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A digitally immune-optimized influenza vaccine broadly neutralizes swine and human H1N1 influenza viruses and protects from heterologous challenge

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ABSTRACT Influenza A virus (IAV) zoonotic transmission and continuous evolution in multiple host species heighten the risk of emerging novel strains at the human-animal interface. Digitally immune-optimized hemagglutinin (HA), neuraminidase (NA), and matrix-2 (M2) vaccine antigens were engineered and combined as a single DNA expression construct (DVX-H1N1) to maximize immune responses to human seasonal, pandemic, and zoonotic H1N1 IAVs. Immunization of mice with DVX antigens induced broad neutralizing antibody responses to human- and swine-origin H1N1 isolates. The DVX-H1N1 candidate was then evaluated in the well-recognized swine challenge model for human influenza in comparison to two benchmark controls representing conventional whole inactivated virus (WIV) vaccines that were either homologous or heterologous to the H1N1 pandemic 2009 subclade 1A.3.3.2 challenge strain, A/swine/England/1353/2009 (H1N1). Serology demonstrated that DVX-H1N1 immunization induced immune responses across a broad spectrum of human and swine H1N1 strains representing antigenic drift and reassortment. Following intranasal virus challenge, nasal shedding of viral RNA was significantly suppressed in the DVX-H1N1 and the homologous control WIV vaccinated groups in comparison to naive controls and the heterologous WIV vaccinated groups. These findings demonstrate that in both swine and mouse models, the DVX-H1N1 vaccine induced broad immune responses and, in pigs, controlled viral infection comparable to conventional strain-matched, but unlike mismatched WIV vaccines.

IMPORTANCE Influenza A virus (IAV) transmission across the animal-human interface is a recognized global health threat. Swine are a key animal reservoir enabling the emergence of novel strains with pandemic potential, posing a threat for animal health and zoonotic spillover to humans. Vaccination remains the most effective intervention against influenza, with current strategies relying on human seasonal strain selection guided by biannual World Health Organization (WHO) recommendations. This approach is challenging owing to viral antigenic drift, reassortment in animal reservoirs, and imperfect antigenic matching between vaccine strains and circulating viruses. These factors reduce vaccine effectiveness and increase threats posed by emerging novel strains, emphasizing the need for next-generation influenza vaccines that improve vaccine-mediated protection and longevity of immunity against an evolving virus. We describe a proof-of-concept digitally immune-optimized antigen vaccine platform with broad immunogenicity and robust efficacy in the swine model, a translational system with the potential to mitigate influenza threats at the human-animal interface.

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KEYWORDS human influenza A virus, broadly protective vaccines, next-generation influenza vaccines, murine, swine, immunogenicity

Influenza A virus (IAV) is a zoonotic virus with human epidemic and pandemic potential (1). IAV infection continues to be a global public health burden, with the World Health Organization (WHO) estimating that a billion people experience seasonal influenza annually, with 3–5 million people affected with severe respiratory illness resulting in 290,000–650,000 deaths (2). Vaccination remains the most effective intervention to mitigate the impact of influenza. Conventional influenza vaccines can limit clinical disease following infection (3, 4) but not prevent nasal shedding of the virus in all vaccinated individuals. Even in years when vaccines are antigenically matched to circulating influenza strains, seasonal influenza vaccines have conferred, on average, only 40% vaccine effectiveness over the last 15 years (5); and despite seasonal immunization, individuals may still develop severe influenza requiring medical intervention. Furthermore, in years when vaccine antigens are poorly matched to circulating viruses, efficacy can be as low as 10% (6).

Influenza vaccine design is challenging because IAVs undergo constant genetic drift due to low-fidelity RNA replication, while the eight-segmented genome enables entire gene segment exchange with co-infecting IAVs (genetic shift). This genetic variability may result in antigenic change if affecting viral envelope proteins hemagglutinin (HA) and neuraminidase (NA), producing viruses with novel gene constellations, and increasing the potential for immune escape, transmission across the animal-human interface, and pandemic emergence (7, 8). Moreover, the HA and NA of an influenza subtype can evolve independently of each other. We constructed a time tree using Nextstrain (9, 10) to show the evolutionary trajectories of H1 and N1 from 2009 to 2025 (Fig. 1). The trees for HA and NA from the same viral strain are connected by lines, and crossed lines suggest different evolutionary paths of HA and NA (Fig. 1).

Swine are important intermediate hosts (17) as they are susceptible to infection by IAVs originating from multiple species, largely due to the presence of both α-2,3- and α-2,6-linked sialic acid receptors, used by avian- and mammalian-origin HA proteins, respectively, for airway cell attachment and infection (7, 18–21). The zoonotic threat posed by swine-origin IAV was exemplified in 2009, when an H1N1 swine-origin reassortant virus spilled over to humans and became pandemic (19, 22, 23). This H1N1 strain, now designated A(H1N1)pdm09 with HA from Clade 1A.3.3.2 (swine-origin IAV nomenclature) (19), rapidly transmitted within human and swine populations globally and continues to be maintained and evolve separately in both host species. Sporadic human infections with so-called variant (v) swine-origin IAV strains from multiple clades continue to be reported and, with continuous genetic diversification, they have the potential to trigger a human pandemic (19, 20, 23).

Following influenza infection or vaccination, the host response is primarily directed against the HA and NA glycoproteins of the virion envelope (6), while other viral components, including the nucleoprotein (NP), non-structural (NS), and matrix proteins (M1 and M2), have less well-characterized roles in the host response to infection and immune-mediated protection (7). Immune responses directed against HA, commonly evaluated using the hemagglutination inhibition (HI) serological test, are used as a surrogate to assess neutralization of virus infectivity. Antibody responses targeting NA also contribute to vaccine protective efficacy in experimental challenge studies (24–26), highlighting the important role both envelope glycoproteins play in immunity, as well as influencing viral fitness, transmission, and immune evasion (27, 28). Moreover, pseudotype virus (PV) assays have provided a convenient, low containment, high-throughput method for evaluating functional antibody-mediated reduction of viral infectivity by HA neutralization or NA inhibition (29–31). The matrix protein 2 (M2) is highly conserved across influenza strains and is frequently incorporated in universal vaccines to enhance immunoprotective responses via non-neutralizing mechanisms and augmentation of HA and NA responses (32–36).

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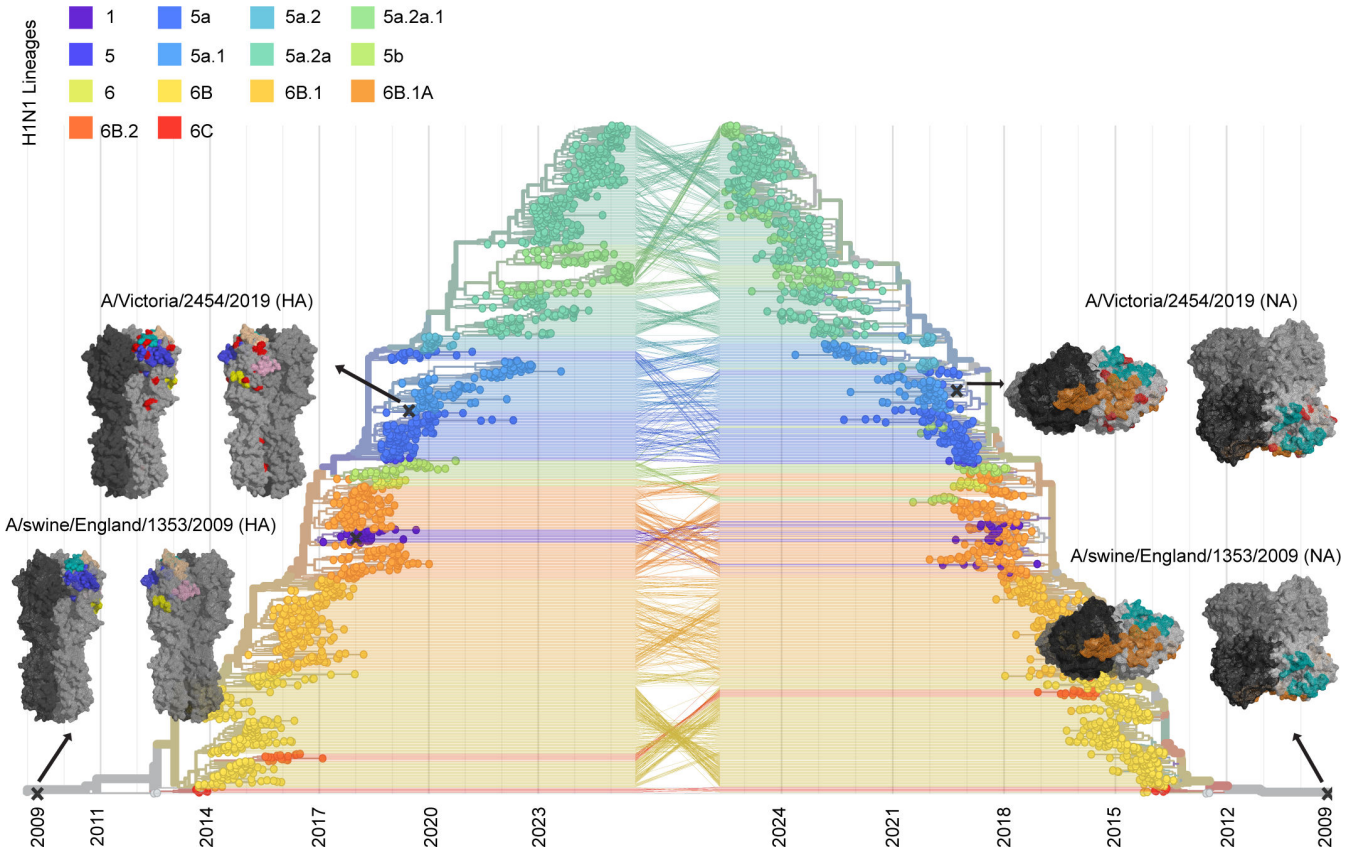


FIG 1 Evolution of HA and NA proteins of the influenza H1N1 pandemic lineage. A time tree representation of HA (left) and NA (right) from H1N1 pandemic lineage viruses circulating in humans since 2009 is depicted. Each sequence is represented as a filled circle and colored according to sub-lineage. H1N1 strains A/swine/England/1353/2009 and A/Victoria/2454/2019, used in this study to produce whole inactivated virus (WIV) vaccines, are indicated by "X" in the time tree to show the evolutionary distance between them. Structures of the ectodomain of HA and the head domain of NA are shown as surface representations, with residues that are different between strains highlighted in red for both HA and NA. The HA antigenic sites (11–13): Sa (light orange), Sb (teal), Cb (yellow), Ca1 (pink), and Ca2 (blue) are also indicated. The antigenic sites of NA are shown in orange and teal. The time trees were generated using Nextstrain (9, 10). Surface representations of HA (14) and NA (15) were generated and rendered using PyMOL (16).

