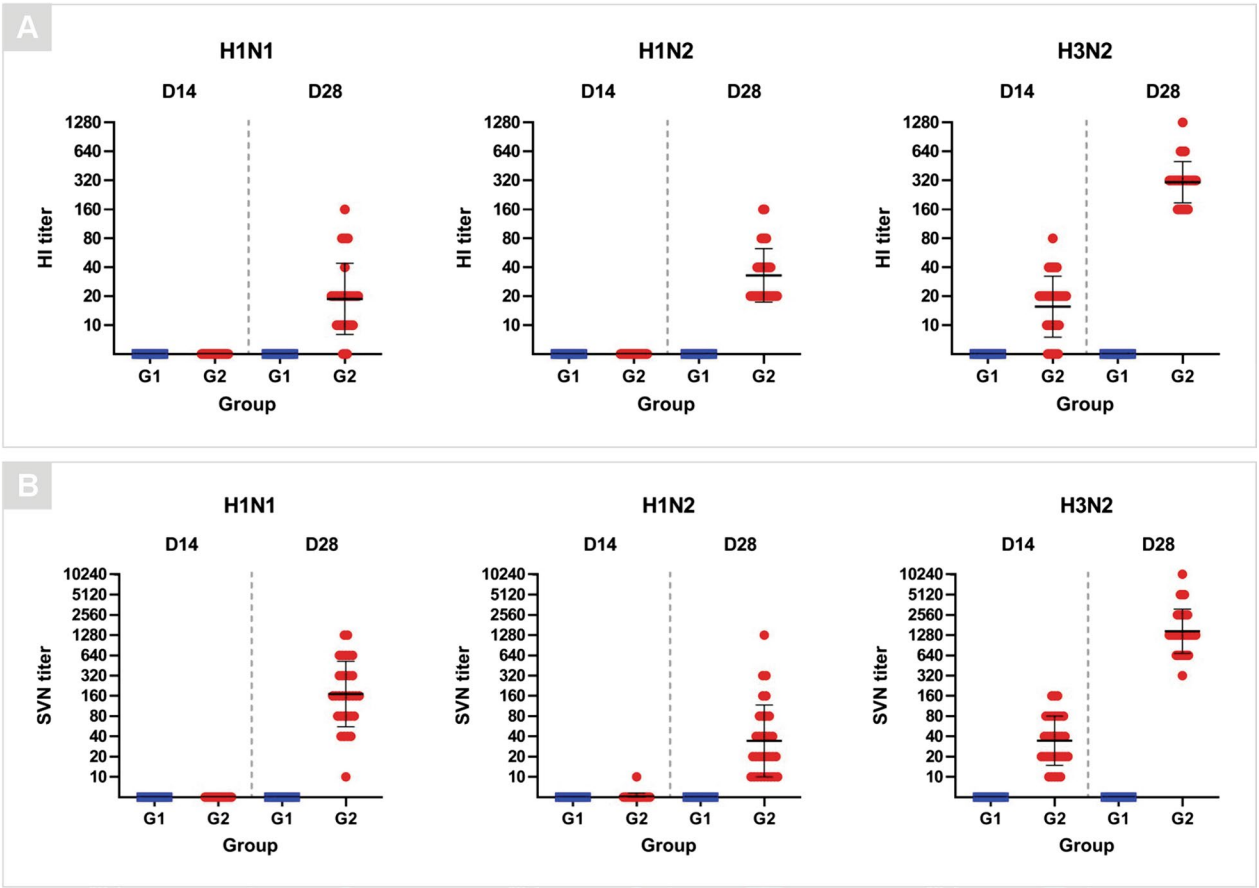

Fig. 3 Hemagglutination inhibition (HI) and serum virus neutralization (SVN) assays. Antibody titers by **A** HI and **B** SVN assays for H1N1, H1N2 and H3N2 subtypes of serum samples collected from pigs in the non-vaccinated (G1) and vaccinated (G2) groups on D14 and D28 post-vaccination. Data are shown for each pig per group and the black lines represent the geometric mean titers ± standard deviation

antibodies titers ranged from 10 to 1280 for H1N1 and H1N2 viruses, and ranged from 320 to 10,240 for H3N2 virus (Fig. 3B). On D90, one out of three pigs from G2 group had antibodies for H1N1 (titer of 320) and H1N2 (titer of 20), and all three pigs had antibodies for H3N2 (titers ranging from 40 to 320) (Additional file 2: Fig. S2B).

Virosomal influenza vaccine elicited cellular immune response in pigs

Significant induction of cell proliferation in vaccinated pigs after in vitro stimulation

Nine different cell subsets were defined in the in vitro stimulated splenocyte proliferation assay: macrophage$^+$ (monocytes/macrophages), CD79a$^+$SWC7$^+$ (B lymphocytes), CD3e$^+$CD4$^+$ (CD4$^+$ T lymphocytes), CD3e$^+$CD4$^+$CD25$^+$ (effector CD4$^+$ T cells), CD3e$^+$CD4$^+$CD27$^+$ (central memory CD4$^+$ T cells), CD3e$^+$CD8α$^+$ (CD8$^+$ T lymphocytes), CD3e$^+$CD8α$^+$CD25$^+$ (effector CD8$^+$ T cells), CD3e$^+$CD8α$^+$CD27$^+$ (central memory CD8$^+$ T cells) and CD3e$^+$CD8α$^+$IFNγ$^+$ (cytotoxic T lymphocytes producing IFN-γ). In all cell subsets evaluated for the three vaccine viruses (H1N1, H1N2 and H3N2), higher cell counts were observed in the G2 group on D28 compared to the G1 group ($P \leq 0.05$) (Fig. 4; Additional file 3: Table S1).

A high level of cellular proliferation was still detected in pigs from the G2 group, three months after the first vaccine dose. Furthermore, on D90, CD79a$^+$SWC7$^+$, CD3e$^+$CD4$^+$CD27$^+$ and CD3e$^+$CD8α$^+$IFNγ$^+$ cell subsets for H1N1, H1N2 and H3N2; CD3e$^+$CD8α$^+$CD27$^+$ cell subset for H1N1 and H3N2; CD3e$^+$CD4$^+$CD25$^+$ cell subset for H1N2; and macrophage$^+$ cell subset for H3N2 were statistically significant ($P \leq 0.05$) (Additional file 4: Fig. S3A; Additional file 3: Table S1).

Cellular profile in vaccinated pigs

Fifteen different cell subsets were defined in the PBMCs: macrophage$^+$ (macrophages), macrophage$^+$SLAII$^+$ (active macrophages), CD14$^+$CD16$^+$ (monocytes/macrophages), CD3e$^-$CD8α^{low}CD335$^+$ (natural killer cells),

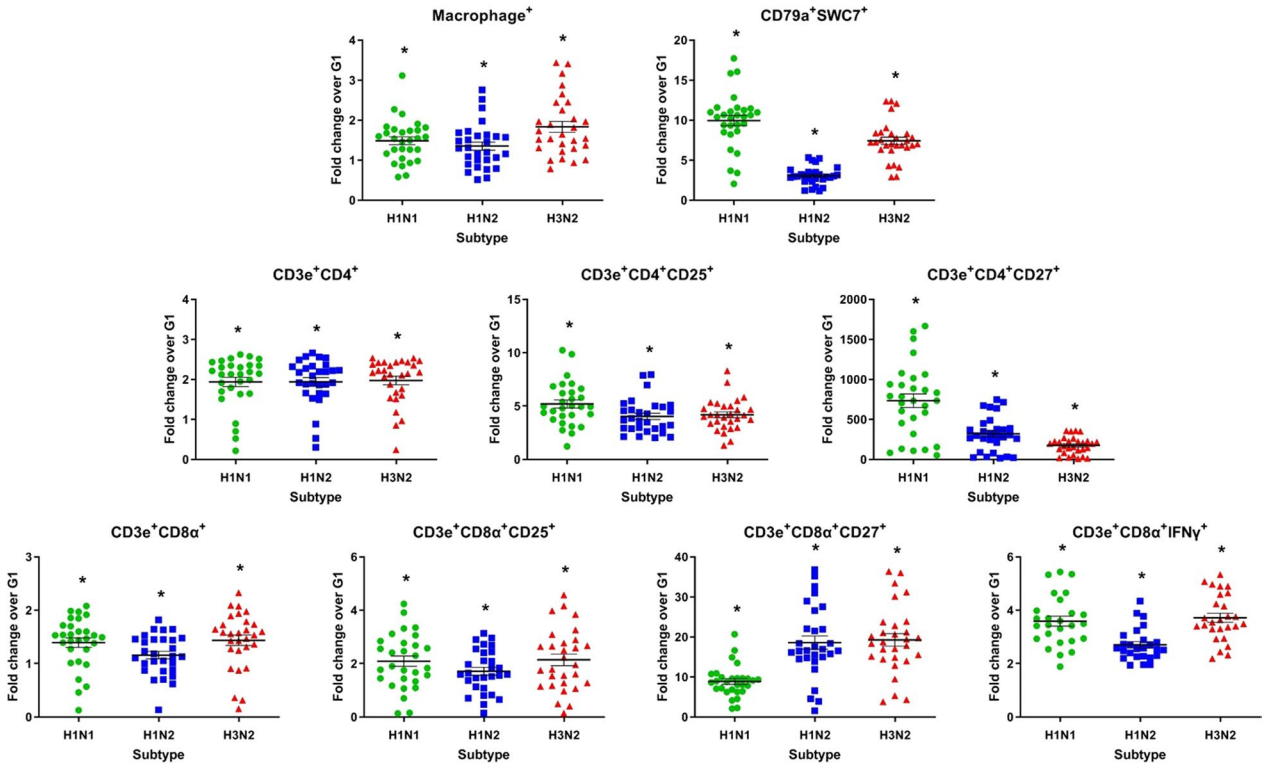

Fig. 4 In vitro cell proliferation assay. Immune cells in the splenocyte proliferation assay stimulated with the vaccine viruses (H1N1, H1N2 and H3N2) were compared as a fold change from the vaccinated group (G2) to the non-vaccinated group (G1) on D28 post-vaccination. Data are shown for each pig per virus subtype, and the black lines represent the mean ± standard error. Asterisks (*) denote significant differences between non-vaccinated (G1) and vaccinated (G2) groups ($P \leq 0.05$)

$CD79a^+$ (total B lymphocytes), $CD79a^+SWC7^+CD5^+$ and $CD79a^+SWC7^+CD5^-$ (conventional B cells), $CD3e^+$ (total T lymphocytes), $CD3e^+CD4^+$ ($CD4^+$ T lymphocytes), $CD3e^+CD8\alpha^+$ ($CD8^+$ T lymphocytes), $CD3e^+CD4^+CD8\alpha^+$ ($CD4^+CD8^+$ double-positive cells), $CD3e^+CD4^+CD25^+$ (effector $CD4^+$ T cells), $CD4^+CD27^+CD45RA^+$ (naive $CD4^+$ T cells), $CD4^+CD27^+CD45RA^-$ (central memory $CD4^+$ T cells), and $CD4^+CD27^-CD45RA^-$ (effector memory $CD4^+$ T cells). A high cell count of macrophage$^+$, macrophage$^+$SLAII$^+$, $CD79a^+$, $CD79a^+SWC7^+CD5^+$, $CD79a^+SWC7^+CD5^-$, $CD3e^+CD8\alpha^+$ and $CD4^+CD27^+CD45RA^-$ was detected in the G2 group on D28 compared to the G1 group ($P \leq 0.05$) (Fig. 5). Moreover, on D90, macrophage$^+$, macrophage$^+$SLAII$^+$, $CD14^+CD16^+$ and $CD3e^+$ cell subsets were significantly higher ($P \leq 0.05$) (Additional file 4: Fig. S3B).

Nine different cell subsets were defined in the BALF cells: $CSF1R^+$ (alveolar macrophages), $CSF1R^+SLAII^+$ (active alveolar macrophages), $CD14^+CD16^+$ (monocytes/macrophages), granulocyte$^+$ (granulocytes), $CD3e^-CD8\alpha^{low}CD335^+$ (natural killer cells), $CD79a^+$ (total B lymphocytes), $CD79a^+CD5^+$ (conventional B cells), $CD3e^+$ (total T lymphocytes) and $CD3e^+CD4^+$ ($CD4^+$ T lymphocytes). A high cell count of $CSF1R^+SLAII^+$, $CD14^+CD16^+$ and $CD79a^+CD5^+$ was observed in the G2 group on D28 compared to the G1 group ($P \leq 0.05$) (Fig. 6). In addition, on D90, $CSF1R^+$, $CSF1R^+SLAII^+$, $CD14^+CD16^+$ and $CD3e^-CD8\alpha^{low}CD335^+$ cell subsets were significantly higher ($P \leq 0.05$) (Additional file 4: Fig. S3C).

Discussion

The genetic and antigenic diversity of swIAVs in Brazil has increased since the emergence of H1N1pdm in 2009 [2, 40–43]. Currently, distinct lineages of H1N1, H1N2 and H3N2 IAVs circulate in swine herds across several Brazilian states [2, 3]. In addition, the Brazilian swIAVs are genetically distinct from the viruses circulating in pigs in other countries [1–3]. Consequently, the development of influenza vaccines that incorporate this genetic and antigenic diversity and can rapidly be updated, including locally adapted swIAVs, is relevant. In our study the vaccine strains represent the most frequently detected influenza virus lineages (H1-1A.3.3.2; H1-1B.2.4; H3-1990.5.2) in pigs in Brazil [1, 3, 4]. As a significant antigenic variation exists between them, no

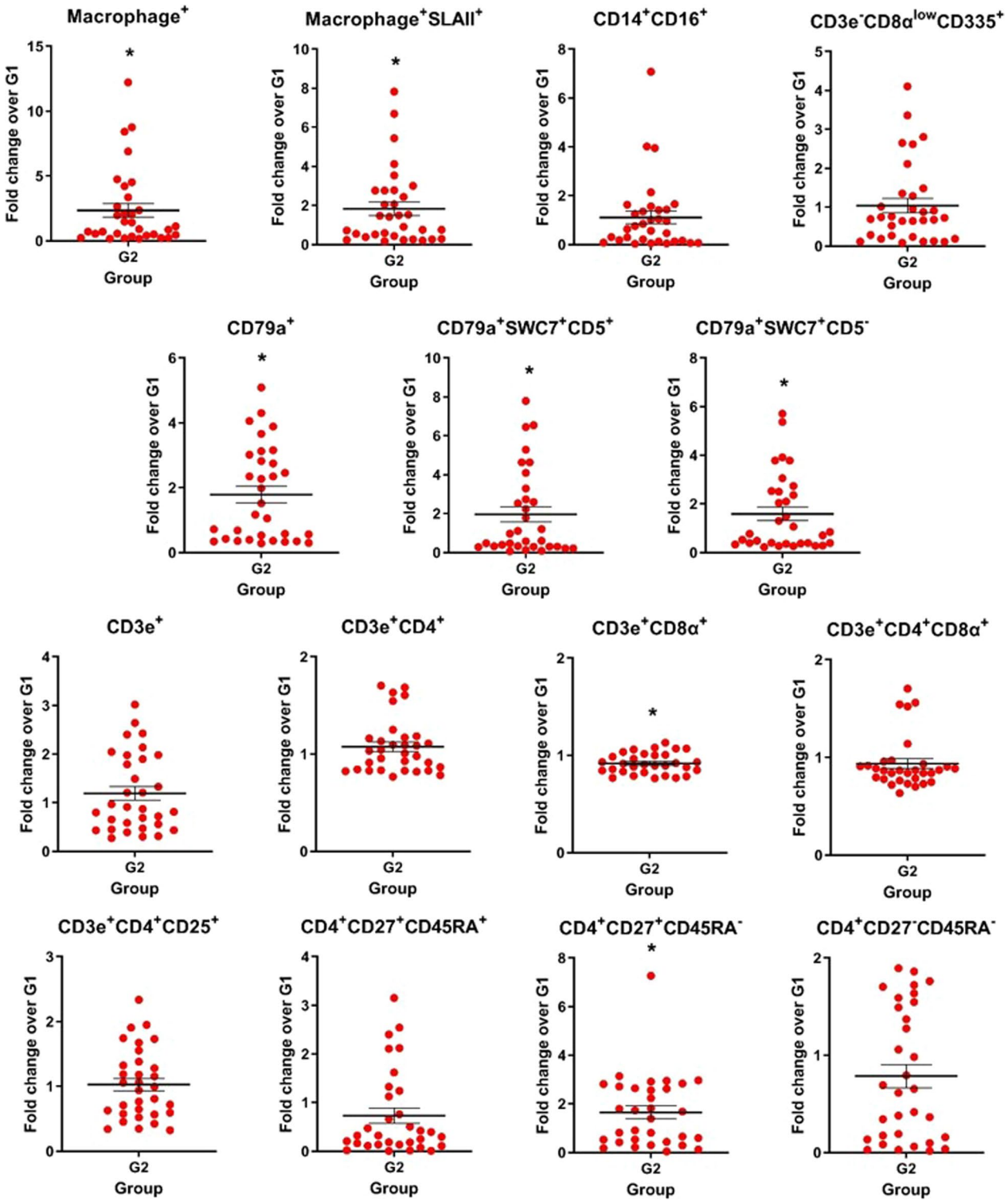

Fig. 5 Cell profile in blood. Immune cells in the peripheral blood mononuclear cells (PBMCs), as a fold change from the vaccinated group (G2) over the non-vaccinated group (G1) on D28 post-vaccination. Data are shown for each pig and the black lines represent the mean ± standard error. Asterisks (*) denote significant differences between non-vaccinated (G1) and vaccinated (G2) groups ($P \leq 0.05$)

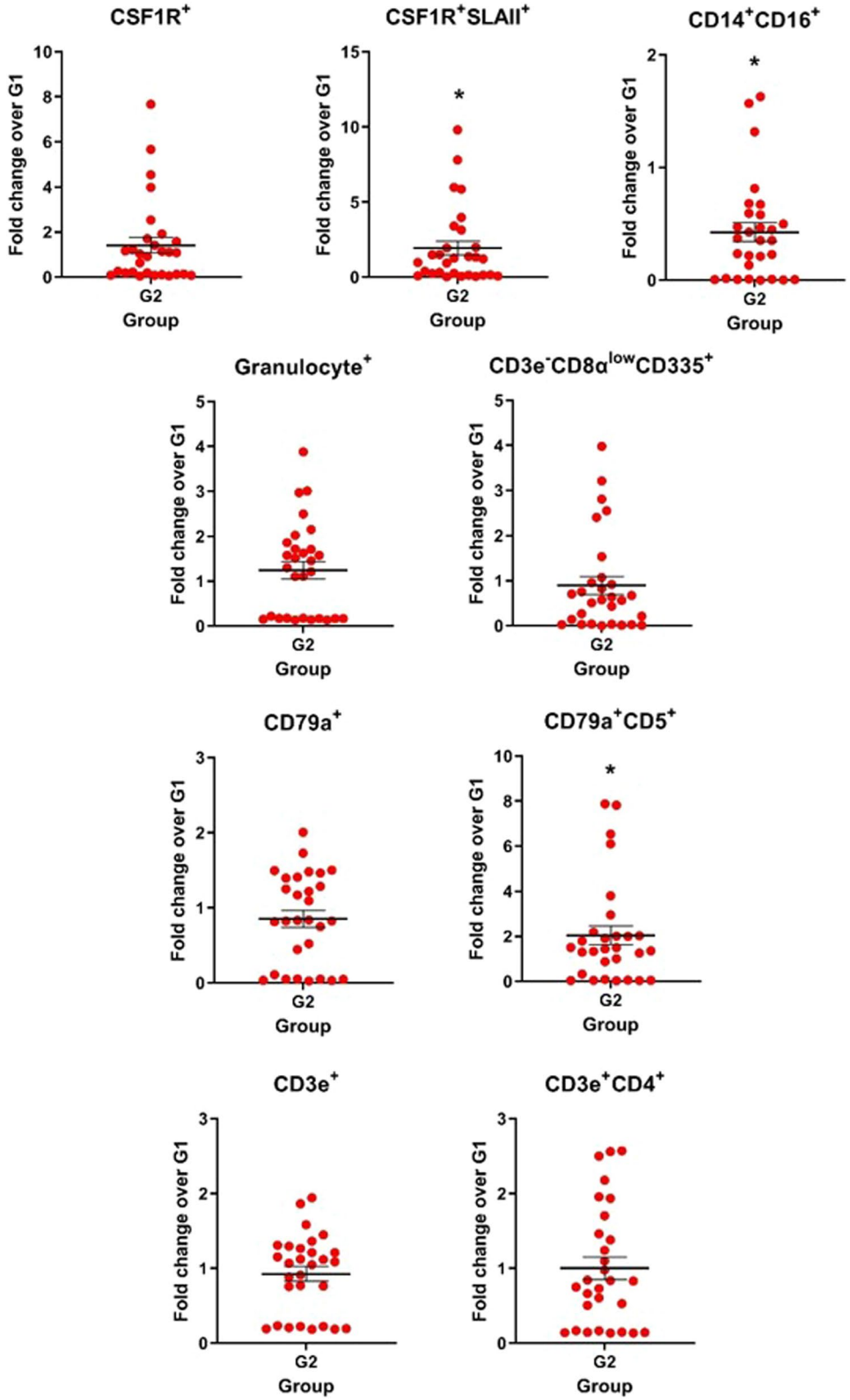

Fig. 6 Cell profile in bronchoalveolar lavage fluid (BALF). Immune cells in the BALF cells, as a fold change from the vaccinated group (G2) over the non-vaccinated group (G1) on D28 post-vaccination. Data are shown for each pig and the black lines represent the mean ± standard error. Asterisks (*) denote significant differences between non-vaccinated (G1) and vaccinated (G2) groups ($P \leq 0.05$)

cross-reactive antibody response in vaccinated pigs was expected.

The virosomal vaccines have been explored as an alternative to the conventional vaccine platforms, such as WIV vaccines, since they elicit both humoral and cellular immune responses while maintaining a favorable safety profile [19, 20]. Research on virosome vaccines, specifically in the context of targeting influenza or swine-related pathogens, remains relatively scarce. Virosome-based influenza vaccines have been reported for use in chickens [22] and humans [21]. Additionally, they have been investigated in mice [44] and ferrets [45] as an experimental model. This vaccine technology has also been successfully applied for other viruses infecting both animals, such as Newcastle disease virus in poultry [46] and avian metapneumovirus in turkeys [47], or humans, as seen with SARS-CoV-2 [48] and hepatitis A [49]. In this study, we demonstrated the immunogenicity in swine of a virosomal influenza vaccine containing the glycoproteins of the most prevalent virus subtypes circulating in Brazilian pig herds. Previously, the vaccine safety was evaluated in mice through the analysis of liver and kidney function, histopathology, and in vitro and in vivo cytotoxicity assays. It was demonstrated that the vaccine formulation was safe and non-cytotoxic for mice [24].

In swine, a robust humoral and cellular immune response was induced after two doses of the vaccine. HI and virus-neutralizing antibodies were detected against H1N1pdm, H1N2 and H3N2 viruses after booster immunization. In humans, the intramuscular administration of a virosomal influenza vaccine induced HI titers like those induced by WIV or subunit vaccines [50]. In poultry vaccinated by a virosomal influenza vaccine, low HI titers were detected seven days post vaccination, showing a significant rise in HI titers 14 days post vaccination, that were maintained at day 28 post vaccination [22]. Antibodies raised against HA are correlated with protection from clinical disease and are important to block the virus attachment and entry, preventing the virus infection [7]. Virus-neutralizing antibodies also have a vital function in preventing the binding of IAV to sialic acid receptors, the fusion process, and the release of newly formed viral particles [51]. A commercial virosomal vaccine for influenza in humans is available in several countries. This vaccine was considered highly effective by mimicking natural infection, and was immunogenic in both healthy and immunocompromised elderly, adults, and children [21].

The induction of influenza HA-specific antibody titers is affected by virus dose [52], and possibly by the virus subtype. Some studies have shown that pigs infected with H3N2 had higher antibody titers than those infected with H1N1 and H1N2 [53, 54]. Moreover, some viruses are more immunogenic than others, have dominant epitopes, and induce higher antibody titers [9]. The process of virus glycoprotein incorporation into the virosome is random, and as this formulation contained the glycoproteins from three different viruses, it is possible that a different concentration of each glycoprotein has been incorporated. According to a previously conducted SDS-PAGE, the virosome formulation used here contained more HA from the H3N2 virus, followed by H1N2 and H1N1 viruses [24]. This could explain high antibody titer for H3N2 virus compared to the antibody titers for H1N1 and H1N2 viruses by HI and SVN assays. Although antibody titers were not high for H1N1 and H1N2 subtypes, a robust cellular immune response was observed for these viruses.

In our study, immunization by a virosome-formulated influenza vaccine primed peripheral blood T and B cell subsets for early recall responses to H1N1pdm, H1N2 and H3N2 strains, including central memory $CD4^+$ T cells ($CD4^+CD27^+CD45RA^-$), cytotoxic T lymphocytes ($CD3e^+CD8^+$), and B lymphocytes ($CD79a^+$, $CD79a^+SWC7^+CD5^+$ and $CD79a^+SWC7^+CD5^-$). As indicated, the virosome vaccine was optimal for stimulation of cell-mediated immunity, cytotoxic T lymphocyte (CTL) activity and in particular, central memory $CD4^+$ T cells. CTL is responsible for elimination of virus-infected cells and clearance of influenza virus infection [55]. The central memory $CD4^+$ T cells and B cells subsets ($CD79a^+$, $CD79a^+SWC7^+CD5^+$ and $CD79a^+SWC7^+CD5^-$) are crucial for the development of memory B cells, antibody production, and antibody class switching [19]. Only the B cell subset $CD79a^+CD5^+$ and macrophages differed in BALF samples from vaccinated pigs, revealing a differentiated cellular response in the mucosa even though the vaccine has been administered by the intramuscular route.

