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A chimeric influenza hemagglutinin delivered by parainfluenza virus 5 vector induces broadly protective immunity against genetically divergent influenza a H1 viruses in swine

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ABSTRACT

Pigs are an important reservoir for human influenza viruses, and influenza causes significant economic loss to the swine industry. As demonstrated during the 2009 H1N1 pandemic, control of swine influenza virus infection is a critical step toward blocking emergence of human influenza virus. An effective vaccine that can induce broadly protective immunity against heterologous influenza virus strains is critically needed. In our previous studies [McCormick et al., 2015; PLoS One, 10(6):e0127649], we used molecular breeding (DNA shuffling) strategies to increase the breadth of the variable and conserved epitopes expressed within a single influenza A virus chimeric hemagglutinin (HA) protein. Chimeric HAs were constructed using parental HAs from the 2009 pandemic virus and swine influenza viruses that had a history of zoonotic transmission to humans. In the current study, we used parainfluenza virus 5 (PIV-5) as a vector to express one of these chimeric HA antigens, HA-113. Recombinant PIV-5 expressing HA-113 (PIV5-113) were rescued, and immunogenicity and protective efficacy were tested in both mouse and pig models. The results showed that PIV5-113 can protect mice and pigs against challenge with viruses expressing parental HAs. The protective immunity was extended against other genetically diversified influenza H1-expressing viruses. Our work demonstrates that PIV5-based influenza vaccines are efficacious as vaccines for pigs. The PIV5 vaccine vector and chimeric HA-113 antigen are discussed in the context of the development of universal influenza vaccines and the potential contribution of PIV5-113 as a candidate universal vaccine.

Abbreviations: HA, hemagglutinin; PIV5, parainfluenza virus 5; PIV5-113, parainfluenza virus 5 expressing the HA from DNA expressing the HA-113 antigen; RSV, respiratory syncytial virus; MERS-CoV, Middle East Respiratory Syndrome Coronavirus; NJ76, A/New Jersey/8/1976; IA06, A/Iowa/01/2006; OH07, A/Ohio/01/2007; ME08, A/Memphis/03/2008; TN09, A/Tennessee/1-560/09; GE81, A/swine/Germany/2/1981; IA92, A/swine/Iowa/40766/1992; BR07, A/Brisbane/59/2007; CA09, A/California/4/2009; MI15, A/Michigan/45/2015; 13E100, A/swine/Iowa/13E100/2013; BALF, bronchoalveolar lavage fluid; HAI, hemagglutination inhibition; ELISA, enzyme-linked immunosorbent assay; RDE, receptor-destroying enzyme; COBRA, computationally optimized broadly reactive antigen; FBS-PBST, Phosphate-buffered saline containing 10 % fetal bovine serum and 0.05 % Tween-20; IACUC, Institutional Animal Care and Use Committee; i.n., intranasal; $TCID_{50}$, 50 % infectious dose in tissue culture; LD_{50}, 50 % lethal dose in mice; MDBK, Madin-Darby bovine kidney; MDCK, Madin-Darby canine kidney; MOI, multiplicity of infection; BHK, baby hamster kidney; NP, nucleoprotein; PR8, A/Puerto Rico/8/34; SH, small hydrophobic; HN, hemagglutinin-neuraminidase; ANOVA, Analysis of variance; IIV, inactivated influenza virus; LAIV, live, attenuated influenza virus.

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1. Introduction

Influenza viruses are associated with approximately 291,000–600,000 deaths annually worldwide (Iuliano et al., 2018), and vaccines remain our best method for limiting influenza virus infections (Bresee et al., 2019; Yamayoshi and Kawaoka, 2019; Grohskopf et al., 2016). In addition to inactivated influenza vaccines, which have been available in a variety of forms since the 1940's (Barberis et al., 2016; Taylor et al., 1945), vaccines created using live, attenuated influenza virus strains (Maassab, 1967; Grohskopf et al., 2018) and recombinant hemagglutinin (HA) proteins are now available (Kuroda et al., 1986; Treanor et al., 1996; Cox et al., 2015). Despite this, influenza seasons can be marred by issues related to vaccine performance (Osterholm et al., 2012), including those associated with vaccine production (Mostafa and Pleschka, 2018; Skowronski et al., 2014), immunogenicity (Cobey et al., 2018; Belongia et al., 2017), and mismatches with the circulating strains (Skowronski et al., 2018). Work with influenza HA proteins, and antibody responses against these proteins, has led to an understanding of the antigenic sites within the globular head (Yewdell et al., 1986; Hensley and Yewdell, 2009; Underwood, 1982; Both et al., 1983; Smith et al., 2004), as well as more conserved epitopes within the stem or stalk domain (Jou et al., 1980; Steel et al., 2010; Ekiert et al., 2009). However, all current vaccines that have been approved for use rely on the expression of HA proteins that match those of circulating virus isolates (Grohskopf et al., 2018). Future influenza vaccines should challenge the concept that the antigen used to induce immunity needs to resemble the HA protein of circulating viruses (Stanekova and Vareckova, 2010; Schepens et al., 2018; Nachbagauer et al., 2014). In designing these new vaccines, we must consider whether host immune responses that target a limited number of conserved epitopes offer sufficient protection against the threat of influenza virus epidemics and pandemics.

Our previous work in influenza vaccine development has focused on application of molecular breeding (DNA shuffling) technology to create chimeric HA proteins that are capable of inducing broad immunity (McCormick et al., 2015). We specifically targeted the HA from the 2009 pandemic virus, as well as HAs from three additional swine influenza viruses that had a history of zoonotic transmission to humans. These four selected parental influenza A H1N1 strains, A/New Jersey/8/76 (NJ76, alpha clade), A/Iowa/1/06 (IA06, beta clade), A/Ohio/1/07 (OH07, gamma clade), and A/Tennessee/1-560/09 (TN09, npdm) viruses, represented distinct phylogenetic clades (Anderson et al., 2016; Zhang et al., 2017). Using the DNA shuffling approach, we generated a library of chimeric HA antigens that, when evaluated in mice using a DNA vaccine approach, yielded three chimeric HAs with the ability to induce immunity against all of the parental HAs. Among these chimeric HAs, the construct HA-113 showed similar levels of antibody response against all four parental HAs, but we were unable to express HA-113 as part of an influenza virion using reverse genetics approaches (McCormick et al., 2015).