In recent years, different influenza vaccine approaches using chimeric HA (37), consensus-based influenza antigens (COBRA) (38), nanoparticles (39–42), and nucleic acid vaccines (43, 44) have been developed. Early DNA vaccine studies in the 1990s examined T-cell immune responses to IAV NP by immunization of mice with DNA, which reduced viral infection following IAV challenge (45). Since these initial studies, DNA immunization has been shown to induce both B-cell and T-cell immune responses to diverse influenza virus antigens (46–49). Compared with conventional vaccines, DNA vaccines offer sequence-defined antigen expression without the adverse impact of anti-vector immunity, favorable safety profiles, and rapid adaptability to emerging viral variants. Additionally, the physiochemical stability of DNA facilitates vaccine scalability of manufacture, storage, and distribution (50–53). However, their translational immunogenicity is critically dependent on efficient delivery to host cells (52). Needle-free injection delivery of DNA has been shown to enhance DNA plasmid dispersion and cellular uptake in skin or muscle, resulting in improved humoral and cellular immune responses relative to conventional needle-syringe administration and approaching those achieved with more invasive physical delivery methods (54, 55).

To provide maximum immunological breadth, we used the Digitally Immune Optimized Synthetic Vaccine (DIOSynVax) platform (56–58) to engineer vaccine antigens informed by widely available IAV sequence data from animal- and human-origin viruses. We focused on HA, NA, and M2 from H1N1 influenza viruses to maximize coverage

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of human seasonal, pandemic, and zoonotic IAV strains. Here, we demonstrate that antigens designed using the DIOSynVax (DVX) platform, delivered by DNA immunization, were immunogenic and produced robust immune responses in both mice and domestic pigs. In pigs, a well-established large animal model for human influenza (59), the DVX-H1N1 vaccine candidate also induced highly efficacious immune protection following a robust A(H1N1)pdm09 swine-origin virus challenge with strain A/swine/England/1353/2009 (59, 60).

RESULTS

In silico DVX HA and NA antigen validation

With the diversity and continuing evolution of both HA and NA components of circulating H1N1 viruses (Fig. 1) as major factors to consider when designing next-generation vaccines, we synthetically engineered gene sequences for HA, NA, and M2 using the Digital Immune Optimized Synthetic Vaccine (DVX) platform (56–58) with the aim of eliciting broad immune responses to A(H1N1)pdm09 influenza viruses. Three antigens were computationally designed separately: a HA antigen—DVX-H1 (Fig. 2A; Fig. S1), NA—DVX-N1 (Fig. 2B; Fig. S2), and M2—DVX-M2 (Fig. 2C; Fig. S3). We utilized data sets up to the year 2018 from the NCBI database of non-redundant HA sequences from the H1N1 pandemic lineage, NA sequences from all HxN1 subtypes, and M2 sequences from all IAV subtypes to design DVX-H1, DVX-N1, and DVX-M2, respectively. Phylogenetic reconstruction of the sequences was performed to engineer antigens that were evolutionarily closest to all the input sequences. Due to the evolutionary relatedness between the input sequences and the nucleotide evolution model used in the DVX pipeline, this new class of computational antigens captures both conserved and distinct broadly reactive epitopes across H1N1 viruses (Fig. 1 and 2A through C). The selected antigens were then functionally validated *in vitro* by transfecting H293T cells with the DVX-H1, DVX-N1, and DVX-M2 DNA expression plasmids. After 24 h, cell surface expression of individual HA (Fig. S4), NA (Fig. S5), and M2 (Fig. S6) antigens was detected by flow cytometry (supplemental methods), verifying translation of these DNA antigens into fully expressed proteins.

DVX-designed HA and NA antigens elicit broad neutralizing responses in mice

Immunogenicity of the individual DVX H1, N1, and M2 antigens was first evaluated in mice by four subcutaneous (SC) immunizations of 50 µg DNA with a 14-day interval, corresponding to 0, 14, 28, and 42 days post-vaccination (dpv) (Table 1). Mice in control groups were immunized with DNA-encoded proteins representing recommended WHO candidate vaccine viruses at the time of DVX antigen design, specifically $H1_{ctrl}$ (A/Michigan/45/2015), $N1_{ctrl}$ (A/Brisbane/2/2018), and $M2_{ctrl}$ (A/Brisbane/2/2018) (Table 1). The empty plasmid vector (pEVAC) was administered to the negative control group.

The functional humoral immune response to HA elicited at study end on 70 dpv was evaluated using neutralization assays (pMN) employing an H1 PV panel (Table 2). All mice immunized with DVX-H1 developed an immune response against all H1 PV tested (Fig. 2D); although titers for individual animals were below 10^3 IC_{50} for PVs representing BR/59/07 and VIC/2570/19. Serum PV neutralizing titers elicited by the $H1_{ctrl}$ antigen could be detected against most human seasonal vaccine H1 strains tested, apart from SC/1/1918, a PV representing the ancestral 1A pandemic influenza virus HA, A/South Carolina/1/1918, the pre-2009 vaccine strain BR/59/07 (HA Clade 1B1.2), and the swine-origin strains sw/GX/13 and sw/HN/18 (HA Clade 1C.2.3). As expected, the pEVAC (empty) plasmid control DNA did not elicit any measurable immune response. HI assays using available, representative H1 viruses (Table 3) were then conducted to evaluate HA head domain-specific antibody responses in the immunization groups. HI titers to representative H1N1 seasonal strains CA/07/09, MI/45/15, and BR/2/18 were detected, as expected, in mice immunized with the matched $H1_{ctrl}$, but only in some of the mice in the group

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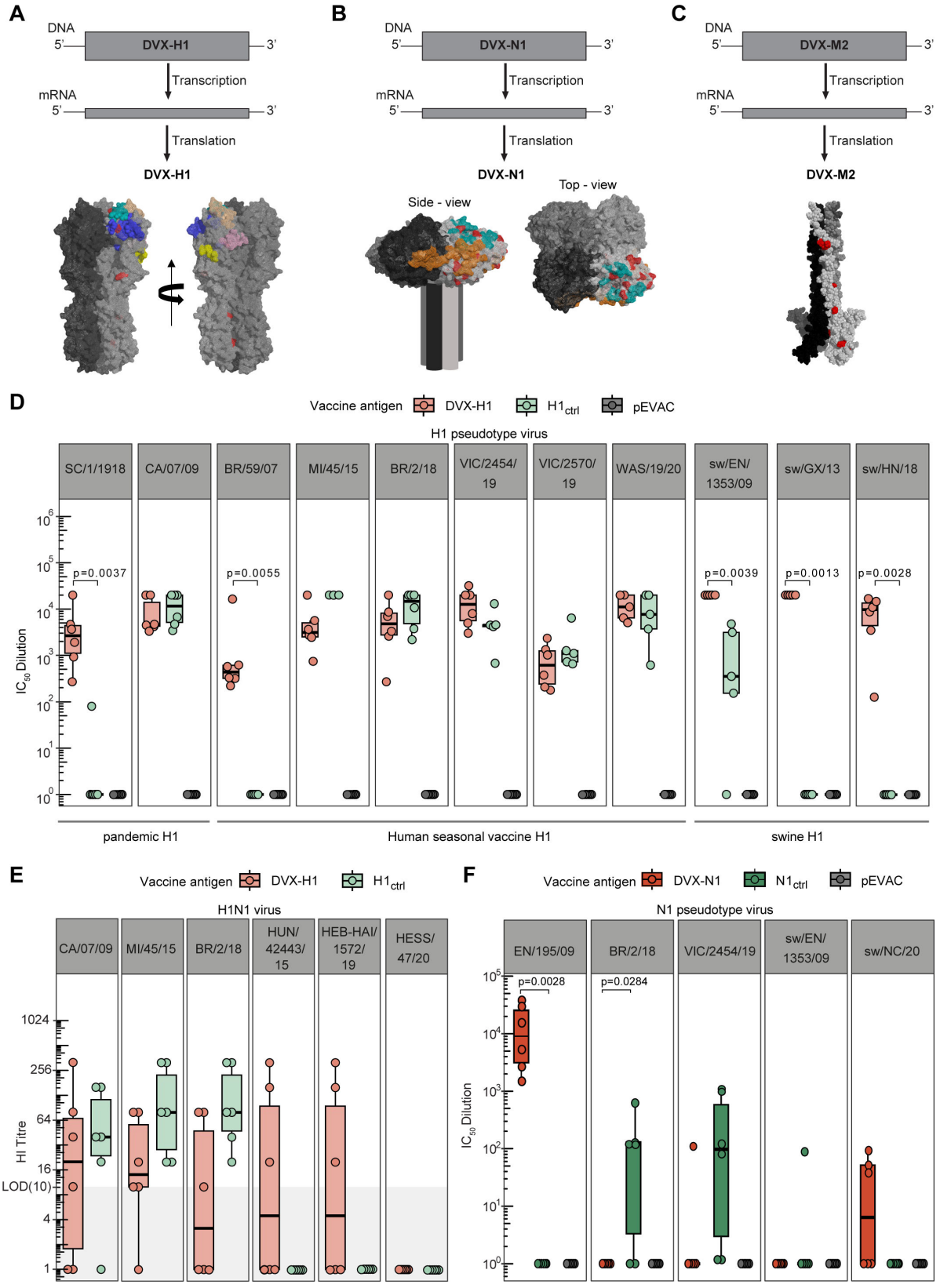