After the in vitro stimulation of splenocytes from the vaccinated group, evidence of cellular immune response was marked by high maturation of macrophages, and high proliferation of B lymphocyte subsets (conventional B cells), T lymphocyte subsets (effector and central memory $CD4^+$ and effector and central memory $CD8^+$), and $CD8^+IFN\gamma^+$ T cells (CTL). The T cell subsets with the most significant responses to viral stimulation were the central memory helper ($CD3e^+CD4^+CD27^+$) and cytotoxic ($CD3e^+CD8\alpha^+CD27^+$) T cells. These cell populations can mediate T helper function and express perforin to mediate cytolytic activity against virus infected cells [56, 57]. Taken together, these populations are required for enduring $CD8^+$ T cell memory [58]. In addition, the virosome influenza vaccine induced significant proliferation of $CD3e^+CD8\alpha^+IFN\gamma^+$ T cells in swine, similar to previous studies in vaccinated mice [44], and pigs

inoculated with a nanoparticle-adjuvanted influenza vaccine [59]. The whole inactivated virus (WIV) vaccine does not induce significant CTL activity, but the virosome is optimal for delivery of the antigen to the cytosol of antigen-presenting cells contributing to the clearance of influenza virus [55]. Nonetheless, higher levels of B cells ($CD79a^+SWC7^+$) proliferated in the splenocytes from the vaccinated pigs. These findings suggest that long-term memory cells preferentially traffic in lymphoid tissues like the spleen [60]. This could explain their low frequency in peripheral blood and BALF after immunization of pigs, in contrast to what was observed in the spleen following the in vitro stimulation. The characteristic recall responses of the two $CD27^+$ populations, following the in vitro stimulation, became noticeably different, indicating that they were likely biased to a T helper 1 (Th1), T helper 2 (Th2) or T follicular helper (Tfh) phenotype. The commitment of memory $CD4^+$ T cells to Th1 or Tfh lineages and memory $CD8^+$ T cells to Th2 lineages provide cells that are poised for the lineage-specific expression of effector molecules upon preexposure to antigen. Upon recognizing virus-infected cells, $CD8^+$ T cells readily respond by killing infected cells by producing antiviral cytokines and promoting the recruitment of immune cells [61]. These findings have important implications for vaccine design, as adjuvanted-virosomal vaccines could promote a higher quantity and quality of memory Tfh cells, potentially allowing for enhanced humoral immunity after prime and boost vaccination. In this way, specific cells generate responses against IAV that can help eliminate the virus from the infected cells. Moreover, they provide protection against future infections with the presence of memory $CD4^+$ and $CD8^+$ T cells that can respond quickly to new virus infections. This broad proliferation of different immune cells, acting in association as observed here, locally, and systemically, is important for the mitigation of influenza infection in swine herds.

Another relevant T-cell response data, after the in vitro stimulation, was that both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells induced an upregulation in CD25 expression. It indicates cell activation [12], which might be associated with a protection response against IAV as observed in clinical challenge studies [62, 63]. These findings indicated that immunization of pigs by an influenza-virosomal vaccine efficiently primes and activates $CD8^+$ T cells that are important for the elimination of virus-infected cells, and reduction of virus shedding [64]. It is not clear why the $CD3e^+CD4^+CD25^+$ and $CD3e^+CD8\alpha^+CD25^+$ responses of pigs vaccinated with H1N1, H1N2 and H3N2 remained lower than $CDe3^+CD4^+CD27^+$ and $CD3e^+CD8\alpha^+CD27^+$ after in vitro stimulation. One possibility is that the IAV antigens activated in vitro the quiescent memory cells, and the analysis time after the activation of memory cells was insufficient to activate and detect the effector cells.

For the design of IAV vaccines, both humoral and cellular immunity should be considered, along with the capability to elicit strong and long-lasting immunity. In the production system in Brazil, the pig market age is around 130–140 days. Here, we assessed the immune response in pigs 90 days after the first vaccine dose, when pigs reached 130 days old. Despite the antibody titers were low at 90 days after vaccination, high proliferation of B lymphocytes, as well as central memory $CD4^+$ and $CD8^+$ T lymphocytes, and CTL ($CD3e^+CD8\alpha^+IFN\gamma^+$) were demonstrated for the three vaccine viruses (H1N1pdm, H1N2 and H3N2) on day 90. Moreover, the specific memory cells detected 90 days' post-vaccination allow rapid clonal expansion in a future exposure to IAV. Although a low number of pigs (n = 3) was evaluated, our results are encouraging since the immune response for influenza after vaccination persisted during the production phase when pigs are more at risk of influenza infection, during the nursery and the finishing phase. Further studies are needed to assess the duration of immunity induced by the virosome vaccine in swine.

Conclusions

In conclusion, the virosomal-based influenza vaccine developed here showed a robust antibody- and cell-mediated immune responses in pigs, with the potential to confer long-lasting immune memory to pigs (until the market age), and proved to be safe. It also allows for the rapid update of vaccine virus components. Additional studies are required to assess whether the vaccine also induced NA-specific antibodies and to ascertain whether this vaccine formulation is protective against the in vivo challenge by swIAV. For vaccine composition, selection of swIAV strains, by antigenic cartography, that better match with circulating viruses in swine in Brazil may contribute to the control of influenza in swine herds, reducing virus transmission among pigs, and the potential likelihood of generation of novel viruses.

Abbreviations

AEC	3-Amino-9-ethylcarbazole
BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
BSL1	Biosafety level 1
CFSE	Carboxyfluorescein succinimidyl ester
CS&T	Cytometer Setup and Tracking
CTL	Cytotoxic T lymphocyte
D	Day
DCPC	1,2-Dicaproyl-sn-glycero-3-phosphocholine
FBS	Fetal bovine serum
FMO	Fluorescence minus one
FSC	Forward scatter
FSC-A	Forward scatter area

FSC-H	Forward scatter height
HA	Hemagglutinin
H&E	Hematoxylin and eosin
HI	Hemagglutination inhibition
IAV	Influenza A virus
IFN	Interferon
LAIV	Live-attenuated influenza vaccine
mAbs	Monoclonal antibodies
MDCK	Madin–Darby canine kidney
MHC	Major histocompatibility complex
NA	Neuraminidase
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PCV2	Porcine circovirus type 2
Pdm	Pandemic
PRRSV	Porcine reproductive and respiratory syndrome virus
SAS	Statistical analysis system
SPF	Specific pathogen-free
SSC	Side scatter
SVN	Serum virus neutralization
swIAV	Swine influenza A virus
Tfh	T follicular helper
Th	T helper
TNE	Tris-NaCl-EDTA
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling
WIV	Whole inactivated virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-023-02153-5.

Additional file 1: **Fig. S1.** TUNEL assay. Number of apoptotic cells observed in the TUNEL assay in different tissues (lung, mediastinal lymph node, spleen, liver and kidney) from pigs in the non-vaccinated (G1) and vaccinated (G2) groups on D28 postvaccination. Data are displayed for each pig per group and the black lines represent the mean ± standard deviation.

Additional file 2: **Fig. S2.** Humoral immune response. Antibody titers by **A** hemagglutination inhibition (HI) and **B** serum virus neutralization (SVN) assays for H1N1, H1N2 and H3N2 subtypes of serum samples collected from pigs in the vaccinated (G2) group on D60 and D90 post-vaccination. Data are shown for each pig and the black lines represent the geometric mean titers ± standard deviation.

Additional file 3: **Table S1**. Data from the in vitro cell proliferation assay. Fold change means and standard errors of immune cells in the splenocyte proliferation assay stimulated with the vaccine viruses (H1N1, H1N2 and H3N2), from the vaccinated group (G2) over the non-vaccinated group (G1) on D28 and D90 post-vaccination.

Additional file 4: **Fig. S3.** Cellular immune response. Immune cells in the **A** in vitro splenocyte proliferation assay stimulated with the vaccine viruses (H1N1, H1N2 and H3N2), **B** peripheral blood mononuclear cells (PBMCs), and C bronchoalveolar lavage fluid (BALF) cells, as a fold change from the vaccinated group (G2) over the non-vaccinated group (G1) on D90 post-vaccination. Data are shown for each pig and the black lines represent the mean ± standard error. Asterisks (*) denote significant differences between non-vaccinated (G1) and vaccinated (G2) groups ($P \leq 0.05$).

Acknowledgements
The authors acknowledge Franciana A. V. Bellaver, Franciele Ianiski, Marisete F. Schiochet and Neide L. Simon for laboratory assistance, and Marina Schmitt and Vivian Fracasso for the graphic design. VH has a grant from Coordination for the Improvement of Higher Education Personnel (CAPES), financial code 001. ACF is a National Council for Scientific and Technological Development (CNPq) 2 fellow.

Author contributions
Conceptualization, RS, APAB, DG and VH; conducting the experiment, VH, APAB, DG and FNF; data analysis and curation, VH, APAB, DG, RS, MAZM and AC; writing—original draft preparation, VH; writing—review and editing, VH, RS, DG, APAB, FNF, MAZM, AC and ACF; funding acquisition, RS and APAB; supervision, RS and ACF. All authors have read and agreed to the published version of the manuscript.

Funding
This study was supported by the Brazilian Agricultural Research Corporation (EMBRAPA, Project SEG 12.13.10.004.00.00 and 22.16.05.004.00.05). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Availability of data and materials
The authors confirm that all relevant data are included in the paper or in the supplementary material. Additional information is available from the authors on reasonable request.

Declarations

Ethics approval and consent to participate
All experimental and animal management procedures were approved by the Animal Use Ethics Committee of Embrapa Swine and Poultry (Protocol no. 001/2017).

Consent for publication
Not applicable.

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

Received: 17 May 2023 Accepted: 6 August 2023
Published online: 16 August 2023

References

1. Anderson TK, Chang J, Arendsee ZW, Venkatesh D, Souza CK, Kimble JB, et al. Swine influenza A viruses and the tangled relationship with humans. Cold Spring Harb Perspect Med. 2021;11:a038737.
2. Nelson MI, Schaefer R, Gava D, Cantão ME, Ciacci-Zanella JR. Influenza A viruses of human origin in swine. Brazil Emerg Infect Dis. 2015;21:1339–47.
3. Tochetto C, Junqueira DM, Anderson TK, Gava D, Haach V, Cantão ME, et al. Introductions of human-origin seasonal H3N2, H1N2 and pre-2009 H1N1 influenza viruses to swine in Brazil. Viruses. 2023;15:576.
4. Junqueira DM, Tochetto C, Anderson TK, Gava D, Haach V, Cantão ME, Baker ALV, Schaefer R, Human-to-swine introductions and onward transmission of 2009 H1N1 pandemic influenza viruses in Brazil. Front Microbiol. 2023;14:1243567.
5. Schaefer R, Lopes S, Gava D, Ciacci-Zanella JR, Anderson TK, Lewis NS, et al. Genetic and antigenic diversity of contemporary influenza A virus in swine in Brazil. Proc Int Pig Vet Soc Congr. Rio de Janeiro; 2020. p. 828.
6. Rajão DS, Walia RR, Campbell B, Gauger PC, Janas-Martindale A, Killian ML, et al. Reassortment between swine H3N2 and 2009 pandemic H1N1 in the United States resulted in influenza A viruses with diverse genetic constellations with variable virulence in pigs. J Virol. 2017;91:e01763-e1816.
7. Krammer F. The human antibody response to influenza A virus infection and vaccination. Nat Rev Immunol. 2019;19:383–97.
8. Koel BF, Mögling R, Chutinimitkul S, Fraaij PL, Burke DF, van der Vliet S, et al. Identification of amino acid substitutions supporting antigenic change of influenza A(H1N1)pdm09 viruses. J Virol. 2015;89:3763–75.
9. Vincent AL, Perez DR, Rajao D, Anderson TK, Abente EJ, Walia RR, et al. Influenza A virus vaccines for swine. Vet Microbiol. 2017;206:35–44.
10. Mancera Gracia JC, Pearce DS, Masic A, Balasch M. Influenza A virus in swine: epidemiology, challenges and vaccination strategies. Front Vet Sci. 2020;7:647.

11. Van Reeth K, Ma W. Swine influenza virus vaccines: to change or not to change—that's the question. Curr Top Microbiol Immunol. 2013;370:173–200.
12. Kappes MA, Sandbulte MR, Platt R, Wang C, Lager KM, Henningson JN, et al. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. Vaccine. 2012;30:280–8.
13. Sharma A, Zeller MA, Li G, Harmon KM, Zhang J, Hoang H, et al. Detection of live attenuated influenza vaccine virus and evidence of reassortment in the U.S. swine population. J Vet Diagnostic Investig. 2020;32:301–11.
14. Braucher DR, Henningson JN, Loving CL, Vincen AL, Kim E, Steitz J, et al. Intranasal vaccination with replication-defective adenovirus type 5 encoding influenza virus hemagglutinin elicits protective immunity to homologous challenge and partial protection to heterologous challenge in pigs. Clin Vaccine Immunol. 2012;19:1722–9.
15. Joshi LR, Knudsen D, Piñeyro P, Dhakal S, Renukaradhya GJ, Diel DG. Protective efficacy of an orf virus-vector encoding the hemagglutinin and the nucleoprotein of influenza A virus in swine. Front Immunol. 2021;12:747574.
16. Borggren M, Nielsen J, Karlsson I, Dalgaard TS, Trebbien R, Williams JA, et al. A polyvalent influenza DNA vaccine applied by needle-free intradermal delivery induces cross-reactive humoral and cellular immune responses in pigs. Vaccine. 2016;34:3634–40.
17. Hernandez LA, Miller CL, Vaughn EM. Particle and subunit-based hemagglutinin vaccines provide protective efficacy against H1N1 influenza in pigs. Vet Microbiol. 2016;191:35–43.
18. Soema PC, Kompier R, Amorij JP, Kersten GFA. Current and next generation influenza vaccines: formulation and production strategies. Eur J Pharm Biopharm. 2015;94:251–63.
19. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM, et al. The virosome concept for influenza vaccines. Vaccine. 2005;23:S38.
20. Wilschut J. Influenza vaccines: the virosome concept. Immunol Lett. 2009;122:118–21.
21. Herzog C, Hartmann K, Künzi V, Kürsteiner O, Mischler R, Lazar H, et al. Eleven years of Inflexal® V-a virosomal adjuvanted influenza vaccine. Vaccine. 2009;27:4381–7.
22. Mallick AI, Parvizi P, Read LR, Nagy É, Behboudi S, Sharif S. Enhancement of immunogenicity of a virosome-based avian influenza vaccine in chickens by incorporating CpG-ODN. Vaccine. 2011;29:1657–65.
23. Zhang J, Gauger PC. Isolation of swine influenza A virus in cell cultures and embryonated chicken eggs. Methods Mol Biol. 2020;2123:281–94.
24. Fonseca F, Haach V, Bellaver F, Bombassaro G, Gava D, Paulino L, et al. Immunological profile of mice immunized with a polyvalent virosome-based influenza vaccine. Preprint at https://doi.org/10.21203/rs.3.rs-2923914/v1 (2023).
25. Zhang J, Harmon KM. RNA extraction from swine samples and detection of influenza A virus in swine by real-time RT-PCR. Methods Mol Biol. 2020;2123:295–310.
26. Lager KM, Vincent AL. In vivo models for pathotyping and vaccine efficacy for swine influenza. Methods Mol Biol. 2020;2123:345–51.
27. Opriessnig T, Yu S, Gallup JM, Evans RB, Fenaux M, Pallares F, et al. Effect of vaccination with selective bacterins on conventional pigs infected with type 2 porcine circovirus. Vet Pathol. 2003;40:521–9.
28. Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, et al. Identification and detection of Actinobacillus pleuropneumoniae by PCR based on the gene apxIVA. Vet Microbiol. 2001;79:47–62.
29. Turni C, Pyke M, Blackall PJ. Validation of a real-time PCR for Haemophilus parasuis. J Appl Microbiol. 2010;108:1323–31.
30. Dubosson CR, Conzelmann C, Miserez R, Boerlin P, Frey J, Zimmermann W, et al. Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. Vet Microbiol. 2004;102:55–65.
31. Townsend KM, Boyce JD, Chung JY, Frost AJ, Adler B. Genetic organization of *Pasteurella multocida cap* loci and development of a multiplex capsular PCR typing system. J Clin Microbiol. 2001;39:924–9.
32. Gava D, Caron L, Schaefer R, Silva VS, Weiblen R, Flores EF, et al. A retrospective study of porcine reproductive and respiratory syndrome virus infection in Brazilian pigs from 2008 to 2020. Transbound Emerg Dis. 2022;69:903–7.
33. Prophet EB, Mills B, Arrington JB, Sobin LH. Laboratory Methods in Histotechnology. Washington: Armed Forces Institute of Pathology; 1992.
34. Gava D, Zanella EL, Morés N, Ciacci-Zanella JR. Transmission of porcine circovirus 2 (PCV2) by semen and viral distribution in different piglet tissues. Pesqui Veterinária Bras. 2008;28:70–6.
35. Kitikoon P, Gauger PC, Vincent AL. Hemagglutinin inhibition assay with swine sera. Methods Mol Biol. 2014;1161:295–301.
36. Gauger PC, Vincent AL. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza A virus in swine. Methods Mol Biol. 2014;1161:313–24.
37. Dawson HD, Lunney JK. Porcine cluster of differentiation (CD) markers 2018 update. Res Vet Sci. 2018;118:199–246.
38. Maciag SS, Bellaver FV, Bombassaro G, Haach V, Morés MAZ, Baron LF, et al. On the influence of the source of porcine colostrum in the development of early immune ontogeny in piglets. Sci Rep. 2022;12:15630.
39. SAS. System for Microsoft Windows. Cary, NC, USA; 2012.
40. Rajão DS, Costa ATR, Brasil BSAF, Del Puerto HL, Oliveira FG, Alves F, et al. Genetic characterization of influenza virus circulating in Brazilian pigs during 2009 and 2010 reveals a high prevalence of the pandemic H1N1 subtype. Influenza Other Respi Viruses. 2013;7:783–90.
41. Schaefer R, Rech RR, Gava D, Cantão ME, da Silva MC, Silveira S, et al. A human-like H1N2 influenza virus detected during an outbreak of acute respiratory disease in swine in Brazil. Arch Virol. 2015;160:29–38.
42. Resende PC, Born PS, Matos AR, Motta FC, Caetano BC, Debur M do C, et al. Whole-genome characterization of a novel human influenza A(H1N2) virus variant, Brazil. Emerg Infect Dis. 2017;23:152–4.
43. Schaefer R, Zanella JRC, Brentano L, Vincen AL, Ritterbusc GA, Silveira S, et al. Isolation and characterization of a pandemic H1N1 influenza virus in pigs in Brazil. Pesqui Veterinária Bras. 2011;31.
44. Kammer AR, Amacker M, Rasi S, Westerfeld N, Gremion C, Neuhaus D, et al. A new and versatile virosomal antigen delivery system to induce cellular and humoral immune responses. Vaccine. 2007;25:7065–74.
45. Lambkin R, Oxford JS, Bossuyt S, Mann A, Metcalfe IC, Herzog C, et al. Strong local and systemic protective immunity induced in the ferret model by an intranasal virosome-formulated influenza subunit vaccine. Vaccine. 2004;22:4390–6.
46. Rasool MH, Mehmood A, Saqalein M, Nisar MA, Almatroudi A, Khurshid M. Development, biological characterization, and immunological evaluation of virosome vaccine against Newcastle disease in Pakistan. Biomed Res Int. 2021.
47. Kapczynski DR. Development of a virosome vaccine against avian metapneumovirus subtype C for protection in turkeys. Avian Dis. 2004;48:332–43.
48. van der Velden YU, Grobben M, Caniels TG, Burger JA, Poniman M, Oomen M, et al. A SARS-CoV-2 Wuhan spike virosome vaccine induces superior neutralization breadth compared to one using the Beta spike. Sci Rep. 2022;12:3884.
49. Van Der Wielen M, Vertruyen A, Froesner G, Ibáñez R, Hunt M, Herzog C, et al. Immunogenicity and safety of a pediatric dose of a virosome-adjuvanted hepatitis A vaccine: a controlled trial in children aged 1–16 years. Pediatr Infect Dis J. 2007;26:705–10.
50. Glück R, Mischler R, Finkel B, Que JU, Cryz SJ, Scarpa B. Immunogenicity of new virosome influenza vaccine in elderly people. Lancet. 1994;344:160–3.
51. Sicca F, Neppelenbroek S, Huckriede A. Effector mechanisms of influenza-specific antibodies: neutralization and beyond. Expert Rev Vaccines. 2018;17:785–95.
52. Waffarn EE, Baumgarth N. Protective B cell responses to flu—no fluke! J Immunol. 2011;186:3823–9.
53. Van Reeth K, Labarque G, Pensaert M. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. Viral Immunol. 2006;19:373–82.
54. Van Reeth K, Gregory V, Hay A, Pensaert M. Protection against a European H1N2 swine influenza virus in pigs previously infected with H1N1 and/or H3N2 subtypes. Vaccine. 2003;21:1375–81.
55. Schmidt ME, Varga SM. The CD8 T cell response to respiratory virus infections. Front Immunol. 2018;9:678.
56. De Bruin TGM, Van Rooij EMA, De Visser YE, Bianchi ATJ. Cytolytic function for pseudorabies virus-stimulated porcine CD4+ CD8dull+ lymphocytes. Viral Immunol. 2000;13:511–20.
57. Denyer MS, Wileman TE, Stirling CMA, Zuber B, Takamatsu HH. Perforin expression can define CD8 positive lymphocyte subsets in pigs allowing

phenotypic and functional analysis of natural killer, cytotoxic T, natural killer T and MHC un-restricted cytotoxic T-cells. Vet Immunol Immunopathol. 2006;110:279–92.
58. Cullen JG, McQuilten HA, Quinn KM, Olshansky M, Russ BE, Morey A, et al. CD4+ T help promotes influenza virus-specific CD8+ T cell memory by limiting metabolic dysfunction. Proc Natl Acad Sci U S A. 2019;116:4481–8.
59. Dhakal S, Lu F, Ghimire S, Renu S, Lakshmanappa YS, Hogshead BT, et al. Corn-derived alpha-D-glucan nanoparticles as adjuvant for intramuscular and intranasal immunization in pigs. Nanomed Nanotechnol Biol Med. 2019;16:226–35.
60. Hale JS, Youngblood B, Latner DR, Mohammed AUR, Ye L, Akondy RS, et al. Distinct memory CD4+ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. Immunity. 2013;38:805–17.
61. Mackay LK, Stock AT, Ma JZ, Jones CM, Kent SJ, Mueller SN, et al. Long-lived epithelial immunity by tissue-resident memory T (TRM) cells in the absence of persisting local antigen presentation. Proc Natl Acad Sci U S A. 2012;109:7037–42.
62. Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, et al. Cellular immune correlates of protection against symptomatic pandemic influenza. Nat Med. 2013;19:1305–12.
63. Wilkinson TM, Li CKF, Chui CSC, Huang AKY, Perkins M, Liebner JC, et al. Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans. Nat Med. 2012;18:274–80.
64. Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. Emerg Infect Dis. 2006;12:48–54.

Publisher's Note

Supplementary information

Additional file 1

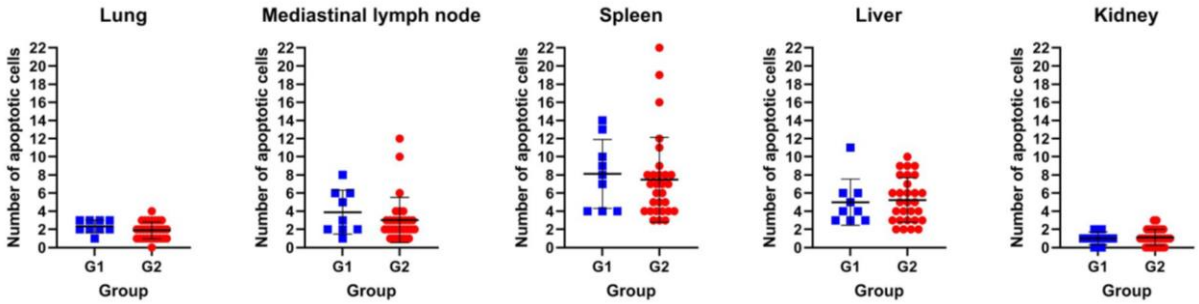

Fig. S1. TUNEL assay. Number of apoptotic cells observed in the TUNEL assay in different tissues (lung, mediastinal lymph node, spleen, liver and kidney) from pigs in the non-vaccinated (G1) and vaccinated (G2) groups on D28 post-vaccination. Data are displayed for each pig per group and the black lines represent the mean ± standard deviation.

Additional file 2

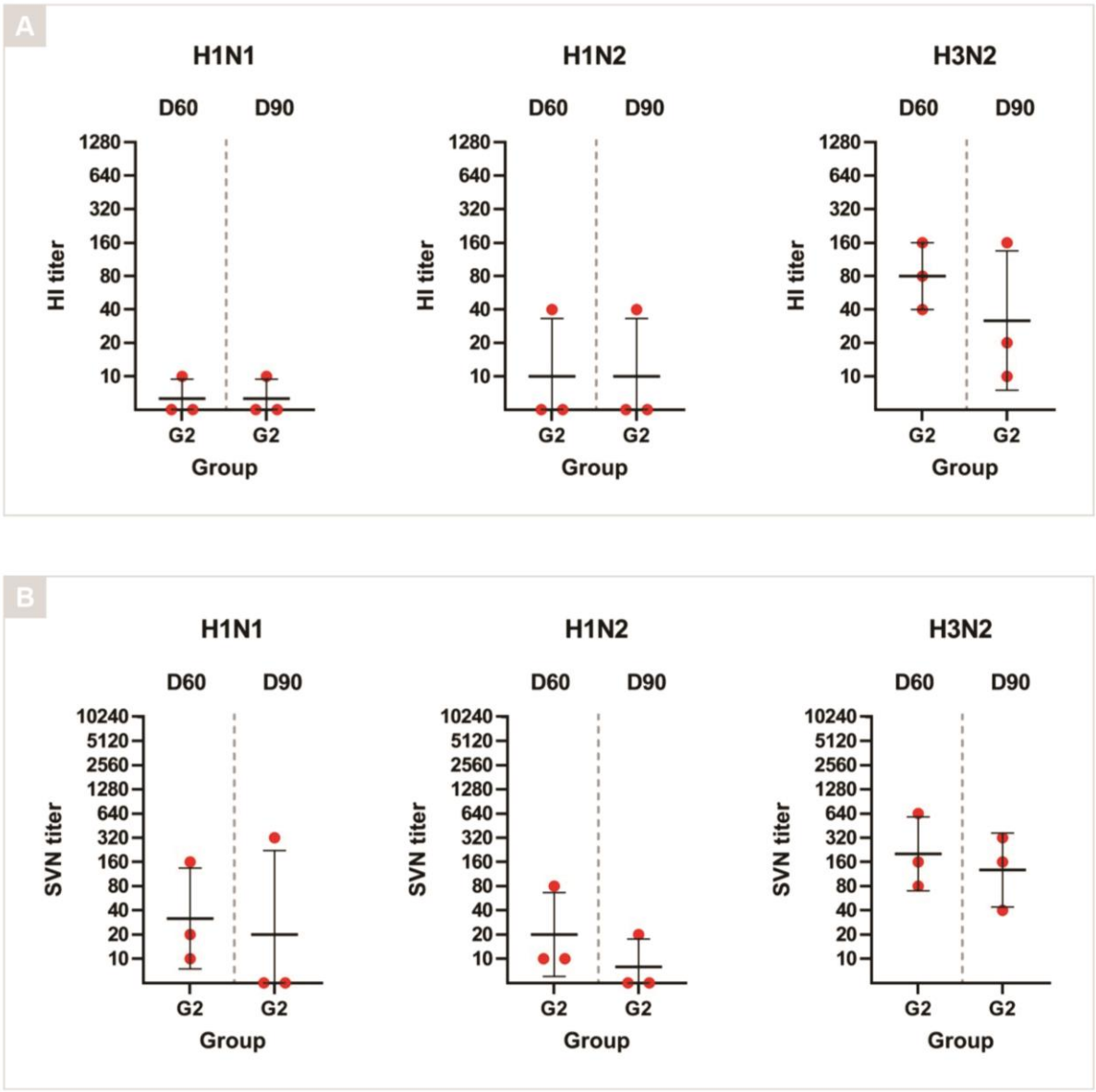

Fig. S2. Humoral immune response. Antibody titers by **(A)** hemagglutination inhibition (HI) and **(B)** serum virus neutralization (SVN) assays for H1N1, H1N2 and H3N2 subtypes of serum samples collected from pigs in the vaccinated (G2) group on D60 and D90 post-vaccination. Data are shown for each pig and the black lines represent the geometric mean titers ± standard deviation.