In order to develop a vaccine product, a protein-expression vector is needed for safe and efficient delivery of the candidate vaccine antigen. Previous studies showed that parainfluenza virus 5 (PIV-5) has several characteristics that make it an attractive vector for vaccine delivery. First, kennel cough vaccines containing live PIV-5 have been used in dogs for over 40 years without safety concern for either humans or animals. Second, PIV-5 can be produced at high titers in many cell types, including Vero cells, which have been approved for vaccine production (Lamb and Kolakofsky, 2001). Third, PIV-5 can infect both human cell lines and primary human cells (Tompkins et al., 2007); and recently PIV5 has been isolated from pigs (Jiang et al., 2018; Lee and Lee, 2013). Fourth, pre-existing immunity against PIV-5 does not negatively affect immunity generated by PIV-5-based vaccines (Chen et al., 2012). Finally, a single-dose immunization of PIV-5 expressing either the HA protein of the H5N1 avian influenza (H5 HA), the G protein of rabies virus, the F protein of respiratory syncytial virus (RSV), or the spike protein of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) provided immunity in mice against avian H5N1, rabies virus, RSV, and MERS-CoV, respectively (Li et al., 2013a; Chen et al., 2013; Phan et al., 2014; Li et al., 2020). Together, these results demonstrate that PIV-5 is an effective vaccine vector.

In the current study, recombinant PIV-5 was engineered to express chimeric HA-113 antigen (PIV5-113). Immunogenicity of PIV5-113 was initially evaluated using a murine challenge model, prior to assessment of protective immunity in a nursery pig model. The results show that PIV5-113 can induce protective immunity against a broad range of influenza viruses. This study opens opportunities to apply this vaccine platform for further development of universal vaccines against influenza virus infection.

2. Methods and materials

2.1. Parental HA genes, DNA shuffling, and viral strains

The HA genes of parental H1N1 influenza A viruses, including A/New Jersey/8/1976 (NJ76; HA gene: CY130118.1, alpha clade), A/Iowa/01/2006 (IA06; HA gene: FJ986618.1, beta clade), A/Ohio/01/2007 (OH07; HA gene: FJ986620.1, gamma clade), and A/Tennessee/1-560/09 (TN09; HA gene: CY040457.1, npdm) were isolated from stock viruses and cloned as described (Hoffmann et al., 2002; Huber et al., 2009). Additional non-parental H1N1 influenza A virus strains, tested for cross reactivity, include A/Memphis/03/2008 (ME08; HA gene: EU779616.1, delta clade), A/swine/Germany/2/1981 (GE81; HA gene: Z30276.1, other/Eurasian swine), A/swine/Iowa/40766/1992 (IA92; HA gene: KP788773, alpha clade), A/Brisbane/59/2007 (BR07; HA gene: CY030232.1, delta-like), A/California/4/2009 (CA09; HA gene: MK159419.1, npdm), A/Michigan/45/2015 (MI15; HA gene: KY117023.1, npdm), and A/swine/Iowa/13E100/2013 (13E100; HA gene: KF885634.1, gamma clade) (Zhang et al., 2017; Anderson et al., 2013). The virus used in the pig challenge study was the A/swine/Alberta/25/2009 isolate (CY097778-CY097785 and JN617971-JN617978, HA: npdm), which has been used previously in pig infection models (Ma et al., 2011).

2.2. Creation of PIV5 vectors expressing influenza hemagglutinin (HA)

The DNA sequence from the shuffled HA gene HA-113 was cloned as described (McCormick et al., 2015). To generate ZL402 (pPIV5--HA113-SH/HN) plasmid, the coding sequence of the chimeric HA protein HA-113 was inserted into the PIV5 genome between the small hydrophobic (SH) gene and the hemagglutinin-neuraminidase (HN) gene. The rescue of infectious recombinant PIV5 was performed as described previously (He et al., 1997). Briefly, the plasmid pZL402, encoding the full-length genome of PIV5 with the HA113 gene inserted between the SH and HN genes, as well as three helper plasmids, pPIV5-NP, pPIV5-P, and pPIV5-L, encoding NP, P, and L proteins, respectively, were co-transfected into BSR T7 cells at 95 % confluence in 6-cm plates. After 72 h of incubation at 37 °C, the media were harvested and cell debris was pelleted by low-speed centrifugation (3000 rpm for 10 min). Plaque assays were performed to obtain a single clone of recombinant viruses. The full-length genome of the plaque-purified single clone of PIV5-113 virus was sequenced as described previously (Li et al., 2013a). Total RNA was purified from the media of PIV5-113-infected Madin-Darby Bovine Kidney (MDBK, American Type Culture Collection (ATCC), Manassas, VA) cells using the viral RNA extraction kit (Qiagen Inc., Valencia, CA). cDNAs were prepared using random hexamers, and aliquots of the cDNA were then amplified by PCR using appropriate oligonucleotide primer pairs. MDBK cells in 6-well plates were infected with PIV5 or PIV5-113 at a multiplicity of infection (MOI) of 0.1. The cells were then washed with PBS and maintained in DMEM supplemented with 2% FBS. Media were collected at 0, 24, 48, 72, 96, and 120 h postinfection (hpi). Virus titers were determined by plaque assay on baby hamster kidney (BHK) cells (ATCC).

2.3. Creation and characterization of HA-expressing virus reassortants

The 8-plasmid reverse genetics system, incorporating the 293 T (ATCC) and Madin-Darby canine kidney (MDCK; ATCC) co-culture condition, was used to create reassortant viruses expressing parental HA genes from either NJ76, IA06, OH07, ME08, or TN09 (McCormick et al., 2015). Briefly, each HA was expressed within a reassortant virus that derived the other 7 genes from the A/Puerto Rico/8/34 (PR8) virus. Viruses rescued from 293 T:MDCK cell co-cultures that expressed the desired HA were propagated in 10-day-old embryonated chicken eggs for 72 h at 35 °C and sequenced to verify appropriate HA genotype. Viruses were characterized for infectivity using MDCK tissue culture ($TCID_{50}$) and lethality in mice (LD_{50}), reported in Table 1, as described previously (McCormick et al., 2015; Huber et al., 2009).

