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Double-attenuated influenza virus elicits broad protection against challenge viruses with different serotypes in swine

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ABSTRACT

Influenza A viruses (IAV) have caused seasonal epidemics and severe pandemics in humans. Novel pandemic strains as in 2009 may emerge from pigs, serving as perpetual virus reservoir. However, reliably effective vaccination has remained a key issue for humans and swine. Here, we generated a novel double-attenuated influenza live vaccine by reverse genetics and subjected immunized mice and pigs to infection with the homologous wild-type, another homosubtypic H1N1, or a heterosubtypic H3N2 virus to address realistic challenge constellations. This attenuated mutant contains an artificial, strictly elastase-dependent hemagglutinin cleavage site and a C-terminally truncated NS1 protein from the IAV A/Bayern/74/2009 ($H1N1_{pdm09}$). Prior to challenge, we immunized mice once and pigs twice intranasally. *In vitro*, the double-attenuated mutant replicated strictly elastase-dependently. Immunized mice and pigs developed neither clinical symptoms nor detectable virus replication after homologous challenge. In pigs, we observed considerably reduced clinical signs and no nasal virus shedding after homosubtypic and reduced viral loads in respiratory tracts after heterosubtypic infection. Protection against homosubtypic challenge suggests that an optimized backbone strain may require less frequent updates with recent HA and NA genes and still induce robust protection in relevant IAV hosts against drifted viruses.

1. Introduction

Influenza A viruses (IAV) cause seasonal epidemics worldwide. Due to antigenic drift, IAV display a broad antigenic variability. Besides seasonal strains, novel pandemic viruses may emerge as in the 2009 H1N1 pandemic. The pig as an intermediate animal host, which is susceptible to both avian and human IAV (Nelson et al., 2012), plays a key role as “mixing vessel” for gene reassortment and spill-over transmissions of novel influenza strains to humans (Brockwell-Staats et al., 2009). Moreover, swine influenza viruses continuously lead to major economic losses in livestock (Brown, 2000). Although inactivated vaccines for humans and swine are available, they often provide insufficient protection due to vaccine mismatching and lack of cross-protection (Schotsaert and Garcia-Sastre, 2017). To reduce disease burden in swine populations and clear target reservoirs preventing emergence of novel zoonotic strains (Olsen, 2002; Van Reeth, 2007), live-attenuated influenza A vaccines (LAIV) are a promising alternative due to elicited broad humoral and cellular immune responses (Jang and Seong, 2013; Schotsaert and Garcia-Sastre, 2017). However, LAIV may bear safety problems because of possible reversion or reassortment with circulating viruses (Rahn et al., 2015).

Previously, we generated influenza A and B mutants carrying an elastase-sensitive hemagglutinin (HA) cleavage site (HACS) motif, which are highly attenuated in mice and offer full protection against lethal challenge with wild-type or heterosubtypic viruses (Stech et al., 2011, 2005). For viral replication, cleavage of the HA precursor HA0 into the HA1 and HA2 fragments is essential and requires host proteases (Bottcher-Friebertshauser et al., 2013). These HACS mutants, however, are dependent on elastase, which is not sufficiently accessible in the respiratory tract, restricting viral replication *in vivo* (Stech et al., 2011, 2005). Follow-up studies of such elastase-dependent mutants in swine confirmed attenuation and efficient protection (Babiuk et al., 2011).

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Another vaccine approach targets the viral non-structural protein 1 (NS1), a multifunctional protein and virulence factor (Hale et al., 2008). Mutants carrying a C-terminally truncated NS1, which are highly attenuated in mice and swine, confer solid protection against challenge (Kappes et al., 2012; Solorzano et al., 2005; Talon et al., 2000). For increased safety against reversion and reassortment of the HA gene, we generated a highly attenuated double-mutant of A/Bayern/74/2009 ($H1N1_{pdm09}$) carrying an elastase-dependent HACS and a C-terminally truncated NS1 protein and investigated it in mice and swine. To address realistic challenge scenarios, we infected the immunized pigs with the homologous wild-type, another homosubtypic H1N1, or a heterosubtypic H3N2 virus.

2. Materials and methods

2.1. Cells and recombinant viruses

Madin-Darby canine kidney cells (MDCK II), porcine kidney cells (PK-15) and human embryonic kidney cells (293 T) were cultivated on minimal essential medium (MEM) containing 10% fetal calf serum (FCS).

We cloned all eight genes of A/Bayern/74/2009 ($H1N1_{pdm09}$) (By09), GISAID (GISAID, 2019) accession numbers EPI1086362-9, by target-primed plasmid amplification (Stech et al., 2008). For generation of an elastase-dependent HACS, we replaced isoleucine, glutamine, serine and arginine at positions four to one upstream to the cleavage site (P4-P1) by four alanines by Quikchange® mutagenesis. For truncation of the NS1 C-terminus after amino acid 99, we removed 141 nucleotides from position 297 and inserted four stop codons in three reading frames. For rescue (Hoffmann et al., 2000) of By09-Ela/NS1-99, we included an NS1 expression plasmid (pcDNA3.0 NS1). Eventually, we cloned all genes of A/Swine/Belzig/02/2001 (H1N1) (SwBel01) and A/Swine/Bissendorf/IDT1864/2003 (H3N2) (SwBiss03) by target-primed plasmid amplification (Stech et al., 2008). After rescues, we excluded unwanted mutations by Sanger sequencing.

By09 and SwBel01 were propagated on MDCK II cells, SwBiss03 on PK-15 cells in serum-free MEM containing 0.2% bovine serum albumin, 1 Unit/ml Penicillin, 1 µg/ml Streptomycin, and 2 µg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (Sigma Aldrich). For propagation of By09-Ela/NS1-99 in MDCK II cells, we added 5 µg/ml porcine pancreatic elastase (Serva). For $TCID_{50}$ assay, we prepared serial tenfold dilutions in infection medium (starting at dilution 1:10) supplemented with 2 µg/ml TPCK-treated trypsin or 5 µg/ml elastase. Dilutions were added to MDCK II cells on 96-well tissue culture plates and incubated 3 days at 37 °C and 5% CO_2. Each well was monitored for cytopathic effect and viral titers were calculated according to Spearman-Kärber (Mahy and Kangro, 1996).