Additional file 3

Table S1. Data from the *in vitro* cell proliferation assay. Fold change means and standard errors of immune cells in the splenocyte proliferation assay stimulated with the vaccine viruses (H1N1, H1N2 and H3N2), from the vaccinated group (G2) over the non-vaccinated group (G1) on D28 and D90 post-vaccination.

Immune cells	D28			D90		
	H1N1	**H1N2**	**H3N2**	**H1N1**	**H1N2**	**H3N2**
$Macrophage^+$ (Myeloid cells)	1.49±0.10 (*P*<0.0001)*	1.35±0.10 (*P*=0.002)*	1.83±0.14 (*P*<0.0001)*	0.98±0.07 (*P*=0.81)	0.98±0.14 (*P*=0.89)	1.52±0.07 (*P*=0.02)*
$CD79a^+SWC7^+$ (B cells)	9.95±0.64 (*P*<0.0001)*	3.05±0.21 (*P*<0.0001)*	7.42±0.46 (*P*<0.0001)*	8.49±0.64 (*P*=0.007)*	2.26±0.28 (*P*=0.04)*	5.79±0.22 (*P*=0.002)*
$CD3e^+CD4^+$ ($CD4^+$ T cells)	1.94±0.12 (*P*<0.0001)*	1.94±0.10 (*P*<0.0001)*	1.97±0.11 (*P*<0.0001)*	1.59±0.34 (*P*=0.22)	1.54±0.32 (*P*=0.23)	1.31±0.28 (*P*=0.39)
$CD3e^+CD4^+CD25^+$ (Effector $CD4^+$ T cells)	5.19±0.38 (*P*<0.0001)*	4.03±0.30 (*P*<0.0001)*	4.18±0.27 (*P*<0.0001)*	3.89±0.85 (*P*=0.07)	2.69±0.41 (*P*=0.05)*	2.49±0.48 (*P*=0.09)
$CD3e^+CD4^+CD27^+$ (Central memory $CD4^+$ T cells)	733.2±83.93 (*P*<0.0001)*	319.2±40.19 (*P*<0.0001)*	176.0±19.72 (*P*<0.0001)*	657.1±29.74 (*P*=0.002)*	267.3±23.39 (*P*=0.007)*	117.7±18.72 (*P*=0.02)*
$CD3e^+CD8\alpha^+$ ($CD8^+$ T cells)	1.39±0.09 (*P*=0.0001)*	1.16±0.07 (*P*=0.03)*	1.44±0.10 (*P*=0.0001)*	1.04±0.25 (*P*=0.88)	0.75±0.16 (*P*=0.26)	0.69±0.25 (*P*=0.34)
$CD3e^+CD8\alpha^+CD25^+$ (Effector $CD8^+$ T cells)	2.09±0.19 (*P*<0.0001)*	1.71±0.15 (*P*<0.0001)*	2.14±0.21 (*P*<0.0001)*	1.04±0.27 (*P*=0.88)	0.67±0.14 (*P*=0.14)	0.69±0.24 (*P*=0.32)
$CD3e^+CD8\alpha^+CD27^+$ (Central memory $CD8^+$ T cells)	8.88±0.73 (*P*<0.0001)*	18.58±1.66 (*P*<0.0001)*	19.26±1.57 (*P*<0.0001)*	5.52±1.02 (*P*=0.04)*	10.77±2.66 (*P*=0.06)	13.64±1.43 (*P*=0.01)*
$CD3e^+CD8\alpha^+IFN\gamma^+$ ($CD8^+$ T cells producing IFN-γ)	3.59±0.18 (*P*<0.0001)*	2.70±0.12 (*P*<0.0001)*	3.72±0.17 (*P*<0.0001)*	3.77±0.41 (*P*=0.02)*	2.94±0.48 (*P*=0.05)*	4.00±0.58 (*P*=0.03)*

Asterisks (*) denote significant differences among non-vaccinated (G1) and vaccinated (G2) groups ($P \leq 0.05$).

Additional file 4

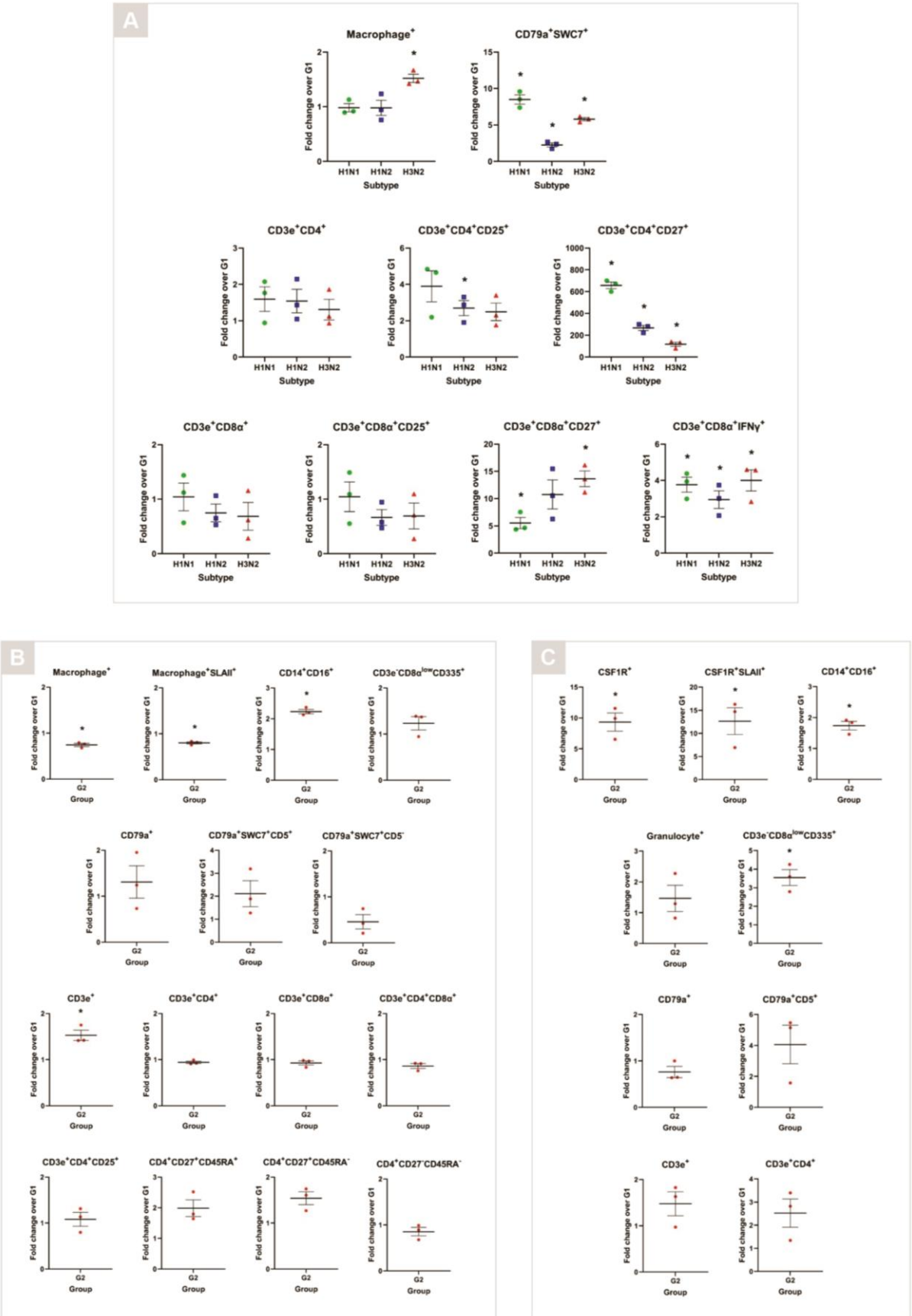

Fig. S3. Cellular immune response. Immune cells in the **(A)** *in vitro* splenocyte proliferation assay stimulated with the vaccine viruses (H1N1, H1N2 and H3N2), **(B)** peripheral blood mononuclear cells (PBMCs), and **(C)** bronchoalveolar lavage fluid (BALF) cells, as a fold change from the vaccinated group (G2) over the non-vaccinated group (G1) on D90 post-vaccination. Data are shown for each pig and the black lines represent the mean ± standard error. Asterisks (*) denote significant differences between non-vaccinated (G1) and vaccinated (G2) groups ($P \leq 0.05$).

4 **ARTIGO CIENTÍFICO 2**

O processo de produção e caracterização da vacina virossomal polivalente para influenza A, bem como a imunogenicidade dessa vacina testada previamente em camundongos, são apresentados a seguir no formato de artigo científico. Este artigo científico foi publicado no periódico *Virology Journal* (20:187, 2023, DOI 10.1186/s12985-023-02158-0) e é intitulado "*Immunological profile of mice immunized with a polyvalent virosome-based influenza vaccine*".

Virology Journal

RESEARCH

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Immunological profile of mice immunized with a polyvalent virosome-based influenza vaccine

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Abstract

Background Influenza A virus (IAV) causes respiratory disease in pigs and is a major concern for public health. Vaccination of pigs is the most successful measure to mitigate the impact of the disease in the herds. Influenza-based virosome is an effective immunomodulating carrier that replicates the natural antigen presentation pathway and has tolerability profile due to their purity and biocompatibility.

Methods This study aimed to develop a polyvalent virosome influenza vaccine containing the hemagglutinin and neuraminidase proteins derived from the swine IAVs (swIAVs) H1N1, H1N2 and H3N2 subtypes, and to investigate its effectiveness in mice as a potential vaccine for swine. Mice were immunized with two vaccine doses (1 and 15 days), intramuscularly and intranasally. At 21 days and eight months later after the second vaccine dose, mice were euthanized. The humoral and cellular immune responses in mice vaccinated intranasally or intramuscularly with a polyvalent influenza virosomal vaccine were investigated.

Results Only intramuscular vaccination induced high hemagglutination inhibition (HI) titers. Seroconversion and seroprotection (> 4-fold rise in HI antibody titers, reaching a titer of ≥ 1:40) were achieved in 80% of mice (intramuscularly vaccinated group) at 21 days after booster immunization. Virus-neutralizing antibody titers against IAV were detected at 8 months after vaccination, indicating long-lasting immunity. Overall, mice immunized with the virosome displayed greater ability for B, effector-T and memory-T cells from the spleen to respond to H1N1, H1N2 and H3N2 antigens.

Conclusions All findings showed an efficient immune response against IAVs in mice vaccinated with a polyvalent virosome-based influenza vaccine.

Keywords Vaccine, Influenza a virus, Vaccination, Seroprotection, Nanovaccine

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Background

The 2009 H1N1 pandemic influenza virus (H1N1pdm09) strongly illustrates the potential of influenza A viruses (IAVs) to cause morbidity and mortality in the human population on a global scale, as well as the importance of swine in the evolution of zoonotic viruses [1]. Pigs are susceptible to infection with both avian and human IAVs, and thus can serve as a "mixing vessel" for the emergence of novel viruses by reassortment of influenza gene segments [2]. IAV is endemic in pigs worldwide, with multiple genetically and antigenically distinct virus lineages of H1N1, H1N2, and H3N2 subtypes circulating in different geographic regions [3, 4]. Clinically, influenza virus infection causes an acute respiratory disease marked by fever, lethargy, coughing, anorexia, and nasal discharge. The IAV disrupts the normal defense system of the respiratory tract and may lead to secondary bacterial infections [5].

The most effective measure to mitigate and control morbidity and mortality associated with IAV in swine populations is vaccination. The swine influenza vaccination is equally crucial for human health, as it reduces swine-to-human and human-to-swine IAV transmission, decreasing the likelihood of pandemic risks and the emergence of new strains [2, 6]. In Brazil, the currently licensed commercial IAV vaccine for pigs is based on the whole inactivated H1N1pdm09 virus (WIV). The protection achieved by this vaccine is primarily mediated by the induction of antibodies targeting the hemagglutinin (HA) and, to a lesser extent, the neuraminidase (NA) viral glycoproteins [7, 8]. However, for a highly effective vaccine, the vaccine antigens must have a close antigenic match with the circulating IAVs in swine herds [9, 10].

The surveillance of IAV in pigs through genetic and antigenic characterization is extremely important for the selection of vaccine candidates. Recently, a great genetic diversity of IAV has been found in the Brazilian pig population, which may have implications for the design of cross-protective vaccines [11]. In this sense, the IAV vaccines for pigs available in the country might provide limited or absent protection against the currently circulating genetically distinct swine IAVs. Furthermore, IAVs have the ability to evade the host immune response through mechanisms known as antigenic drift and antigenic shift, which require a regular update of the viruses that compose the vaccine to match the circulating viruses [12].

Several studies have been conducted in the last few years aiming to develop broadly protective vaccines that induce both humoral [13, 14] and cellular immune responses [3, 7, 15–17]. In general, these vaccines target antigenically conserved epitopes on the HA [18–20], expressed by a virus-like particle (VLP) [21]. Nevertheless, none of the proposed solutions has produced a practical vaccine that induces broad heterosubtypic protection or achieves the desired sterilizing immunity. Broad protection against IAVs can be achieved with either polyvalent vaccines of mixed subtype-specific immunogens or the use of a good immunogen conserved among circulating IAV subtypes [8]. A polyvalent influenza vaccine could decrease the inherent limitations of influenza vaccines because they are designed to protect against different influenza viruses that circulate in swine herds [17, 22]. Furthermore, to achieve effective immunity through immunization, the target of virus-neutralizing antibodies needs to antigenically match the circulating IAVs, which in pigs consist of isolates from distinct lineages of H1N1, H1N2, and H3N2 subtypes [23, 24].

Virosomes are VLPs produced in vitro from purified envelope components; nevertheless, they lack the genetic material and internal proteins of the native virus. Influenza virosomes combine the technical benefits of a well-regulated composition with the immunological advantages of VLPs [25]. Incorporated within the phospholipid bilayer of the virosomes are the functional IAV envelope glycoproteins, HA and NA. These viral proteins not only offer structural stability and homogeneity to the virosomal formulations, but they also contribute significantly to the immunological features of the virosomes, which distinguish them from other liposomal systems [26]. The fully functional fusion activity of virosomes containing the HA protein permits receptor-mediated uptake and natural intracellular processing of the antigen, thereby triggering both humoral and cellular immune responses [27]. In this study, a trivalent virosomal swIAV vaccine based on a H1N1pdm, H1N2 and H3N2 viruses was constructed using a dialyzable short-chain phospholipid (1,2-Dicaproyl-sn-Glycero-3-Phosphocholine, DCPC) as a solubilizing agent, since this surfactant presents the best performance for viral solubilization; it maintains the envelope proteins functionality, and is completely removed by dialysis during virosome production [28]. In addition, virosomes efficacy was demonstrated in mice by measuring the cellular immune response and the serum antibody response against the IAV vaccine strains.

Methods

Viruses

Three IAVs isolated from swine were selected for the preparation of the influenza vaccine: A/swine/Brazil/025−15/2015 1 A.3.3.2 (H1N1pdm; NCBI GenBank Accession HA=MH559931 and NA=MH559933; BRMSA 1710), A/swine/Brazil/223-15-1/2015 1B.2.4 (H1N2; NCBI GenBank Accession HA=MH560035 and NA=MH560037; BRMSA 1698) and A/swine/Brazil/028-15-8/2015 (H3N2; NCBI GenBank Accession HA=MH559963 and NA=MH559965; BRMSA 1697). H1N1 and H1N2 viral samples were propagated in SPF

(Specific Pathogen-Free) embryonated chicken eggs, and H3N2 virus was inoculated into Madin-Darby Canine Kidney (MDCK; BCRJ) cells, according to Zhang and Gauger [29]. To confirm the IAV presence, the cell supernatant and chorioallantoic fluid harvested from eggs were tested by hemagglutination assay [30] and by RT-qPCR [31].

Virus concentration

Approximately 1 L of each virus was individually concentrated by tangential ultrafiltration using a flow pump coupled to a cassette containing a dialysis membrane consisting of polyethersulfone (PES) with a cut-off of 100 kDa (Vivaflow 200 System, Sartorius, Germany). Then, 50 mL of each viral concentrate was ultracentrifuged at 100,000 x g for 4 h at 4 °C (Optima LE 80 K, Beckman Coulter, USA). The supernatants were discharged, and the pellets of each virus were diluted to a final volume of 5 mL in TNE buffer (10 mM Tris, 100 mM NaCl and 1 mM EDTA, pH 7.4). The concentrated viruses were titrated by hemagglutination assay [30], and the hemagglutinin content was determined by SDS-PAGE (NuPAGE™ Bis-Tris, Thermo Fisher, USA) using acrylamide gel plate (4–12%) and silver staining according to the manufacturer's recommendations. The HA concentration was calculated from the intensity of the bands (obtained with the ImageJ software) using an equation obtained from a standard calibration curve of albumin.

Virosome preparation and characterization

The multivalent virosome was prepared according to de Jonge, Leenhouts [32] with modifications. Briefly, the same volume of each virus was mixed (1:1:1) and diluted in a 200 mM solution of 1,2-dicaproyl-sn-glycero-3-phosphocholine (DCPC, Avanti Polar Lipids, USA). After gentle homogenization, the pooled viruses were further diluted 1:2 (v/v) in the TNE buffer, and the final mixture was kept in an ice bath for 30 min to ensure the dissolution of the viral envelopes. Afterward, the mixture was ultracentrifuged at 100,000 x g for 30 min at 4 °C to remove the viral nucleocapsids. The supernatant was extensively dialyzed in a cellulose dialysis tube (cut-off 10 kDa, SpectraPor, USA) against TNE buffer for 48 h at 4 °C to remove the surfactant (DCPC), which led to the self-assembling of the virosome vesicles. Subsequently, the physical-chemical characteristics of the dialyzed virosome formulation were determined, measuring the particle size and zeta potential by dynamic laser scattering and laser Doppler electrophoresis (Zetasizer, Malvern). The HA antigen content from H1N1, H1N2, and H3N2 was determined by SDS-PAGE.

Electron microscopy

Transmission electron microscopy (TEM) was used to examine the morphology and ultrastructure of the virosomes using the microscope JEOL JEM-1011 (Jeol, Japan). The samples were used pure or at a 50% dilution in ultrapure water. Before analysis, 3 µL of virosome suspension was deposited into a copper grid covered with Formvar®. The copper grids were fixed and after they had completely dried, they were contrasted with 1% Osmium Tetroxide vapor. Thus, 40 µL of Osmium Tetroxide were placed at the bottom of a petri dish containing the copper grids for 40 min. Virosomes were digitized using an UltraScan® camera connected to Digital Micrograph 3.6.5® computer software (Gatan, USA). To eliminate any doubt about what were virosomes and what were artifacts from the contrast with 1% Osmium Tetroxide, a negative control was created during the analysis that used only ultrapure water and Osmium Tetroxide [33].

Assessment of the infectivity and cytotoxicity of influenza virosomes

In order to evaluate the infectivity of virosome, it was inoculated into embryonated chicken eggs and incubated at 37 °C for 4 days. Virosome was also inoculated into MDCK cells and monitored for 7 days.

For the in vitro cytotoxicity assays, immortalized macrophage lines (RAW 264.7 cells, BCRJ-0212) were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) modified to contain 4 mM GlutaMAX (Gibco), 4500 mg/L glucose, 1 mM sodium pyruvate (Sigma-Aldrich), and 1500 mg/L sodium bicarbonate (Sigma-Aldrich) with 10% of fetal bovine serum (FBS) (Sigma-Aldrich). RAW 264.7 cells were cultured and maintained at 37 °C and 5% CO_2. After the formation of the cell monolayer, the adherent cells were detached by scraping. Initially, RAW 264.7 cells (2×10^5 cells/well) were seeded in 96-well plates, cultured for 24 h for adhesion and then treated with different dilutions of the virosome formulation (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256, v/v) for 24, 48 and 72 h. This procedure was repeated for different passages of the cell culture until the desired sample size ($n=8$) was reached. The control cells received the same volume of a simple liposome (prepared with phospholipids - Lipoid® S100, Lipoid). The cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay. At the end of each incubation time, the medium was removed, and the cells were washed twice with DPBS (Sigma-Aldrich) and incubated for 3 h with 5 mg/mL MTT solution at 37 °C. After incubation, the precipitated formazan crystals were dissolved in 200 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich). Optical densities (OD) were measured at 540 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher

Scientific). The absorbance values recorded for untreated cells (negative control) represent 100% of cell viability and were used as a reference to calculate the percentage of cell viability in the presence of each sample concentration. Complementary cytotoxicity analysis was performed using the enzyme terminal deoxynucleotidyl transferase (TdT) and the propidium iodide (PI) staining kit (APO-DIRECT™, BD Biosciences). RAW 264.7 cells were plated and treated with the virosome dilutions (1:32 and 1:64) as described above. The assay was done according to the manufacturer's guidelines at 24, 48 and 72 h after exposure to the virosomes [33]. The staining protocol consisted of cell incubation with TdT-FITC enzyme and staining with propidium iodide. After 24 and 48 h of exposure, cells were analyzed using a flow cytometer (Accuri C6 Plus, Becton-Dickinson, USA), and the percentage of intact cell membranes per group was determined. The percentage of live cells was calculated from the fluorescence readings defined according to the kit instructions.

Immunization of mice with multivalent influenza virosomes

The protocols and the use of animals for this research complied with the Animal Use Ethics Committee of Embrapa Swine and Poultry (protocol number 001/2016). C57BL/6 mice (female, 6–8 weeks old) were reared under SPF conditions and divided into 3 groups as follows: non-vaccinated control (NV, $n=20$), intranasal vaccinated (5 µL/nostril or 10 µL/animal, IN, $n=20$), intramuscular vaccinated (100 µL/animal, IM, $n=20$). An additional group, non-vaccinated liposome control ($n=20$), served as a control for virosomes. The G4 group did not show any difference in the analyzes performed when compared to the animals in the NV group (data not shown). A mucoadhesive adjuvant (carboxymethyl cellulose – CMC) was added to the formulation (0.125%, m/v) for intranasal administration, and Emulsigen-D® (MVP Adjuvants, USA) for intramuscular administration (20%, v/v) [34]. The experimental protocol consisted of the administration of two doses of the vaccine 2 weeks apart (days 1 and 15). At 21 days (day 36) and eight months later (day 255) after the second vaccine dose, ten animals/group from all three groups were euthanized using intraperitoneal injection of sodium pentobarbital (80 µg/g body weight).

Biochemical determinations

For blood collection, mice were anesthetized with intraperitoneal ketamine-xylazin (ketamine 60 µg/g body weight, and xylazin 10 µg/g body weight). Blood samples were drawn by retro-orbital bleeding on days 0 (before vaccination), 3 and 17 (two days after each immunization). In order to assess the possible toxicity of the vaccine, quantification of biochemical markers from the serum samples was performed, evaluating the hepatic (AST and ALT) and renal (urea and creatinine) functions. These assays were performed with colorimetric kits, according to the manufacturer's instructions (Labtest, Brazil).

Morphologic assessment

For histopathology analysis, liver, kidney and lung tissue samples were collected at necropsy and fixed with 4% buffered paraformaldehyde, dehydrated in a graded series of ethanol, paraffin-embedded and sectioned at 4 µm. This material was stained with hematoxylin-eosin (H&E). Furthermore, to assess the in vivo cytotoxicity of the virosome, staining for apoptosis was performed using the In Situ Cell Death Detection kit (Roche, Germany), according to the manufacturer's instructions. Nuclei were counterstained with 3,3-diaminobenzidine (Sigma-Aldrich, USA). The TUNEL assay is employed to identify and quantify apoptotic nuclei by an in situ reaction involving TdT-mediated dUTP-X nick end labeling. TUNEL-positive nuclei were quantified using light microscopy under magnification of 400x. The degree of TUNEL expression was calculated in 25 distinct fields (corresponding to a total area of 0.08 mm^2). Results were expressed as cells/mm^2.

Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) from mice was obtained at necropsy (days 36 and 255), through a tracheostomy procedure in a biosafety cabinet. BALF was collected by flushing the lungs four times with 0.2 mL sterile physiological saline (0.9% NaCl) via the tracheal cannula. After BALF collection, a protease inhibitor cocktail was added to a final concentration of 1x and also phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mM. One portion of the BALF samples was stored at -80 °C for ELISA assay. ELISA was performed to quantify total IgA immunoglobulin in the BALF supernatant using the Invitrogen kit (Thermo Fisher, USA) in accordance with the manufacturer's recommendations. Another part of the BALF samples was used for cell quantification. Trypan blue exclusion method using a Neubauer chamber was applied for cell quantification. For differential cytological analysis, a dried cell smear was prepared with an aliquot of the suspension, and stained with May-Grünwald-Giemsa staining. The slides were analyzed by light optical microscopy. At least 500 leukocytes were counted per high-power field, and the absolute differential cell counts were calculated by multiplying the percentage of each given cell type by the total cell count.

Serology

Mice were anesthetized and blood was collected, through the retro-orbital plexus, at days 0, 36 and 255. Then, sera were evaluated for the presence of IAV-specific antibodies by hemagglutination inhibition (HI) assay [35]. The same IAV strains used in the virosomal vaccine composition were used as antigens in the HI assay. Results were reported as geometric mean antibody titers.

Isolation of white blood cells from the spleen

The complete spleen from each mouse was aseptically collected in RPMI 1640 medium (Gibco) at necropsy (days 36 and 255). Each spleen was mechanically dissociated, and filtered through a nylon filter (70 µm). Then, red blood cells were lysed with Pharm Lyse™ buffer (BD Biosciences). The lysis reaction was stopped by adding of RPMI 1640 medium with 2% FBS, and the cells were washed twice. The cells were resuspended in complete RPMI 1640 medium, supplemented with 10% FBS (Gibco, Brazil), 1 mM GlutaMAX (Gibco, Brazil), 25 mM HEPES (Sigma-Aldrich, USA), 1 mM sodium pyruvate (Sigma-Aldrich, USA), 50 M 2-mercaptoethanol (Gibco, USA) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, USA). Finally, the cell number was counted with 0.4% trypan blue to determine the viable cell concentration. In general, the mice spleens yielded around $8–10\times10^7$ viable splenocytes. The cells were resuspended in 95% FBS+5% DMSO (Sigma-Aldrich) and cryopreserved at a final concentration of 2×10^6 cells/mL.