2.4. Mouse immunization and challenge

A total of 98 adult (6–8-week-old) female BALB/cJ mice were obtained from Harlan Laboratories (Indianapolis, IN) and housed in groups of four, with 24 -h access to food and water. All mouse experiments were performed following the guidelines established and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Dakota (Vermillion, SD). Mice were immunized intranasally with either PBS, PIV5 vector (10^6 PFU), or PIV5-113 (10^6 PFU), and boosted on day 28. Sera were collected three weeks after boosting and analyzed by hemagglutination inhibition and enzyme-linked immunosorbent assay, as described below. All 98 mice were challenged intranasally with 10 LD_{50} of the indicated influenza viruses four weeks after boosting, and monitored daily for signs of morbidity and mortality, as described (Huber et al., 2001; Huber et al., 2006; Chaussee et al., 2011). Mice were euthanized when they achieved humane endpoints, including weights equal to or below 80 % of their initial body weight.

2.5. Hemagglutination inhibition assay

Hemagglutination inhibition (HAI) assays were performed, with receptor-destroying enzyme (RDE, Accurate Chemical, Westbury, NY) being used to treat serum samples prior to use in the HAI assay, as described previously (Cwach et al., 2011). RDE-treated serum samples from pigs (1 mL volume) were incubated with chicken red blood cells (50 μL) to remove nonspecific agglutinins in the serum, as described (Truelove et al., 2016; Wibawa et al., 2012). All RDE-treated sera were diluted, and 4 HA units of virus were added to each well. The virus:sera mixtures were incubated for one hour at 4 °C, and a 0.5 % solution of chicken red blood cells (Lampire Biological Laboratories, Pipersville, PA) was added to each well. Titers are reported as the reciprocal of the final serum dilution that inhibited hemagglutination. A titer of 5 was assigned to serum samples that did not demonstrate HAI at the starting serum dilution of 1:10.

Table 1
Characteristics of reassortant viruses expressing parental HAs.

Virus Name	Abbreviation	$TCID_{50}$ in MDCK[a]	LD_{50} in Mice[b]
A/New Jersey/8/76 HA:PR8 (1:7)	NJ76	9.0	5.0
A/Iowa/1/06 HA:PR8 (1:7)	IA06	7.625	3.625
A/Ohio/1/07 HA:PR8 (1:7)	OH07	8.25	3.5
A/Tennessee/1-560/09 HA:PR8 (1:7)	TN09	7.5	2.5

[a] Values are reported as $\log_{10}$ $TCID_{50}$ per mL.
[b] Values are reported as the $\log_{10}$ $TCID_{50}$ that represented 1 LD_{50} in BALB/c mice.

2.6. Antibody detection by enzyme-linked immunosorbent assay (ELISA)

Serum antibodies were detected using an ELISA, as described previously (McCormick et al., 2015; Huber et al., 2009; Huber et al., 2006). Briefly, 96-well flat bottom plates (NUNC, Thermo Fisher Scientific) were coated with concentrated, formalin-inactivated reassortant viruses expressing individual HAs (1 μg mL^{-1}). Sera were serially diluted in PBS containing 10 % FBS (Atlanta Biologicals, Lawrenceville, GA) and 0.05 % (v/v) Tween-20 (Sigma, St. Louis, MO) (FBS-PBST). Alkaline phosphatase-conjugated goat anti-mouse IgG (H + L), IgG1, and IgG2a (Southern Biotechnology, Inc., Birmingham, AL) antibodies were diluted in FBS-PBST and added to the plates. Plates were washed, and 1 mg mL^{-1} *p*-nitrophenyl phosphate substrate (Sigma, St. Louis, MO) in diethanolamine buffer was added. One hour after substrate addition, the OD was measured at 405 nm using a BioTek ELx808 plate reader (BioTek Instruments, Inc., Winooski, VT). Reciprocal serum antibody titers for individual sera are reported at 50 % maximal binding on the individual titration curves. Individual sera with starting dilution OD_{405} values that were less than 2 times the OD_{405} of negative control sera were assigned a titer of one dilution below the starting serum dilution.

2.7. Pig vaccination and challenge

The pig experiment was performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Kansas State University. A total of eighteen 3–4 weeks old pigs were obtained from a commercial vendor. They were free of swine influenza virus, porcine reproductive and respiratory syndrome virus, and *Mycoplasma hypopneumoniae*. Pigs were randomly divided into three groups (n = 6), and housed separately in animal isolation facilities at Kansas State University. After a 4-day acclimation period, pigs were immunized intranasally with 10^6 PFU PIV5 vector virus or recombinant PIV5-113, and this day was referred to as day 0 post-vaccination (DPV 0). As a negative control, a third group of pigs were inoculated with the same volume of PBS. Fourteen days after primary inoculation (DPV 14), pigs were boosted with the same material used at initial inoculation. At 14 days after the second immunization (DPV 28), serum samples were collected and pigs were challenged intratracheally with 10^6 PFU A/swine/Alberta/25/09-H1N1 influenza virus and clinical signs were monitored daily. Nasal swab samples were collected on days 1, 3, and 5 post-challenge (DPC 1, 3, and 5), and bronchoalveolar lavage (BAL) fluid was collected after euthanasia on DPC 5, as described previously (McCormick et al., 2015).

2.8. Data analysis

Graphing and statistical analyses were performed using GraphPad Prism version 8 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Significance is reported at $P < 0.05$, using the tests indicated.