FIG 2 DVX immunogen designs and evaluation of mouse serological responses. (A) Mice were immunized with pEVAC DNA plasmid encoding DVX-H1, expressing full-length trimeric HA upon translation. Surface representation of the ectodomain of DVX-H1 is shown, and amino acid differences with the challenge strain A/swine/England/1353/2009 HA are shown in red. The HA antigenic sites (11–13) are highlighted: Sa (light orange), Sb (teal), Cb (yellow), Ca1

(Continued on next page)

Fig 2 (Continued)

(pink), and Ca2 (blue). (B) Mice were immunized with pEVAC DNA plasmid encoding DVX-N1, expressing full-length tetrameric NA upon translation. Surface representation of the ectodomain of DVX-N1 is shown, and amino acid differences with A/swine/England/1353/2009 NA are highlighted in red. (C) Mice were immunized with pEVAC DNA plasmid encoding DVX-M2, expressing full-length tetrameric M2 upon translation. Surface representation of full-length DVX-M2 is shown, and amino acid differences with A/swine/England/1353/2009 M2 are highlighted in red. Surface representations of DVX-H1, DVX-N1, and DVX-M2 were generated and rendered using Pymol (16). (D) Neutralization of a panel of H1 PVs representing pandemic, human seasonal, and swine-origin viruses (Table 2) tested using serum samples obtained at 70 dpv. Serum neutralizing titers are represented as IC_{50} values (fold dilution of sera that resulted in 50% PV neutralization). (E) HI titers measured using a panel of H1N1 virus strains (Table 3). The lower limit of detection (LOD) is indicated at 10, and all values within the shaded gray area are considered negative for HI. (F) Inhibition of N1 PVs determined via pELLA. IC_{50} values (fold dilution of sera that resulted in 50% PV inhibition) are shown. For panels D–F, the median and interquartile range of individual mouse serum samples are shown (n = 6) per vaccination group. For plot D, due to serum availability, five samples (n = 5) from $H1_{ctrl}$ were tested against VIC/2454/19, WAS/19/20, VIC/2570/19, sw/EN/1353/09; and three samples (n = 3) against MI/45/15. Only significant comparisons ($P < 0.05$) between DVX-H1 vs $H1_{ctrl}$ (D and E), and DVX-N1 vs $N1_{ctrl}$ (F) as determined by Wilcoxon signed-rank test are shown for brevity. All other P values are tabulated in Table S1.

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immunized with DVX-H1 (Fig. 2E). Three of six mice immunized with DVX-H1 also developed HI responses to viruses representing zoonotic swine-origin human variant strains HUN/42443/15, and HEB-HAI/1572/19 (HA from Clade H1C.2.3). In contrast, HI titers to these A(H1)v strains were not detected in $H1_{ctrl}$ immunized mice. Neither DVX-H1 nor $H1_{ctrl}$ immunization elicited a serological response to the HESS/47/20 (clade 1C.2.2) strain. The variation in antibody titers between individual animals in a group likely reflects the limitations associated with DNA SC immunization of mice, which we have observed in previous studies.

Neuraminidase inhibition (NAI) activity of serum samples obtained at 70 dpv from mice immunized with DVX-N1, $N1_{ctrl}$, and pEVAC plasmid-only control was assessed by pseudotype enzyme-linked lectin assay (pELLA) using an N1 PV panel (Table 2). Both DVX-N1 and $N1_{ctrl}$ sera showed limited responses to the N1 PV tested. Serum NAI activity was detected in mice immunized with DVX-N1 only to EN/195/09, and in 3/6 mice to sw/NC/20 PVs (Fig. 2F). $N1_{ctrl}$ immunization elicited NAI activity to the homologous BR/2/18 strain and lower inhibition (~10^2 IC_{50}) to another human seasonal strain, VIC/2454/19. Sera from mice immunized with DVX-M2 and $M2_{ctrl}$ produced anti-M2 binding titers; however, there was no significant difference in the results between the DNA immunogens and the pEVAC control (data not shown). Based on the reported enhanced immunogenicity of influenza vaccines incorporating multiple antigens (38, 43), we then designed a DNA vaccine for characterization in a large animal model for human influenza.

DVX-H1N1 elicits immune responses comparable to adjuvanted whole inactivated virus vaccines in pigs

To assess potential enhancement of immunogenicity and facilitate production, DVX-H1, DVX-N1, and DVX-M2 were combined into a single DNA antigen string linked by 2A peptide sequences (Fig. 3A) to allow expression of multiple proteins from a single open reading frame (61). This DVX-H1N1 vaccine candidate was characterized in the well-recognized pig model for influenza by comparing immunogenicity and efficacy with

TABLE 1 DNA immunogens used in mouse immunogenicity studies[a]

Vaccine ID	Antigen	Component	Animals per group	Dose (µg)	Delivery	Days post-vaccination (dpv)
DVX-H1	Hemagglutinin (H1)	DNA	6	50	50 µL SC	0, 14, 28, and 42
DVX-N1	Neuraminidase (N1)	DNA	6	50	50 µL SC	0, 14, 28, and 42
DVX-M2	M2 protein (M2)	DNA	6	50	50 µL SC	0, 14, 28, and 42
$H1_{ctrl}$	A/Michigan/45/2015 (H1)	DNA	6	50	50 µL SC	0, 14, 28, and 42
$N1_{ctrl}$	A/Brisbane/2/2018 (N1)	DNA	6	50	50 µL SC	0, 14, 28, and 42
$M2_{ctrl}$	A/Brisbane/2/2018 (M2)	DNA	6	50	50 µL SC	0, 14, 28, and 42
pEVAC	Plasmid vector with no insert	DNA	6	50	50 µL SC	0, 14, 28, and 42

[a]SC, subcutaneous; dpv, days post-vaccination.

TABLE 2 Virus strains and sequences used to generate HA and NA pseudotype viruses and $H1_{ctrl}$, $N1_{ctrl}$, and $M2_{ctrl}$

Virus strain	Designation	Abbreviation	Accession no.	HA clade
HA				
A/South Carolina/1/1918 (H1)	Pandemic 1918	SC/1/1918	AF117241	1A.1
A/California/07/2009 (H1)	Seasonal pdm09	CA/07/09	MN596845	1A.3.3.2
A/England/195/2009 (H1)	Seasonal pdm09	EN/195/09	GQ166661.1	1A.3.3.2
A/Brisbane/59/2007 (H1)	Seasonal pre-2009	BR/59/07	CY058487	1B.2
A/Michigan/45/2015 (H1)	Seasonal pdm09	MI/45/15	EPI662594	1A.3.3.2
A/Brisbane/2/2018 (H1)	Seasonal pdm09	BR/2/18	EPI1383389	1A.3.3.2
A/Victoria/2454/2019 (H1)	Seasonal pdm09	VIC/2454/19	EPI1701884	1A.3.3.2
A/Victoria/2570/2019 (H1)	Seasonal pdm09	VIC/2570/19	EPI1741926	1A.3.3.2
A/Washington/19/2020 (H1)	Seasonal pdm09	WAS/19/20	MT331513	1A.3.3.2
A/swine/England/1353/2009 (H1)	Swine pdm09	sw/EN/1353/09	KR701097.1	1A.3.3.2
A/swine/Guangxi/BB1/2013 (H1)	Swine	sw/GX/13	KJ725056	1C.2.3
A/swine/Henan/SN/10/2018 (H1)	Swine	sw/HN/18	MN416619	1C.2.3
NA				
A/England/195/2009 (N1)	Seasonal pdm09	EN/195/09	GQ166659	
A/Brisbane/2/2018 (N1)	Seasonal pdm09	BR/2/18	EPI1312565	
A/Victoria/2454/2019 (N1)	Seasonal pdm09	VIC/2454/19	EPI1701883	
A/swine/England/1353/2009 (N1)	Swine pdm09	sw/EN/1353/09	KR701099.1	
A/swine/North Carolina/A02478985/2020 (N1)	Swine	sw/NC/20	MT020063	
M2				
A/Brisbane/2/2018 (M2)	Seasonal pdm09	BR/2/18	EPI1312561	

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conventional, WIV vaccines derived from strains A/swine/England/1353/2009 (WIV_{1353}) and A/Victoria/2454/2019 (WIV_{Vic}) formulated in a light paraffin oil adjuvant licensed for pig vaccination (Table 4). Using a prime-boost schedule with a 28-day interval (Fig. 3B), groups of pigs (n = 5/group) were immunized by intradermal Needle-free Injection System (NFIS) Tropis (PharmaJet, Golden, CO) administration of DVX-H1N1 DNA. Comparator adjuvanted benchmark WIV vaccines were administered intramuscularly according to standard approaches for pig vaccination. No adverse clinical signs were observed after immunization. A fourth group of naive control pigs was not immunized.