2.2. Virus passaging monitored by immunofluorescence of expressed NP

A 6-well plate of confluent MDCK-II cells was infected with By09-Ela/NS1-99 in phosphate buffered saline (PBS) (Multiplicity of infection (MOI) 0.5, corresponding to 6×10^5 $TCID_{50}$) for one hour at 37 °C and 5% CO_2. After two washing steps with PBS, 3 ml infection medium supplemented with either 2 µg/ml TPCK-treated trypsin, 5 µg/ml elastase, or without protease was added to separate wells in duplicate. After 12 h, we added 1 ml supernatant to another 6-well plate of confluent MDCK-II cells. After one hour, we washed the cells with PBS and added infection medium with the same protease. For immunofluorescence, we fixated cells with 3.7% formaldehyde and permeabilized them with 0.5% Triton X-100 in PBS. Nonspecific binding sites were blocked with 1% skimmed milk in PBS. Subsequently, plates were incubated with rabbit antisera against Influenza virus NP (Gene Tax) 1:1000 diluted in 1% skimmed milk one hour at room temperature and then with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Invitrogen) (1:1000) in 1% skimmed milk for 45 min at room temperature. After each step, cells were washed with PBS. Samples were evaluated by a fluorescence microscope (Leica DMi8 automated) for green fluorescence excited at 488 nm.

2.3. Mice and pigs

Four-week old, female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) were housed in individually ventilated cages under BSL3 conditions. Mice were anesthetized intraperitoneally with Xylazine (Rompun®, Bayer) and intranasally infected with 50 µl of diluted virus or PBS. Over a period of 14 days, we observed each mouse daily regarding body weight or death. By $TCID_{50}$ assay, entire organ homogenates of lungs, hearts, and brains were titrated.

Thirty-nine four-week old German landrace pigs were obtained from a commercial high health status herd in Germany (BHZP Garlitz, Langenheide, Germany) in which no vaccines against swine influenza A virus are administered routinely. Prior to immunization, all pigs were tested negative for acute influenza virus infection by matrix gene quantitative RealTime-PCR (AgPath.ID™ One-Step RT-PCR Kit, Applied Biosystems) on nasal swabs (modified from (Spackman et al., 2002)). Moreover, we tested sera for IAV antibodies by a competitive ELISA (ID-Vet) and hemagglutinin inhibition assay (HI) with 3 different viruses as antigen: By09 ($H1N1_{pdm09}$), SwBelz01 (H1N1) and SwBiss03 (H3N2). Pigs were immunized at six weeks of age and challenged six weeks later intranasally by a Mucosal Atomization Device (Prosys International Ltd) (Hemmink et al., 2016).

2.4. Serology

Sera (25 µl) were pretreated with cholera filtrate (Sigma-Aldrich) in calcium salt solution (100 µl) for 16 h at 37 °C and then added to sodium citrate solution (125 µl). Afterwards those samples were heat-inactivated at 56 °C for 30 min. We added 100 µl 1% chicken erythrocytes to 250 µl of the diluted sera, incubated the samples for 30 min and centrifuged them at 14.000 rpm for 5 s. For HI assay, supernatants were serially diluted two-fold (starting at 1:2) on 96-well plates in PBS. Afterwards, we added 4 hemagglutinating units of the virus to each well and incubated plates 45 min. Eventually, 1% chicken erythrocytes were added and the plates were incubated for 30 min to read out the HI titers. Pig sera (10 µl) were tested for IAV nucleoprotein-specific antibodies by the competitive ID Screen ELISA (ID-Vet).

2.5. Gross pathology and histopathology

We euthanized two pigs per group on day 4 after challenge to examine the entire respiratory tract macroscopically. Additionally, we took samples from nasal cavity, pharynx, trachea, *lymphonodi tracheobronchiales*, and 7 standardized locations within the lung for histopathological examination by HE staining and assessment of virus topography by immunohistochemistry. Virus antigen detection *in situ* was performed with the avidin–biotin–peroxidase complex method (Vectastain PK 6100, Vector, Burlingame, CA, USA) using a polyclonal rabbit IAV (A FPV/Rostock/34) nucleoprotein antiserum (dilution 1:750) and a secondary biotinylated goat anti-rabbit IgG1 (Vector, Burlingame, CA, USA) antibody (dilution 1:200) with 3-amino-9-ethylcarbazol as chromogen and hematoxylin counterstain.

2.6. Flow cytometry

We prepared single cell suspensions from spleen and the right tracheobronchial lymph node from pigs using 100 µm cell strainers and isolated leukocytes from blood by density gradient centrifugation using Pancoll (Pan Biotech) for cryo preservation. Immune cell subsets in organ single cell suspension as well as whole blood were identified using these fluorescent dye-labelled antibodies: mouse anti-pig CD4 PerCp-Cy5.5 (clone 74-12-4), rat anti-human CD197 AlexaFluor 647