3. Results

3.1. Expression of HA-113 using recombinant PIV5 does not reduce PIV5 propagation kinetics in tissue culture

Recombinant PIV5-113 was constructed by inserting the HA113 gene into a plasmid containing the full-length cDNA genome of PIV5 at the junction between the SH and HN genes (Fig. 1A). Infectious PIV5-113 virus was recovered by transfecting the plasmid into BSR T7 cells along with plasmids carrying NP, P, and L genes as described previously (Li et al., 2013a). The recovered virus was plaque-purified and grown in MDBK cells, and the full-length genome of plaque-purified virus was sequenced using direct reverse transcription-PCR (RT-PCR) sequencing. A single plaque-purified clone matching the exact cDNA of the genome sequence was used for subsequent experiments. To compare growth of

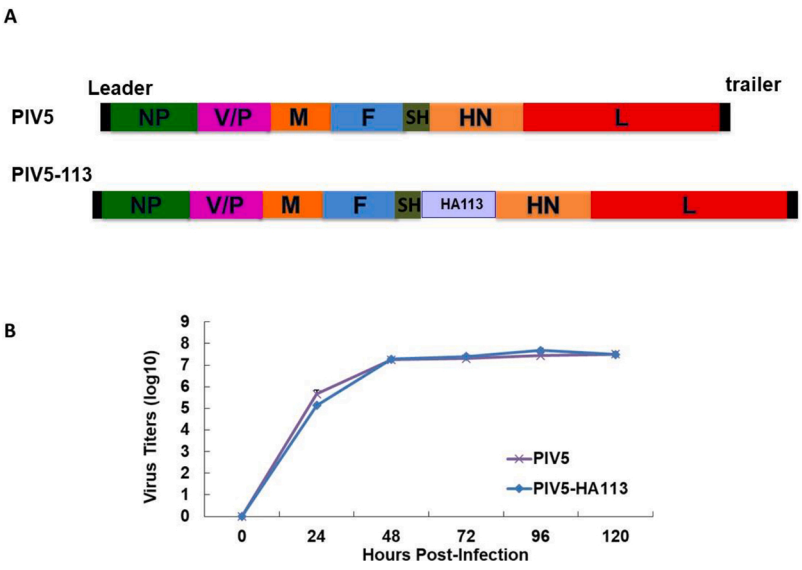

Fig. 1. Generation and analysis of PIV5-HA113. (A). Schematic of PIV5-113. The PIV5 genome contains seven genes in the order of 3′-NP-V/P-M-F-SH-HN-L-5′, with leader and trailer regions located at the ends of the genome. The HA113 gene was inserted into the PIV5 genome between the SH and HN genes. (B). Multiple-step growth curves of PIV5 and PIV5-113 in tissue culture. MDBK cells were infected with PIV5 or PIV5-113 at an MOI of 0.1, and the media were collected at 24-h intervals. Virus titers were determined by plaque assay on BHK cells.

PIV5 and PIV5-113 in tissue culture, multiple-step growth curves were performed using MDBK cells. PIV5-113 grew to similar titers as PIV5 at the indicated time points (Fig. 1B).

3.2. PIV5-113-induces antibodies and protects against infection in a mouse model

To assess the immunogenicity of PIV5-113, we initially tested this recombinant vaccine in mice. Three groups of mice were immunized with either PBS (negative control), PIV5 (vector control), or PIV5-113. Mice were boosted on day 28, and sera were collected three weeks after boosting. HAI assay results showed significantly increased titers ($P < 0.05$) of HA-reactive antibodies against the parental viruses of chimeric HA113, including NJ76, IA06, OH07, and TN09 viruses in the group of mice vaccinated with PIV5-113, in comparison with serum from mice inoculated with either PBS or PIV5 vector alone (Fig. 2). Sera were tested for reactivity with a non-parental virus, ME08 (delta clade) to determine the breadth of vaccine-induced immunity in PIV5-113-vaccinated mice. Vaccine-induced HA-reactive antibodies against ME08 were significantly increased in PIV5-vaccinated animals, but these levels were lower than those induced against the NJ76, IA06, OH07, and TN09 parental HAs.

To confirm results generated from HAI, we used an isotype-specific ELISA, which included tests for total antibody (IgG (H + L)), IgG1, and IgG2a (Fig. 3). ELISA results showed a significant increase ($P < 0.05$) in titers of IgG (H + L), IgG1, and IgG2a antibodies against NJ76, IA06, OH07, and TN09 viruses in PIV5-113-vaccinated mice compared to antibody responses in groups of mice inoculated with either PBS or PIV5 vector alone. Similar to the results from the HAI assay, antibodies against ME08 were detected by ELISA, this time at levels that were similar to those induced against IA06 and TN09.

Next, we challenged mice with the 4 different influenza viruses individually expressing the parental HA proteins used to create HA-113 (Table 1). These viruses were created by reverse genetics as described above, and they were designed so that they differ only in the HA expressed, with internal genes and neuraminidase being provided by the mouse-adapted influenza virus strain PR8 (Huber et al., 2009). Our results show that PIV5-113-vaccinated mice were protected from challenge with viruses expressing the parental HAs of NJ76, IA06, OH07, and TN09. This was observed in both the weight loss (Fig. 4) and Kaplan-Meier survival curves (Fig. 5) generated after challenge. In addition to the PIV5-113-vaccinated mice that survived lethal challenge, 2 out of 8 mice in the PIV5 alone group survived challenge with NJ76. This result might be due to increases in cell-mediated immunity stimulated by PIV5, which is consistent with a result that had been previously reported in PIV5-immunized mice (Li et al., 2013b). Mice that were vaccinated with PIV5-113 were not protected from challenge with the non-parental ME08 virus, which did not show strong antibody reactivity in the HAI assay (Fig. 2).

3.3. PIV5-113 induces antibodies and protects pigs against infection

Since PIV5-113 induced both antibody responses (Figs. 2 and 3) and protection (Figs. 4 and 5) in vaccinated mice, we evaluated its

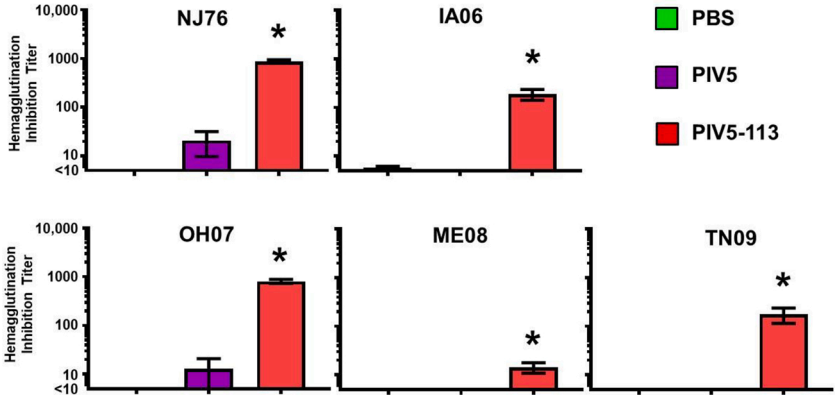

Fig. 2. Evaluation of PIV5-113-induced antibodies against parental HA, using the HAI assay. Mice were vaccinated with either PBS, PIV5, or PIV5-113, and sera were collected after delivery of a boost, as described above. Results were obtained using sera from PBS-inoculated mice (n = 20), PIV5-inoculated mice (n = 40), and PIV5-113-inoculated mice (n = 38). *Indicates a significant difference ($P < 0.05$) upon individual comparison with PBS-inoculated mice and PIV5-inoculated mice, determined using One Way ANOVA with a Tukey post-hoc test.