In vitro cell proliferation assay

Viable spleen cells were thawed and suspended in DPBS at a concentration of 5×10^6 cells/mL and labeled with 2.5 µM carboxyfluorescein succinimidyl ester (CFSE) by applying the CellTrace™ CFSE Cell Proliferation kit (Invitrogen), according to previous reports [36]. After CFSE labeling, splenocytes were resuspended in complete RPMI 1640 medium, plated in 24-well plates (5×10^6 cells/well). Subsequently, the cells were stimulated in vitro by adding 8000 $TCID_{50}$/mL of the three vaccine viruses (H1N1, H1N2 and H3N2) for 96 h at 37 °C, under 5% CO_2, in the dark. For the negative control, only culture medium was added to cells (non-virus-stimulated cells), and for the positive control, the cells were stimulated separately with 5 µg/mL of Concanavalin A from *Canavalia ensiformis* (Sigma-Aldrich). After in vitro stimulation of cells with H1N1, H1N2 and H3N2 viruses, lymphocyte proliferation from spleens was measured as an indicator of T and B-cell responses at 21 days after the boost immunization.

Table 1 Panel of fluorochrome-labeled monoclonal antibodies

Panel	Antibody
Mouse Naïve/Memory T cell (cod 561,609, BD Becton Dickinson)	anti-CD44 / PE anti-CD4 / PerCP-Cy™5.5 anti-CD62L / APC anti-CD3e / APC-CY™7
Mouse T Lymphocyte Activation Antibody Cocktail (cod 557,908, BD Becton Dickinson)	anti-CD25 / PE-Cy™7 anti -CD69 / PE anti-CD3e / APC
Mouse T Lymphocyte Subset Antibody Cocktail (cod 558,431, BD Becton Dickinson)	anti-CD3e / PE-Cy™7 anti-CD4 / PE anti-CD8α / APC
Mouse B Lymphocyte Subset Antibody Cocktail (cod 558,332, BD Becton Dickinson)	anti-CD45R / B220 / PE-Cy™7 anti-CD23 (FcεRII) / PE anti-sIgM / APC
Mouse B Lymphocyte Activation Antibody Cocktail (cod 558,063, BD Becton Dickinson)	anti-CD25 / PE-Cy™7 anti-CD69 / PE anti-CD19 / APC

Monoclonal antibodies used to evaluate the cellular immune response of spleen cells from immunized mice in the in vitro stimulation assay

Cell staining and flow cytometry

CFSE in combination with monoclonal antibodies (mAbs) enabled concomitant access to cell proliferation and activation status of cell subpopulations. Proliferation was detected by loss of CFSE fluorescence [36]. Flow cytometry analysis was performed to identify and quantify lymphocyte subpopulations (CD3e, CD4, CD8α, CD19, CD45R/B220 and sIgM mAbs), to measure the levels of cellular activation and mature resting marker expression (CD23, CD25 and CD69 mAbs), and cellular memory marker expression (CD62L and CD44 mAbs) (Becton Dickinson; Table 1). Cell densities were calculated and transferred to flow cytometry tubes (approximately 1×10^6/well). Cells were treated with a blocking solution (10% v/v normal mouse serum) to block unoccupied binding sites on the second antibody, and thus cells were labeled for 30 min at room temperature in a dark room with a cocktail of specific mAbs (Table 1), 7-aminoactinomycin D (7-AAD) and isotype controls (BD Biosciences). Antibody concentrations used were in accordance with the manufacturer's instructions.

Before sample analysis, the flow cytometer settings were checked using Cytometer Setup and Tracking beads (CS&T beads, BD), as described in the manufacturer's instructions. Compensation beads were used with single stains of each antibody to establish the compensation settings. The compensation matrix was identically applied to all samples. The side scatter (SSC) threshold level was set at 8,000 units to eliminate debris. Gates considered indicating positive and negative staining cells were set based on fluorescence minus one (FMO) tests of samples and these gates were applied consistently to each sample, allowing minor adjustments for SSC variability. 7-Aminoactinomycin D (7-AAD) staining was used to distinguish dead from viable cells by flow cytometry. In the preliminary procedure to set up instrument technical

parameters, isotype controls were used to evaluate fluorochrome unspecific staining. Buffer for flow cytometry was prepared in PBS containing 0.01% w/v sodium azide (Sigma-Aldrich), 2% v/v FBS (Gibco) and 2% w/v bovine serum albumin (BSA, Sigma-Aldrich).

A total of 100,000 events per tube were acquired in the flow cytometer (Accuri C6 Plus and FACSCanto, Becton-Dickinson, USA) and analyzed using the FlowJo software (Becton-Dickinson, USA). The lymphocyte gate was set on light-scatter properties (Forward Scatter vs. Side Scatter). Proliferation by CFSE (reflected by successive reduction of fluorescence intensities by dye distribution to daughter cells) was measured by flow cytometry. Results were expressed as percentages of stained cells.

Statistical analysis

Differences between vaccinated groups (intranasal – IN and intramuscular – IM routes) and non-vaccinated groups (NV) in biochemical data, immunoglobulins, apoptosis rate, and BALF cell count were analyzed through ANOVA, using the MIXED procedure of Statistical Analysis System (SAS - Cary, North Carolina, USA). In addition, differences between these groups in the in vitro cell proliferation assay were evaluated using the two-sided Student's t test. Analysis of variance (F test) was carried out to assess the effect of the administration route and age in the in vitro cell proliferation assay, applying the Tukey test whenever a significant effect ($P \leq 0.05$) of virosome was detected. For the analysis of HI, the descriptive level of probability of Fisher's exact test was used; percentages followed by distinct letters on the lines differ significantly according to Fisher's exact test. *P* values ≤ 0.05 were considered statistically significant [37].

Table 2 Hemagglutination titer and HA content of IAVs used to produce the virosome vaccine formulation

Subtype	Strain identification	Hemagglutination titer	Hemagglutinin content (µg/mL) *
H1N1	A/swine/Brazil/25 – 15/2015	1:81920	36.2
H1N2	A/swine/Brazil/223-15-1/2015	1:40960	139.1
H3N2	A/swine/Brazil/28-15-8/2015	1:20480	478.9

* Hemagglutinin content was estimated by SDS-PAGE using a bovine serum albumin curve as standard for calculations

Results

Multivalent virosome preparation, SDS-PAGE, and size distribution of particle diameters

The propagated influenza viruses were concentrated approximately 500x after concentration by ultracentrifugation and purification using ultrafiltration. Table 2 presents the hemagglutination titers and HA content of the IAVs prior to the preparation of the multivalent virosomes. A pre-formulation study was carried out, and we found that using equivalent volumes of each virus strain (1:1:1) led to a more stable formulation under refrigerated storage.

The purified virosomes were loaded on a polyacrylamide gel for electrophoresis. SDS-PAGE analysis (semi-quantitative method using albumin as standard; Fig. 1) showed that the purified virosomes contained mainly HA, based on the expected molecular weight of each IAV glycoprotein. The total HA content of the virosomes was nearly 160 µg/mL. Besides, considering the HA content for each virus, we estimated that the total HA presented in the formulation corresponds to 6, 21 and 73% for H1N1, H1N2 and H3N2, respectively, due to the differences on HA content for each strain.

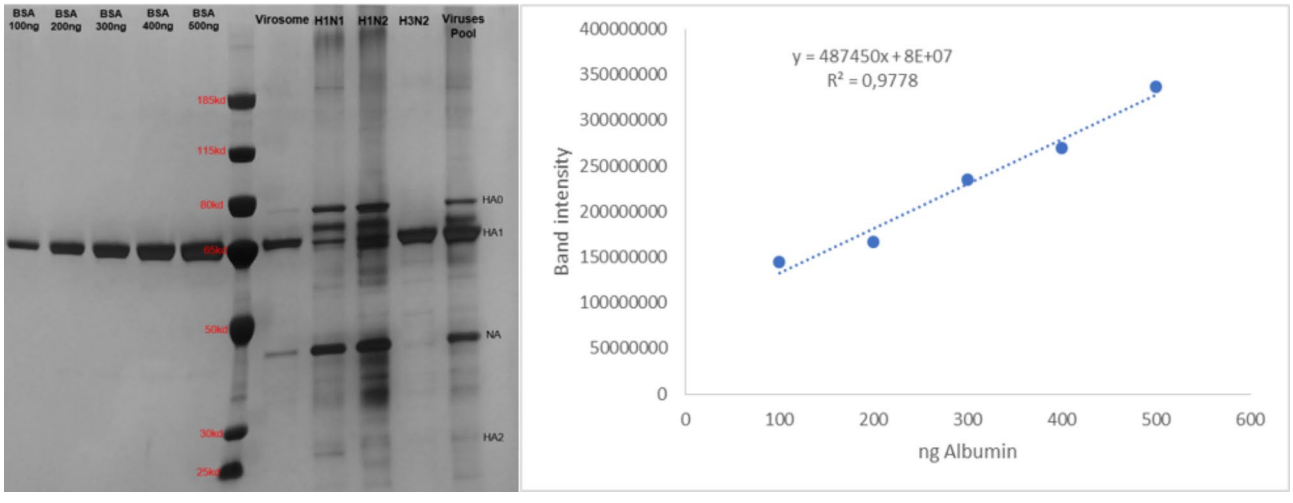

Fig. 1 SDS-PAGE gel and standard curve of bovine serum albumin (approximately 66.5 kDa). Used to quantify the hemagglutinin of the viral strains and multivalent virosomes. On the SDS-PAGE gel it is visible: HA composed of two forms, uncleaved HA (HA0 ≈ 75–65 kDa) and cleaved HA (HA1 ≈ 55 kDa and HA2 ≈ 27 kDa); and NA (≈ 45–50 kDa). The band intensities were acquired using the ImageJ software

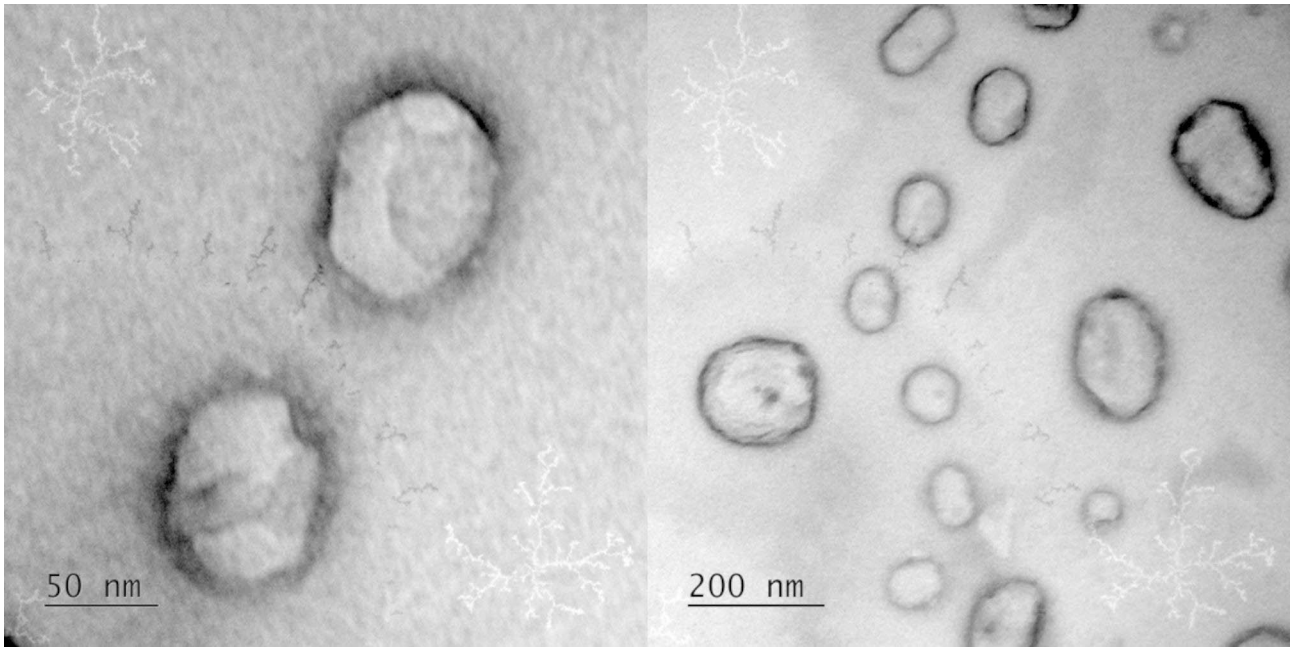

Fig. 2 Transmission electron microscopy (TEM) image of multivalent virosomes produced with H1N1, H1N2 and H3N2 influenza strains. Image captured at 80 kV

Table 3 Cell viability of macrophages (RAW 264.7 cell line) exposed to different dilutions of the virosome formulation

Dilution of the virosome formulation	Time (hours)		
	24	48	72
1:256	98.33 ± 0.31 [a]	95.97 ± 0.58 [a]	91.11 ± 0.33 [a]
1:128	97.51 ± 0.81 [ab]	94.38 ± 0.88 [a]	89.56 ± 0.68 [ab]
1:64	97.75 ± 0.44 [ab]	89.27 ± 0.64 [b]	86.69 ± 0.61 [b]
1:32	95.20 ± 0.96 [abc]	90.23 ± 0.57 [b]	87.48 ± 0.32 [b]
1:16	94.15 ± 0.20 [bc]	84.27 ± 1.05 [c]	80.07 ± 1.09 [c]
1:8	92.89 ± 0.21 [c]	82.58 ± 0.43 [c]	78.94 ± 0.22 [cd]
1:4	93.25 ± 0.87 [c]	82.48 ± 0.44 [c]	77.95 ± 0.07 [cd]
1:2	92.38 ± 0.88 [c]	82.20 ± 0.63 [c]	76.39 ± 0.75 [d]
Pr > F	< 0.0001	< 0.0001	< 0.0001

Based on membrane damage (MTT assay) after 24, 48 and 72 h

Data represent mean ± standard error ($n = 8$). The analysis was performed with a one-way ANOVA and with Tukey's post-test

[a,b,c,d] Different superscript letters indicate significant statistical differences between virosome dilutions ($P \leq 0.0001$)

The virosome formulation was characterized in terms of particle size and zeta potential, which were around 110 nm (PDI = 0.19) and −6.2, respectively. TEM images displayed circular and ellipsoid structures as shown in Fig. 2. The virosomes presented an average diameter of 100 nm and unilamellar membrane, sometimes followed by blebs near it.

In vitro cytotoxicity

At first, to ensure that the virosome consisted of a non-infective nanoparticle, the infectivity of virosome was assessed in embryonated chicken eggs and in MDCK cells. No viral replication was observed in embryonated chicken eggs inoculated with virosomes, and there was no visible cytopathic effect on cell line in comparison to control cells.

The viability of RAW 264.7 cells was evaluated after exposing the cells for 24, 48 and 72 h to different dilutions 1:2 to 1:256 (v/v) of the virosome (Table 3). After 24 and 48 h of incubation, the cells presented cell viability above 80% for all virosome dilutions, indicating the safety of virosomes. However, after 72 h of exposure of the cells to the virosome dilutions of 1:2 up to 1:8, cell viability ranged between 75 and 80%, showing that long-term exposure was slightly cytotoxic. Besides, when compared to the negative control in the MTT assay, exposure to the other virosome dilutions evaluated presented no effect on cell viability (> 80%) at this interval of incubation. In general, the virosome formulation was well tolerated at 1:16 to 1:256 dilutions at all exposure times. In terms of cell apoptosis, the cytotoxicity rate (apoptotic cells) determined by flow cytometry ranged from 1 to 2% for both virosome dilutions evaluated (1:32 and 1:64) during the same time periods (24, 48 and 72 h) [38].

Biochemical and apoptosis analyses

No significant injury signs or differences were detected among non-vaccinated and vaccinated mice, regardless of the virosomal vaccine administration route (Table S1, Additional file 1). The hepatic (AST and ALT) and renal (urea and creatinine) values were within the normal range [39]. Furthermore, mice showed no adverse

reactions associated with the immunization using the virosomes.

Regarding the histopathological analysis, the different tissues evaluated (kidney, lung and liver) did not show relevant microscopic changes. Furthermore, the TUNEL assay revealed no difference in the rate of cellular apoptosis in the kidney and liver of animals immunized with the virosome, which was similar to the control group at 36 days (Figure S1, Additional file 2).

Cytological findings in BALF

On average, the BALF volume recovered was 82%. Mean values for total cell counts of BALF at 36 days from mice in the intranasal vaccinated, intramuscular vaccinated, and non-vaccinated groups were $8.8 \pm 1.2 \times 10^5$ cells/mL, $7.65 \pm 0.9 \times 10^5$ cells/mL, and $5.7 \pm 0.7 \times 10^5$ cells/mL, respectively. In all groups, the cellular composition of BALF consisted predominantly of alveolar macrophages (>53%) and fewer numbers of lymphocytes (<43%) and neutrophils (<1%). Both groups of immunized mice (intranasally and intramuscularly) showed an increase in the population of lymphocytes ($P \leq 0.0001$) when compared to the non-immunized animals (Table 4). Intranasally immunized mice also had a greater increase in total cell population.

Immunogenicity assessment

Hemagglutination inhibition (HI) titers for the three IAV subtypes (H1N1, H1N2 and H3N2) were measured in serum samples obtained on days 21 (day 36) and 240 (day 255) after booster immunization for both intramuscular and intranasal immunization. The sera of mice immunized with virosomes by intramuscular route showed a significantly greater increase in HI titers than intranasal immunization for the three vaccine strains ($P \leq 0.0001$; Fig. 3). In the follow-up evaluations (days 36), all mice in the intramuscularly vaccinated group had vaccine-induced HI antibody titers (˃1:40) to H3N2. The weak responses to H1N1 and H1N2 antibody titers (˂1:40) were detected in a few intramuscularly immunized mice. Nevertheless, the intramuscular vaccine was immunogenic in mice and elicited significant HI antibody responses to H1N1, H1N2 and H3N2. The seroconversion to intramuscular immunization 21 days after the booster immunization was 90% H1N1, 80% H1N2, 100% H3N2.

Table 4 Differential leukocyte counts in the BALF of mice at 21 days after two doses of virosomes

Groups	Macrophages	Lymphocytes	Neutrophils
Non-vaccinated	$5.06 \pm 0.58 \times 10^5$	$0.30 \pm 0.04 \times 10^{5b}$	$0.28 \pm 0.14 \times 10^5$
Intranasally vaccinated	$4.74 \pm 0.62 \times 10^5$	$3.70 \pm 0.05 \times 10^{5a}$	$0.34 \pm 0.10 \times 10^5$
Intramuscular vaccinated	$4.34 \pm 0.94 \times 10^5$	$2.82 \pm 0.04 \times 10^{5a}$	$0.42 \pm 0.08 \times 10^5$

BALF = bronchoalveolar lavage fluid

Values represent mean ± standard deviation ($n = 10$)

[a] Different superscript letter indicates significant statistical differences between groups ($P \leq 0.0001$). The analysis was performed with the Tukey test

Intranasally vaccinated mice, on the other hand, did not develop detectable HI antibody titers 21 days after the booster immunization (day 36). Only one mouse vaccinated intranasally had HI-antibodies to H1N1 (titer 1:40), H1N2 (1:40) and H3N2 (1:40) viruses 8 months after the second immunization, as shown in Fig. 3. The intramuscular immunization elicited a greater rate of seroconversion (90% H1N1, 40% H1N2, 100% H3N2) compared with the intranasal immunization (10% H1N1, 10% H1N2, 10% H3N2) 8 months after the booster immunization.

The total immunoglobulin IgA was quantified in the BALF of mice. ELISA assays for total antibodies revealed that, regardless of the administration route, vaccinated mice had a higher IgA concentration than non-vaccinated mice (6.85 ± 0.98 mg/dL) on day 21 post-vaccination ($P \leq 0.0001$). Animals intranasally vaccinated with the virosome (57.84 ± 3.61 mg/dL) had higher IgA concentrations than intramuscularly vaccinated mice (36.81 ± 5.39 mg/dL, $P \leq 0.0001$; Fig. 4).

Virosomes are an effective vaccine for inducing H1N1, H1N2 and H3N2-specific recall T-cell responses

To examine whether the virosome induces cell-mediated immunity, T cell suspensions were obtained from vaccinated mice on days 21 and 240 following the second vaccination. Gates were set using the non-virus-stimulated sample for each individual mouse. To summarize, the gate was based on forward scatter (FSC) and side scatter (SSC) features in order to estimate the lymphocyte population and exclude debris. The doublet cells were subjected to doublet plotting showing forward scatter height (FSC-H) against forward scatter area (FSC-A). Dead cells were excluded from the analysis using 7-AAD staining. The proliferation of lymphocytes was determined by $CFSE^{low}$, which allowed the evaluation of the specific proliferation induced in each experimental group by each virus (H1N1, H1N2 and H3N2). Counterstaining with CD3e and CD45R/B220 allowed us to gate on T and B cells, respectively. Among T lymphocytes ($CD3e^+$), subsets of $CD4^+$ T cells, $CD8\alpha^+$ T cells, CD62L, CD44, CD25, and CD69 were distinguished using panels. In the first analysis, gates were used in the $CD4^+$ and $CD8^+$ populations for the analysis of T lymphocyte subgroups. In the second analysis, gates were used in the $CD69^+$ and $CD69^+CD25^+$ populations, and in the third analysis, gates were used in the $CD3e^+CD4^+CD44^{high}CD62L^{high}$ and $CD3e^+CD4^+CD44^{high}CD62L^{low}$ populations. Among B-lymphocyte cells, activated lymphoblasts were stained with a mouse B-lymphocyte activation antibody cocktail and a mouse B-lymphocyte subset antibody cocktail.

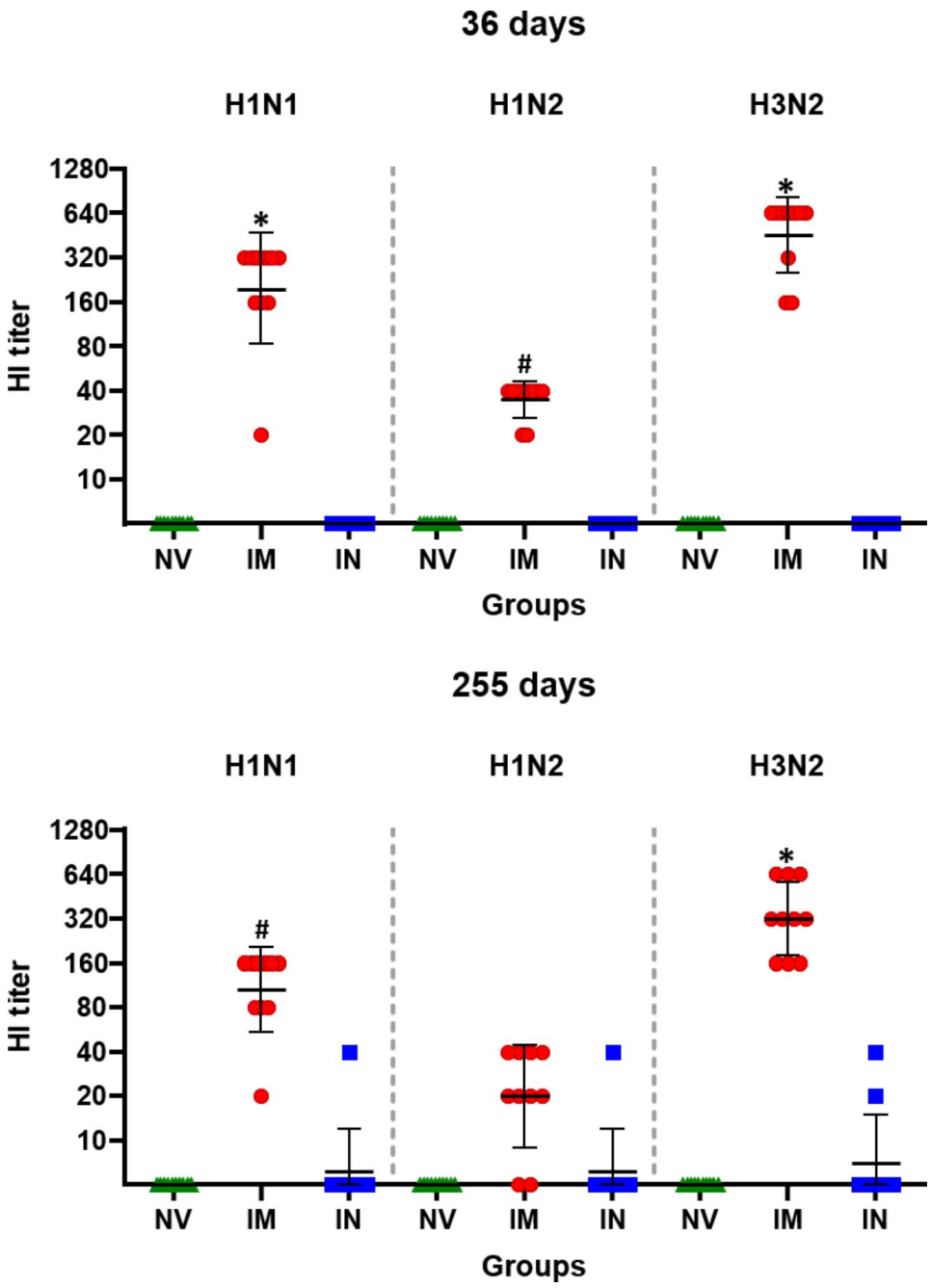

Fig. 3 Hemagglutination inhibition (HI) assay. (NV) non-vaccinated, (IM) intramuscularly vaccinated, and (IN) intranasally vaccinated. Antibody titers by HI test for H1N1, H1N2 and H3N2 subtypes in serum samples from mice on day 21 and 240 post-vaccination. Data are shown for each mouse per group and the black lines represent the geometric mean titers ± standard deviation

The in vitro stimulated lymphocyte proliferation assay identified ten distinct cell subsets: $CD3e^{+}CD4^{+}$ ($CD4^{+}$ T lymphocytes), $CD3e^{+}CD8\alpha^{+}$ ($CD8^{+}$ T lymphocytes), $CD3e^{+}CD69^{+}$ (very early activation T-cell), $CD3e^{+}CD69^{+}CD25^{+}$ (effector T cells), $CD3e^{+}CD4^{+}CD44^{high}CD62L^{high}$ (central memory $CD4^{+}$ T lymphocytes), $CD3e^{+}CD4^{+}CD44^{high}CD62L^{low}$ (effector memory $CD4^{+}$ T lymphocytes), $CD19^{+}CD69^{+}$ (very early activation B-cell), $CD19^{+}CD69^{+}CD25^{+}$ (effector B cells), $CD45R/B220^{+}sIgM^{+}$ (immature and mature B cells or transitional B cells), and $CD45R/B220^{+}sIgM^{+}CD23^{+}$ (mature resting conventional B cells).