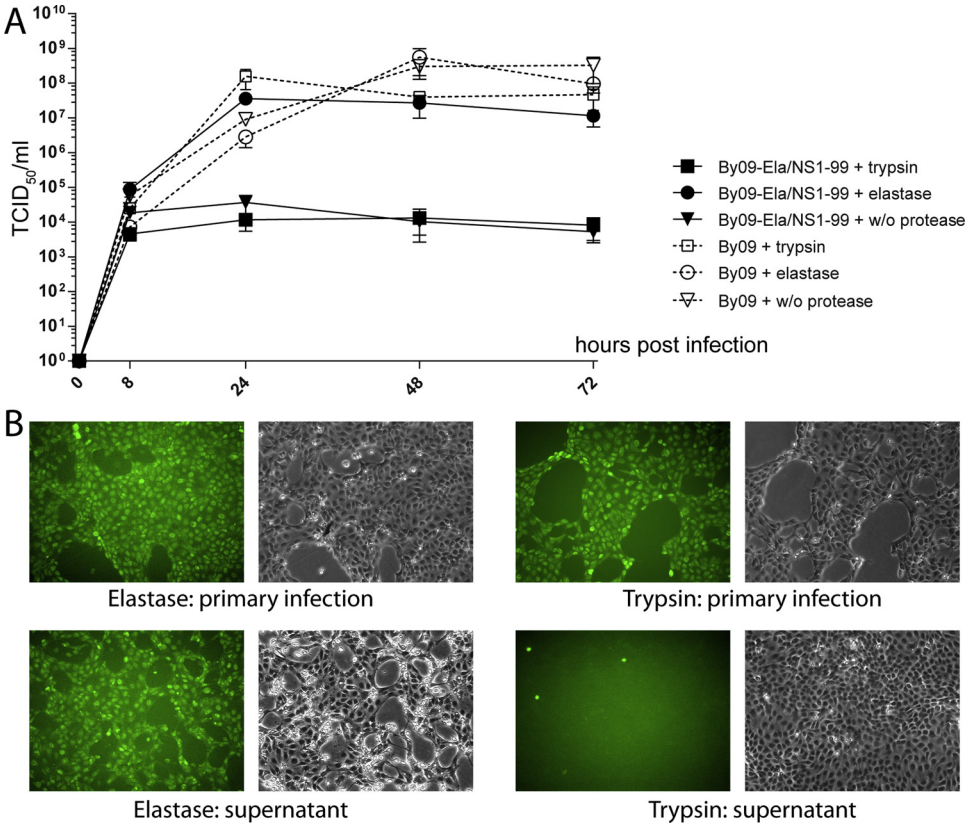


Fig. 1. Strict elastase dependency of By09-Ela/NS1-99. (A) Growth curves of By09-Ela/NS1-99 (black graphs, black symbols) and By09 (dotted graphs, hollow symbols) in the absence of an exogenous protease or in the presence of either trypsin or elastase. Per each time point, means with standard deviation were determined from two independently infected cell cultures, each titrated in duplicate (Downer detection limit: dilution factor 1:10). (B) Immunofluorescence and bright field images of MDCK II cells 20 h after infection with 0.5 MOI By09-Ela/NS1-99 in the presence of either elastase or trypsin. After primary infection, supernatants were used to infect fresh MDCK II cells in the presence of the corresponding protease.

(clone 3D12, both BD Biosciences), goat anti-pig CD45RA FITC (clone MIL13, Bio-Rad), mouse anti-pig CD8α PE (clone 74-2-11), and mouse anti-pig CD27 (IgG1, clone b30c7, both in-house); the latter stained with secondary rat anti-mouse IgG1 BrilliantViolet421 (clone RMG1-1, Biolegend). All analyses were run on BD Canto II flow cytometer, FACS DIVA (BD Bioscience) and FlowJo software (Tree Star Inc.). From singlets, all leukocytes were gated and CD4+/CD8α- leukocytes are termed T-helper cells. The term CD8+ effector memory cells denotes CD8α+/CD4-/CD45RA-/CD27-/CCR7- cells from leukocytes.

3. Results

3.1. By09-Ela/NS1-99 is strictly elastase-dependent

By reverse genetics and site-directed mutagenesis, we obtained the double-attenuated mutant By09-Ela/NS1-99 carrying an artificial elastase dependent HACS motif and a C-terminally truncated NS1 protein. To determine growth curves, we infected MDCK II cells with By09-Ela/NS1-99 or By09 in the presence of trypsin or elastase, or in the absence of any added protease (Fig. 1A). In the presence of elastase, the mutant reached the wild-type titer (1.6×10^8 $TCID_{50}$/ml) within one magnitude after 24 h. However, in the presence of trypsin or absence of any protease, the mutant stagnated after 8 h at low titers of 10^3 to 10^4 $TCID_{50}$/ml, indicating single-cycle replication.

To demonstrate strict elastase-dependency of By09-Ela/NS1-99 for multicycle replication, we performed two subsequent passages on MDCKII cells in presence of elastase, trypsin or no protease. By09-Ela/NS1-99 was able to infect MDCK II cells in the presence of trypsin but the subsequent passage of supernatant eventually resulted in no visible infection (Fig. 1B). Correspondingly, the titers of elastase-dependent virus after the second passage in the presence of trypsin or no exogenous protease were very low: 1.4×10^3 $TCID_{50}$/ml and 1.6×10^3 $TCID_{50}$/ml, respectively (Table 1). Those resulting levels lie far below the inocula for the second passage being in the range of 10^5 $TCID_{50}$, indicating the absence of further virus replication. Correspondingly, no virus in supernatants from first and second passage with trypsin or without exogenous protease could be titrated in the presence of trypsin. Therefore, only in the permanent presence of elastase, By09-Ela/NS1-99 is able to sustain multicycle replication (Fig. 1B). Taken together, By09-Ela/NS1-99 is strictly elastase-dependent *in vitro*.

Table 1
Titration of By09-Ela/NS1-99 after infection in the presence of elastase, trypsin, or no protease.

Passage	Elastase	Trypsin	No Protease
#1	7.2×10^7	1.1×10^6	8.9×10^5
#2	4.7×10^7	1.4×10^3	1.6×10^3

Titers ($TCID_{50}$/ml) were determined from two independently infected cell cultures in the presence of elastase, each titrated in duplicate (Downer detection limit: dilution factor 1:10).

3.2. By09-Ela/NS1-99 is fully attenuated in mice and induces effective protection

To investigate the attenuation of By09-Ela/NS1-99 *in vivo*, we intranasally infected mice with 10^6 $TCID_{50}$ of By09-Ela/NS1-99, By09 (each n = 5) or PBS (n = 3). In contrast to the wild-type, By09-Ela/NS1-99 did not cause weight loss (Fig. 2A) or any other signs of disease. To assess the viral loads in organs, we sacrificed two mice 12, 24, or 72 h post infection and removed lungs, hearts and brains for virus titration. Whereas By09 displayed increasing virus loads in the lung, By09-Ela/NS1-99 titers stagnated and declined considerably at 72 h (Fig. 2B), suggesting clearance of the inoculum. To investigate the protection induced by By09-Ela/NS1-99, we intranasally immunized animals with 10^6, 10^5, 10^4 or 10^3 $TCID_{50}$ By09-Ela/NS1-99, or PBS (n = 4, n = 3, n = 2, n = 2, n = 3, respectively) and challenged them 3 weeks later with 10^6 $TCID_{50}$ of By09. In stark contrast to the PBS-