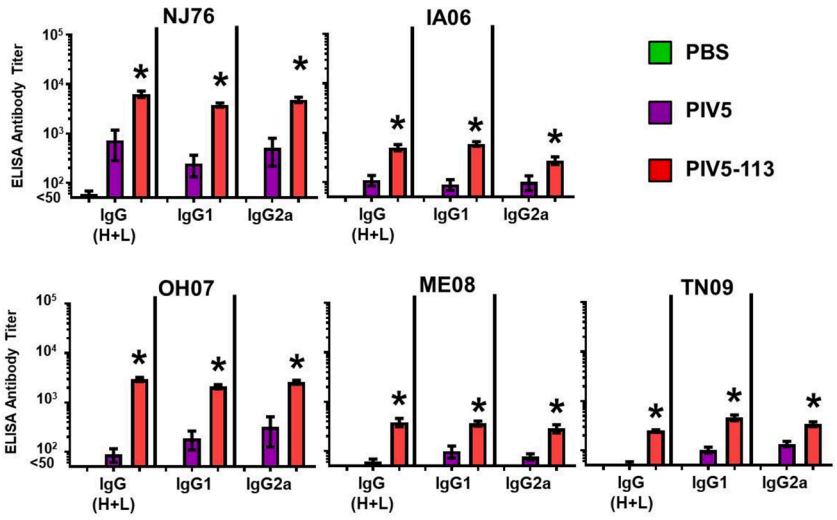

Fig. 3. Evaluation of PIV5-113-induced antibodies against parental HA, using ELISA: IgG (H + L), IgG1, and IgG2a. Mice were vaccinated with either PBS, PIV5, or PIV5-113, and sera were collected after delivery of a boost, as described above. Results were obtained using sera from PBS-inoculated mice (IgG (H + L): n = 18 for NJ76 and IA06, n = 20 for OH07, ME08, and TN09, IgG1: n = 18 for NJ76, IA06, OH07, ME08, and TN09, IgG2a: n = 18 for NJ76, IA06, OH07, ME08, and TN09), PIV5-inoculated mice (n = 40), and PIV5-113 (n = 38). *Indicates a significant difference ($P < 0.05$) upon individual comparison with PBS-inoculated mice and PIV5-inoculated mice, determined using One Way ANOVA with a Tukey post-hoc test.

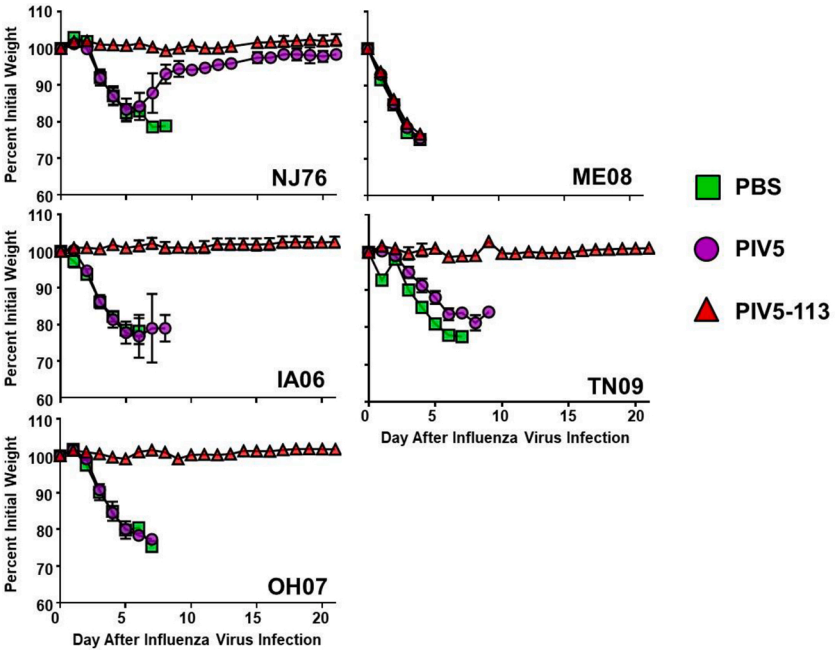

Fig. 4. Weight loss in PIV5-113-vaccinated mice that are challenged with mouse-adapted influenza viruses expressing NJ76, IA06, OH07, ME08, or TN09 HAs. Mice were vaccinated using PIV5 and challenged after a booster inoculation. Mice were challenged with the individual viruses indicated in the panels on Day 0, and weight changes were tracked for 21 days post-challenge. Groups of PBS-inoculated mice (n = 4 per virus), PIV5-inoculated mice (n = 7 for NJ76 and OH07, n = 8 for IA06, ME08, and TN09), and PIV5-113-inoculated mice (n = 7 for NJ76 and OH07, n = 8 for IA06, ME08, and TN09) were evaluated.

application as a vaccine candidate against influenza infection in pigs. Since pigs are natural hosts for influenza virus, we tested PIV5-113 in a nursery pig model. Three groups of pigs were immunized with PBS, PIV5, and recombinant PIV5-113 and boosted with the same dose of inoculant at 14 DPV. At 28 DPV, pigs were challenged with the wild-type A/swine/Alberta/25/09-H1N1 influenza virus (Alb09), which is a member of the npdm H1 clade, similar to TN09. Sera collected at DPV28 did not show significant increases in HAI antibodies, a result that could be explained by the use of the intranasal route for vaccination (Fig. 6A). At 5 days post-challenge (DPC5), HAI assay results show that the PIV5-113-vaccinated pigs had significantly increased ($P < 0.05$) antibody titers against viruses expressing the parental NJ76, IA06, OH07, and TN09 HAs, in comparison to antibodies detected in both control groups. Interestingly, similar to our findings in mice, antibodies against the non-parental ME08 were also significantly increased in the PIV5-113 vaccine group at DPC5, albeit at a lower level than antibodies against the parental HAs (Fig. 6A).