Notably, intramuscular and intranasal immunizations induced a robust T cell and B cell response (Fig. 5). For H1N1, H1N2 and H3N2 viruses, virosome-vaccinated (intramuscular and intranasal immunization) mice had more than 2-fold higher levels of T-cell subsets than non-vaccinated or naive mice (Fig. 5). Both

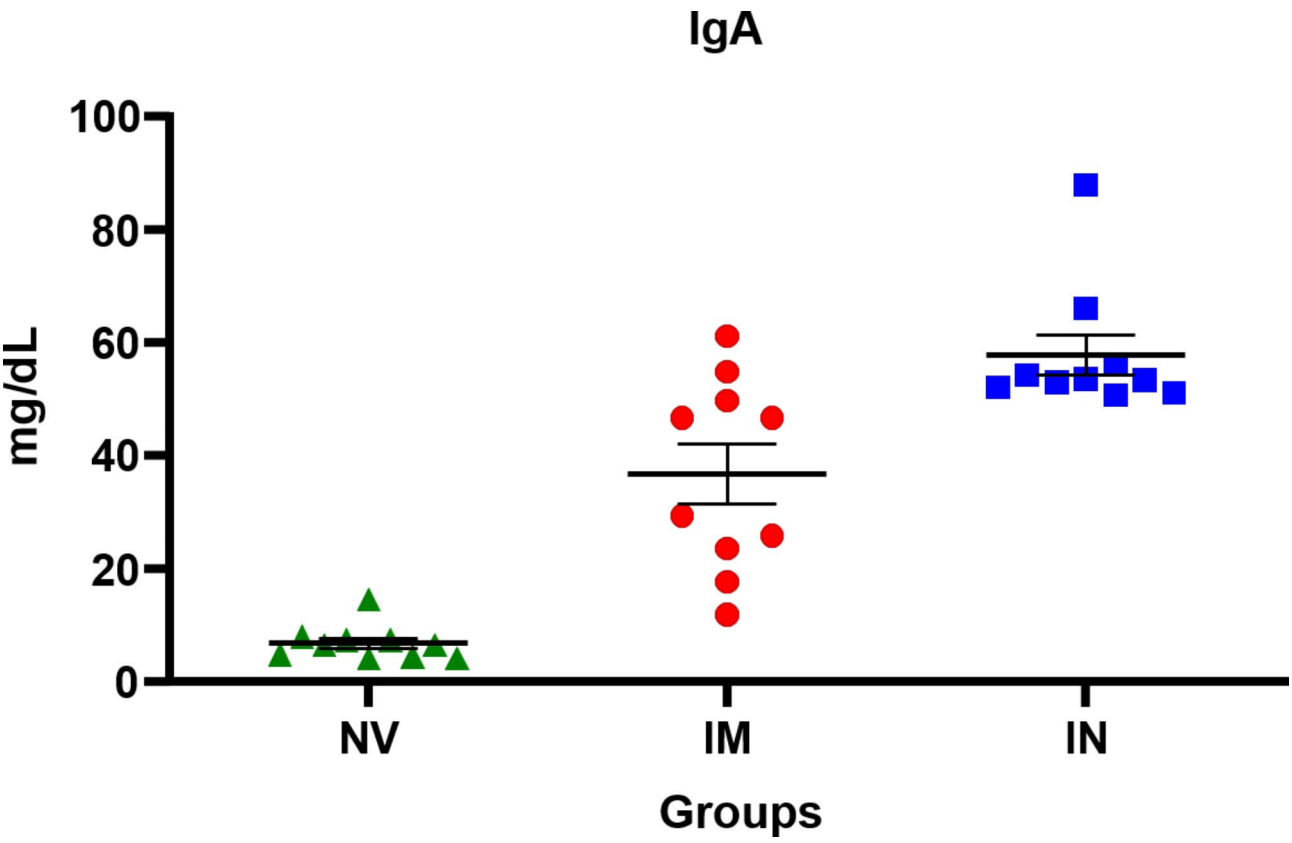

Fig. 4 Quantification of total IgA antibody in the bronchoalveolar lavage fluid (BALF) of mice by ELISA assay. Data are shown for each mouse per group and the black lines represent the mean ± standard error. NV = non-vaccinated; IM = intramuscularly vaccinated; IN = intranasally vaccinated

intramuscular and intranasal immunization resulted in higher $CD3e^+CD4^+$, $CD3e^+CD8\alpha^+$, $CD3e^+CD69^+$, $CD3e^+CD69^+CD25^+$, $CD3e^+CD4^+CD44^{high}CD62L^{high}$, and $CD3e^+CD4^+CD44^{high}CD62L^{low}$ T cell subsets for H1N1 ($P \leq 0.0001$), H1N2 ($P \leq 0.001$) and H3N2 ($P \leq 0.001$) (Fig. 5 and Table S2, Additional file 1). The administration route influenced the amount of some cell subsets, with intranasal immunization producing more $CD3e^+CD69^+$ cells ($P \leq 0.05$) and intramuscular administration producing more $CD3e^+CD69^+CD25^+$ cells ($P \leq 0.05$; Table 5).

Mice immunized (intramuscular and intranasal) with virosomes showed a significant increase at 21 days' post-vaccination (day 36) in the absolute numbers of effector ($CD19^+CD69^+$ and $CD19^+CD69^+CD25^+$), transitional and mature B cells ($CD45R/B220^+sIgM^+$) compared to baseline ($P \leq 0.05$) for H1N1, H1N2, and H3N2 (Fig. 5; Table 5). In contrast, intranasal immunization favored a greater establishment of B cells, $CD45R/B220^+sIgM^+$, $CD19^+CD69^+$ and $CD19^+CD69^+CD25^+$ cell subsets, for H1N1, H1N2, and H3N2 than intramuscular immunization (Table 5).

Virosomes induce efficiently long-lived immunity to influenza

Inducing long-lasting protective immunity is one of the objectives of vaccination. Hence, we assessed the longevity of H1N1, H1N2 and H3N2 antibody responses induced by virosome vaccination. The H1N1, H1N2 and H3N2-specific HI antibody responses to virosome-immunized mice were maintained at significantly higher levels (Fig. 5) than those of non-vaccinated mice for over 8 months, indicating that influenza virus immunity can be long-lived.

To assess the long-term protective efficacy, cellular immune responses were also analyzed in mice groups that were intramuscularly and intranasally immunized with multivalent virosomes at 8 months after vaccination.

As shown in Table 5 (comparison between routes of administration) and Table S2, Additional file 1 (comparison between non-vaccinated versus intramuscularly vaccinated group and non-vaccinated versus intranasally vaccinated group), 8 months after boost immunization, immunized mice showed a route-dependent increase of $CD45R/B220^+sIgM^+CD23^+$ compared to the non-vaccinated group ($P \leq 0.0001$). In comparison to non-vaccinated mice, intramuscular and intranasal immunized mice exhibited significant T- and B-cell proliferation in

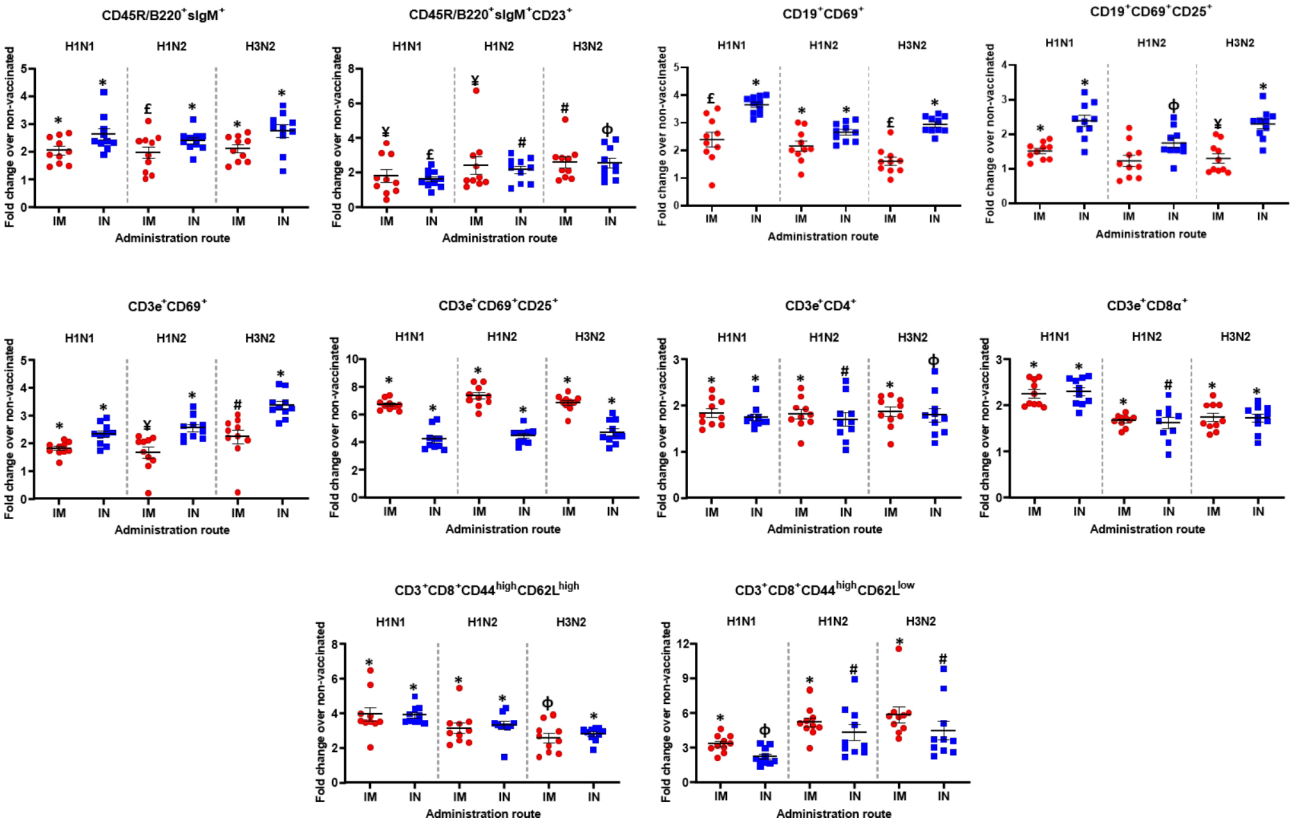

Fig. 5 In vitro cell proliferation assay. Immune cells in the splenocytes proliferation assay stimulated with the vaccine viruses (H1N1, H1N2 and H3N2) were compared as a fold change from the intranasal (IN) and intramuscular (IM) vaccinated group over the non-vaccinated group (NV) on day 21 post-vaccination. Data shown are the fold increase in the mean percentage and standard error of indicated immune cells from vaccinated mice versus those of non-vaccinated (NV) mice. $^{¥}P \leq 0.05$ versus NV, $^{£}P \leq 0.005$ versus NV, $^{\#}P \leq 0.001$ versus NV, $^{\varphi}P \leq 0.0005$ versus NV, and $^{*}P \leq 0.0001$ versus NV.

response to H1N1, H1N2, and H3N2 ($P \leq 0.05$; Table S2, Additional file 1). However, mice immunized intranasally displayed a higher level of T-cell subset ($CD3e^{+}CD69^{+}$; Table 5) proliferation than mice immunized intramuscularly. The $CD3e^{+}CD4^{+}CD44^{high}$ $CD62L^{high}$ and $CD3e^{+}CD4^{+}CD44^{high}$ $CD62L^{low}$ cell subsets for H1N1, H1N2 and H3N2 were significantly higher when immunized intramuscularly than intranasally ($P \leq 0.05$), as well as the $CD3e^{+}CD69^{+}CD25^{+}$ and cell subsets for H1N1 (Table 5). Intramuscular immunizations induced greater memory-related central responses in tissues other than the respiratory mucosa, such as the spleen.

Discussion

Designing novel vaccine candidates that closely mimic the native morphology of the specific virus without being pathogenic themselves remains a major challenge in the development of influenza vaccines [40]. Virosomes are tightly controlled virus-like particles that can be used in vaccine formulation [41]. In general, vaccination or infection fail conferring long-lasting protection due to the appearance of new or antigenically distinct influenza A virus strains [4]. Thus, the development of new, highly effective, and well-tolerated vaccines is essential. In a human safety and immunogenicity study, a prototype trivalent virosome influenza vaccine was compared to commercial whole inactivated virus and subunit vaccines [42]. The virosome vaccine produced higher protective titers and was less reactogenic and more immunogenic than either the whole-inactivated virus or subunit influenza vaccines. The synthesis of liposomes using envelopes from influenza A virus (virosome), which contain the main viral antigens (HA and NA), has yielded encouraging findings [4, 27]. In the current investigation, we designed a multivalent virosome from a cocktail of surface glycoproteins from H1N1, H1N2, and H3N2 viruses using the reconstitution technique with DCPC as detergent, and its immunogenicity as a swIAV vaccine candidate was investigated in mice. de Jonge, Holtrop [43] demonstrated that the use of DCPC as a viral membrane solubilizer has significant advantages over the conventional approach of virosome synthesis, which involves the solubilization of the viral envelope with Triton X-100, followed by its removal with polystyrene beads. Considering the high critical micelle concentration of DCPC, dialysis is an efficient procedure for the removal of

Table 5 H1N1, H1N2 and H3N2-specific recall T and B-cell responses after intramuscular and intranasal immunization with virosomes

Immune cells		36 days		255 days	
		Intramuscular	Intranasal	Intramuscular	Intranasal
			H1N1		
B cells	$CD19^+CD69^+$	2.394 ± 0.261	3.657 ± 0.098^a	3.204 ± 0.134^b	3.221 ± 0.182
	$CD19^+CD69^+CD25^+$	1.515 ± 0.076	2.391 ± 0.162^a	2.759 ± 0.102^b	2.534 ± 0.200
	$CD45R/B220^+sIgM^+$	2.032 ± 0.149	2.615 ± 0.206^a	2.399 ± 0.192	2.335 ± 0.201
	$CD45R/B220^+sIgM^+CD23^+$	1.785 ± 0.350	1.601 ± 0.153	6.717 ± 0.530^b	7.896 ± 0.554^b
T cells	$CD3e^+CD4^+$	1.839 ± 0.091	1.743 ± 0.077	1.474 ± 0.053^b	1.329 ± 0.070^b
	$CD3e^+CD8\alpha^+$	2.249 ± 0.088	2.293 ± 0.091	1.667 ± 0.082^b	1.406 ± 0.137^b
	$CD3e^+CD69^+$	1.809 ± 0.074	2.324 ± 0.119^a	1.397 ± 0.082^b	2.040 ± 0.070^a
	$CD3e^+CD69^+ CD25^+$	6.717 ± 0.122	4.210 ± 0.245^a	1.882 ± 0.174^b	1.299 ± 0.120^{ab}
	$CD3e^+CD4^+CD44^{high}CD62L^{high}$	3.946 ± 0.393	3.893 ± 0.155	3.555 ± 0.205	2.526 ± 0.116^{ab}
	$CD3e^+CD4^+CD44^{high}CD62L^{low}$	3.325 ± 0.237	2.231 ± 0.230^a	1.938 ± 0.177^b	1.226 ± 0.052^{ab}
			H1N2		
B cells	$CD19^+CD69^+$	2.161 ± 0.184	2.663 ± 0.105^a	2.749 ± 0.107^b	2.037 ± 0.218^{ab}
	$CD19^+CD69^+CD25^+$	1.231 ± 0.161	1.748 ± 0.134^a	2.141 ± 0.135^b	1.875 ± 0.176^b
	$CD45R/B220^+sIgM^+$	1.958 ± 0.219	2.397 ± 0.117^a	2.641 ± 0.197^b	2.156 ± 0.178
	$CD45R/B220^+sIgM^+CD23^+$	2.393 ± 0.526	2.138 ± 0.221	12.70 ± 0.99^b	12.51 ± 1.14^b
T cells	$CD3e^+CD4^+$	1.813 ± 0.104	1.695 ± 0.148	1.332 ± 0.078^b	1.313 ± 0.059^b
	$CD3e^+CD8\alpha^+$	1.664 ± 0.041	1.617 ± 0.120	1.074 ± 0.076^b	1.035 ± 0.095^b
	$CD3e^+CD69^+$	1.658 ± 0.201	2.543 ± 0.127^a	1.504 ± 0.089	1.982 ± 0.092^a
	$CD3e^+CD69^+ CD25^+$	7.326 ± 0.234	4.458 ± 0.180^a	1.347 ± 0.082^b	1.097 ± 0.120^b
	$CD3e^+CD4^+CD44^{high}CD62L^{high}$	3.136 ± 0.300	3.302 ± 0.236	3.518 ± 0.348	2.395 ± 0.130^{ab}
	$CD3e^+CD4^+CD44^{high}CD62L^{low}$	5.184 ± 0.411	4.293 ± 0.683	2.893 ± 0.130^b	2.087 ± 0.176^{ab}
			H3N2		
B cells	$CD19^+CD69^+$	1.608 ± 0.148	2.942 ± 0.095^a	2.703 ± 0.092^b	2.421 ± 0.189^b
	$CD19^+CD69^+CD25^+$	1.303 ± 0.136	2.298 ± 0.136^a	2.434 ± 0.132^b	2.322 ± 0.126
	$CD45R/B220^+sIgM^+$	2.110 ± 0.142	2.735 ± 0.225^a	2.712 ± 0.118^b	2.426 ± 0.110
	$CD45R/B220^+sIgM^+CD23^+$	2.558 ± 0.327	2.527 ± 0.282	10.63 ± 0.93^b	13.02 ± 0.94^{ab}
T cells	$CD3e^+CD4^+$	1.860 ± 0.107	1.793 ± 0.145	1.462 ± 0.054^b	1.252 ± 0.068^b
	$CD3e^+CD8\alpha^+$	1.741 ± 0.093	1.724 ± 0.091	1.107 ± 0.054^b	1.200 ± 0.085^b
	$CD3e^+CD69^+$	2.226 ± 0.249^c	3.361 ± 0.146^a	2.088 ± 0.125	2.610 ± 0.122^{ab}
	$CD3e^+CD69^+ CD25^+$	6.848 ± 0.181	4.711 ± 0.264^a	1.447 ± 0.176^b	1.737 ± 0.254^b
	$CD3e^+CD4^+CD44^{high}CD62L^{high}$	2.571 ± 0.268	2.796 ± 0.125	2.566 ± 0.178	2.138 ± 0.124^{ab}
	$CD3e^+CD4^+CD44^{high}CD62L^{low}$	5.857 ± 0.679	4.471 ± 0.801	2.959 ± 0.120^b	2.155 ± 0.110^{ab}

Data are shown as fold change means ± standard errors

[a] Superscript indicate a significant difference by the F test between the routes of administration within the age groups ($P \leq 0.05$)

[b] Superscript indicate a significant difference by the F test between ages groups within the routes of administration ($P \leq 0.05$)

DCPC, allowing the self-assembling of virosome vesicles with the benefit of not altering the viral antigens (HA and NA), a common issue related to Triton X-100 (alteration of the protein conformation and loss of antigenicity) [44, 45].

SDS-PAGE analysis revealed that multivalent virosomes contained both HA and NA protein bands and lacked virus nucleocapsid complexes. The average diameter of the virosomes was about 110 nm, which is consistent with the observations made in previous experiments [46]. Regarding the confirmation of HA (and to a lesser extent NA) by electrophoresis, this may result in the presentation of additional antigens by major histocompatibility complex classes I and II (MHC-I and MHC-II) proteins, resulting in the activation of T- and B-cells and dendritic cells, as seen during viral infection [47, 48]. In addition, influenza virosomes are a very promising strategy for antigen dose sparing, because it leads to high immunogenic response at low doses without affecting the protective effect of the vaccine [49]. We demonstrated that the swIAV virosomes are highly immunogenic and may elicit a strong humoral and cellular immune response against H1N1, H1N2 and H3N2 viruses.

Assessing the in vitro safety of virosome is essential to consider it as new vaccine prototype prior to its administration to animals [48]. For this, to assure that the virosome has no infectivity, the nanoformulation was inoculated in embryonated chicken eggs revealing the absence of viable virus, but with presence of HA agglutination activity suggesting that the virus disruption and

virosome reconstitution have succeeded. In addition, cell viability assays were performed, revealing that no significant alterations were detected in any of the assessed virosome dilutions. This outcome served as confirmation of their safety for in vivo experiments.

The cytological examination of BALF revealed that the infiltration of lymphocyte cells into the airways was significantly higher in the vaccinated mice compared to the non-vaccinated mice. In previous studies, this lymphocyte infiltration in BALF suggested that the cellular immune response caused by the vaccination could be linked to the inflammatory cellular responses seen in the lungs [50]. However, the infiltration of lymphocytes into the bronchi and alveoli and lymphoid hyperplasia around the bronchi and blood vessels were not observed in the vaccinated groups as well as non-vaccinated group. In addition, no change in the rate of cellular apoptosis was observed in the lungs of vaccinated mice.

The literature lacks established operational definitions for the waning of influenza immunity; therefore, immunogenicity in this study was assessed using the criteria of the European Agency for the Evaluation of Medicinal Products (EMEA) [51]. Antibodies directed against the HA protein measured by hemagglutination inhibition (HI) assay are correlated with protection against influenza [3]. In the mouse model, even low levels of pre-existing influenza immunity (immunological memory) have an immunostimulating effect, as they enhance the antibody response against unrelated antigens delivered by influenza virosomes [48]. Hence, to confirm protective immunogenicity, at least one of the three EMEA requirements should be achieved: (i) seroconversion defined by ˃4-fold increase in HI antibody titer, reaching a HI titer of ≥1:40, in ˃40% of immunized subjects; (ii) an increase in geometric mean titers (GMT) of 2.5-fold; and (iii) seroprotection defined by the achievement of an HI titer of ≥1:40 in ˃70% of subjects. Liposomes containing no influenza virus proteins were administered to an additional control group (data not shown). This group exhibited similar HI antibody results as the non-vaccinated group. An important finding was that the polyvalent influenza-based virosome engendered protective HI titers against the three IAV strains that were significantly higher in the intramuscularly immunized mice compared to the intranasally immunized mice. According to EMEA criteria, seroconversion and seroprotection were achieved for three IAV strains by virosome administered via the intramuscular route. Regarding the fact that the mice used were specific pathogen-free and isogenic, even without the evaluation of antibodies prior to immunization, the fold-change was compared to the antibody titers of non-vaccinated mice, which were considered baseline levels. On the other hand, intranasally immunized mice had non-protective antibody titers at baseline, which were similar to previous studies [52]. Only one out of the intranasal-vaccinated animals displayed HI antibodies eight months after vaccination. According to this animal, to obtain a quality mucosal vaccine response against IAV, it will be necessary to reevaluate the formulation and consider new strategies for eliciting systemic and mucosal immune responses, including the use of appropriate vaccine adjuvants. Almost certainly, the antibody levels increased more gradually, the post-vaccination peak was later and was not detected in the initial analysis. However, our findings indicate that immunization with multivalent swIAV virosomes administered by nasal route is able to generate local immune responses, stimulating greater production of IgA in BALF, which may contribute to the protection of mice against a subsequent challenge. Therefore, intranasal immunization with the virosome-based swIAV did not induce a systemic humoral immune response but could induce a local humoral immune response. Besides, we highlight that the intranasal formulation consisted of a simple mucoadhesive system (inclusion of carboxymethyl cellulose), and possibly the immunological results could be improved with the design of a more sophisticated intranasal delivery system.

The lymphocyte proliferation from the spleen is significantly higher in the vaccinated group than in the non-vaccinated group. Our findings showed that the multivalent swIAV virosomal vaccine was able to induce effector, transitional, and mature B cells, as well as memory and effector T cells, with increased proliferation after virus stimulation. Nanoparticles and possibly virosomes often encapsulate ligand molecules for pattern recognition receptors (PRRs; e.g., Toll-like receptors, TLRs) that are expressed in dendritic cells and B cells [53, 54]. Expressed virosomes sometimes contain viral DNA or RNA, which has the potential to engage DNA or RNA sensors (i.e., TLR9 and TRL7) in B cells and dendritic cells [55]. Thus, we speculate that TLR signals might play antibody-enhancing roles during booster immunization of memory B cells in our experimental system. Further, nanovaccines may directly act on P-binding memory B cells; they eventually receive robust B cell receptor (BCR) signals or increased T cell help as a result of strong BCR cross-linking [54]. The stimulation of splenocytes from vaccinated mice with H1N1, H1N2 and H3N2 viruses also elicited higher cell proliferation of effector, transitional, and mature B cells under these conditions.

T-cell responses are known to help in the expansion of cross-protective immunity [56]. The cellular proliferation response of the liposome control group (data not shown) was the same as that of the non-vaccinated group. Our findings showed higher $CD3e^{+}CD4^{+}$ T helper, $CD3e^{+}CD8a^{+}$ T cytotoxic and $CD3e^{+}CD69^{+}CD25^{+}$ T effector cells in vaccinated mice. The multivalent swIAV virosomes elicited a robust cytotoxic T lymphocyte

(CTL) response mediated by $CD8^+$ T lymphocytes, which is important for virus clearance [57]. The induction of a T-helper response is required for the antigen-specific B-cell and/or cytotoxic T-lymphocyte response to be supported [48]. Of note, intranasal and intramuscular immunization were associated with greater acquisition of CD69, residence marker associated with retention in lymphoid tissues [58].

Evidence observed in this study suggests that immunization with the virosome induced central memory and effector memory $CD4^+$ T cells. These memory subsets, both short- and long-term, arise after antigenic stimulation with increased proliferative and reconstitutive capacities in immunized mice. Vaccine-induced memory T cells may be decisive in generating long-lasting immunity and inducing viral destruction. We observed that effector and central memory $CD4^+$ T cells were abundant 21 days after booster immunization but declined over time, even though, they were still higher when compared to non-vaccinated mice. Thus, central memory $CD4^+$ T cells induced by virosome immunization have a long duration and may be able to provide sustained help for $CD8^+$ T cells [59]. The long-lived memory T cell population has an enhanced capacity for self-renewal and multipotency to generate all memory (central memory and effector memory) and effector T cell subsets in vitro [60]. Mice were vaccinated twice, which was sufficient to elicit an H1N1, H1N2, and H3N2 IAV-specific memory T-cell response, eliminating the need for heterologous prime-boost approaches. Therefore, avoiding continuous antigenic stimulation that can lead to progressive loss of memory potential, as an undesirable consequence, will drive T cells toward terminal differentiation, which compromises their capacity to clear systemic infections [61, 62]. The significant presence of memory cells after vaccination and their enhanced proliferative capacity can sustain the generation of all subsets of effector and memory T cells.