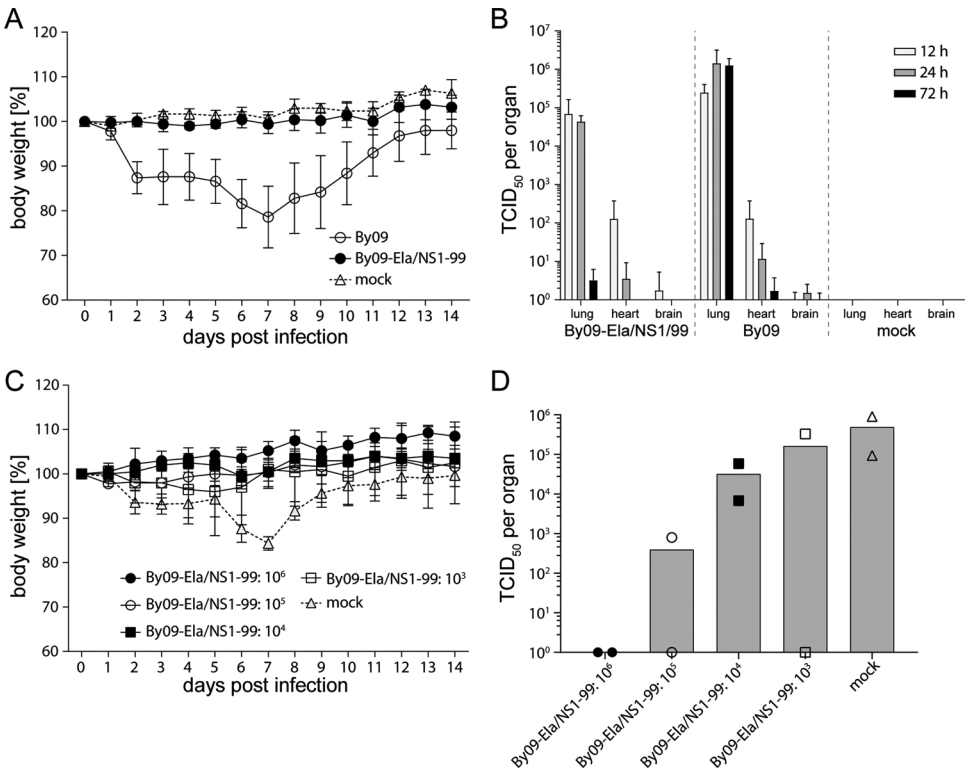


Fig. 2. Attenuation and elicited protection of By09-Ela/NS1-99 in mice. (A) Weight from mice inoculated intranasally with 10^6 $TCID_{50}$ of either By09, By09-Ela/NS1-99, or PBS. (B) $TCID_{50}$ titers from lungs, hearts, and brains, taken 12, 24 or 72 h after intranasal inoculation with 10^6 $TCID_{50}$ of either By09-Ela/NS1-99, By09, or PBS. (C) Average weight loss of mice challenged with 10^6 $TCID_{50}$ of By09 three weeks after intranasal immunization with 10^6, 10^5, 10^4 or 10^3 $TCID_{50}$ By09-Ela/NS1-99, or PBS. (D) Virus titers of lungs from two mice each group at day 3 after challenge. Downer detection limit: dilution factor 1:10.

inoculated control animals displaying considerable weight losses, all vaccinated mice developed no or less than 5% weight loss (Fig. 2C). On day 3 post challenge, we sacrificed two additional mice for each immunization dose and removed the lungs for virus titration. In contrast to all other groups, the highest immunization dosage of 10^6 $TCID_{50}$ By09-Ela/NS1-99 resulted in prohibition of challenge virus replication (Fig. 2D). Taken together, the vaccine candidate By09-Ela/NS1-99 elicits strong protection in mice.

3.3. Boost immunization of pigs with By09-Ela/NS1-99 offers protection against homologous challenge and elicits decreased clinical signs after homosubtypic challenge

To determine protection elicited by By09-Ela/NS1-99 in pigs, we performed a challenge study with three immunized groups (each n = 7) and three mock groups (n = 6, n = 6, and n = 5, Supplementary Table 1). Thirty-three animals were tested antibody-negative whereas five animals were found seropositive (Supplementary Tables 1 and 2), most likely due to maternally derived antibodies. One of these five seropositive animals was added to each of five of the six experimental groups (Supplementary Table 1).

We immunized 21 pigs with 1.6×10^6 $TCID_{50}$ of By09-Ela/NS1-99 in 4 ml inoculation volume and mock-immunized 17 pigs with PBS. After the immunization, three of 21 pigs developed short fever (≥ 40 °C) on days three and four but without other clinical signs. We could not detect any infectious virus in nasal swabs from day one to seven. Three weeks after vaccination, the immunized animals received a booster immunization (control groups received PBS again) without further clinical signs or viral shedding. Another three weeks later, we challenged both vaccinated and mock-immunized animals with 10^6 $TCID_{50}$ of the homologous wild-type By09 ($H1N1_{pdm09}$), 10^6 $TCID_{50}$ of homosubtypic SwBel01 (H1N1), or 4×10^5 $TCID_{50}$ of heterosubtypic SwBiss03 (H3N2). After homologous challenge, none of the immunized animals developed any clinical signs, while the respective control group showed mild fatigue on day three to five post challenge (pc) (Fig. 3). Additionally, we detected no nasal viral shedding in five pigs of the

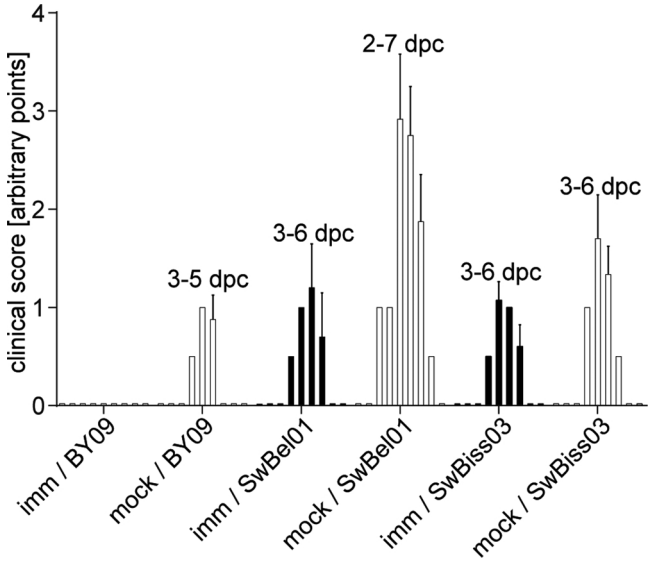


Fig. 3. Clinical scores after challenge in pigs. Homologous challenge: By09 ($H1N1_{pdm09}$), homosubtypic challenge: SwBel01 (H1N1) and heterosubtypic challenge: SwBiss03 (H3N2). Total score for one individual is added according to the severity of symptoms in each of these categories: general status, respiration, nasal discharge, or conjunctivitis as 0 none, 1 light, 2 medium, 3 severe. Mean daily group scores with standard deviation of immunized groups (imm) and mock-immunized groups (mock) are represented by black or empty bars, respectively, from days post challenge (dpc) 0 to 8. Labels above bars describe duration of symptoms in dpc.