Based on this finding, we decided to determine the breadth of PIV5-113-induced immunity by further testing the antibody response against an extended panel of viruses (Fig. 6B). These viruses represent isolates that had been previously circulating in both pigs and humans, including the IA92 and CA09 viruses we tested previously (McCormick et al., 2015). In addition, we included the MI15 H1N1 virus isolate that was used in the 2018−19 Northern Hemisphere seasonal influenza vaccine (Grohskopf et al., 2018) and a primary swine H1N1 virus (13E100) that was isolated during surveillance of pigs at a swine abattoir (Zhang et al., 2019). The HAI assay was performed using serum samples from all three groups of pigs, collected at DPC5. As shown in Fig. 6B, serum antibodies from PIV5-113-vaccinated pigs showed a significant increase ($P < 0.05$) in HAI titer against the IA92, CA09, MI15, and 13E100 influenza viruses, suggesting the PIV5-113 is able to induce broad immunity against genetically diversified H1 viruses that circulate in both pigs and humans, including additional representatives of the alpha (IA92), npdm (CA09 and MI15), and gamma (13E100) H1 clades.

After challenge, PIV5-113-vaccinated pigs appeared to be protected from the challenge with the npdm H1 clade Alb09 virus, as evidenced by the absence of virus shedding in nasal secretions (Fig. 7). In contrast, challenge virus was detected in pigs in the PBS and PIV5 vector groups

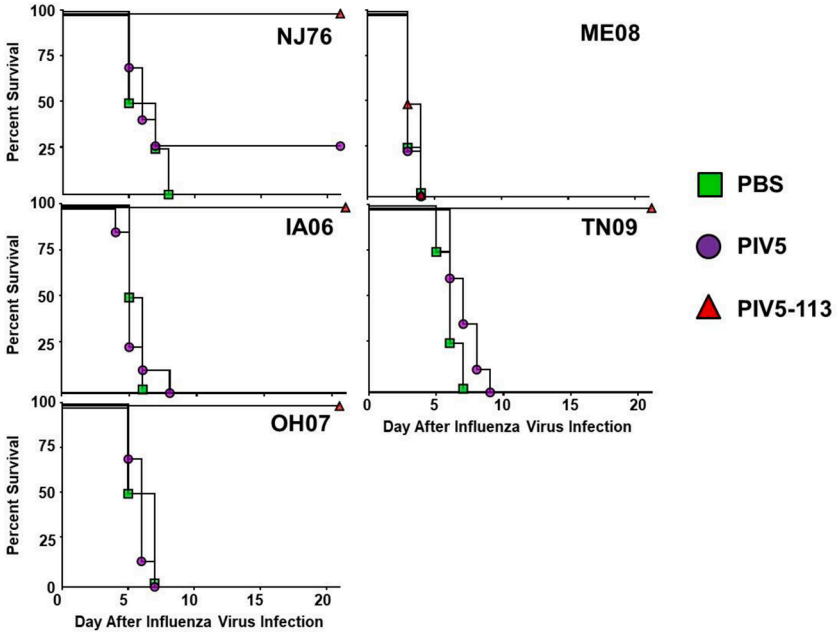

Fig. 5. Survival in PIV5-113-vaccinated mice that are challenged with mouse-adapted influenza viruses expressing NJ76, IA06, OH07, ME08, or TN09 HAs. Mice were vaccinated using PIV5 and challenged after a booster inoculation. Mice were challenged with the individual viruses indicated in the panels on Day 0, and survival is reported for 21 days post-challenge using Kaplan-Meier plots. Groups of PBS-inoculated mice (n = 4 per virus), PIV5-inoculated mice (n = 7 for NJ76 and OH07, n = 8 for IA06, ME08, and TN09), and PIV5-113-inoculated mice (n = 7 for NJ76 and OH07, n = 8 for IA06, ME08, and TN09) were evaluated.

as early as Day 3 post-challenge (Fig. 7B). Subsequently, a significant increase ($P < 0.05$) in virus was detected in nasal swab samples from all control pigs at Day 5 post-challenge (Fig. 7C). To confirm virus infection of the lower respiratory tract, bronchoalveolar lavage fluid (BALF) was collected from all animals at DPC5 (Fig. 7D). Pigs in the PBS and PIV5 groups showed detectable levels of virus in the BALF. In contrast, virus was not detected in BALF collected from PIV5-113-vaccinated pigs. Together, results from the nasal swab and BALF samples indicate that PIV5-113 induced protective immunity in vaccinated pigs, which protected pigs against influenza infection in both the upper and the lower respiratory tracts.

4. Discussion

The current influenza vaccine selection process relies on surveillance to predict viruses that will circulate during the next influenza season, with selection of viruses that are predicted to match these viruses (Francis et al., 1947a; Francis et al., 1947b; Ziegler et al., 2018). This process can be time-consuming and has not always been able to match viruses that arise from either antigenic drift during seasonal influenza virus circulation, or from the antigenic shift that initiates pandemics (Meiklejohn, 1983; Kilbourne et al., 2002; Dowdle et al., 1974). In pigs, annual updates for swine influenza vaccines is not economically feasible. Since influenza viruses associated with pandemics often come from a known source, with representative viruses circulating in birds and pigs prior to emergence in humans (Scholtissek, 1987; Trebbien et al., 2011; Eriksson et al., 2018), there is often knowledge of the viruses circulating in all three dominant species infected with influenza viruses (Webster et al., 1992). This presents an opportunity to use this knowledge to design vaccines that prevent infection from interspecies transmission events. To use this knowledge to our advantage, our previous work showed that DNA shuffling could yield chimeric HAs as candidate vaccine antigens against genetically diversified influenza viruses that circulate in human and pigs. Using reverse genetics, we advanced one chimeric HA, identified as HA-129 into both the PR8 and the A/swine/Texas/4199-2/98 backbone (TX98-129) to generate inactivated influenza virus (IIV) and live, attenuated influenza virus (LAIV) candidate vaccines (McCormick et al., 2015). Results from other chimeric HA proteins created from gene shuffling, including the chimeric HA-113, showed improved breadth of antigenicity, but we were unable to express these HAs using conventional IIV and LAIV vaccine production methods.