Serum samples obtained at 42 dpv were used to assess the humoral responses induced by DVX-H1N1 and WIV immunizations using panels of H1 and N1 PV (Table 2). Anti-H1 serum neutralizing responses ($IC_{50} \geq 10^3$) were detected in all immunized pigs against the majority of H1 (Fig. 3C). However, DVX-H1N1 and WIV_{Vic} immunization elicited low responses ($IC_{50} < 10^3$) in some animals when evaluated with the pre-2009 human seasonal influenza vaccine virus BR/59/07 H1 PV, while no neutralizing responses to sw/GX/13 H1 PV were detected in the majority of animals (Fig. 3C). All immunized

TABLE 3 Panels of virus strains used in the hemagglutination inhibition (HI) assay

Virus strain	Abbreviation	H1N1 clade information (swine nomenclature extended to human isolates)
Murine panel		
A/California/07/2009 (H1N1)	CA/07/09	H1A.3.3.2 human seasonal vaccine strain 2010–2016
A/Michigan/45/2015 (H1N1)	MI/45/15	H1A.3.3.2 human seasonal vaccine strain 2017–2019
A/Brisbane/2/2018 (H1N1)	BR/2/18	H1A.3.3.2 human seasonal vaccine strain 2019–2020
A/Hunan/42443/2015 (A(H1)v)	HUN/42443/15	H1C.2.3 Eurasian avian-like variant strain
A/Hebei-Haigang/1572/2019 (A(H1)v)	H-H/1572/219	H1C.2.3 Eurasian avian-like variant strain
A/Hessen/47/2020 (H1N1)	HESS/47/20	H1C.2.2 Eurasian avian-like variant strain
Swine panel		
A/Victoria/2454/2019 (H1N1)	VIC/2454/19	H1A.3.3.2 human seasonal vaccine strain 2020-2021, homologous to WIV_{VIC}
A/England/195/2009 (H1N1)	EN/195/09	H1A.3.3.2 variant and human seasonal vaccine candidate
A/swine/England/1353/2009 (H1N1)	sw/EN/1353/09	H1A.3.3.2 challenge strain homologous to WIV_{1353} (Table 4)
A/swine/England/13C4WA/2022 (H1N1)	sw/EN/13C4WA/22	H1A.3.3.2 contemporary swine-origin H1N1 strain

TABLE 4 Antigens used in swine immunogenicity and challenge study[b]

Vaccine group	Antigen	Component	Adjuvant	Pigs per group	Antigen amount	Total dose volume	Delivery[a]
DVX-H1N1	Multivalent H1, N1, M2	DNA	None	5	800 µg	400 µL	ID 4 × 100 µL
WIV_{1353}	A/swine/England/1353/2009 (H1N1)	Inactivated whole virus	Light paraffin oil	5	1,280 HAU	3 mL	IM 2 × 1.5 mL
WIV_{Vic}	A/Victoria/2454/2019 (H1N1)	Inactivated whole virus	Light paraffin oil	5	1,280 HAU	3 mL	IM 2 × 1.5 mL
Naive	Not vaccinated	–	None	5	–	–	–

[a]Prime (0 dpv) and boost (28 dpv) immunizations were identical.
[b]WIV, whole inactivated virus; HAU, hemagglutination units; ID, intradermal; IM, intramuscular; dpv, days post-vaccination.

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groups showed serological NAI activity to human seasonal and swine N1 PV tested (Fig. 3D). WIV_{1353} immunization produced significantly higher NAI titers compared to DVX-H1N1 immunization when evaluated using the seasonal N1 PVs. As expected, sera from the WIV_{Vic}-immunized group demonstrated notably higher peak N1 NAI activity to the homologous human seasonal N1 PV VIC/2454/19 and related BR/2/18. Taken together, these data demonstrated the immunogenicity of DVX-H1N1.

DVX-H1N1 vaccine efficacy in swine correlates with both humoral and cell-mediated immune responses

We then investigated the vaccine efficacy of DVX-H1N1 immunization compared to adjuvanted, strain-based, whole inactivated control vaccines (WIV_{Vic} and WIV_{1353}) in the well-recognized pig challenge model for human influenza. All pigs were challenged intranasally with a swine-origin A(H1N1)pdm09 strain, A/swine/England/1353/2009 (62), on 69 dpv (corresponding to 0 days post-challenge [dpc]) (Fig. 3B). Clinical signs were monitored daily after virus inoculation until study end using an established clinical scoring system (63). Signs were not apparent or mild and transiently reached no more than 3 out of a possible maximum of 21, largely owing to a slight increase in rectal temperatures in all animals on 4–5 dpc (Fig. S7A).

Viral RNA was monitored in longitudinal nasal samples by real-time reverse transcription PCR (RRT-qPCR) to provide a rapid and sensitive measure of nasal shedding. Pigs immunized with DVX-H1N1 and the WIV_{1353} vaccine homologous to the challenge virus showed considerably reduced nasal shedding of viral RNA (Fig. 4A), corresponding to levels near the lower limit of infectious virus quantification by conventional titration (60, 62). In comparison, nasal shedding in WIV_{Vic}-immunized and naive animals was detected between 2 and 5 dpc (Fig. 4A) and had a biphasic profile typical of a productive challenge with a moderate dose of virus infecting target cells in successive waves, as previously demonstrated (60, 62). Interestingly, animals immunized with the heterologous adjuvanted WIV_{Vic}, a human seasonal vaccine strain, failed to protect against infection with elevated viral RNA shedding at similar levels to non-vaccinated animals. Area under the curve analysis showed this post-challenge reduction in RNA shedding of the DVX-H1N1 and WIV_{1353} immunized animals to be significant ($P < 0.05$) compared to naive or mismatched control WIV_{Vic} vaccines (Fig. 4B), indicating that the DVX-H1N1 vaccine markedly suppressed viral replication over time as efficiently as a homologous strain-matched adjuvanted WIV_{1353} vaccine. Necropsy samples obtained on 8 or 9 dpc revealed that viral RNA in nasal swabs or respiratory tract samples from any vaccinated group was below the limit of quantification (LOQ) threshold for the assay apart from detection at very low levels in the nasal turbinate sample from one unvaccinated pig (Fig. S7B), indicating resolution of infection.

The humoral immune responses in all immunization groups were then characterized by analyzing influenza virus-specific serum antibody levels monitored longitudinally on 0, 28, 42, and 63 dpv by HI, PV HA neutralization, and NA inhibition assays, and ELISA to detect NP antibodies (Fig. 5).

The HI panel included human- and swine-origin HA 1A.3.3.2 strains spanning 13 years (Table 3). HI titers in all immunized groups increased between 28 and 42 dpv for all virus antigens tested (Fig. 5A through D). The control WIV_{1353} and WIV_{Vic} immunogens

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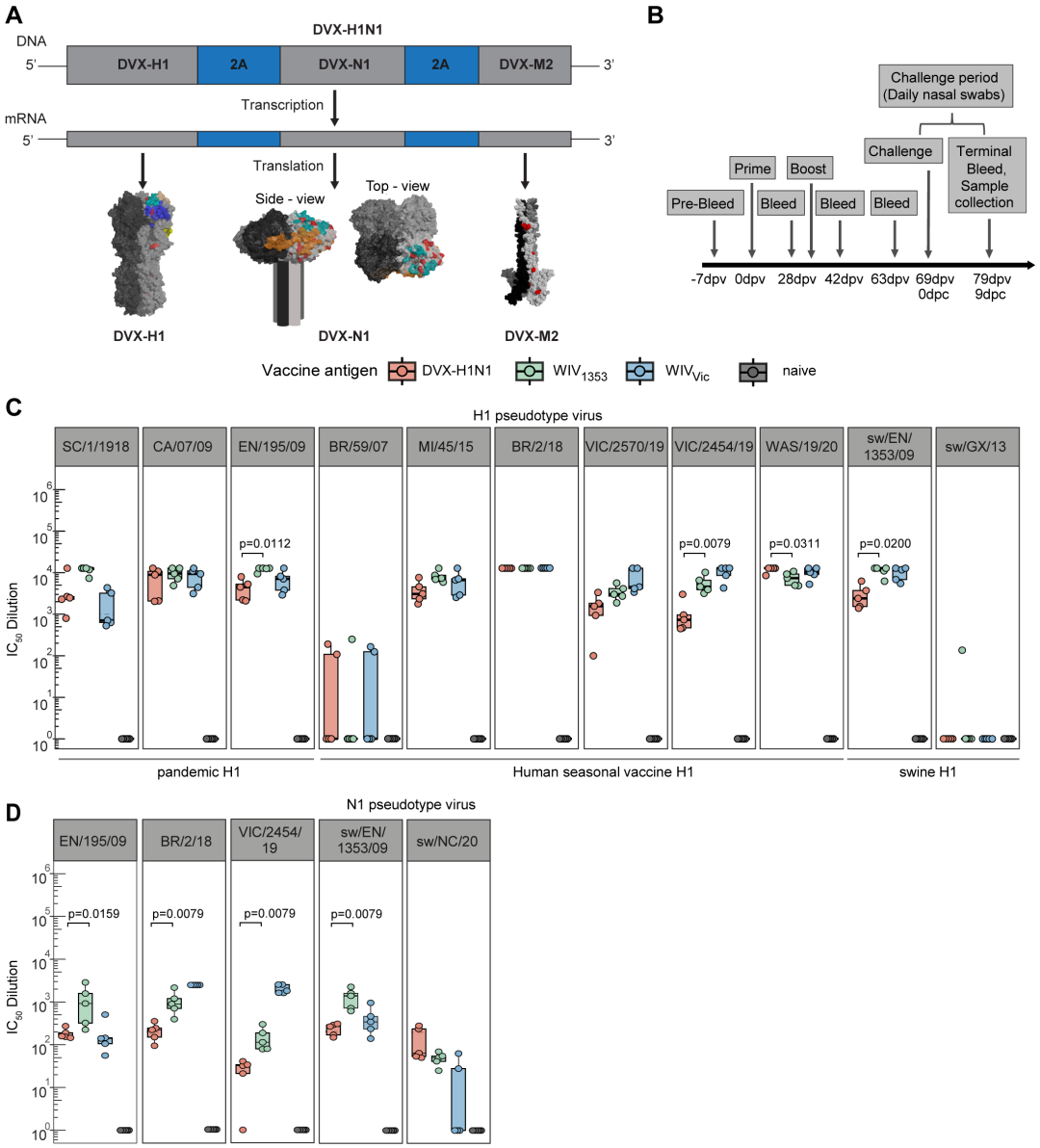