immunized group (Fig. 4A) except for one animal (#7), which was IAV antibody-positive prior to immunization (Supplementary Fig. 1 and Supplementary Table 2); another animal was euthanized because of an unrelated injury (Supplementary Table 1). By contrast, we found nasally shed virus in all mock-immunized animals (Fig. 4A), being statistically significant (Table 2). After homosubtypic challenge, we

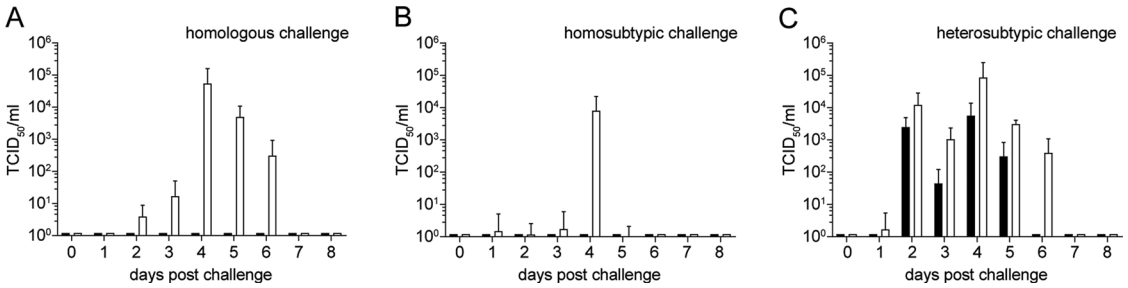


Fig. 4. Nasal virus shedding of immunized pigs is inhibited after homologous and homosubtypic challenge. Daily means and standard deviation of viral titers from nasal swabs per group. Immunized groups and mock-immunized groups are represented by black and empty bars, respectively. (A) Homologous challenge By09 ($H1N1_{pdm09}$) except animal #7 ab+, which displayed putative maternal antibodies reactive to By09 prior immunization (Supplementary Fig. 1). (B) Homosubtypic challenge SwBel01 (H1N1). (C) Heterosubtypic challenge SwBiss03 (H3N2). Downer detection limit: dilution factor 1:10.

Table 2
Statistical Comparison of Nasal Viral Shedding of Immunized versus Mock Pigs after Challenge.

Challenge Infection	Significantly different ($P < 0.05$)
homologous	significant at days 3 ($P = 0.0476$), 4 ($P = 0.0079$), and 5 ($P = 0.0286$) after challenge
homosubtypic	no
heterosubtypic	no

We performed Mann-Whitney test using GraphPad Prism 7.00; the primarily antibody-positive animals were excluded. The pairwise comparison of the other pig challenge data (immunized vs. mock): clinical score (Fig. 3), and HI titers (Fig. 6) revealed no significant differences.

detected mild fatigue in immunized animals from day three to six pc (Fig. 3), but no infectious virus (Fig. 4B). The corresponding control group, however, developed lethargy and predominantly respiratory symptoms such as nasal discharge, abdominal breathing and wheezing from day two pc but shed only small amounts of infectious virus (Fig. 3 and 4B). With both heterosubtypic challenged groups, the immunized animals developed slightly weaker but comparable clinical symptoms (slight fatigue and abdominal breathing) and slight lower nasal viral titers (Fig. 3 and 4C). Overall, we observed full protection against homologous challenge and notably decreased clinical signs and no detectable viral shedding after homosubtypic challenge.

3.4. Boost immunization with By09-Ela/NS1-99 prevents or reduces viral load in the respiratory tract after different challenge infections

To assess information about viral load and pathological changes in the respiratory tract after challenge, we performed necropsy on two pigs per group on day 4 pc. After homologous challenge with By09 ($H1N1_{pdm09}$), the immunized pigs did not exhibit any virus-positive tissue samples (Fig. 5A-C) or any macroscopic and histopathological lung lesions (Supplementary Table 3). However, both mock-immunized animals were virus-positive (Fig. 5A-C), and one pig developed mild bronchiolointerstitial pneumonia with intralesional influenza A nucleoprotein-positive bronchiolar and bronchial epithelia, alveolar macrophages and luminal debris (Supplementary Table 3).

Furthermore after homosubtypic challenge with SwBel01 (H1N1), we found only in the pharynx of one immunized pig a few foci of virus-infected cells (Fig. 5C, Supplementary Table 3), but no other virus-positive or antigen-positive tissue samples (Fig. 5A,B). By contrast, we obtained virus-positive samples from one mock-immunized pig (Fig. 5A,C). This animal displayed oligofocal atelectasis in the accessory and middle lung lobes (Supplementary Fig. 2) accompanied by characteristic moderate bronchiolointerstitial pneumonia with intralesional influenza A nucleoprotein-positive bronchiolar and bronchial epithelia and luminal debris (Supplementary Table 3 and Supplementary Fig. 3,4, 5A-D).