Conclusions

These findings together have significant implications for the design of T cell–based vaccines that target intracellular pathogens, like influenza virus [60]. In addition, we suspect that the immunity measures observed here are lower than the described immunity demonstrated by viral challenge. HI is well accepted as a parameter to define protection induced by vaccination. Besides, HI quantifies the antibody response to the globular head of influenza hemagglutinin, but it does not evaluate the ability of the antibodies to neutralize virus infection [63, 64]. Nonetheless, there is a strong correlation between HI and functional viral neutralizing antibodies [63]. Finally, even if seroprotection is achieved in >70% of isogenic mice vaccinated intramuscularly for subtypes H1N1, H1N2 and H3N2, future seroprotection studies in the target species (swine) are crucial. Antibody titers can fluctuate pre- and post-vaccination due to a variety of circumstances, including previous influenza infections and immunizations, genetic variations, and prior heterologous infections [63], but our mice had no antibody titers prior to the first immunization. According to the results obtained, the multivalent swIAV virosome designed was immunogenic in mice when intramuscularly administered, inducing systemic antibodies, and the potential for protection against influenza infection.

Abbreviations

7-AAD	7-aminoactinomycin D
BALF	Bronchoalveolar lavage fluid
BCR	B cell receptor
BSA	Bovine serum albumin
CFSE	Carboxyfluorescein succinimidyl ester
CMC	Carboxymethyl cellulose
CS&T	Cytometer Setup and Tracking
CTL	Cytotoxic T lymphocyte
DCPC	1,2-dicaproyl-sn-glycero-3-phosphocholine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EMEA	European Medicines Evaluation Agency
FBS	Fetal bovine serum
FMO	Fluorescence minus one
FSC	Forward scatter
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GMT	Geometric mean titers
HA	Hemagglutinin
H&E	Hematoxylin and eosin
HI	Hemagglutination inhibition
H1N1pdm	H1N1 pandemic
IAV	Influenza A virus
IM	Intramuscular
IN	Intranasal
mAbs	Monoclonal antibodies
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Neuraminidase
NV	Non-vaccinated
OD	Optical densities
PES	Polyethersulfone
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern recognition receptor
SAS	Statistical Analysis System
SPF	Specific pathogen-free
SSC	Side scatter
swIAV	Swine influenza A virus
TdT	Terminal deoxynucleotidyl transferase
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNE	Tris-NaCl-EDTA
VLP	Virus-like particle
WIV	Whole inactivated virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-023-02158-0.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
The authors acknowledge Arlei Coldebella for the statistical analysis. VH has a grant from Coordination for the Improvement of Higher Education Personnel (CAPES), financial code 001.

Authors' contributions
Conceive and design the study, FNF and APB; performed the experiment, FNF, VH, FVB, GB, DG, LSP, LFB, MS and APB; data analysis and curation, FNF, VH, FVB, DG, WCA, RS and APB; writing – original draft preparation, FNF and APB; writing – review and editing, FNF, VH, DG, WCA, RS and APB; funding acquisition, APB; supervision of the research, RS and APB. All authors read and approved the final manuscript.

Funding
This work was supported by Brazilian Agricultural Research Corporation (Embrapa, 12.13.10.004.00.00) and Foundation for Support of Research and Innovation of the State of Santa Catarina (FAPESC, project accession no. 2017TR1738). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Availability
The data that support this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Declarations

Competing interests
The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate
All experimental and animal management procedures were approved by the Animal Use Ethics Committee of Embrapa Swine and Poultry (protocol number 001/2016).

Consent for publication
Not applicable.

Received: 24 May 2023 / Accepted: 11 August 2023
Published online: 21 August 2023

References

1. Dawood FS, Iuliano AD, Reed C, Meltzer MI, Shay DK, Cheng PY, et al. Estimated global mortality associated with the first 12 months of 2009 pandemic influenza A H1N1 virus circulation: a modelling study. Lancet Infect Dis. 2012;12(9):687–95.
2. Ma W, Lager KM, Vincent AL, Janke BH, Gramer MR, Richt JA. The role of swine in the generation of novel influenza viruses. Zoonoses Public Health. 2009;56(6–7):326–37.
3. Van Reeth K, Ma W. Swine influenza virus vaccines: to change or not to change-that's the question. Curr Top Microbiol Immunol. 2013;370:173–200.
4. Anderson TK, Chang J, Arendsee ZW, Venkatesh D, Souza CK, Kimble JB et al. Swine Influenza A Viruses and the tangled relationship with humans. Cold Spring Harbor perspectives in medicine. 2021;11(3).
5. Rech RR, Gava D, Silva MC, Fernandes LT, Haach V, Ciacci-Zanella JR, et al. Porcine respiratory disease complex after the introduction of H1N1/2009 influenza virus in Brazil. Zoonoses Public Health. 2018;65(1):e155–e61.
6. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009;325(5937):197–201.
7. Mancera Gracia JC, Pearce DS, Masic A, Balasch M. Influenza A virus in Swine: Epidemiology, Challenges and Vaccination strategies. Front Vet Sci. 2020;7:647.
8. Tan GS, Leon PE, Albrecht RA, Margine I, Hirsh A, Bahl J, et al. Broadly-reactive neutralizing and non-neutralizing antibodies Directed against the H7 Influenza Virus Hemagglutinin Reveal Divergent Mechanisms of Protection. PLoS Pathog. 2016;12(4):e1005578.
9. Babiuk S, Masic A, Graham J, Neufeld J, van der Loop M, Copps J, et al. An elastase-dependent attenuated heterologous swine influenza virus protects against pandemic H1N1 2009 influenza challenge in swine. Vaccine. 2011;29(17):3118–23.
10. de Jong JC, Heinen PP, Loeffen WL, van Nieuwstadt AP, Claas EC, Bestebroer TM, et al. Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. Vaccine. 2001;19(31):4452–64.
11. Tochetto C, Junqueira DM, Anderson TK, Gava D, Haach V, Cantao ME et al. Introductions of human-origin Seasonal H3N2, H1N2 and Pre-2009 H1N1 influenza viruses to Swine in Brazil. Viruses. 2023;15(2).
12. Vijaykrishna D, Poon LL, Zhu HC, Ma SK, Li OT, Cheung CL, et al. Reassortment of pandemic H1N1/2009 influenza a virus in swine. Science. 2010;328(5985):1529.
13. Bosworth B, Erdman MM, Stine DL, Harris I, Irwin C, Jens M, et al. Replicon particle vaccine protects swine against influenza. Comp Immunol Microbiol Infect Dis. 2010;33(6):e99–e103.
14. Heinen PP, van Nieuwstadt AP, de Boer-Luijtze EA, Bianchi AT. Analysis of the quality of protection induced by a porcine influenza a vaccine to challenge with an H3N2 virus. Vet Immunol Immunopathol. 2001;82(1–2):39–56.
15. Kappes MA, Sandbulte MR, Platt R, Wang C, Lager KM, Henningson JN, et al. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. Vaccine. 2012;30(2):280–8.
16. Masic A, Booth JS, Mutwiri GK, Babiuk LA, Zhou Y. Elastase-dependent live attenuated swine influenza a viruses are immunogenic and confer protection against swine influenza a virus infection in pigs. J Virol. 2009;83(19):10198–210.
17. Soema PC, Kompier R, Amorij JP, Kersten GF. Current and next generation influenza vaccines: Formulation and production strategies. Eur J Pharm biopharmaceutics: official J Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2015;94:251–63.
18. Holzer B, Rijal P, McNee A, Paudyal B, Martini V, Clark B, et al. Protective porcine influenza virus-specific monoclonal antibodies recognize similar haemagglutinin epitopes as humans. PLoS Pathog. 2021;17(3):e1009330.
19. Sistere-Oro M, Lopez-Serrano S, Veljkovic V, Pina-Pedrero S, Vergara-Alert J, Cordoba L, et al. DNA vaccine based on conserved HA-peptides induces strong immune response and rapidly clears influenza virus infection from vaccinated pigs. PLoS ONE. 2019;14(9):e0222201.
20. Zost SJ, Wu NC, Hensley SE, Wilson IA. Immunodominance and antigenic variation of Influenza Virus Hemagglutinin: implications for design of Universal Vaccine Immunogens. J Infect Dis. 2019;219(Suppl1):38–S45.
21. Cai M, Gan P, Hu X, Mai Z, Ji C, Yi H, et al. Protective effect of bivalent H1N1 and H3N2 VLP vaccines against eurasian avian-like H1N1 and recent human-like H3N2 influenza viruses in a mouse model. Vet Microbiol. 2022;266:109370.
22. Schwartzman LM, Cathcart AL, Pujanauski LM, Qi L, Kash JC, Taubenberger JK. An Intranasal Virus-Like particle vaccine broadly protects mice from multiple subtypes of Influenza A Virus. mBio. 2015;6(4):e01044.
23. Kallewaard NL, Corti D, Collins PJ, Neu U, McAuliffe JM, Benjamin E, et al. Structure and function analysis of an antibody recognizing all Influenza A Subtypes. Cell. 2016;166(3):596–608.
24. Vincent AL, Ma W, Lager KM, Janke BH, Richt JA. Swine influenza viruses a north american perspective. Adv Virus Res. 2008;72:127–54.
25. Moser C, Amacker M, Zurbriggen R. Influenza virosomes as a vaccine adjuvant and carrier system. Expert Rev Vaccines. 2011;10(4):437–46.
26. Wilschut J. Influenza vaccines: the virosome concept. Immunol Lett. 2009;122(2):118–21.
27. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM, et al. The virosome concept for influenza vaccines. Vaccine. 2005;23(Suppl 1):26–38.
28. de Jonge J, Schoen P, ter Veer W, Stegmann T, Wilschut J, Huckriede A. Use of a dialyzable short-chain phospholipid for efficient solubilization and reconstitution of influenza virus envelopes. Bba-Biomembranes. 2006;1758(4):527–36.
29. Zhang J, Gauger PC. Isolation of Swine Influenza A Virus in cell cultures and Embryonated Chicken Eggs. Methods Mol Biol. 2020;2123:281–94.
30. Killian ML. Hemagglutination Assay for Influenza Virus. Methods Mol Biol. 2020;2123:3–10.

31. Zhang J, Harmon KM. RNA extraction from Swine samples and detection of Influenza A Virus in Swine by Real-Time RT-PCR. Methods Mol Biol. 2020;2123:295–310.
32. de Jonge J, Leenhouts JM, Holtrop M, Schoen P, Scherrer P, Cullis PR, et al. Cellular gene transfer mediated by influenza virosomes with encapsulated plasmid DNA. Biochem J. 2007;405(1):41–9.
33. Baron LF, Fonseca FND, Maciag SS, Bellaver FAV, Ibeli AMG, Mores MAZ et al. Toltrazuril-Loaded Polymeric Nanocapsules as a Promising Approach for the Preventive Control of Coccidiosis in Poultry. Pharmaceutics. 2022;14(2).
34. Souza CK, Rajao DS, Sandbulte MR, Lopes S, Lewis NS, Loving CL, et al. The type of adjuvant in whole inactivated influenza a virus vaccines impacts vaccine-associated enhanced respiratory disease. Vaccine. 2018;36(41):6103–10.
35. Kitikoon P, Gauger PC, Vincent AL. Hemagglutinin inhibition assay with swine sera. Methods Mol Biol. 2014;1161:295–301.
36. Maciag SS, Bellaver FV, Bombassaro G, Haach V, Mores MAZ, Baron LF, et al. On the influence of the source of porcine colostrum in the development of early immune ontogeny in piglets. Sci Rep. 2022;12(1):15630.
37. SAS. System for Microsoft Windows, Release 9.4. In: System for Microsoft Windows R, editor. 2002–2012. NC, USA2012.
38. Jung O, Smeets R, Hartjen P, Schnettler R, Feyerabend F, Klein M et al. Improved in Vitro Test Procedure for full Assessment of the cytocompatibility of degradable Magnesium based on ISO 10993-5/-12. Int J Mol Sci. 2019;20(2).
39. Silva-Santana G, Bax JC, Fernandes DCS, Bacellar DTL, Hooper C, Dias AASO, et al. Clinical hematological and biochemical parameters in Swiss, BALB/c, C57BL/6 and B6D2F1 Mus musculus. Anim Model Exp Med. 2020;3(4):304–15.
40. Asadi K, Gholami A. Virosome-based nanovaccines; a promising bioinspiration and biomimetic approach for preventing viral diseases: a review. Int J Biol Macromol. 2021;182:648–58.
41. Moser C, Muller M, Kaeser MD, Weydemann U, Amacker M. Influenza virosomes as vaccine adjuvant and carrier system. Expert Rev Vaccines. 2013;12(7):779–91.
42. Gluck R, Mischler R, Finkel B, Que JU, Scarpa B, Cryz SJ. Jr. Immunogenicity of new virosome influenza vaccine in elderly people. Lancet. 1994;344(8916):160–3.
43. de Jonge J, Holtrop M, Wilschut J, Huckriede A. Reconstituted influenza virus envelopes as an efficient carrier system for cellular delivery of small-interfering RNAs (vol 13, pg 995, 2006). Gene Ther. 2006;13(12):995.
44. Stegmann T, Kamphuis T, Meijerhof T, Goud E, de Haan A, Wilschut J. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: a safe and immunogenic non-replicating vaccine formulation. Vaccine. 2010;28(34):5543–50.
45. Kheiri MT, Jamali A, Shenagari M, Hashemi H, Sabahi F, Atyabi F, et al. Influenza virosome/DNA vaccine complex as a new formulation to induce intra-subtypic protection against influenza virus challenge. Antivir Res. 2012;95(3):229–36.
46. Ando S, Tsuge H, Mayumi T. Preparation of influenza virosome vaccine with muramyldipeptide derivative B30-MDP. J Microencapsul. 1997;14(1):79–90.
47. Huckriede A, Bungener L, Daemen T, Wilschut J. Influenza virosomes in Vaccine Development. Method Enzymol. 2003;373:74–91.
48. Moser C, Amacker M, Kammer AR, Rasi S, Westerfeld N, Zurbriggen R. Influenza virosomes as a combined vaccine carrier and adjuvant system for prophylactic and therapeutic immunizations. Expert Rev Vaccines. 2007;6(5):711–21.
49. Liu H, de Vries-Idema J, Ter Veer W, Wilschut J, Huckriede A. Influenza virosomes supplemented with GPI-0100 adjuvant: a potent vaccine formulation for antigen dose sparing. Med Microbiol Immunol. 2014;203(1):47–55.
50. Torrieri-Dramard L, Lambrecht B, Ferreira HL, Van den Berg T, Klatzmann D, Bellier B. Intranasal DNA vaccination induces potent mucosal and systemic Immune responses and cross-protective immunity against Influenza Viruses. Mol Ther. 2011;19(3):602–11.
51. EMEA EAftEoMP-. Ad Hoc Working Party on Influenza Vaccines: summary record of meeting on the choice of strains for influenza vaccine for the 1995–1996 vaccination campaign. 1995.
52. Cusi MG, Lomagistro MM, Valassina M, Valensin PE, Gluck R. Immunopotentiating of mucosal and systemic antibody responses in mice by intranasal immunization with HLT-combined influenza virosomal vaccine. Vaccine. 2000;18(25):2838–42.
53. Onodera T, Hosono A, Odagiri T, Tashiro M, Kaminogawa S, Okuno Y, et al. Whole-virion Influenza Vaccine recalls an early burst of High-Affinity memory B cell response through TLR Signaling. J Immunol. 2016;196(10):4172–84.
54. Onodera T, Hashi K, Shukla RK, Miki M, Takai-Todaka R, Fujimoto A, et al. Immune-Focusing Properties of Virus-like particles improve protective IgA responses. J Immunol. 2019;203(12):3282–92.
55. Abe T, Hemmi H, Miyamoto H, Moriishi K, Tamura S, Takaku H, et al. Involvement of the toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus. J Virol. 2005;79(5):2847–58.
56. Petrova G, Ferrante A, Gorski J. Cross-Reactivity of T cells and its role in the Immune System. Crit Rev Immunol. 2012;32(4):349–72.
57. Blom RAM, Amacker M, van Dijk RM, Moser C, Stumbles PA, Blank F et al. Pulmonary delivery of virosome-bound Antigen enhances Antigen-Specific CD4(+) T cell proliferation compared to liposome-bound or Soluble Antigen. Front Immunol. 2017;8.
58. Takamura S. Niches for the long-term maintenance of Tissue-Resident memory T cells. Front Immunol. 2018;9.
59. Ahrends T, Busselaar J, Severson TM, Babala N, de Vries E, Bovens A et al. CD4(+) T cell help creates memory CD8(+) T cells with innate and help-independent recall capacities. Nat Commun. 2019;10.
60. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, et al. A human memory T cell subset with stem cell-like properties. Nat Med. 2011;17(10):1290–U325.
61. Wirth TC, Xue HH, Rai D, Sabel JT, Bair T, Harty JT, et al. Repetitive Antigen Stimulation induces stepwise transcriptome diversification but preserves a core signature of memory CD8(+) T cell differentiation. Immunity. 2010;33(1):128–40.
62. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. Immunity. 2010;33(4):451–63.
63. Jacobson RM, Grill DE, Oberg AL, Tosh PK, Ovsyannikova IG, Poland GA. Profiles of influenza A/H1N1 vaccine response using hemagglutination-inhibition titers. Hum Vacc Immunother. 2015;11(4):961–9.
64. Wilson JR, Tzeng WP, Spesock A, Music N, Guo Z, Barrington R, et al. Diversity of the murine antibody response targeting influenza A(H1N1pdm09) hemagglutinin. Virology. 2014;458:114–24.

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Supplementary information

Additional file 1

Table S1. Biochemical parameters of renal and liver function of mice after two doses of multivalent vaccine for swine influenza.

Variable	Groups			Pr>F
	G1	G2	G3	
ALT	50.76±2.05[b]	59.41±2.29 [a]	56.31±2.22 [ab]	0.0298
AST	80.30±5.06	71.35±4.78	73.90±5.08	0.4346
Creatinine	0.445±0.043	0.332±0.033	0.339±0.032	0.0669
BUN	17.19±1.29[b]	18.14±0.71[a]	18.70±0.7 [a]	<0.0001

Means followed by distinct letters in the lines differ significantly by Tukey's test ($P \leq 0.05$).

Table S2. Stimulation proliferation index for lymphocytes from splenocytes for H1N1, H1N2 and H3N2 among all groups at 21 days (52 days) and 8 months (255 days) after booster immunization.

Immune cells		Intramuscular	Intranasal	Intramuscular	Intranasal	Intramuscular	Intranasal
				36 days			
		H1N1		H1N2		H3N2	
B cells	$CD19^+CD69^+$	2.394 ± 0.261^d	3.657 ± 0.098^e	2.161 ± 0.184^e	2.663 ± 0.105^e	1.608 ± 0.148^b	2.942 ± 0.095^e
	$CD19^+CD69^+CD25^+$	1.515 ± 0.076^e	2.391 ± 0.162^e	1.231 ± 0.161	1.748 ± 0.134^d	1.303 ± 0.136^a	2.298 ± 0.136^e
	$CD45R/B220^+sIgM^+$	2.032 ± 0.149^d	2.615 ± 0.206^e	1.958 ± 0.219^b	2.397 ± 0.117^e	2.110 ± 0.142^e	2.735 ± 0.225^e
	$CD45R/B220^+sIgM^+CD23^+$	1.785 ± 0.350^a	1.601 ± 0.153^b	2.393 ± 0.526^a	2.138 ± 0.221^d	2.558 ± 0.327^c	2.527 ± 0.282^d
T cells	$CD3e^+CD4^+$	1.839 ± 0.091^e	1.743 ± 0.077^e	1.813 ± 0.104^e	1.695 ± 0.148^c	1.860 ± 0.107^e	1.793 ± 0.145^d
	$CD3e^+CD8\alpha^+$	2.249 ± 0.088^e	2.293 ± 0.091^e	1.664 ± 0.041^e	1.617 ± 0.120^c	1.741 ± 0.093^e	1.724 ± 0.091^e
	$CD3e^+CD69^+$	1.809 ± 0.074^e	2.324 ± 0.119^e	1.658 ± 0.201^a	2.543 ± 0.127^e	2.226 ± 0.249^c	3.361 ± 0.146^e
	$CD3e^+CD69^+ CD25^+$	6.717 ± 0.122^e	4.210 ± 0.245^e	7.326 ± 0.234^e	4.458 ± 0.180^e	6.848 ± 0.181^e	4.711 ± 0.264^e
	$CD3e^+CD4^+CD44^{high}$ $CD62L^{high}$	3.946 ± 0.393^e	3.893 ± 0.155^e	3.136 ± 0.300^e	3.302 ± 0.236^e	2.571 ± 0.268^d	2.796 ± 0.125^e
	$CD3e^+CD4^+CD44^{high}$ $CD62L^{low}$	3.325 ± 0.237^e	2.231 ± 0.230^d	5.184 ± 0.411^e	4.293 ± 0.683^d	5.857 ± 0.679^e	4.471 ± 0.801^e
				255 days			
		H1N1		H1N2		H3N2	
B cells	$CD19^+CD69^+$	3.204 ± 0.134^e	3.221 ± 0.182^e	2.749 ± 0.107^e	2.037 ± 0.218^c	2.703 ± 0.092^e	2.421 ± 0.189^e
	$CD19^+CD69^+CD25^+$	2.759 ± 0.102^e	2.534 ± 0.200^e	2.141 ± 0.135^e	1.875 ± 0.176^c	2.434 ± 0.132^e	2.322 ± 0.126^e
	$CD45R/B220^+sIgM^+$	2.399 ± 0.192^e	2.335 ± 0.201^e	2.641 ± 0.197^e	2.156 ± 0.178^e	2.712 ± 0.118^e	2.426 ± 0.110^e
	$CD45R/B220^+sIgM^+CD23^+$	6.717 ± 0.530^e	7.896 ± 0.554^e	12.70 ± 0.99^e	12.51 ± 1.14^e	10.63 ± 0.93^e	13.02 ± 0.94^e
T cells	$CD3e^+CD4^+$	1.474 ± 0.053^e	1.329 ± 0.070^c	1.332 ± 0.078^b	1.313 ± 0.059^d	1.462 ± 0.054^e	1.252 ± 0.068^b
	$CD3e^+CD8\alpha^+$	1.667 ± 0.082^e	1.406 ± 0.137^a	1.074 ± 0.076	1.035 ± 0.095	1.107 ± 0.054	1.200 ± 0.085^a
	$CD3e^+CD69^+$	1.397 ± 0.082^c	2.040 ± 0.070^e	1.504 ± 0.089^d	1.982 ± 0.092^e	2.088 ± 0.125^e	2.610 ± 0.122^e
	$CD3e^+CD69^+ CD25^+$	1.882 ± 0.174^c	1.299 ± 0.120^a	1.347 ± 0.082^b	1.097 ± 0.120	1.447 ± 0.176^a	1.737 ± 0.254^a
	$CD3e^+CD4^+CD44^{high}$ $CD62L^{high}$	3.555 ± 0.205^e	2.526 ± 0.116^e	3.518 ± 0.348^e	2.395 ± 0.130^e	2.566 ± 0.278^d	2.138 ± 0.124^e
	$CD3e^+CD4^+CD44^{high}$ $CD62L^{low}$	1.938 ± 0.177^d	1.226 ± 0.052^b	2.893 ± 0.330^d	2.087 ± 0.176^d	2.959 ± 0.320^d	2.155 ± 0.110^e

Data are shown as fold change means ± standard error.

^{a,b,c,d,e} Different superscript letters indicate significant statistical difference between groups (a: $P\leq0.05$, b: $P\leq0.005$, c: $P\leq0.001$, d: $P\leq0.0005$, e: $P\leq0.0001$).

Additional file 2

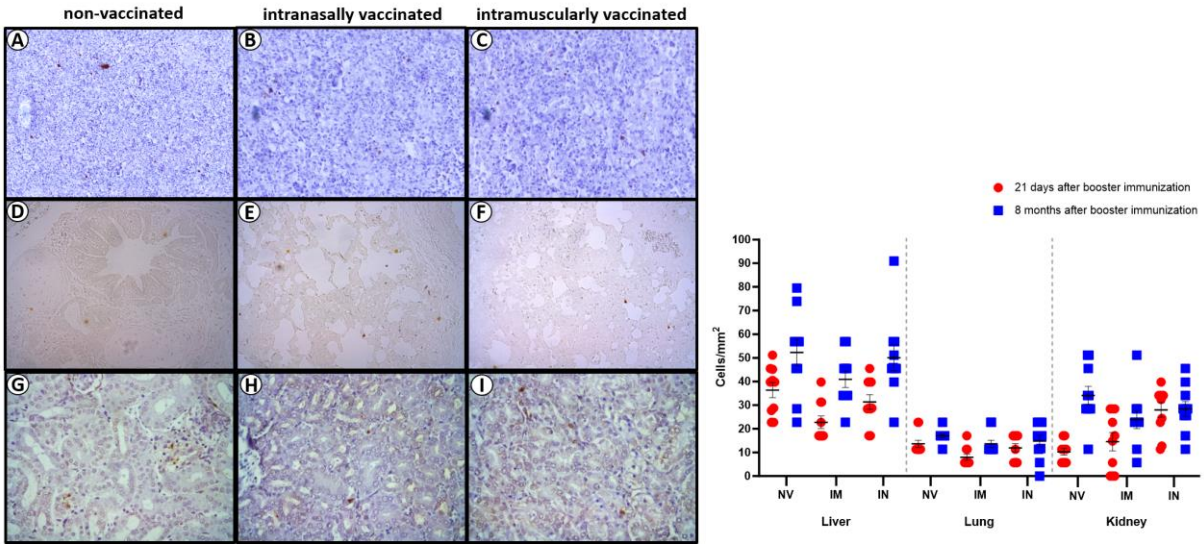


Figure S1. TUNEL assay. Number of apoptotic cells observed in the TUNEL assay in different tissues (liver, lung and kidney) from non-vaccinated (NV), intranasally vaccinated (IN) and intramuscularly vaccinated (IM) mice on D21, D240 post-vaccination. Non-paired Student's *t* test, with the data expressed as mean ± standard error. A, B, C: liver; D, E, F: lung; and G, H, I: kidney at 21 days after booster immunization.