FIG 3 DVX-H1N1 vaccine design, study schedule for pigs immunized with DVX-H1N1, positive control WIV vaccines, WIV_{1353} and WIV_{Vic}, as well as naive, non-vaccinated animals, and evaluation of serological responses. (A) Pigs were immunized with a single DNA plasmid encoding DVX-H1, DVX-N1, and DVX-M2, linked by 2A peptides and expressed as individual trimeric HA, tetrameric NA, and tetrameric M2, respectively, and shown as surface representations. (B) Immunization, sampling, and challenge schedule of pigs immunized with DVX-H1N1 and control WIV vaccines (Table 4). Animals were challenged with 1.67×10^6 $TCID_{50}$ A/swine/England/1353/2009 (H1N1) 69 dpv. (C) Neutralization of H1 PVs representing pandemic, seasonal, and swine zoonotic H1 strains (Table 2) evaluated using serum samples obtained at 42 dpv. Neutralizing titers are represented as IC_{50} dilution values. (D) Inhibition of N1 PV (Table 2) with serum obtained at 42 dpv. IC_{50} dilution values were determined via pELLA. For panels C and D, the median and interquartile range of individual pig serum samples are shown ($n = 5$) per vaccination group. Only significant comparisons (P values < 0.05) between DVX-H1N1 vs WIV_{1353} as determined by the Wilcoxon signed-rank test are shown for brevity. All other P values are tabulated in Table S1.

induced significantly higher HI titers compared to DVX-H1N1 when measured on 28 dpv, and these titers increased by 42 dpv. As expected, the highest titers were elicited by the homologous antigens A/swine/England/1353/2009 and A/Victoria/2454/2019 in the WIV_{1353} and WIV_{Vic}-immunized groups, respectively. Titers in all immunized groups were also higher when evaluated using A(H1N1)pdm09 human seasonal vaccine strain A/England/195/2009 antigen compared to the swine-origin antigen A/swine/England/13C4WA/2022. Serum neutralizing HA titers monitored using PVs homologous

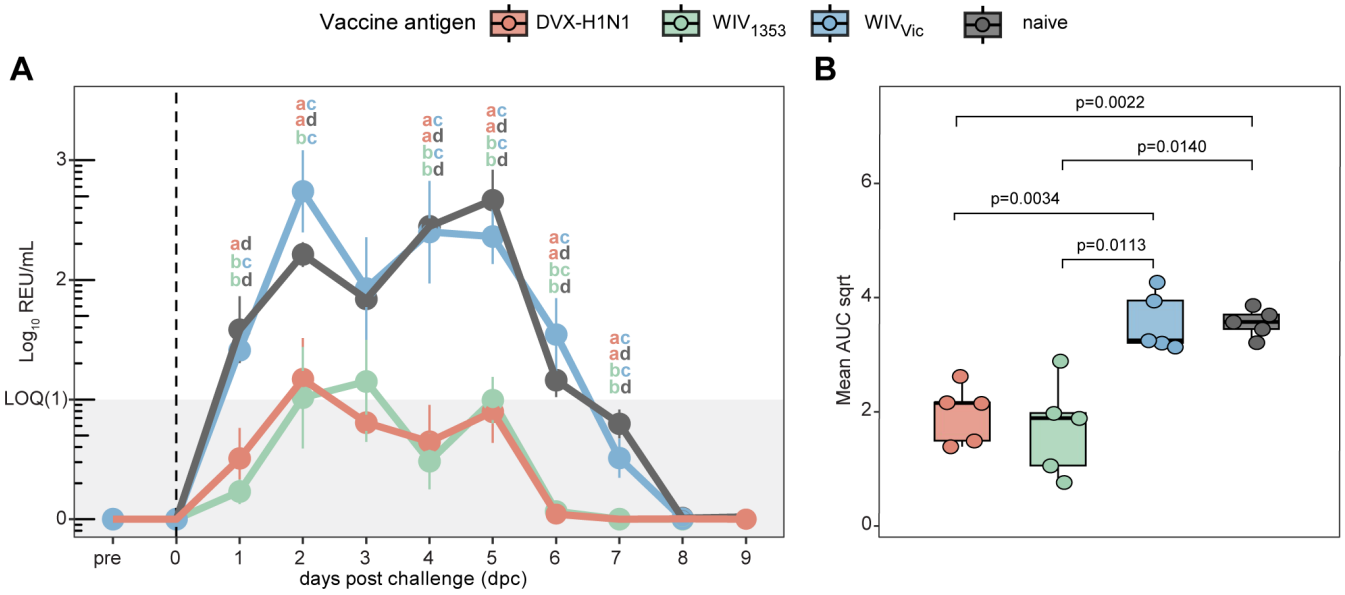


FIG 4 Viral RNA shedding following challenge of all pigs with the swine-origin A(H1N1)pdm09 virus A/swine/England/1353/2009. RNA quantified by RRT-qPCR in daily nasal swab samples is expressed as (A) mean $\log_{10}$ relative equivalent units (REU)/mL (± SEM) over time (x-axis: dpc), with the area below the LOQ shaded in gray; and (B) the square root of the area under the curve (AUC sqrt). For panel A, significant comparisons between groups (a [orange] = DVX-H1N1, b [green] = WIV_{1353}, c [blue] = WIV_{Vic}, and d [gray] = naive) were calculated by Kruskal-Wallis analysis, followed by the post hoc Dunn's test, and are denoted as letters (ac: DVX-H1N1 vs WIV_{Vic}; ad: DVX-H1N1 vs naive; bc: WIV_{1353} vs WIV_{Vic}; bd: WIV_{1353} vs naive). Actual *P* values are tabulated in Table S3. For panel B, comparisons with significant differences are denoted as *P* values calculated using the Brown-Forsythe and Welch ANOVA and Dunnett's T3 comparisons test.

to the conventional WIV vaccines showed a similar humoral response over time (Fig. 5E and F), with the highest immunization phase titers observed at 42 dpv with minimum IC_{50} values of 10^3. Similarly, NAI activity specific for A/swine/England/1353/2009 (N1 PV) (Fig. 5G) and A/Victoria/2454/2019 (N1 PV) (Fig. 5H) was detected. As expected, in all groups, titers were higher for homologous antigen and immunogen combinations. Additionally, during the immunization phase, NP-specific antibody responses were detected in the WIV-vaccinated groups, but not in the group that received DVX-H1N1, which does not contain NP antigen (Fig. 5I). This distinct response is advantageous for Differentiating Infected from VAccinated (DIVA) vaccine strategies (64).

Following challenge, the HI antibody response to all viruses tested increased. Elevated HI titers specific for the challenge strain were detected at 8 dpc in all groups, including non-immunized naive animals (Fig. 5A through D), indicative of an effective challenge. HA serum neutralizing and NAI titers obtained using PV assays (Fig. 5E through H) showed similar patterns to the HI assays, with a marked increase in IC_{50} titers following challenge. In all groups, serological responses to antigens homologous to the challenge strain were higher compared to heterologous antigens [belonging to the same A(H1N1)pdm09 clade but drifted over time] in the vaccination-virus combinations. Notably, despite high anti-HA and NAI titers elicited by immunization with the WIV_{Vic} vaccine, representing a human seasonal vaccine from 2019 that is antigenically drifted but belongs to the same clade as the challenge strain, nasal shedding of viral RNA was not suppressed following challenge in this group (Fig. 4). The NP antibody titers remained high in both WIV vaccinated groups at 8 dpc. As expected, anti-NP titers rose rapidly in the non-vaccinated group upon challenge, following a similar trend with the DVX-H1N1 vaccinated group (Fig. 5I), indicating a robust immunological challenge and reinforcing the effectiveness of DVX-H1N1 in reducing nasal shedding of viral RNA post-challenge (Fig. 4A).