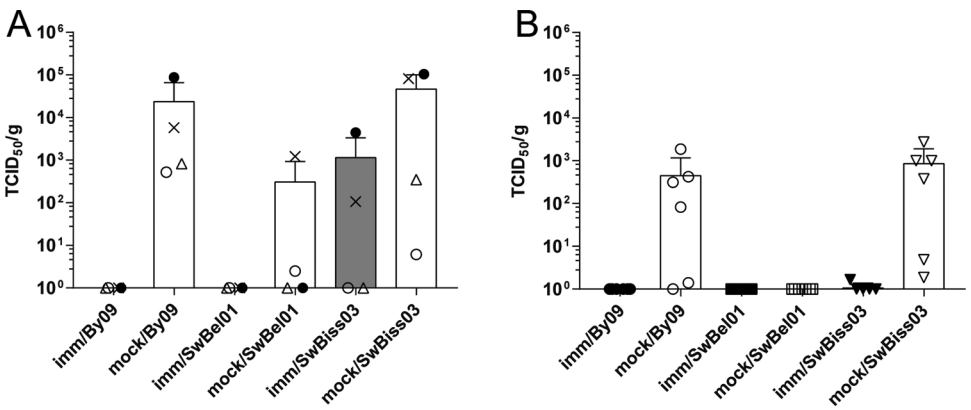
After heterosubtypic challenge with SwBiss03 (H3N2), two animals in both mock-immunized and immunized groups displayed mild bronchiolointerstitial pneumonia with intralesional IAV nucleoprotein-positive bronchiolar, bronchial, and bronchial gland epithelia plus few alveolar macrophages (Supplementary Table 3). Nonetheless, we observed in the immunized animals a lower virus load in the upper respiratory tract (Fig. 5A) and a strongly reduced amount of virus in lung samples (Fig. 5B), compared to the mock-immunized pigs. Correspondingly, semiquantitative immunohistochemistry revealed a considerably reduced virus antigen score in vaccinated animals (Fig. 5C). Taken together, we observed a reduced or no apparent viral load of vaccinated animals after the different challenge infections.

3.5. Humoral and cellular immune responses after first and boost immunization and challenge

We detected a very low HI serum antibody titer in one vaccinated animal three weeks after first immunization (up to 1:28, Supplementary Fig. 6) and found 10 of the 21 vaccinated animals HI antibody-positive three weeks after boost immunization (Fig. 6A). In NP antibody ELISA, 18 were positive at day 21 after boost immunization (Supplementary Fig. 7A). However, after homologous or homosubtypic challenge on day 8 pc, all immunized animals were tested HI antibody-positive against By09 with considerably increased HI titers (Fig. 6B). In both homosubtypic challenged groups, few animals developed weak HI titers against SwBel01 at that time (Supplementary Fig. 8A), whereas all heterosubtypic challenged animals developed already moderate antibody titers against SwBiss03 (Supplementary Fig. 8B). Remarkably, all immunized pigs displayed higher NP antibody levels than mock-immunized animals irrespective a seropositive status prior to immunization (Supplementary Fig. 7 and Supplementary Table 4) or the choice of the challenge virus (Supplementary Fig. 7B), somewhat corresponding to the conservation of the NP (Supplementary Fig. 11 and 19).

To determine cellular immune responses, we investigated different samples by flow cytometry. During immunization period, we found no detectable differences in T and B cell responses between vaccinated and mock-immunized animals in blood samples (day 0, 2, 8 after first and boost immunization and on challenge day). Then, we determined T cell responses in organ samples from animals sacrificed on day 4 pc. In immunized animals challenged with By09, we detected a two-fold higher percentage of CD4+ helper cells in the blood and a considerable increase in spleen compared to the mock-immunized group (Fig. 7A).

However, we found no differences in percentage of CD8+ memory cells in lymphoid organs of homologous challenged animals. In contrast, immunized animals challenged with homosubtypic virus showed considerably more CD8+ effector memory cells in spleen samples but not in the draining lymph node. Compared to the control group, immunized animals challenged with SwBiss03 displayed a two-fold higher percentage of CD8+ effector memory cells in both spleen and lung lymph node (Fig. 7B). In summary, initial low HI antibody titers against



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group	lnn	lung	trachea	pharynx	nose	total
imm/By09	0	0	0	0	0	**0**
mock/By09	0	7	2	2	0	**11**
imm/SwBel01	0	0	0	1	0	**1**
mock/SwBel01	0	4	1	0	0	**5**
imm/SwBiss03	0	3	0	0	1	**4**
mock/SwBiss03	1	7	2	2	3	**15**

Fig. 5. Immunization reduces viral load in the respiratory tract in challenged pigs. Mean group virus titers ($TCID_{50}$) with standard deviation (bars) and specific titers (symbols) from different organ samples (mean titer of samples from two animals per group): (A) nasal mucosa (filled circles), tonsils (open circles), trachea (crosses), and tracheobronchial lymph node (open triangles) (n = 4); (B) lung (n = 6). Immunized groups (imm) and mock-immunized groups (mock) are represented by grey or empty bars, respectively. (C) Total group scores of tissue samples detected positive for influenza A virus nucleoprotein by immunohistochemistry. Score: negative 0, focal 1, multifocal 2, confluent or diffuse 3. The lung score represents the sum of the scores of the seven standardized lung locations. lnn: middle tracheobronchial lymph nodes. All titers were determined in duplicates. Downer detection limit: dilution factor 1:10.

By09 immediately after boost immunization increased after homologous and homosubtypic challenge. Moreover, we found differentially increased CD4+ and CD8+ memory T cell responses in the immunized groups.

4. Discussion

Previously established single-attenuated mutants carrying either an elastase-dependent HACS or a C-terminally truncated NS1 protein display full attenuation and confer after immunization strong protection in mice and swine (Babiuk et al., 2011; Kappes et al., 2012; Solorzano et al., 2005; Stech et al., 2011, 2005; Talon et al., 2000). Here, we aimed to combine both promising attenuation features and generated the double-attenuated mutant By09-Ela/NS1-99 based on an $H1N1_{pdm09}$ strain. Like the single-attenuated elastase mutants (Stech et al., 2011, 2005), By09-Ela/NS1-99 is strictly dependent on elastase, forcing single-cycle replication in the absence of this protease. Combined with a deficient NS1 protein, By09-Ela/NS1-99 represents a promising candidate for a further attenuated, but still immunogenic LAIV with increased safety features. Because the initial vaccination of mice resulted in full protection against homologous challenge, we further investigated the immunization potential of By09-Ela/NS1-99 in a relevant IAV host and an authentic outbred influenza animal model, the swine. To address three different challenge scenarios, we immunized pigs with By09-Ela/NS1-99 twice and challenged them with moderate doses of the homologous wild-type By09 ($H1N1_{pdm09}$), the homosubtypic SwBel01 (H1N1), and the heterosubtypic SwBiss03 (H3N2).