5 CONCLUSÕES

A infecção causada pelo IAV é considerada um dos principais problemas sanitários na produção de suínos. A implementação de medidas de prevenção e controle da doença nos rebanhos suínos, como a vacinação, é de extrema importância por reduzir as perdas na produção, bem como minimizar o risco de surgimento de vírus com potencial pandêmico. Neste estudo foi avaliada a imunogenicidade de uma vacina virossomal trivalente para influenza em suínos. Houve indução de resposta imune humoral e celular robusta para os três subtipos virais que compõem a vacina (H1N1, H1N2 e H3N2), após a administração de duas doses vacinais. A vacina virossomal induziu anticorpos específicos para HA e vírus-neutralizantes, bem como a indução de diferentes células imunológicas, como a maturação de macrófagos, e proliferação de linfócitos B, linfócitos T $CD4^+$ e $CD8^+$ efetores e de memória central. Além disso, foi demonstrado potencial para conferir imunidade duradoura nos suínos vacinados até a idade de abate dos suínos, com a detecção de linfócitos B, linfócitos T $CD4^+$ e $CD8^+$ de memória. Ademais, a vacina se mostrou segura e não citotóxica para os suínos. O uso da plataforma virossomal permite flexibilidade para atualização dos IAVs presentes na vacina, e desta forma, refletir a diversidade genética e antigênica dos vírus influenza circulantes em suínos no Brasil. A vacinação de suínos contra o IAV poderá reduzir o impacto da doença na produção de suínos, pela redução da transmissão viral entre suínos, e o potencial de geração de novos vírus. Ademais, contribui para a saúde humana, reduzindo a transmissão zoonótica de influenza, bem como a emergencia de pandemias.

REFERÊNCIAS

ABDOLI, A. et al. An H1-H3 chimeric influenza virosome confers complete protection against lethal challenge with PR8 (H1N1) and X47 (H3N2) viruses in mice. **Pathogens and Disease**, v. 72, n. 3, p. 197–207, 2014.

ABENTE, E. J. et al. Alphavirus-vectored hemagglutinin subunit vaccine provides partial protection against heterologous challenge in pigs. **Vaccine**, v. 37, n. 11, p. 1533–1539, 2019.

ABPA (Associação Brasileira de Proteína Animal). **Relatório anual 2022**. Disponível em: <https://abpa-br.org/wp-content/uploads/2022/05/Relatorio-Anual-ABPA-2022-vf.pdf>. Acesso em: 8 jan. 2023.

ALBRECHT, M.; ARCK, P. C. Vertically transferred immunity in neonates: mothers, mechanisms and mediators. **Frontiers in Immunology**, v. 11, n. 555, 2020.

ALLERSON, M. et al. The impact of maternally derived immunity on influenza A virus transmission in neonatal pig populations. **Vaccine**, v. 31, n. 3, p. 500–505, 2013.

ALLIE, S. R.; RANDALL, T. D. Pulmonary immunity to viruses. **Clinical Science**, v. 131, n. 14, p. 1737–1762, 2017.

ANDERSON, T. K. et al. Swine influenza A viruses and the tangled relationship with humans. **Cold Spring Harbor Perspectives in Medicine**, v. 11, n. 3, p. a038737, 2021.

ANDRADE, F. Non-cytotoxic antiviral activities of granzymes in the context of the immune antiviral state. **Immunological Reviews**, v. 235, n. 1, p. 128–146, 2010.

ARKEMA, A. et al. Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes. **Vaccine**, v. 18, n. 14, p. 1327–1333, 2000.

ARPAIA, N. et al. A distinct function of regulatory T cells in tissue protection. **Cell**, v. 162, n. 5, p. 1078–1089, 2015.

BAHADORAN, A. et al. Immune responses to influenza virus and its correlation to age and inherited factors. **Frontiers in Microbiology**, v. 7, n. 1841, 2016.

BARRÍA, M. I. et al. Localized mucosal response to intranasal live attenuated influenza vaccine in adults. **Journal of Infectious Diseases**, v. 207, n. 1, p. 115–124, 2013.

BASHA, S. et al. Comparison of antibody and T-cell responses elicited by licensed inactivated- and live-attenuated influenza vaccines against H3N2 hemagglutinin. **Human Immunology**, v. 72, n. 6, p. 463–469, 2011.

BAUDON, E. et al. Epidemiological features of influenza circulation in swine populations: a systematic review and meta-analysis. **PLoS ONE**, v. 12, n. 6, p. e0179044, 2017.

BERNASCONI, V. et al. Mucosal vaccine development based on liposome technology. **Journal of Immunology Research**, v. 2016, n. 5482087, 2016.

BETTS, R. J. et al. Influenza A virus infection results in a robust, antigen-responsive, and widely disseminated Foxp3+ regulatory T cell response. **Journal of Virology**, v. 86, n. 5, p. 2817–2825, 2012.

BORGGREN, M. et al. A polyvalent influenza DNA vaccine applied by needle-free intradermal delivery induces cross-reactive humoral and cellular immune responses in pigs. **Vaccine**, v. 34, n. 32, p. 3634–3640, 2016.

BOSWORTH, B. et al. Replicon particle vaccine protects swine against influenza. **Comparative Immunology, Microbiology and Infectious Diseases**, v. 33, n. 6, p. e99–e103, 2010.

BRAUCHER, D. R. et al. Intranasal vaccination with replication-defective adenovirus type 5 encoding influenza virus hemagglutinin elicits protective immunity to homologous challenge and partial protection to heterologous challenge in pigs. **Clinical and Vaccine Immunology**, v. 19, n. 11, p. 1722–1729, 2012.

BRAUN, M. et al. Virus-like particles induce robust human T-helper cell responses. **European Journal of Immunology**, v. 42, n. 2, p. 330–340, 2012.

BRAVO-VASQUEZ, N. et al. Swine influenza virus (H1N2) characterization and transmission in ferrets, Chile. **Emerging Infectious Diseases**, v. 23, n. 2, p. 241–251, 2017.

BROCKMEIER, S. L.; HALBUR, P. G.; THACKER, E. L. Porcine respiratory disease complex. In: BROGDEN, K. A.; GUTHMILLER, J. M. (Eds.). **Polymicrobial Diseases**. Washington (DC): ASM Press, 2002. p. 231–258.

BROWN, I. H. The epidemiology and evolution of influenza viruses in pigs. **Veterinary Microbiology**, v. 74, n. 1–2, p. 29–46, 2000.

BUSQUETS, N. et al. Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. **Veterinary Research**, v. 41, n. 5, p. 74, 2010.

CAMPOS, A. C. A. et al. Bat influenza A(HL18NL11) virus in fruit bats, Brazil. **Emerging Infectious Diseases**, v. 25, n. 2, p. 333–337, 2019.

CAO, X. Self-regulation and cross-regulation of pattern-recognition receptor signalling in health and disease. **Nature Reviews Immunology**, v. 16, n. 1, p. 35–50, 2016.

CAPPUCCIO, J. A. et al. Outbreak of swine influenza in Argentina reveals a non-contemporary human H3N2 virus highly transmissible among pigs. **Journal of General Virology**, v. 92, n. 12, p. 2871–2878, 2011.

CHEN, X. et al. Host immune response to influenza A virus infection. **Frontiers in Immunology**, v. 9, n. 320, p. 1–13, 2018.

CHENG, X. et al. Evaluation of the humoral and cellular immune responses elicited by the live attenuated and inactivated influenza vaccines and their roles in heterologous protection in ferrets. **Journal of Infectious Diseases**, v. 208, n. 4, p. 594–602, 2013.

CHOI, Y. S.; BAUMGARTH, N. Dual role for B-1a cells in immunity to influenza virus infection. **Journal of Experimental Medicine**, v. 205, n. 13, p. 3053–3064, 2008.

CIACCI-ZANELLA, J. R. et al. Influenza A virus infection in Brazilian swine herds following the introduction of pandemic 2009 H1N1. **Veterinary Microbiology**, v. 180, n. 1–2, p. 118–122, 2015.

CIMICA, V.; GALARZA, J. M. Adjuvant formulations for virus-like particle (VLP) based vaccines. **Clinical Immunology**, v. 183, p. 99–108, 2017.

CORREIA, V.; ABECASIS, A. B.; REBELO-DE-ANDRADE, H. Molecular footprints of selective pressure in the neuraminidase gene of currently circulating human influenza subtypes and lineages. **Virology**, v. 522, p. 122–130, 2018.

CORTI, D. et al. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. **Science**, v. 333, n. 6044, p. 850–856, 2011.

COX, N. J.; SUBBARAO, K. Global epidemiology of influenza: past and present. **Annual Review of Medicine**, v. 51, n. 1, p. 407–421, 2000.

CRISCI, E. et al. Review: influenza virus in pigs. **Molecular Immunology**, v. 55, n. 3–4, p. 200–211, 2013.

CULHANE, M.; GARRIDO-MANTILLA, J.; TORREMORELL, M. Specimen types, collection, and transport for influenza A viruses of swine. **Methods in Molecular Biology**, v. 2123, p. 273–280, 2020.

DE, A. Molecular evolution of hemagglutinin gene of influenza A virus. **Frontiers in Bioscience**, v. 10, p. 101–118, 2018.

DE BRUIN, T. G. M. et al. Cytolytic function for pseudorabies virus-stimulated porcine CD4+ CD8dull+ lymphocytes. **Viral Immunology**, v. 13, n. 4, p. 511–520, 2000.

DE VEER, M.; MEEUSEN, E. New developments in vaccine research - unveiling the secret of vaccine adjuvants. **Discovery Medicine**, v. 12, n. 64, p. 195–204, 2011.

DE VLEESCHAUWER, A. R. et al. Cross-protection between antigenically distinct H1N1 swine influenza viruses from Europe and North America. **Influenza and Other Respiratory Viruses**, v. 5, n. 2, p. 115–122, 2011.

DETMER, S. et al. Diagnostics and surveillance for swine influenza. **Current Topics in Microbiology and Immunology**, v. 370, p. 85–112, 2013.

DIEBOLD, S. S. Recognition of viral single-stranded RNA by Toll-like receptors. **Advanced Drug Delivery Reviews**, v. 60, n. 7, p. 813–823, 2008.

DUNBAR, P. et al. Pulmonary monocytes promote the establishment of lung tissue-resident CD8 T cell memory following influenza infection. **The Journal of Immunology**, v. 198, n. 1, p. 153.18, 2017.

EKIERT, D. C. et al. Antibody recognition of a highly conserved influenza virus epitope. **Science**, v. 324, n. 5924, p. 246–251, 2009.

EL BAKKOURI, K. et al. Universal vaccine based on ectodomain of matrix protein 2 of influenza A: Fc receptors and alveolar macrophages mediate protection. **The Journal of Immunology**, v. 186, n. 2, p. 1022–1031, 2011.

ERTL, H. C. Viral vectors as vaccine carriers. **Current Opinion in Virology**, v. 21, p. 1–8, 2016.

EWER, K. J. et al. Viral vectors as vaccine platforms: from immunogenicity to impact. **Current Opinion in Immunology**, v. 41, p. 47–54, 2016.

FOTOUHI, F. et al. Adjuvant use of the NKT cell agonist alpha-galactosylceramide leads to enhancement of M2-based DNA vaccine immunogenicity and protective immunity against influenza A virus. **Archives of Virology**, v. 162, n. 5, p. 1251–1260, 2017.

GAMBLIN, S. J.; SKEHEL, J. J. Influenza hemagglutinin and neuraminidase membrane glycoproteins. **Journal of Biological Chemistry**, v. 285, n. 37, p. 28403–28409, 2010.

GARTEN, R. J. et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. **Science**, v. 325, n. 5937, p. 197–201, 2009.

GAUGER, P. C. et al. Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (δ-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus. **Vaccine**, v. 29, n. 15, p. 2712–2719, 2011.

GAUGER, P. C. et al. Kinetics of lung lesion development and pro-inflammatory cytokine response in pigs with vaccine-associated enhanced respiratory disease induced by challenge with pandemic (2009) A/H1N1 influenza virus. **Veterinary Pathology**, v. 49, n. 6, p. 900–912, 2012.

GAUGER, P. C. et al. Live attenuated influenza A virus vaccine protects against A(H1N1)pdm09 heterologous challenge without vaccine associated enhanced respiratory disease. **Virology**, v. 471–473, p. 93–104, 2014.

GAUGER, P. C.; VINCENT, A. L. Serum virus neutralization assay for detection and quantitation of serum neutralizing antibodies to influenza A virus in swine. **Methods in Molecular Biology**, v. 2123, p. 321–333, 2020a.

GAUGER, P. C.; VINCENT, A. L. Enzyme-linked immunosorbent assay for detection of serum or mucosal isotype-specific IgG and IgA whole-virus antibody to influenza A virus in swine. **Methods in Molecular Biology**, v. 2123, p. 311–320, 2020b.

GAYMARD, A. et al. Functional balance between neuraminidase and haemagglutinin in influenza viruses. **Clinical Microbiology and Infection**, v. 22, n. 12, p. 975–983, 2016.

GEURTS VAN KESSEL, C. H. et al. Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. **Journal of Experimental Medicine**, v. 205, n. 7, p. 1621–1634, 2008.

GONEAU, L. W. et al. Zoonotic influenza and human health—part 1: virology and epidemiology of zoonotic influenzas. **Current Infectious Disease Reports**, v. 20, n. 10, p. 37, 2018.

GOODELL, C. K. et al. Evaluation of screening assays for the detection of influenza A virus serum antibodies in swine. **Transboundary and Emerging Diseases**, v. 63, n. 1, p. 24–35, 2016.

GORRES, J. P. et al. DNA vaccination elicits protective immune responses against pandemic and classic swine influenza viruses in pigs. **Clinical and Vaccine Immunology**, v. 18, n. 11, p. 1987–1995, 2011.

GRANT, E. J. et al. Human influenza viruses and CD8+ T cell responses. **Current Opinion in Virology**, v. 16, p. 132–142, 2016.

GUNSON, R. N.; COLLINS, T. C.; CARMAN, W. F. Practical experience of high throughput real time PCR in the routine diagnostic virology setting. **Journal of Clinical Virology**, v. 35, n. 4, p. 355–367, 2006.

GUO, H.; KUMAR, P.; MALARKANNAN, S. Evasion of natural killer cells by influenza virus. **Journal of Leukocyte Biology**, v. 89, n. 2, p. 189–194, 2011.

HAACH, V. et al. One-step multiplex RT-qPCR for the detection and subtyping of influenza A virus in swine in Brazil. **Journal of Virological Methods**, v. 269, p. 43–48, 2019.

HAMPSON, A. W. Influenza virus antigens and 'antigenic drift'. **Perspectives in Medical Virology**, v. 7, p. 49–85, 2002.

HEER, A. K. et al. CD4+ and CD8+ T cells exhibit differential requirements for CCR7-mediated antigen transport during influenza infection. **The Journal of Immunology**, v. 181, n. 10, p. 6984–6994, 2008.

HEINEN, P. P. et al. Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection. **Viral Immunology**, v. 13, n. 2, p. 237–247, 2000.

HEINEN, P. P.; DE BOER-LUIJTZE, E. A.; BIANCHI, T. J. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. **Journal of General Virology**, v. 82, n. Pt 11, p. 2697–2707, 2001.

HERNANDEZ, L. A.; MILLER, C. L.; VAUGHN, E. M. Particle and subunit-based hemagglutinin vaccines provide protective efficacy against H1N1 influenza in pigs. **Veterinary Microbiology**, v. 191, p. 35–43, 2016.

HERZOG, C. et al. Eleven years of Inflexal® V-a virosomal adjuvanted influenza vaccine. **Vaccine**, v. 27, p. 4381–4387, 2009.

HERZOG, C.; METCALFE, I. C.; SCHAAD, U. B. Virosome influenza vaccine in children. **Vaccine**, v. 20, n. 5, p. B24–B28, 2002.

HILLAIRE, M. L. B. et al. Human T-cells directed to seasonal influenza A virus cross-react

with 2009 pandemic influenza A (H1N1) and swine-origin triple-reassortant H3N2 influenza viruses. **Journal of General Virology**, v. 94, n. Pt 3, p. 583–592, 2013.

HINTZEN, G. et al. Induction of tolerance to innocuous inhaled antigen relies on a CCR7-dependent dendritic cell-mediated antigen transport to the bronchial lymph node. **The Journal of Immunology**, v. 177, n. 10, p. 7346–7354, 2006.

HISCOTT, J. et al. MasterCARD: a priceless link to innate immunity. **Trends in Molecular Medicine**, v. 12, n. 2, p. 53–56, 2006.

HO, A. W. S. et al. Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. **The Journal of Immunology**, v. 187, n. 11, p. 6011–6021, 2011.

HOFT, D. F. et al. Comparisons of the humoral and cellular immune responses induced by live attenuated influenza vaccine and inactivated influenza vaccine in adults. **Clinical and Vaccine Immunology**, v. 24, n. 1, p. e00414-16, 2017.

HOLT, P. G. et al. Regulation of immunological homeostasis in the respiratory tract. **Nature Reviews Immunology**, v. 8, n. 2, p. 142–152, 2008.

HOLZER, B. et al. T and B cell immune responses to influenza viruses in pigs. **Frontiers in Immunology**, v. 10, n. 98, 2019.

HUCKRIEDE, A. et al. The virosome concept for influenza vaccines. **Vaccine**, v. 23, n. S1, p. S38, 2005.

HUMPHREYS, I. R.; SEBASTIAN, S. Novel viral vectors in infectious diseases. **Immunology**, v. 153, n. 1, p. 1–9, 2018.

ICTV (International Committee on Taxonomy of Viruses). **Virus taxonomy: 2021 release**. Disponível em: <https://ictv.global/taxonomy>. Acesso em: 8 jan. 2023.

IMAI, M.; KAWAOKA, Y. The role of receptor binding specificity in interspecies transmission of influenza viruses. **Current Opinion in Virology**, v. 2, n. 2, p. 160–167, 2012.

ITO, T. et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. **Journal of Virology**, v. 72, n. 9, p. 7367–7373, 1998.

ITO, T. Interspecies transmission and receptor recognition of influenza A viruses. **Microbiology and Immunology**, v. 44, n. 6, p. 423–430, 2000.

JANKE, B. H. Diagnosis of swine influenza. **Swine Health and Production**, v. 8, n. 2, p. 79–84, 2000.

JANKE, B. H. Clinicopathological features of swine influenza. **Current Topics in Microbiology and Immunology**, v. 370, p. 69–83, 2013.

JANKE, B. H. Influenza A virus infections in swine: pathogenesis and diagnosis. **Veterinary Pathology**, v. 51, n. 2, p. 410–426, 2014.

JOHNSON, N. P. A. S.; MUELLER, J. Updating the accounts: global mortality of the 1918-1920 “Spanish” influenza pandemic. **Bulletin of the History of Medicine**, v. 76, n. 1, p. 105–115, 2002.

JOSHI, L. R. et al. Protective efficacy of an orf virus-vector encoding the hemagglutinin and the nucleoprotein of influenza A virus in swine. **Frontiers in Immunology**, v. 12, p. 747574, 2021.

JUNG, H. E.; LEE, H. K. Host protective immune responses against influenza A virus infection. **Viruses**, v. 12, n. 5, p. 504, 2020.

JUNG, K.; HA, Y.; CHAE, C. Pathogenesis of swine influenza virus subtype H1N2 infection in pigs. **Journal of Comparative Pathology**, v. 132, n. 2–3, p. 179–184, 2005.

JUNQUEIRA, D. M. et al. Human-to-swine introductions and onward transmission of 2009 H1N1 pandemic influenza viruses in Brazil. **Frontiers in Microbiology**, v. 14, n. 1243567, 2023.

KAMMER, A. R. et al. A new and versatile virosomal antigen delivery system to induce cellular and humoral immune responses. **Vaccine**, v. 25, n. 41, p. 7065–7074, 2007.

KAPPES, M. A. et al. Vaccination with NS1-truncated H3N2 swine influenza virus primes T cells and confers cross-protection against an H1N1 heterosubtypic challenge in pigs. **Vaccine**, v. 30, n. 2, p. 280–288, 2012.

KESHAVARZ, M. et al. Influenza vaccine: where are we and where do we go? **Reviews in Medical Virology**, v. 29, n. 1, p. e2014, 2019.

KHATRI, M. et al. Swine influenza H1N1 virus induces acute inflammatory immune responses in pig lungs: a potential animal model for human H1N1 influenza virus. **Journal of Virology**, v. 84, n. 21, p. 11210–11218, 2010.

KIM, J. H.; JACOB, J. DNA vaccines against influenza viruses. **Current Topics in Microbiology and Immunology**, v. 333, p. 197–210, 2009.

KIM, M. C. et al. Multiple heterologous M2 extracellular domains presented on virus-like particles confer broader and stronger M2 immunity than live influenza A virus infection. **Antiviral Research**, v. 99, n. 3, p. 328–335, 2013.

KITIKOON, P. et al. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. **Veterinary Immunology and Immunopathology**, v. 112, n. 3–4, p. 117–128, 2006.

KITIKOON, P. et al. Swine influenza matrix 2 (M2) protein contributes to protection against infection with different H1 swine influenza virus (SIV) isolates. **Vaccine**, v. 28, n. 2, p. 523–531, 2009.

KITIKOON, P. et al. Swine influenza virus vaccine serologic cross-reactivity to contemporary US swine H3N2 and efficacy in pigs infected with an H3N2 similar to 2011-2012 H3N2v. **Influenza and Other Respiratory Viruses**, v. 7, n. (Suppl. 4), p. 32–41, 2013.

KITIKOON, P.; GAUGER, P. C.; VINCENT, A. L. Hemagglutinin inhibition assay with swine sera. **Methods in Molecular Biology**, v. 1161, p. 295–301, 2014.

KLEIN, E. Y. et al. Influenza A H1N1 pandemic strain evolution - divergence and the potential for antigenic drift variants. **PLoS ONE**, v. 9, n. 4, p. e93632, 2014.

KRAMMER, F. et al. Chimeric hemagglutinin influenza virus vaccine constructs elicit broadly protective stalk-specific antibodies. **Journal of Virology**, v. 87, n. 12, p. 6542–6550, 2013.

KRAMMER, F. The human antibody response to influenza A virus infection and vaccination. **Nature Reviews Immunology**, v. 19, n. 6, p. 383–397, 2019.

KREIJTZ, J. H. C. M.; FOUCHIER, R. A. M.; RIMMELZWAAN, G. F. Immune responses to influenza virus infection. **Virus Research**, v. 162, n. 1–2, p. 19–30, 2011.

KUMAR, H.; KAWAI, T.; AKIRA, S. Pathogen recognition by the innate immune system. **International Reviews of Immunology**, v. 30, n. 1, p. 16–34, 2011.

KUNTZ-SIMON, G.; MADEC, F. Genetic and antigenic evolution of swine influenza viruses in Europe and evaluation of their zoonotic potential. **Zoonoses and Public Health**, v. 56, n. 6–7, p. 310–325, 2009.

KUTZLER, M. A.; WEINER, D. B. DNA vaccines: ready for prime time? **Nature Reviews Genetics**, v. 9, n. 10, p. 776–788, 2008.

LAMB, J. R. et al. Antigen-specific human T lymphocyte clones: induction, antigen specificity, and MHC restriction of influenza virus-immune clones. **The Journal of Immunology**, v. 128, n. 1, p. 233–238, 1982.

LANDOLT, G. A. et al. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. **Journal of Clinical Microbiology**, v. 41, n. 5, p. 1936–1941, 2003.

LANDOLT, G. A.; OLSEN, C. W. Up to new tricks – a review of cross-species transmission of influenza A viruses. **Animal Health Research Reviews**, v. 8, n. 1, p. 1–21, 2007.

LANGE, E. et al. Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs. **Journal of General Virology**, v. 90, n. 9, p. 2119–2123, 2009.

LARSEN, D. L. et al. Systemic and mucosal immune responses to H1N1 influenza virus infection in pigs. **Veterinary Microbiology**, v. 74, n. 1–2, p. 117–131, 2000.

LARSEN, D. L.; OLSEN, C. W. Effects of DNA dose, route of vaccination, and coadministration of porcine interleukin-6 DNA on results of DNA vaccination against influenza virus infection in pigs. **American Journal of Veterinary Research**, v. 63, n. 5, p. 653–659, 2002.

LAVER, W. G.; WEBSTER, R. G. Preparation and immunogenicity of a purified influenza

virus haemagglutinin and neuraminidase subunit vaccine. **Postgraduate Medical Journal**, v. 52, p. 373–378, 1976.

LEE, B. W. et al. Class specific antibody response to influenza A H1N1 infection in swine. **Veterinary Microbiology**, v. 43, n. 2–3, p. 241–250, 1995.

LEE, D. H. Complete genome sequencing of influenza A viruses using next-generation sequencing. **Methods in Molecular Biology**, v. 2123, p. 69–79, 2020.

LEE, J. H.; GRAMER, M. R.; JOO, H. S. Efficacy of swine influenza A virus vaccines against an H3N2 virus variant. **Canadian Journal of Veterinary Research**, v. 71, n. 3, p. 207–212, 2007.

LEE, L. Y. H. et al. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. **Journal of Clinical Investigation**, v. 118, n. 10, p. 3478–3490, 2008.

LEE, Y. N. et al. Co-immunization with tandem repeat heterologous M2 extracellular proteins overcomes strain-specific protection of split vaccine against influenza a virus. **Antiviral Research**, v. 122, p. 82–90, 2015.

LEWIS, N. S. et al. The global antigenic diversity of swine influenza A viruses. **eLife**, v. 5, n. e12217, p. 1–17, 2016.

LI, L.; PETROVSKY, N. Molecular mechanisms for enhanced DNA vaccine immunogenicity. **Expert Review of Vaccines**, v. 15, n. 3, p. 313–329, 2016.

LI, X. et al. Influenza virus-like particles harboring H9N2 HA and NA proteins induce a protective immune response in chicken. **Influenza and Other Respiratory Viruses**, v. 11, n. 6, p. 518–524, 2017.

LILLIE, P. J. et al. Preliminary assessment of the efficacy of a T-cell-based influenza vaccine, MVA-NP+M1, in humans. **Clinical Infectious Diseases**, v. 55, n. 1, p. 19–25, 2012.

LOEFFEN, W. L. A. et al. Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. **Veterinary Immunology and Immunopathology**, v. 92, n. 1–2, p. 23–35, 2003.

LOEFFEN, W. L. A. et al. Vaccination with a soluble recombinant hemagglutinin trimer protects pigs against a challenge with pandemic (H1N1) 2009 influenza virus. **Vaccine**, v. 29, n. 8, p. 1545–1550, 2011.

LÓPEZ, A. Respiratory system. In: MCGAVIN, M. D.; ZACHARY, J. F. (Eds.). **Pathologic Basis of Veterinary Disease**. 4th. ed. Saint Louis: Mosby Elsevier, 2007. p. 463–558.