IFN-γ cell-mediated immune responses were also assessed by collecting PBMCs longitudinally post-vaccination and post-challenge from all groups and stimulating them with the same whole inactivated vaccine antigens used to immunize the positive control

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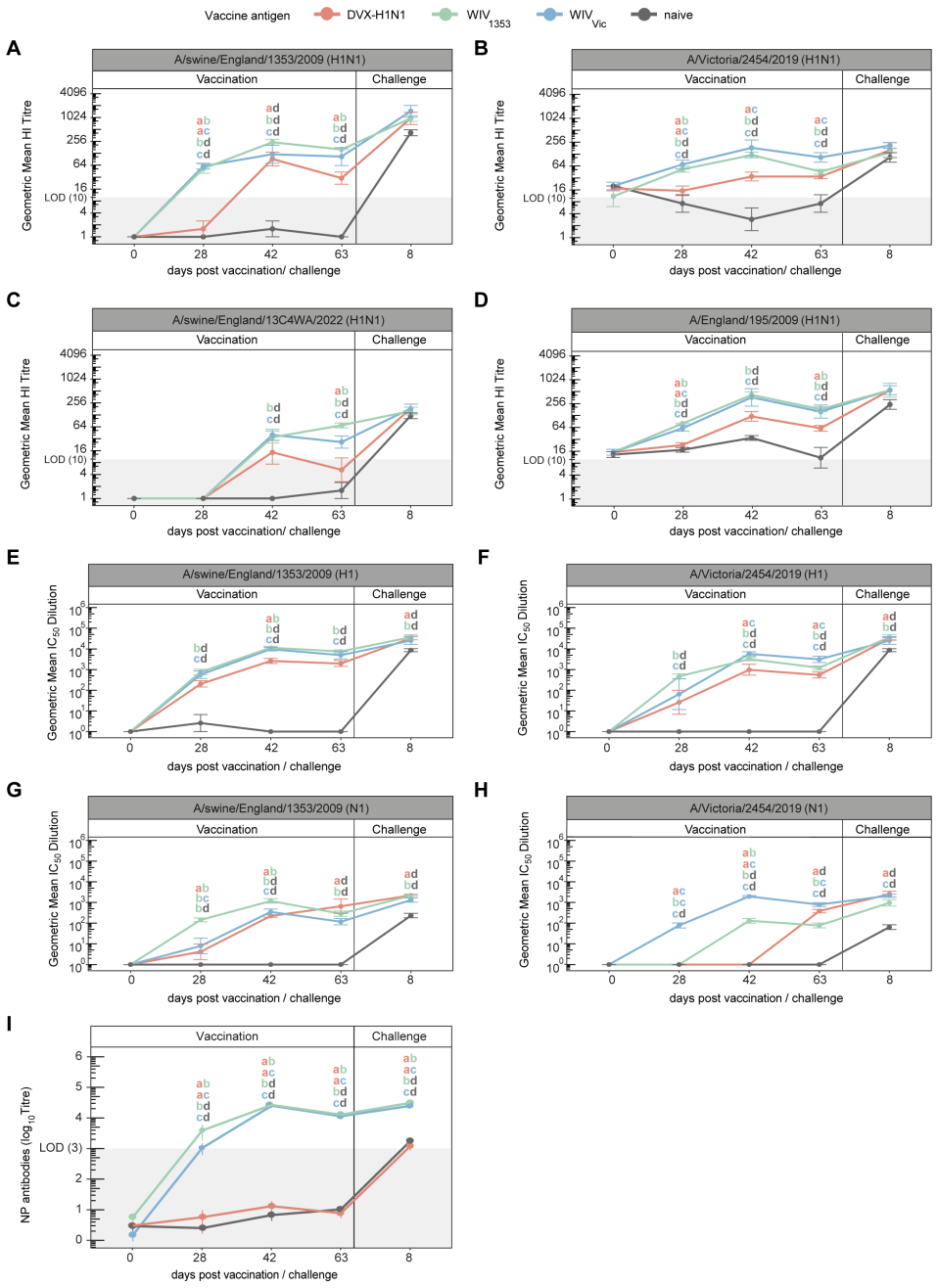


FIG 5 Longitudinal humoral immune responses in vaccinated-challenged pigs evaluated at 0, 28, 42, and 63 dpv, and terminally at 8 dpc. Geometric mean HI titers measured using the pandemic 2009 A(H1N1)pdm09 virus antigens; (A) A/swine/England/1353/2009 (WIV_{1353}), (B) A/Victoria/2454/2019 (WIV_{Vic}), (C) a contemporary swine-origin strain, A/swine/England/13C4WA/2022, and (D) A/England/195/2009. Area below the LOD is shaded in gray, and values within this range are considered negative. Geometric mean serum neutralizing IC_{50} titers evaluated using HA PV assays based on (E) A/swine/England/1353/2009 and (F) A/Victoria/2454/2019. Geometric mean of NA inhibition IC_{50} values monitored via pELLA using NA PV based on (G) A/swine/England/1353/2009 and (H) A/Victoria/2454/2019. (I) Serum antibody titers (mean and standard error) specific for influenza A virus NP evaluated using an indirect ELISA (IDvet). Area below the LOD is shaded in gray, and values within this range are considered negative. For panels A–H, the geometric mean and standard error of individual pig serum samples are shown (n = 5 per vaccination group). Significant comparisons between groups (a [orange] = DVX-H1N1, b [green] = WIV_{1353}, c [blue] = WIV_{Vic}, and d [gray] = naive) were calculated by Kruskal-Wallis analysis, followed by post hoc Dunn's test and are denoted as letters (ab: DVX-H1N1 vs WIV_{1353}; ac: DVX-H1N1 vs WIV_{VIC}; ad: DVX-H1N1 vs naive; bc: WIV_{1353} vs WIV_{VIC}; bd: WIV_{1353} vs naive; cd: WIV_{VIC} vs naive). Actual P values are tabulated in Table S4.

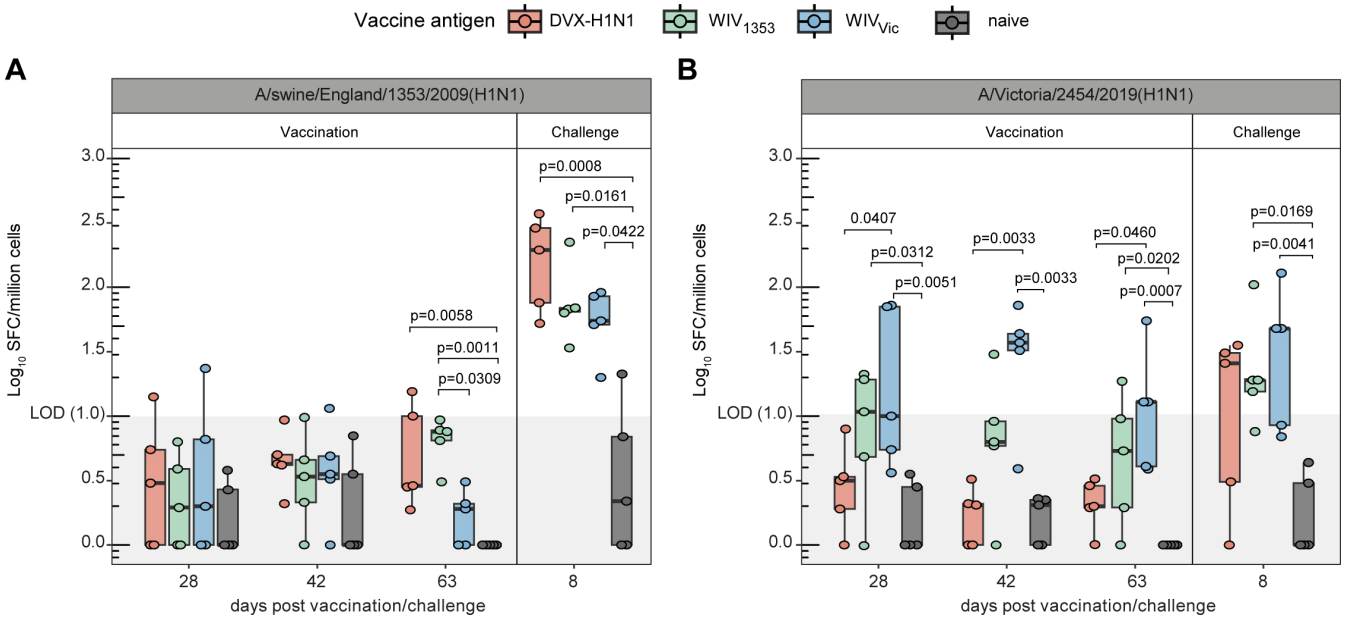


FIG 6 Cellular immune responses in vaccination groups monitored during the immunization period and post-challenge. IFN-γ responses of PBMCs isolated 28 days post-vaccination (28 dpv), 2 weeks after boost immunization (42 dpv), 1 week prior to challenge (63 dpv), and 8 dpc were evaluated by ELISpot following stimulation with positive control WIV vaccine strains (A) WIV_{1353} and (B) WIV_{Vic}. Results are expressed as the median and interquartile range of log_{10}-transformed spot-forming cells (SFC)/million cells (± SEM), with a value below 1 Log_{10} (LOD) shaded gray and considered negative for SFCs. Only significant comparisons ($P < 0.05$), as determined via a Kruskal-Wallis test, followed by post hoc Dunn's test, are indicated. All other *P* values are tabulated in Table S5.

groups, specifically WIV_{1353} homologous to the challenge strain, or the heterologous WIV_{Vic} antigen. Stimulation by WIV_{1353} did not elicit any appreciable IFN-γ cellular responses (median levels all below limit of detection [LOD]) in samples taken during the immunization window in any of the vaccinated groups (Fig. 6A). However, the cellular response in DVX-H1N1 immunized animals increased at 8 dpc and was not significantly different from the WIV positive control vaccine groups, including the group immunized with the homologous WIV_{1353} antigen, while no responses were detected in naive animals. IFN-γ responses in PBMCs obtained from WIV_{Vic}-immunized animals and stimulated with the homologous WIV_{Vic} antigen showed values that were significantly higher than DVX-H1N1 and naive groups during the immunization phase (Fig. 6B). Following challenge, at 8 dpc, cells from both WIV-vaccinated animal groups showed a slight increase in IFN-γ expression, while 3/5 DVX-H1N1 immunized animals responded to WIV_{Vic} stimulation (Fig. 6B). As expected, IFN-γ responses were not significant in PBMCs collected from unvaccinated control animals, even after challenge.