In the current study, we utilized PIV5 as a vector for delivery of HA-113 as a candidate vaccine, and evaluated induction of protective immunity in both mice and pigs. Results from vaccination of mice show that HA-113 can induce significant increases in the level of antibodies against the parental NJ76, IA06, OH07, and TN09 viruses, as well as the non-parental ME08, albeit at a lower level. Subsequently, PIV5-113-vaccinated mice were protected against challenge with NJ76, IA06, OH07, and TN09 viruses, but not ME08. Similarly, when we evaluated PIV5-113 in pigs, we showed that immunity induced by PIV5-113 yielded undetectable virus titers in nasal swab and BALF samples collected in the days after challenge with the Alb09 influenza virus. We were able to detect antibodies against the OH07 and IA06 viruses prior to challenge and against all 4 parental viruses after challenge. Our results also showed high antibody titers against genetically diversified H1 strains, including ME08, IA92, CA09, MI15, and 13E100 viruses.

Interestingly, serum antibodies from PIV5-113-vaccinated pigs were able to recognize viruses that circulated in humans (MI15) and in pigs (13E100) after 2009. This is an important finding because the lastest strain used to create HA-113 was from a 2009 H1N1 isolate (TN09), and MI15 represents a more recent H1N1 isolate that was included in human vaccines. PIV5-113 focuses on the H1 HA, and shows that broad immunity can be induced after vaccination with this single HA. We are using this evidence as a proof-of-concept approach to show that chimeric HA proteins can be created to incorporate epitopes from both swine and human influenza viruses. The DNA-shuffled HA products can be designed based on the dominant HAs circulating in animals and humans, including other subtypes of influenza viruses like H3, H5, and H7 HAs.

DNA shuffling represents an approach to generate universal influenza vaccines by creating random recombinants of distinct HA proteins (McCormick et al., 2015). This approach works by incorporating a broad range of epitopes into the HA protein itself, which differs from some of the more recent efforts that focus on conserved immune targets within the HA protein. Specifically, HA stalk domain vaccine constructs (Steel et al., 2010; Mullarkey et al., 2016; Nachbagauer et al., 2015) and computationally optimized broadly reactive antigen (COBRA) vaccines (Allen et al., 2018; Giles and Ross, 2011) attempt to focus on conserved, broadly-reactive epitopes, including those that are not often highly immunogenic during natural infection (Schepens et al., 2018; Klausberger et al., 2016; Tao et al., 2009). The HA-113 construct randomly incorporates epitopes from both the immunodominant globular head

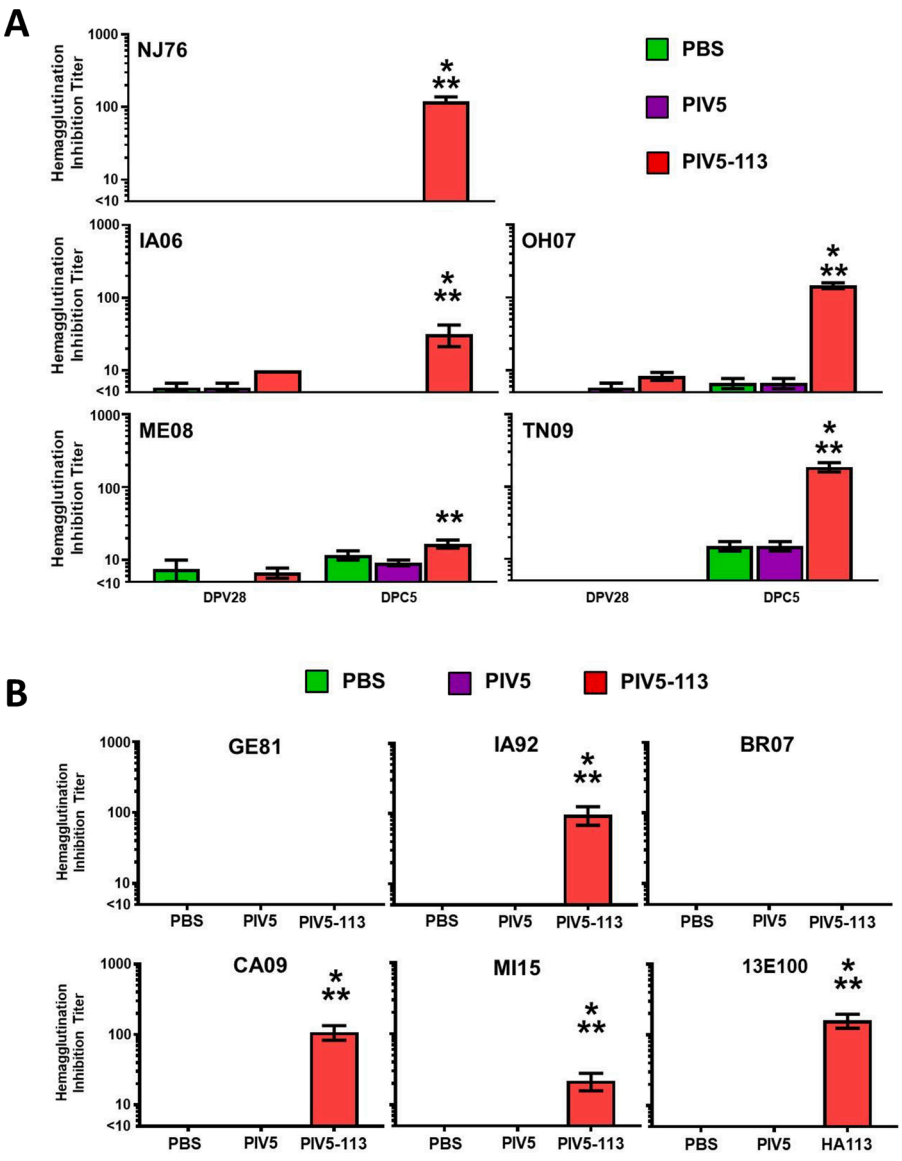

Fig. 6. Evaluation of PIV5-113-induced antibodies against parental and non-parental HAs, using the HAI assay. Pigs were vaccinated with either PBS, PIV5, or PIV5-113, and sera were collected after delivery of a boost, as described above. Results were obtained using sera from PBS-inoculated pigs (n = 6), PIV5-inoculated pigs (n = 6), and PIV5-113-inoculated pigs (n = 6) at day 28 post-vaccination (DPV28) or day 5 post-challenge (DPC5). Panel A shows HAI titers against parental (NJ76, IA06, OH07, and TN09) and the non-parental ME08 HAs presented previously in mice. Panel B shows HAI titers against the non-parental GE80, IA92, BR07, CA09, MI15, and 13E100 HAs. *Indicates a significant difference ($P < 0.05$) compared to PBS-inoculated pigs at the indicated time after vaccination or challenge, determined using One Way ANOVA with a Tukey post-hoc test. **Indicates a significant difference ($P < 0.05$) compared to PIV5-inoculated pigs at the indicated time after vaccination or challenge, determined using One Way Analysis of Variance with a Tukey post-hoc test.