LOVING, C. L. et al. Heightened adaptive immune responses following vaccination with a temperature-sensitive, live-attenuated influenza virus compared to adjuvanted, whole-inactivated virus in pigs. **Vaccine**, v. 30, n. 40, p. 5830–5838, 2012.

LOVING, C. L. et al. Efficacy in pigs of inactivated and live attenuated influenza virus

vaccines against infection and transmission of an emerging H3N2 similar to the 2011-2012 H3N2v. **Journal of Virology**, v. 87, n. 17, p. 9895–9903, 2013.

LOW, J. G. H. et al. Safety and immunogenicity of a virus-like particle pandemic influenza A (H1N1) 2009 vaccine: results from a double-blinded, randomized Phase I clinical trial in healthy Asian volunteers. **Vaccine**, v. 32, n. 39, p. 5041–5048, 2014.

LOWEN, A. C. It's in the mix: reassortment of segmented viral genomes. **PLoS Pathogens**, v. 14, n. 9, p. e1007200, 2018.

LUCKHEERAM, R. V. et al. CD4+T cells: differentiation and functions. **Clinical and Developmental Immunology**, v. 2012, n. 925135, 2012.

LUKENS, M. V. et al. Respiratory syncytial virus-induced activation and migration of respiratory dendritic dells and subsequent antigen presentation in the lung-draining lymph node. **Journal of Virology**, v. 83, n. 14, p. 7235–7243, 2009.

MA, W.; KAHN, R. E.; RICHT, J. A. The pig as a mixing vessel for influenza viruses: human and veterinary implications. **Journal of Molecular and Genetic Medicine**, v. 3, n. 1, p. 158–166, 2009.

MA, W.; RICHT, J. A. Swine influenza vaccines: current status and future perspectives. **Animal Health Research Reviews**, v. 11, n. 1, p. 81–96, 2010.

MALLICK, A. I. et al. Enhancement of immunogenicity of a virosome-based avian influenza vaccine in chickens by incorporating CpG-ODN. **Vaccine**, v. 29, n. 8, p. 1657–1665, 2011.

MALLICK, A. I. et al. Vaccination with CpG-adjuvanted avian influenza virosomes promotes antiviral immune responses and reduces virus shedding in chickens. **Viral Immunology**, v. 25, n. 3, p. 226–231, 2012.

MAPA (Ministério da Agricultura Pecuária e Abastecimento). **Regulamento técnico para produção, controle e emprego de vacinas autógenas**. Disponível em: <https://www.gov.br/agricultura/pt-br/acesso-a-informacao/participacao-social/consultas-publicas/documentos/Minutaautgenas.pdf>. Acesso em: 23 jan. 2023.

MARKOWSKA-DANIEL, I.; POMORSKA-MÓL, M.; PEJSAK, Z. The influence of age and maternal antibodies on the postvaccinal response against swine influenza viruses in pigs. **Veterinary Immunology and Immunopathology**, v. 142, n. 1–2, p. 81–86, 2011.

MASIC, A.; BABIUK, L. A.; ZHOU, Y. Reverse genetics-generated elastase-dependent swine influenza viruses are attenuated in pigs. **Journal of General Virology**, v. 90, n. 2, p. 375–385, 2009.

MATROSOVICH, M. et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. **Journal of Virology**, v. 74, n. 18, p. 8502–8512, 2000.

MEDINA, R. A.; GARCÍA-SASTRE, A. Influenza A viruses: new research developments. **Nature Reviews Microbiology**, v. 9, n. 8, p. 590–603, 2011.

MOHSEN, M. O. et al. Major findings and recent advances in virus–like particle (VLP)-based vaccines. **Seminars in Immunology**, v. 34, p. 123–132, 2017.

MORGAN, S. B. et al. Aerosol delivery of a candidate universal influenza vaccine reduces viral load in pigs challenged with pandemic H1N1 virus. **The Journal of Immunology**, v. 196, n. 12, p. 5014–5023, 2016.

NELLI, R. K. et al. Comparative distribution of human and avian type sialic acid influenza receptors in the pig. **BMC Veterinary Research**, v. 6, n. 4, 2010.

NELSON, M. I. et al. Introductions and evolution of human-origin seasonal influenza A viruses in multinational swine populations. **Journal of Virology**, v. 88, n. 17, p. 10110–10119, 2014.

NELSON, M. I. et al. Influenza A viruses of human origin in swine, Brazil. **Emerging Infectious Diseases**, v. 21, n. 8, p. 1339–1347, 2015a.

NELSON, M. I. et al. Global migration of influenza A viruses in swine. **Nature Communications**, v. 6, n. 6696, p. 1–11, 2015b.

NELSON, M. I. et al. Novel human-like influenza A Viruses circulate in swine in Mexico and Chile. **PLoS Currents**, v. 7, 2015c.

NELSON, M. I.; VINCENT, A. L. Reverse zoonosis of influenza to swine: new perspectives on the human-animal interface. **Trends in Microbiology**, v. 23, n. 3, p. 142–153, 2015.

NEUMANN, G.; KAWAOKA, Y. Host range restriction and pathogenicity in the context of influenza pandemic. **Emerging Infectious Diseases**, v. 12, n. 6, p. 881–886, 2006.

NEUMANN, G.; KAWAOKA, Y. Transmission of influenza A viruses. **Virology**, v. 479–480, p. 234–246, 2015.

NEURATH, A. R. et al. The effect of nonaqueous solvents on the quaternary structure of viruses: a procedure for the simultaneous concentration, purification and disruption of influenza viruses. **Microbios**, v. 4, n. 14, p. 145–150, 1971.

NICHOLLS, J. M. et al. Evolving complexities of influenza virus and its receptors. **Trends in Microbiology**, v. 16, n. 4, p. 149–157, 2008.

OUYANG, J. et al. NRAV, a long noncoding RNA, modulates antiviral responses through suppression of interferon-stimulated gene transcription. **Cell Host and Microbe**, v. 16, n. 5, p. 616–626, 2014.

PANTIN-JACKWOOD, M. J. Immunohistochemical staining of influenza virus in tissues. **Methods in Molecular Biology**, v. 2123, p. 29–36, 2020.

PARDI, N. et al. mRNA vaccines - a new era in vaccinology. **Nature Reviews Drug Discovery**, v. 17, n. 4, p. 261–279, 2018.

PARYS, A. et al. Alternating 3 different influenza vaccines for swine in Europe for a broader

antibody response and protection. **Veterinary Research**, v. 53, n. 1, p. 44, 2022.

PENA, L. et al. Strain-dependent effects of PB1-F2 of triple-reassortant H3N2 influenza viruses in swine. **Journal of General Virology**, v. 93, n. 10, p. 2204–2214, 2012.

PEREDA, A. et al. Evidence of reassortment of pandemic H1N1 influenza virus in swine in Argentina: are we facing the expansion of potential epicenters of influenza emergence? **Influenza and Other Respiratory Viruses**, v. 5, n. 6, p. 409–412, 2011.

PETSCH, B. et al. Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. **Nature Biotechnology**, v. 30, n. 12, p. 1210–1216, 2012.

PILLET, S. et al. A plant-derived quadrivalent virus like particle influenza vaccine induces cross-reactive antibody and T cell response in healthy adults. **Clinical Immunology**, v. 168, p. 72–87, 2016.

PIPKIN, M. E. et al. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. **Immunity**, v. 32, n. 1, p. 79–90, 2010.

PIZZOLLA, A. et al. Resident memory CD8+ T cells in the upper respiratory tract prevent pulmonary influenza virus infection. **Science Immunology**, v. 2, n. 12, p. eaam6970, 2017.

PYO, H. M. et al. Pandemic H1N1 influenza virus-like particles are immunogenic and provide protective immunity to pigs. **Vaccine**, v. 30, n. 7, p. 1297–1304, 2012.

QIU, Y.; DE HERT, K.; VAN REETH, K. Cross-protection against European swine influenza viruses in the context of infection immunity against the 2009 pandemic H1N1 virus: studies in the pig model of influenza. **Veterinary Research**, v. 46, p. 105, 2015.

RAJÃO, D. S. et al. Genetic characterization of influenza virus circulating in Brazilian pigs during 2009 and 2010 reveals a high prevalence of the pandemic H1N1 subtype. **Influenza and Other Respiratory Viruses**, v. 7, n. 5, p. 783–790, 2013a.

RAJÃO, D. S. et al. Diagnosis and clinic-pathological findings of influenza virus infection in Brazilian pigs. **Pesquisa Veterinária Brasileira**, v. 33, n. 1, p. 30–36, 2013b.

RAJÃO, D. S. et al. Pathogenesis and vaccination of influenza A virus in swine. **Current Topics in Microbiology and Immunology**, v. 385, p. 307–326, 2014.

RAJÃO, D. S. et al. Vaccine-associated enhanced respiratory disease is influenced by haemagglutinin and neuraminidase in whole inactivated influenza virus vaccines. **Journal of General Virology**, v. 97, n. 7, p. 1489–1499, 2016.

RAJÃO, D. S.; PÉREZ, D. R. Universal vaccines and vaccine platforms to protect against influenza viruses in humans and agriculture. **Frontiers in Microbiology**, v. 9, n. 123, p. 1–21, 2018.

RAJÃO, D. S.; VINCENT, A. L.; PEREZ, D. R. Adaptation of human influenza viruses to swine. **Frontiers in Veterinary Science**, v. 5, n. 347, p. 1–12, 2019.

RANGEL-MORENO, J. et al. B cells promote resistance to heterosubtypic strains of influenza via multiple mechanisms. **The Journal of Immunology**, v. 180, n. 1, p. 454–463, 2008.

RECH, R. R. et al. Porcine respiratory disease complex after the introduction of H1N1/2009 influenza virus in Brazil. **Zoonoses and Public Health**, v. 65, n. 1, p. e155–e161, 2018.

RICHT, J. A. et al. Pathogenic and antigenic properties of phylogenetically distinct reassortant H3N2 swine influenza viruses cocirculating in the United States. **Journal of Clinical Microbiology**, v. 41, n. 7, p. 3198–3205, 2003.

RICHT, J. A. et al. Real-time reverse transcription-polymerase chain reaction assays for the detection and differentiation of North American swine influenza viruses. **Journal of Veterinary Diagnostic Investigation**, v. 16, n. 5, p. 367–373, 2004.

ROMAGOSA, A. et al. Vaccination of influenza A virus decreases transmission rates in pigs. **Veterinary Research**, v. 42, n. 1, 2011.

SANDBULTE, M. R. et al. Optimal use of vaccines for control of influenza A virus in swine. **Vaccines**, v. 3, n. 1, p. 22–73, 2015.

SCHAEFER, R. et al. Isolation and characterization of a pandemic H1N1 influenza virus in pigs in Brazil. **Pesquisa Veterinária Brasileira**, v. 31, n. 9, 2011.

SCHAEFER, R. et al. Orientações para o diagnóstico de influenza em suínos. **Pesquisa Veterinária Brasileira**, v. 33, n. 1, p. 61–73, 2013.

SCHAEFER, R. et al. **Genetic and antigenic diversity of contemporary influenza A virus in swine in Brazil**. Proceedings of the International Pig Veterinary Society Congress. Rio de Janeiro: 2020.

SCHMIDT, C. et al. Swine influenza virus and association with the porcine respiratory disease complex in pig farms in Southern Brazil. **Zoonoses and Public Health**, v. 63, n. 3, p. 234–240, 2016.

SCHMIDT, M. E.; VARGA, S. M. The CD8 T cell response to respiratory virus infections. **Frontiers in Immunology**, v. 9, n. 678, 2018.

SHARMA, A. et al. Detection of live attenuated influenza vaccine virus and evidence of reassortment in the U.S. swine population. **Journal of Veterinary Diagnostic Investigation**, v. 32, n. 2, p. 301–311, 2020.

SHAW, M. L.; PALESE, P. Orthomyxoviridae. In: KNIPE, D. M.; HOWLEY, P. M. (Eds.). **Fields Virology**. 6th. ed. Philadelphia: Lippincott Williams and Wilkins, 2013. p. 1151–1185.

SHORT, K. R. et al. One health, multiple challenges: the inter-species transmission of influenza A virus. **One Health**, v. 1, p. 1–13, 2015.

SMITH, G. J. D. et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. **Nature**, v. 459, n. 7250, p. 1122–1125, 2009.

SOEMA, P. C. et al. Current and next generation influenza vaccines: formulation and production strategies. **European Journal of Pharmaceutics and Biopharmaceutics**, v. 94, p. 251–263, 2015a.

SOEMA, P. C. et al. Influenza T-cell epitope-loaded virosomes adjuvanted with CpG as a potential influenza vaccine. **Pharmaceutical Research**, v. 32, n. 4, p. 1505–1515, 2015b.

SOLÓRZANO, A. et al. Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. **Journal of Virology**, v. 79, n. 12, p. 7535–7543, 2005.

SOUZA, C. K. et al. The type of adjuvant in whole inactivated influenza A virus vaccines impacts vaccine-associated enhanced respiratory disease. **Vaccine**, v. 36, n. 41, p. 6103–6110, 2018.

SRETA, D. et al. Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial. **Virology Journal**, v. 6, n. 34, 2009.

SRIDHAR, S. Heterosubtypic T-cell immunity to influenza in humans: challenges for universal T-cell influenza vaccines. **Frontiers in Immunology**, v. 7, n. 195, 2016.

STEEL, J. et al. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. **mBio**, v. 1, n. 1, p. e00018-10, 2010.

SUN, J. et al. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. **Nature Medicine**, v. 15, n. 3, p. 277–284, 2009.

SWAIN, S. L.; MCKINSTRY, K. K.; STRUTT, T. M. Expanding roles for CD4+ T cells in immunity to viruses. **Nature Reviews Immunology**, v. 12, n. 2, p. 136–148, 2012.

TANG, X. et al. Recombinant adenoviruses displaying matrix 2 ectodomain epitopes on their fiber proteins as universal influenza vaccines. **Journal of Virology**, v. 91, n. 7, p. e02462-16, 2017.

THACKER, E. L.; THACKER, B. J.; JANKE, B. H. Interaction between Mycoplasma hyopneumoniae and swine influenza virus. **Journal of Clinical Microbiology**, v. 39, n. 7, p. 2525–2530, 2001.

TOCHETTO, C. et al. Introductions of human-origin seasonal H3N2, H1N2 and pre-2009 H1N1 influenza viruses to swine in Brazil. **Viruses**, v. 15, n. 2, p. 576, 2023.

TONG, S. et al. A distinct lineage of influenza A virus from bats. **Proceedings of the National Academy of Sciences of the United States of America**, v. 109, n. 11, p. 4269–4274, 2012.

TONG, S. et al. New world bats harbor diverse influenza A viruses. **PLoS Pathogens**, v. 9, n. 10, 2013.

TORREMORELL, M. et al. Procedures to eliminate H3N2 swine influenza virus from a pig herd. **Veterinary Record**, v. 165, n. 3, p. 74–77, 2009.

TORREMORELL, M. et al. Transmission of influenza A virus in pigs. **Transboundary and Emerging Diseases**, v. 59, n. Suppl. 1, p. 68–84, 2012.

TRILLA, A.; TRILLA, G.; DAER, C. The 1918 "Spanish flu" in Spain. **Clinical Infectious Diseases**, v. 47, n. 5, p. 668–673, 2008.

TUMPEY, T. M. et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. **Science**, v. 310, n. 5745, p. 77–80, 2005a.

TUMPEY, T. M. et al. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. **Journal of Virology**, v. 79, n. 23, p. 14933–14944, 2005b.

TUTYKHINA, I. L. et al. Development of adenoviral vector-based mucosal vaccine against influenza. **Journal of Molecular Medicine**, v. 89, p. 331–341, 2011.

VALERO-PACHECO, N. et al. Antibody persistence in adults two years after vaccination with an H1N1 2009 pandemic influenza virus-like particle vaccine. **PLoS ONE**, v. 11, n. 2, p. e0150146, 2016.

VAN BRAECKEL-BUDIMIR, N.; HARTY, J. T. Influenza-induced lung Trm: not all memories last forever. **Immunology and Cell Biology**, v. 95, n. 8, p. 651–655, 2017.

VAN DE SANDT, C. E.; KREIJTZ, J. H. C. M.; RIMMELZWAAN, G. F. Evasion of influenza A viruses from innate and adaptive immune responses. **Viruses**, v. 4, n. 9, p. 1438–1476, 2012.

VAN DOMSELAAR, R.; BOVENSCHEN, N. Cell death-independent functions of granzymes: hit viruses where it hurts. **Reviews in Medical Virology**, v. 21, n. 5, p. 301–314, 2011.

VAN GISBERGEN, K. P. J. M. et al. The costimulatory molecule CD27 maintains clonally diverse CD8+ T cell responses of low antigen affinity to protect against viral variants. **Immunity**, v. 35, n. 1, p. 97–108, 2011.

VAN POUCKE, S. G. M. et al. Replication of avian, human and swine influenza viruses in porcine respiratory explants and association with sialic acid distribution. **Virology Journal**, v. 7, n. 38, 2010.

VAN REETH, K. et al. Protection against a European H1N2 swine influenza virus in pigs previously infected with H1N1 and/or H3N2 subtypes. **Vaccine**, v. 21, n. 13–14, p. 1375–1381, 2003.

VAN REETH, K.; LABARQUE, G.; PENSAERT, M. Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. **Viral Immunology**, v. 19, n. 3, p. 373–382, 2006.

VAN REETH, K.; MA, W. Swine influenza virus vaccines: to change or not to change - that's the question. **Current Topics in Microbiology and Immunology**, v. 370, p. 173–200, 2013.

VAN REETH, K.; VAN GUCHT, S.; PENSAERT, M. Investigations of the efficacy of European H1N1- and H3N2-based swine influenza vaccines against the novel H1N2 subtype. **The Veterinary Record**, v. 153, n. 1, p. 9–13, 2003.

VAN REETH, K.; VINCENT, A. L. Influenza viruses. In: ZIMMERMAN, J. J. et al. (Eds.). **Diseases of Swine**. 11th. ed. Hoboken: Wiley-Blackwell, 2019. p. 576–593.

VAN REETH, K.; VINCENT, A. L.; LAGER, K. M. Vaccines and vaccination for swine influenza: differing situations in Europe and the USA. In: SWAYNE, D. E. (Ed.). **Animal Influenza**. 2nd. ed. Hoboken: John Wiley & Sons, 2016. p. 480–501.

VANDER VEEN, R. L. et al. Haemagglutinin and nucleoprotein replicon particle vaccination of swine protects against the pandemic H1N1 2009 virus. **Veterinary Record**, v. 173, n. 14, p. 344, 2013.

VANDER VEEN, R. L.; HARRIS, D. L. H.; KAMRUD, K. I. Alphavirus replicon vaccines. **Animal Health Research Reviews**, v. 13, n. 1, p. 1–9, 2012.

VAZQUEZ-PAGAN, A.; SCHULTZ-CHERRY, S. Serological responses to influenza vaccination during pregnancy. **Microorganisms**, v. 9, n. 11, p. 2305, 2021.

VINCENT, A. L. et al. Evaluation of hemagglutinin subtype 1 swine influenza viruses from the United States. **Veterinary Microbiology**, v. 118, n. 3–4, p. 212–222, 2006.

VINCENT, A. L. et al. Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. **Vaccine**, v. 25, n. 47, p. 7999–8009, 2007.

VINCENT, A. L. et al. Swine influenza viruses: a North American perspective. **Advances in Virus Research**, v. 72, p. 127–154, 2008a.

VINCENT, A. L. et al. Failure of protection and enhanced pneumonia with a US H1N2 swine influenza virus in pigs vaccinated with an inactivated classical swine H1N1 vaccine. **Veterinary Microbiology**, v. 126, n. 4, p. 310–323, 2008b.

VINCENT, A. L. et al. Characterization of a newly emerged genetic cluster of H1N1 and H1N2 swine influenza virus in the United States. **Virus Genes**, v. 39, n. 2, p. 176–185, 2009.

VINCENT, A. L. et al. Experimental inoculation of pigs with pandemic H1N1 2009 virus and HI cross-reactivity with contemporary swine influenza virus antisera. **Influenza and Other Respiratory Viruses**, v. 4, n. 2, p. 53–60, 2010a.

VINCENT, A. L. et al. Efficacy of inactivated swine influenza virus vaccines against the 2009 A/H1N1 influenza virus in pigs. **Vaccine**, v. 28, n. 15, p. 2782–2787, 2010b.

VINCENT, A. L. et al. Review of influenza A virus in swine worldwide: a call for increased surveillance and research. **Zoonoses and Public Health**, v. 61, n. 1, p. 4–17, 2014.

VINCENT, A. L. et al. Influenza A virus vaccines for swine. **Veterinary Microbiology**, v. 206, p. 35–44, 2017.

VINCENT, A. L.; ANDERSON, T. K.; LAGER, K. M. A brief introduction to influenza A virus in swine. **Methods in Molecular Biology**, v. 2123, p. 249–271, 2020.

VOSKOBOINIK, I.; WHISSTOCK, J. C.; TRAPANI, J. A. Perforin and granzymes: function, dysfunction and human pathology. **Nature Reviews Immunology**, v. 15, n. 6, p. 388–400, 2015.

WAFFARN, E. E.; BAUMGARTH, N. Protective B cell responses to flu—no fluke! **The Journal of Immunology**, v. 186, n. 7, p. 3823–3829, 2011.

WANG, J. et al. Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-λ1) in response to influenza A infection. **The Journal of Immunology**, v. 182, n. 3, p. 1296–1304, 2009.

WANG, S. et al. Transport of influenza virus neuraminidase (NA) to host cell surface is regulated by ARHGAP21 and Cdc42 proteins. **Journal of Biological Chemistry**, v. 287, n. 13, p. 9804–9816, 2012.

WANG, X. et al. Computational approach for predicting the conserved B-cell epitopes of hemagglutinin H7 subtype influenza virus. **Experimental and Therapeutic Medicine**, v. 12, n. 4, p. 2439–2446, 2016.

WATANABE, T. T. N. et al. Histopathological and immunohistochemical findings of swine with spontaneous influenza a infection in Brazil, 2009-2010. **Pesquisa Veterinária Brasileira**, v. 32, n. 11, p. 1148–1154, 2012.

WEBSTER, R. G. et al. Evolution and ecology of influenza A viruses. **Microbiological Reviews**, v. 56, n. 1, p. 152–79, 1992.

WESLEY, R. D.; TANG, M.; LAGER, K. M. Protection of weaned pigs by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of H3N2 swine influenza virus. **Vaccine**, v. 22, n. 25–26, p. 3427–3434, 2004.

WHITE, L. A.; TORREMORELL, M.; CRAFT, M. E. Influenza A virus in swine breeding herds: combination of vaccination and biosecurity practices can reduce likelihood of endemic piglet reservoir. **Preventive Veterinary Medicine**, v. 138, p. 55–69, 2017.

WHITMIRE, J. K.; TAN, J. T.; WHITTON, J. L. Interferon-γ acts directly on CD8+ T cells to increase their abundance during virus infection. **The Journal of Experimental Medicine**, v. 201, n. 7, p. 1053–1059, 2005.

WILSCHUT, J. Influenza vaccines: the virosome concept. **Immunology Letters**, v. 122, n. 2, p. 118–121, 2009.

WOHLBOLD, T. J. et al. Vaccination with adjuvanted recombinant neuraminidase induces broad heterologous, but not heterosubtypic, cross-protection against influenza virus infection in mice. **mBio**, v. 6, n. 2, p. e02556, 2015.

WU, F. et al. Characterization of immunity induced by M2e of influenza virus. **Vaccine**, v. 25, n. 52, p. 8868–8873, 2007.

WU, T. et al. Lung-resident memory CD8 T cells (TRM) are indispensable for optimal cross-protection against pulmonary virus infection. **Journal of Leukocyte Biology**, v. 95, n. 2, p. 215–224, 2014a.

WU, Y. et al. Bat-derived influenza-like viruses H17N10 and H18N11. **Trends in Microbiology**, v. 22, n. 4, p. 183–191, 2014b.

YAMAYOSHI, S.; KAWAOKA, Y. Current and future influenza vaccines. **Nature Medicine**, v. 25, n. 2, p. 212–220, 2019.

ZENS, K. D.; CHEN, J. K.; FARBER, D. L. Vaccine-generated lung tissue–resident memory T cells provide heterosubtypic protection to influenza infection. **JCI Insight**, v. 1, n. 10, p. e85832, 2016.

ZHANG, C. et al. Advances in mRNA vaccines for infectious diseases. **Frontiers in Immunology**, v. 10, n. 594, 2019.

ZHANG, H. et al. Viral and host factors required for avian H5N1 influenza A virus replication in mammalian cells. **Viruses**, v. 5, n. 6, p. 1431–1446, 2013.

ZHANG, J.; GAUGER, P. C. Isolation of swine influenza A virus in cell cultures and embryonated chicken eggs. **Methods in Molecular Biology**, v. 2123, p. 281–294, 2020.

ZHANG, J.; GAUGER, P.; HARMON, K. Swine Influenza A Virus. In: LIU, D. (Ed.). **Molecular Detection of Animal Viral Pathogens**. Boca Raton: CRC Press, 2016. p. 399–406.

ZHANG, J.; HARMON, K. M. RNA extraction from swine samples and detection of influenza A virus in swine by real-time RT-PCR. **Methods in Molecular Biology**, v. 2123, p. 295–310, 2020.

ZHU, J.; YAMANE, H.; PAUL, W. E. Differentiation of effector CD4 T cell populations. **Annual Review of Immunology**, v. 28, p. 445–489, 2010.

ZUCKERMANN, F. A. Extrathymic CD4/CD8 double positive T cells. **Veterinary Immunology and Immunopathology**, v. 72, n. 1–2, p. 55–66, 1999.

ZURBRIGGEN, R. Immunostimulating reconstituted influenza virosomes. **Vaccine**, v. 21, n. 9–10, p. 921–924, 2003.

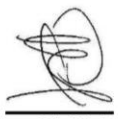
ANEXO A – Aprovação da Comissão de Ética no Uso de Animais (CEUA)

Embrapa Suínos e Aves	Certificado*	**ETICA** **1/1**

**Em concordância com a Orientação Técnica CONCEA no 8, de 18 de março de 2016 (Anexo I)*

Certificamos que a proposta intitulada **"Diversidade genética e antigênica dos vírus influenza A e eficácia de métodos de diagnóstico e vacina nanotecnológica para o controle da influenza em suínos"**, registrada com o nº **001/2017**, sob a responsabilidade de **Rejane Schaefer** – que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DO(A) Embrapa Suínos e Aves, em reunião de **07/07/2017**.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da Autorização	14/07/2017 – 01/06/2021
Espécie/linhagem/raça	*Sus domesticus* (Suíno)
Nº de animais	58
Peso/Idade	6-7kg/ 28 e 35 dias
Sexo	Machos
Origem	Granja Embrapa Suínos e Aves



Presidente CEUA/CNPSA