DISCUSSION

Pandemic influenza remains one of the most serious threats to human and animal health worldwide. Swine influenza viruses contribute to heightened zoonotic and pandemic risk (19, 20, 65). Here, we describe next-generation influenza vaccine technology using the DIOSynVax platform (56–58) to engineer *in silico* designed immunogens capable of eliciting broad immune responses against human- and swine-origin H1N1 viruses. Our findings demonstrate that digitally designed immune-optimized (DVX) antigens are immunogenic in mice and pigs, and in the pig animal model of human influenza, immunity induced by the DVX-H1N1 vaccine candidate is effective in reducing viral shedding following intranasal challenge.

DVX HA, NA, and M2 antigens were first characterized individually in the mouse model to establish proof-of-concept immunoreactivity. The DVX-H1 antigen elicited a detectable humoral response, although it had lower HI antibody titers compared to the control wild-type strain-based H1 antigen ($H1_{ctrl}$) expressed in the same DNA vaccine

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platform (Fig. 2E). Nonetheless, high-throughput HA neutralization assays (29–31) using a PV panel representing diverse strains drifted over time revealed markedly increased breadth of DVX-H1 responses compared to $H1_{ctrl}$ (Fig. 2D).

Given these findings and the growing body of evidence that other viral proteins, such as NA, in addition to HA, improve the immunogenicity of candidate influenza vaccines, DVX-H1 was incorporated into a single DVX-H1N1 DNA vaccine candidate together with DVX-N1 and DVX-M2. The immunogenicity of DVX-H1N1 was assessed in domestic pigs and was found to elicit comparable humoral immunity when evaluated in parallel with conventional, adjuvanted (light paraffin oil) strain-based WIV vaccines (Fig. 3 and 5). Furthermore, using PV panels representing diverse H1 and N1 antigens, DVX-H1N1 immunization of pigs demonstrated functional HA neutralization and NA inhibition antibody responses, with increased NA responses in comparison to the response elicited by DVX-N1 immunization of mice (Fig. 2F vs Fig. 3D). Antibody titers varied between individual animals, as commonly observed in outbred pig populations (66). Nonetheless, compared to DVX-H1N1 vaccinated pigs, the antibody responses in both WIV-vaccinated groups showed higher HA neutralization and NA inhibition titers, particularly when evaluated using the homologous antigen (Fig. 3C, D, and 5E through H). Interestingly, although not suitable for immunization of mice, the intradermal administration of DVX-H1N1 using an NFIS was found to be highly effective in domestic pigs, as well as being applicable for use in human immunization (67–69). Importantly, hemagglutination inhibition titers revealed that the DVX-H1N1 candidate elicited antibody responses to pandemic 2009 1A.3.3.2 strains, demonstrating breadth covering a decade of genetic diversification (Table 3). Not surprisingly, the adjuvanted strain-based whole inactivated virus vaccines, WIV_{1353} and WIV_{Vic}, administered intramuscularly, induced greater strain-specific HI titers in the immunization phase compared to the computationally engineered DVX-H1N1 vaccine candidate (Fig. 5A through D).

We then assessed the efficacy of the immune response by challenging pigs, a well-recognized large animal model for human influenza, with a swine-origin A(H1N1)pdm09 strain, A/swine/England/1353/2009, that is highly similar to human-origin strains from 2009. Pigs immunized with DVX-H1N1 or with the conventional, adjuvanted WIV_{1353} vaccine antigenically matched to the challenge strain showed significant reduction in nasal shedding of viral RNA, an observation which we have demonstrated correlates to levels below the accurate limit of detection of infectious virus (60). In contrast, challenge of the strain-mismatched WIV_{Vic} and unvaccinated control animals resulted in significantly higher levels of nasal shedding of viral RNA (Fig. 4); levels that in previous studies were shown to correlate with productive infection and infectious virus transmission (60, 66). Despite a comparable humoral response to WIV_{1353} vaccination (Fig. 3C and D, Fig. 5), the WIV_{Vic} vaccine failed to demonstrate a protective immune response in contrast to the DVX-H1N1 and WIV_{1353} vaccines (Fig. 4). This is reminiscent of findings from the post-2009 pandemic H1N1 field where A(H1N1)pdm09 infection resulted in atypical infections and higher susceptibility in some middle-aged or older adults with measurable antibody titers to the current vaccine strain at that time that failed to effectively protect against circulating contemporary H1N1 strains (70–73). This highlighted limitations of the traditional HI-focused correlate of protection paradigm (71, 73).

Hemagglutinin remains the major target of neutralizing antibodies. However, antigenically distinct emergent viruses may not be recognized by existing immunological memory, rendering traditional vaccines that primarily elicit humoral responses ineffective, necessitating vaccine strain reformulation and re-vaccination in the case of seasonal vaccines (4, 70, 74). Although an HI titer of 40 to a specific challenge virus is widely recognized as an important correlate of immune protection against influenza in humans, these results indicate the need for continued monitoring of seasonal viruses for immune escape. Our findings are consistent with these observations and support the concept that, although HI titers remain a valuable surrogate marker of immunity, they do not fully capture all protective immune mechanisms or all biologically relevant antigenic

changes. This also emphasizes the limitations of seasonal, strain-based influenza vaccines and the susceptibility to ongoing genetic drift and antigenic diversification of envelope glycoproteins (6, 75).

Notably, despite apparent reduced HI-mediated protection, the DVX-H1N1 vaccine conferred protection against A/swine/England/1353/2009 challenge. Although this study was not designed to identify the immune correlates responsible for protection, several alternative mechanisms may have contributed. The N1 component of DVX-H1N1 only shares 90.0% sequence identity with WIV_{1353} (47 aa difference) and 88.3% with WIV_{VIC} (55 aa difference); while WIV_{1353} and WIV_{VIC} share 94.7% (25 aa difference) sequence identity with each other (Fig. 2B; Fig. S5). These multiple sequence alignments reveal that, while traditional strain-matching of HA, and to a certain extent NA, is important for generating targeted immune responses, it does not guarantee vaccine efficacy in the case of WIV_{Vic}. Additionally, DVX-H1N1-immunized animals showed a marked increase in antigen-specific cellular immune responses following challenge, indicating effective boosting of cellular responses to virus exposure (Fig. 6A). Collectively, these findings suggest that DVX-H1N1 may induce a broader and more functionally diverse immune response than what is reflected by HI titers alone.

In this context, computationally immune-optimized vaccine candidates, such as DVX-H1N1, offer additional advantages for improved efficacy of vaccines for the control of influenza. Specifically, nucleic acid sequences can be rapidly adjusted to account for emerging strains of concern based on seasonal virus surveillance sequence information. Coupled with high-throughput functional assays, such as pseudotype virus-based neutralizing screens, this strategic approach allows for rapid evaluation of efficacy, as well as differentiation by DIVA diagnostics in animal health applications.

The evolving nature of influenza viruses in animals with spillovers to humans highlights the importance of a "One Health" approach to infectious disease control across both human and veterinary medicine to better address infectious disease threats. Benefits include more effective global surveillance, cooperation, and response for the early intervention and prevention of disease outbreaks while strengthening pandemic preparedness at the international level (76, 77). Lessons learned from the last (COVID-19) pandemic suggest that more effective vaccines are required earlier for emergency implementation in the event of an outbreak in humans of a novel virus with pandemic potential. The DVX platform as presented in this study has been developed not only to prevent early vaccine escape with sequelae of cycles of new variants, but also to respond to high-priority virus threats with clinically developed vaccine candidates that can be readily deployed when pandemic threats arise.

MATERIALS AND METHODS

In silico design of vaccine antigens

Nucleotide sequences of the HA of pandemic lineage of the H1N1 subtype, NA of HxN1 subtype, and M2 from all influenza A subtypes were downloaded from the NCBI database (78). Utilizing the data set of sequences of H1N1 and HxN1 collected from 1918 to 2018, unique antigen sequences were generated for HA, NA, and M2 that were phylogenetically closest to all the input sequences in comparison to any sequence in the downloaded data set. Briefly, the sequences were trimmed to the coding regions, respectively, for HA, NA, and M2, and were filtered for redundancy at 95% nucleotide sequence identity. Multiple sequence alignments of HA, NA, and M2 were generated using the MAFFT algorithm (15) with default parameters. Phylogenetic trees were produced using the previously generated multiple sequence alignment (MSA) as an input to the IQ-tree algorithm (79). The optimal nucleotide model for phylogenetic tree generation was chosen according to the Bayesian information criteria score. The phylogenetic tree and MSA were used as input to Hyphy (80) to generate antigens (56, 58).

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Production of DNA vaccines

For DNA vaccine production, selected antigen sequences were codon-optimized for human expression via the GeneOptimizer algorithm (81), synthesized as individual sequences or as a DNA string, and cloned into pEVAC (GeneArt, Germany). Plasmids were transformed via heat shock into competent DH5-α *Escherichia coli* (Invitrogen, EC0112) cells. DNA was extracted from bacterial cultures using the EndoFree Plasmid Mega Kit (QIAGEN, 12381) for production of DNA for immunogenicity studies (Tables 1 and 4). Plasmid DNA was quantified using UV spectrophotometry (Nanodrop 1000, ThermoFisher, UK) and sequence integrity was verified by Sanger sequencing (Source Bioscience, UK). Endotoxin levels were verified to not exceed acceptable limits (≤0.01 Endotoxin Units/µg) for *in vivo* studies using the Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher, USA).