Undesired reassortment between the attenuated vaccine virus and circulating swine viruses under field conditions is conceivable. Correspondingly, in-vitro co-infection experiments on an elastase HA cleavage site mutant from A/Hong Kong/1/1968 (H3N2) and two avian H3 strains revealed that HA reassortants (carrying other genes as well) could be selected in the absence of elastase (Kreibich et al., 2013). However, because the strictly elastase-dependent HA cleavage site leads to restriction to single-cycle replication in the absence of the appropriate protease, the time window for such reassortment events would be rather narrow in vivo. Such a reassortment scenario may occur if an already infected pig would be immunized by an LAIV. Therefore, the live vaccine virus should be derived from a recent circulating swine strain being genetically very close. Alternatively, reassortment of genes other than HA or NS could be restricted by introducing micro RNA response elements into the open reading frames (Perez et al., 2009; Waring et al., 2018).

For virus administration to pigs, we chose the intranasal route by a

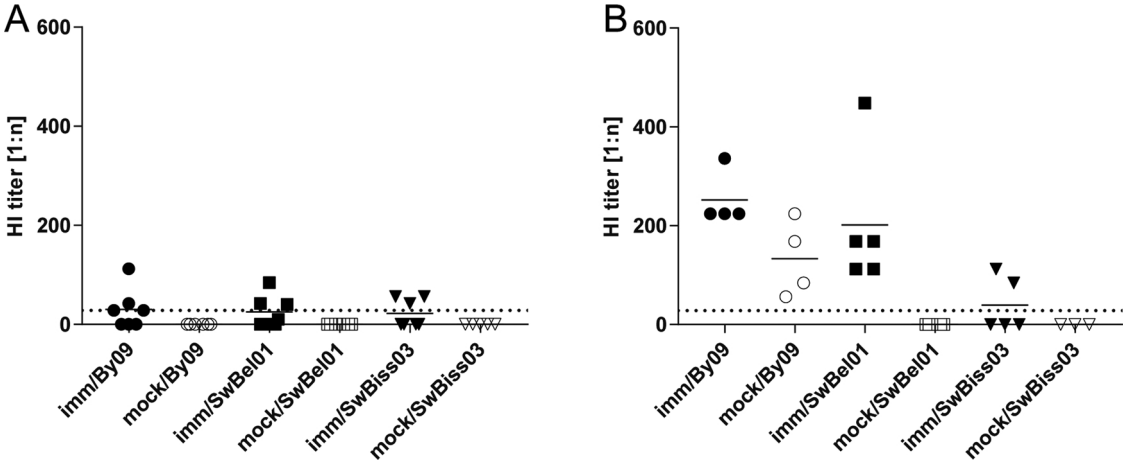

Fig. 6. HI serum antibody titers against By09 in pigs. Mean (line) and individual values (symbols) of immunized groups (imm) and mock-immunized groups (mock) after homologous challenge By09 ($H1N1_{pdm09}$), homosubtypic challenge SwBel01 (H1N1) and heterosubtypic challenge SwBiss03 (H3N2). (A) HI titers on 21 days after boost immunization, (B) HI titers 8 days after challenge. Dotted line indicates the detection limit. HI titers were determined in duplicates.

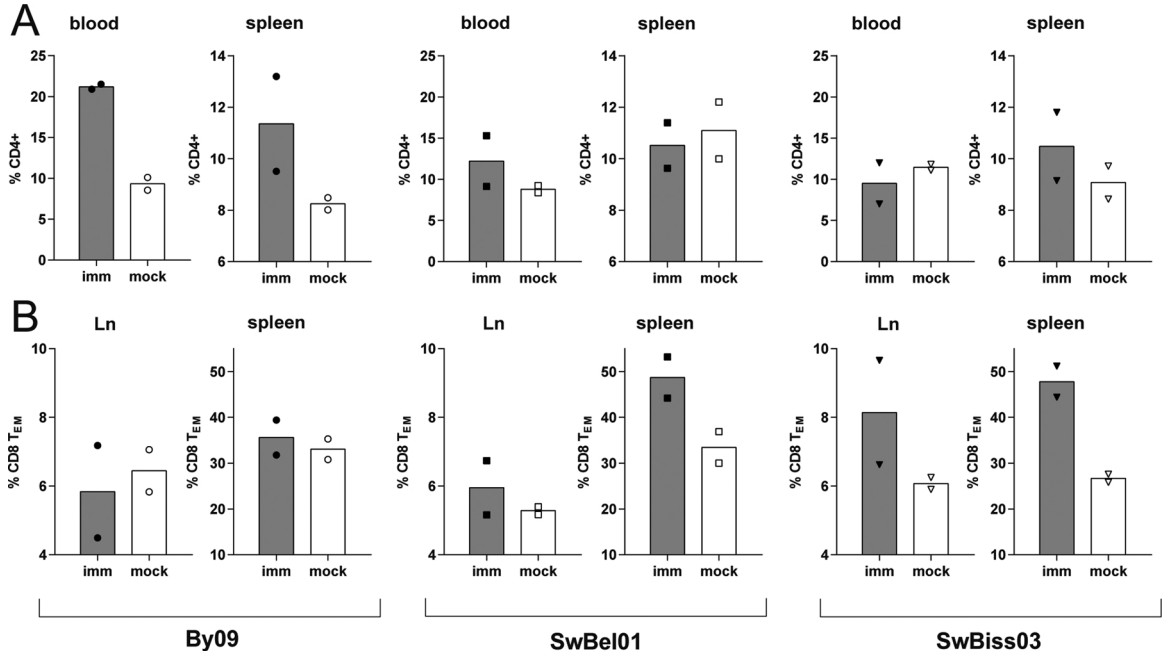

Fig. 7. Cellular immune responses in pigs after different challenges. Animals were infected with homologous By09 ($H1N1_{pdm09}$), homosubtypic SwBel01 (H1N1), or heterosubtypic SwBiss03 (H3N2). (A) CD4 helper cells (CD4 +) in percent of leukocytes versus mock in blood and spleen. (B) CD8 + effector memory (T_{EM}) in percent of leukocytes in lymphoid organs versus mock, Ln: right tracheobronchial lymph node. Mean (bars) and individual values (black symbols: immunized animals; hollow symbols mock-immunized animals). Two animals per group sacrificed on day 4.

mucosal atomization device (Hemmink et al., 2016). Although being somewhat more time-consuming than intramuscular injection, the intranasal administration is the most convenient and applicable vaccination route for LAIV likely to stimulate the local immune response (Vincent et al., 2007).