and the stalk domain, which may contribute to the broad antigenicity and protection that we report in this study. As evidenced by the detection of ME08 antibody reactivity at a level that does not yet yield protection, future studies are warranted to improve both the strength and the breadth of PIV5-113-induced immunity. These efforts could include either the addition of adjuvants to improve antibody levels or inclusion of additional virus isolates into the HA construct itself to improve the breadth of immunity induced.

We have chosen PIV5 as a vector for antigen delivery based on a number of characteristics of this paramyxovirus (Tompkins et al., 2007; Jiang et al., 2018; Lee and Lee, 2013; Chen et al., 2012; Li et al., 2013a; Chen et al., 2013). Our previous work has shown that a single immunization with PIV5 expressing either the HA protein of H5N1 avian influenza, the G protein of rabies virus, the F protein of RSV, or the spike protein of MERS-CoV induces immunity against the corresponding pathogens (Li et al., 2013a; Chen et al., 2013; Phan et al., 2014; Li et al., 2020). In addition, PIV5 vectors expressing the influenza virus nucleoprotein can protect against different influenza A virus subtypes in mice (Li et al., 2013c). It will be interesting to evaluate a combination of PIV5-113 and PIV5-NP as a candidate vaccine to increase the breadth of protection. Previously, we have tested PIV5-based candidate vaccines in mice, hamsters, guinea pigs, ferrets, dogs, and monkeys and found that PIV5-based candidate vaccines are effective in those animals (Tompkins et al., 2007; Chen et al., 2012, 2013; Li et al., 2013b,c; Mooney et al., 2017; Mooney et al., 2013; Chen et al., 2008; Chen et al., 2015; Wang et al., 2017). Importantly, this is the first report showing the efficacy of PIV5 as a vector for swine vaccine development. The fact that PIV5-HA113 provided nearly sterilizing immunity against influenza virus challenge in pigs demonstrates that PIV5 is an excellent vector for vaccine development in this species.

In summary, our previous work proved the concept that chimeric HA antigens generated by DNA shuffling could be used to induce broad immunity against genetically diversified influenza viruses that circulate in both pigs and humans; however, we were limited in our ability to express some of the best candidates within intact influenza virions (McCormick et al., 2015). In the current study, we demonstrate that PIV5 can be used to express a chimeric antigen, HA-113, and that immune responses induced by PIV5-113 are protective in both mice and pigs. We expect that the vaccine platform and technologies established in our studies can be applied to HA and NA proteins within different influenza A virus subtypes, including those that remain on our pandemic radar like H2N2, H5N1, and H7N9 (Webby and Webster, 2003; Freidl et al., 2014).

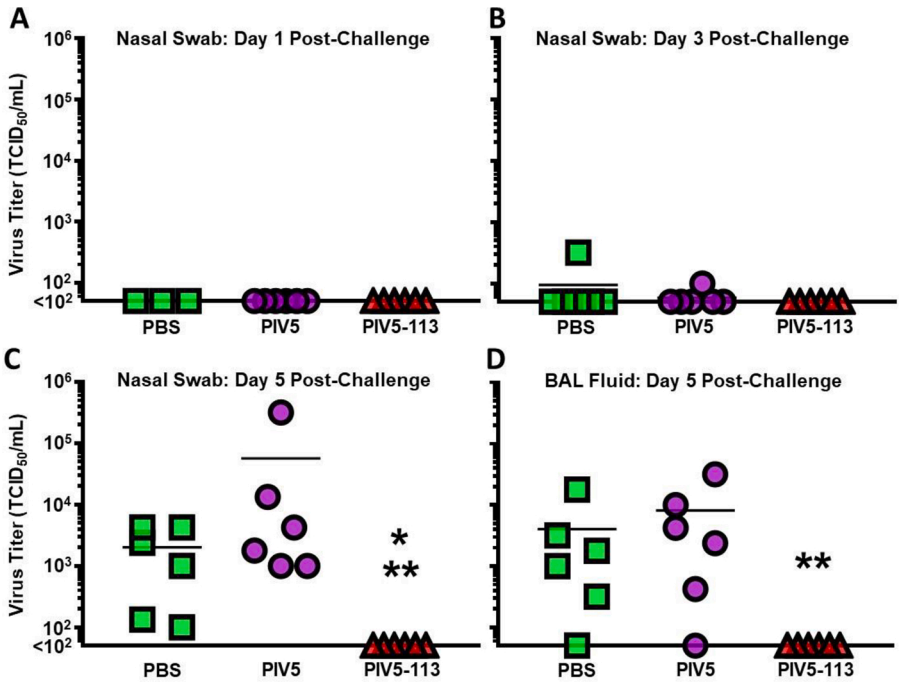

Fig. 7. Virus titers from nasal swab and BALF samples taken from pigs after influenza virus challenge. Nasal swab samples were taken on Days 1 (Panel A), 3 (B), and 5 (C) post-infection and BALF (D) was collected on Day 5 post-infection. Pigs were initially inoculated with either PBS (n = 6), PIV5 (n = 6), or PIV5-113 (n = 6), and were challenged after a boost was delivered. *Indicates a significant difference ($P < 0.05$) compared to PBS-inoculated pigs, determined by Kruskal-Wallis one-way ANOVA. **Indicates a significant difference ($P < 0.05$) compared to PIV5-inoculated pigs, determined using Kruskal-Wallis one-way ANOVA.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108859.

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