Overall, we detected only negligible clinical signs and no viral shedding after immunization indicating that replication of By09-Ela/NS1-99 is severely restricted *in vivo* and highly attenuated in swine.

Although HI serum antibodies were elicited only to a very limited extent after booster immunization, we observed different efficiencies against the three challenge viruses. Therefore, neutralizing serum antibody titers did not corroborate with the protection level paralleling other LAIV studies (Loving et al., 2013; Morgan et al., 2016), which emphasizes that serum antibody titers prior to challenge may not serve as reliable correlates of protection of LAIV.

Against homologous challenge with By09 in antibody-negative pigs, we observed full protection, indicated by the absence of clinical symptoms, no detectable viral shedding, and no virus load in the respiratory tract. Neutralizing antibodies were increased in immunized animals compared to the mock group. Accordingly, we detected a two-fold higher percentage of CD4 + T helper cells that get B cells to produce specific antibodies. The observed humoral immune response could arise from the full identity of all epitopes in By09 to those in the LAIV By09-Ela/NS1-99. Although investigations for mucosal antibodies were not included in this study, a protective local immune response is very likely because mucosal IgA were demonstrated to be induced by single-attenuated NS1-truncated LAIV in swine (Richt et al., 2006). Therefore, a subsequent follow-up study should address the local mucosal immunity.

After heterosubtypic challenge with SB03 (H3N2), we detected a considerably decreased viral load in the lungs of immunized animals along with an increased percentage of CD8 + effector memory cells in spleen and in the draining lung lymph node. We speculate that these findings indicate systemic and local cellular immune responses triggered by vaccination.

Although the homosubtypic challenge with SwBel01 (H1N1) resulted in mild, distinct clinical disease in the unvaccinated control group, we only detected very low amounts of infectious virus in collected samples of this group. We speculate that viral replication of SwBel01 occurred predominantly in the pharyngeal and tracheal epithelium and at very limited extents in the nasal tissue. At least in one of two sacrificed animals of this group, we found considerable pathological changes and influenza A nucleoprotein-positive tracheal, bronchiolar and bronchial epithelia cells. Therefore, further investigations would require homosubtypic challenge viruses in high dose and with stronger virulence. However, the immunized group showed decreased clinical symptoms, no detectable viral shedding, or virus in lung samples indicating some protection against virus replication. Interestingly, we found increased antibody titers against By09 ($H1N1_{pdm09}$), the parental strain of By09-Ela/NS1-99, in sera of immunized animals 8 days pc, possibly corresponding to the original antigenic sin (Vatti et al., 2017). Those induced antibodies may bind less efficiently to differing epitope(s) of SwBel01 and were probably less protective. Because mucosal antibodies (Vincent et al., 2017) and neuraminidase-inhibiting antibodies may confer broader protection (Marcelin et al., 2011, 2012), they should be addressed in follow-up studies. Previous studies in mice and swine demonstrated potential protection by T cells during IAV infection with different subtypes (Benton et al., 2001; Talker et al., 2016; Tchilian and Holzer, 2017). The observed increased percentage of CD8 + effector memory cells might be an indication for a systemic immune response. Correspondingly, the peptide alignments of the HA and NA of By09 and SwBel01 indicated several amino acid substitutions (Supplementary Fig. 9 and 10) in known mapped antibody epitopes (Job et al., 2018; Retamal et al., 2014; Wan et al., 2013), suggesting that the protection against the homosubtypic challenge was highly unlikely to be antibody-mediated. Eventually, elucidation of the specific protection mechanism would require further immunological studies with an increased number of sacrificed animals on different sampling days.

Prior to immunizations, we found five animals seropositive, most likely due to maternally derived antibodies. One of these animals developed nasal shedding after homologous challenge infection following immunization. Such maternally derived antibodies can impair the development of active immunity after infection as well as vaccination

(Loeffen et al., 2003; Salmon et al., 2009; Sandbulte et al., 2015). By contrast, other studies demonstrated that LAIVs conferred protection (Pyo et al., 2015) or reduction of viral shedding (Genzow et al., 2017) despite the presence of maternally derived antibodies. Although we observed some interference of pre-existing antibodies with the vaccine virus in one animal, this problem would require targeted investigations to elucidate frequency and extent of interference due to maternally derived antibodies.

In this pilot study, we generated a highly attenuated but still immunogenic double-mutated LAIV, which offers increased safety. Recently, a novel live vaccine based on NS1 truncation (Genzow et al., 2017) has been introduced to the US market for usage in swine (Ingelvac Provenza™), indicating the applicability of LAIV in the field. An additional HA elastase cleavage site mutation as in By09-Ela/NS1-99 may result in two additional beneficial features: Secondary attenuation leading to increased safety and prevention of transmission of the HA gene of the vaccine virus into field strains via reassortment. In pigs, we observed differential efficiencies after immunization with By09-Ela/NS1-99 ($H1N1_{pdm09}$) against moderately dosed challenge with three different viruses having a homologous ($H1N1_{pdm09}$), homosubtypic (H1N1), or heterosubtypic (H3N2) serotype.

Follow-up studies should elucidate the detailed immune mechanisms involved in those three different challenge scenarios as well as the administration of challenge viruses with increased virulence and in high dose. In particular, we found full protection against homologous challenge and indications for strong protection conferred by By09-Ela/NS1-99 ($H1N1_{pdm09}$) against a different virus of the same HA/NA subtype, a realistic vaccine/challenge constellation both in swine and humans. Therefore, we suggest that an optimized backbone based on established field strains or carrying microRNA responsive elements (Perez et al., 2009; Waring et al., 2018) may offer increased safety and requires less frequent updates with recent HA and NA genes but still induce broader protection in relevant IAV hosts against drifted viruses.

Conflict of interests

The authors declare that they have no conflict of interests.

Ethical approval

All animal experiments on mice and pigs were approved by the responsible ethics committee of the State Office for Agriculture, Food Safety and Fishery in Mecklenburg-Western Pomerania (LALFF M-V) (reference numbers 7221.3-1.1-051/12 and 7221.3-1004/16). All methods and procedures were carried out in accordance with the relevant guidelines and regulations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2019.03.013.

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