Production of the challenge strain and whole inactivated virus vaccines

The positive control WIV vaccines were prepared from two A(H1N1)pdm09 strains with HA clade 1A.3.3.2; A/swine/England/1353/2009 (H1N1) (82) and a representative human seasonal vaccine strain A/Victoria/2454/2019 (H1N1), reference stock IVR-207, kindly provided by the Worldwide Influenza Center (WIC, The Francis Crick Institute, London, UK). Virus stocks were propagated in embryonated hens' eggs and inactivated using beta-propiolactone at 1:2,000 (vol/vol) dilution for 2 h as previously described (83). The resulting WIV_{1353} and WIV_{Vic} antigens (Table 4) were quantified using the HA assay (84), and in order to standardize antigen payload, each dose was formulated to contain 1,280 HA units (HAU) in 1 mL using endotoxin-free PBS (Gibco) as previously described for WIV vaccine formulation (51, 60). Immediately prior to administration, the positive control WIV vaccines were prepared in a 3 mL dose volume as a 1:2 antigen:adjuvant mixture using a licensed, commercially available light paraffin oil adjuvant (https://www.noahcompendium.co.uk/?id=-453390, Ceva) as described previously (60). The challenge strain, A/swine/England/1353/2009 (H1N1), was propagated in Madin-Darby canine kidney cells and titrated using the 50% tissue culture infectious dose ($TCID_{50}$) method (84).

Pseudotype virus production

H1 and N1 sequences (Table 2) were produced as HA or NA lentiviral PV as described previously (29–31) by plasmid transfection via FuGENE HD (ProMega, USA) of HEK 293T/17 cells in 6-well plates with four plasmids: pEVAC encoding HA or NA, respectively, pCSFLW encoding a luciferase reporter gene, p8.91 gag-pol encoding lentiviral Gag antigen and polymerase, and a plasmid encoding type II transmembrane protease serine 4 (TMPRSS4). NA PV production required a fifth plasmid, pEVAC HA, encoding H11 HA from A/red shoveler/Chile/C14653/2016 (sequence accession MH134837). Only for HA pseudotype viruses, 1 unit of exogenous *Vibrio cholerae* neuraminidase in 50 µL PBS (Sigma-Aldrich N2876) was added per well 24 h post-transfection. Supernatant was collected 48 h post-transfection, passed through a 0.45 µm filter, and stored at −80°C. Titration was carried out as described previously (29–31).

Vaccine immunogenicity in the murine model

Six- to eight-week-old female BALB/c mice were obtained from Charles River Laboratories and housed in seven groups of six mice each at the University Biomedical Services, University of Cambridge. DNA at a concentration of 50 µg suspended in 50 µL of endotoxin-free PBS (Gibco) was administered subcutaneously (SC) on the rear flank of each mouse. Following the initial prime at 0 dpv, immunizations were boosted on 14, 28, and 42 dpv. The respective groups (Table 1) received DNA encoding a DVX antigen HA (H1), NA (N1), or M2 (M2), or the respective control immunogens HA ($H1_{ctrl}$), NA ($N1_{ctrl}$), or M2 ($M2_{ctrl}$) derived from specific wild-type sequences. One group was immunized with 50 µL of plasmid pEVAC (no insert) as a negative control. All mice were humanely

euthanized under non-recovery isoflurane (5%) anesthesia on 70 dpv, and terminal blood samples were collected. Serum samples were stored at −20°C for later determination of the humoral immune responses induced by vaccination.

Immunogenicity and efficacy study in pigs

Five-week-old Landrace cross male pigs (n = 20) were obtained from a commercial high health status herd and housed in four groups (n = 5 per group). Pigs were screened for absence of IAV RNA by matrix gene RRT-qPCR (85) and absence of IAV antibodies to H1N1 1A.3.3.2 antigens by HI (86, 87). Throughout the study, all pigs received weaner/grower feed and had access to water *ad libitum*. For all vaccines, pigs were immunized (0 dpv) at 7 weeks of age and boosted 28 days later. The DVX-H1N1 DNA vaccine was administered intradermally (ID) by delivery of four 100 µL doses of 200 µg (two doses posterior to both left and right ears) using the NFIS Tropis (PharmaJet, Golden, CO). The monovalent positive control WIV_{1353} or WIV_{Vic} vaccines formulated in light paraffin oil adjuvant were administered intramuscularly (IM) into the trapezius muscle 25–30 mm posterior to each ear as two 1.5 mL injections per pig (Table 4). A negative control group was not vaccinated.

All pigs were challenged intranasally (IN) on 69 dpv (corresponding to 0 dpc) with 4 mL (2 mL per nostril) of A/swine/England/1353/2009 (H1N1) virus delivered as an atomized spray of droplets with diameters ranging from 30 to 100 µm using a MAD300 device (Teleflex, USA). Back-titration of the challenge stock confirmed that 1.67×10^6 $TCID_{50}$ was administered to each pig. Animals were monitored daily, and clinical signs including demeanor, appetite, rectal temperature, and any respiratory signs, such as coughing and sneezing, were recorded using a clinical scoring system (63). Clotted and heparin-anticoagulated blood samples using SST and Heparin vacutainers (BD BioSciences) were obtained prior to immunization at 28, 42, and 63 dpv, as well as after challenge at 8 dpc. Serum samples obtained from clotted blood were archived at −20°C, and PBMCs were isolated from anticoagulated blood and cryopreserved as described previously (60). Nasal swabs (one per nostril) were obtained from each pig prior to vaccination and challenge, and then daily post-challenge until the end of the study, and stored dry at −80°C (60). Animals were humanely euthanized with an overdose of intravenous pentobarbital sodium at the study end on 8 dpc for WIV-vaccinated groups, and 9 dpc for DVX-H1N1 and unvaccinated naive control groups. Bronchioalveolar lavage (BAL) necropsy samples were obtained in Dulbecco's PBS (Gibco), and cellular and supernatant fractions were archived as described above.

Real-time reverse transcription PCR

To evaluate viral RNA shedding by RRT-qPCR, nasal swabs obtained at specified time points from each pig were immersed in 1 mL of Leibovitz L-15 medium (ThermoFisher Scientific), containing 1% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, 5,000 U/mL). Total RNA was extracted from the swab suspensions and BAL fluid using the QIAamp Viral RNA Biorobot Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Viral RNA was quantified by RRT-qPCR targeting the swine IAV M-gene (60, 66, 85). The amount of RNA present is expressed as relative equivalent units (REU) of RNA correlated to a standard 10-fold dilution series of RNA purified from the same batch of virus used for challenge with a known $TCID_{50}$ titer. Although these units measure the amount of viral RNA present and not infectious virus, it may be inferred from the linear relationship of the dilution series that REU are proportional to the amount of infectious virus present. As described previously (60), this approach allows real-time monitoring of virus shedding, even at low levels where conventional titration of infectious virus does not yield consistent results.

Vaccine-induced humoral immune response

HA neutralizing titers, evaluated using a H1 PV panel (Table 2) in a PV microneutralization assay, were determined for both mouse and pig serum (30). For mice, serum samples

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were diluted 1:50, and for pigs 1:100; responses were normalized against virus-only (0% neutralization) and cell-only (100% neutralization) controls and plotted on GraphPad Prism 11. Values were fitted using nonlinear regression (curve fit), where x is log fold-dilution of sera, and y is normalized response. Results are expressed as the IC_{50} fold dilution of sera that resulted in 50% neutralization of virus.

Neuraminidase inhibition titers evaluated using an N1 PV panel (Table 2) were determined for both mice and pigs using a pELLA (31). Mouse and pig sera were initially diluted 1:20 and then serially diluted twofold in duplicate. The IC_{50} was calculated as the inverse dilution of serum or antibody concentration that resulted in 50% inhibition of NA activity as determined via GraphPad Prism 11.

Mouse (88) and pig (87) antisera were assessed by HI assay using an H1N1 reference virus panel (Table 3). Additionally, pig antibody NP titers were measured longitudinally using an indirect multi-species ELISA directed to the IAV NP (ID Screen IDvet).

Porcine IFN-γ ELISpot assay

Antigen-specific IFN-γ immune responses in PBMCs were measured following immunization and virus challenge using a swine-specific ELISpot assay (89). PBMCs were stimulated with the identical antigens used to produce the WIV_{1353} and WIV_{Vic} positive control vaccines. The number of spot-forming cells (SFC) per 10^6 cells was counted by an automated ELISpot reader (AID) and corrected for background (tissue culture medium-stimulated). Assay and viability controls included responses to Pokeweed mitogen (Sigma) as a positive control for the functional capability of cells to produce IFN-γ.

Statistical analysis

All statistical analyses were performed with RStudio for Windows (RStudio 2024.09.0 Posit Software, PBC). The following tests: Wilcoxon signed-rank, Kruskal-Wallis, followed by post hoc Dunn's test, Brown-Forsythe and Welch ANOVA, and Dunnett's T3 comparisons were used to determine statistically significant differences between two or more groups in comparison to the control groups. Only statistically significant *P* values are displayed in the figures.

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than L. Heeney, Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Visualization.

DATA AVAILABILITY

All data supporting the findings of this study are available within the paper and its supplemental material.

ETHICS APPROVAL

In vivo studies were conducted in accordance with UK Home Office regulations under the Animals (Scientific Procedures) Act 1986 (ASPA) following ethical approval. Mouse studies were approved by the Animal Welfare Ethical Review Body (AWERB), University of Cambridge (Project license P8143424B). Pig study number PP9878849-2-002 was approved by the AWERB of the Animal and Plant Health Agency (APHA). Results are reported according to the ARRIVE Guidelines (90).

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (JVI01073-26-s0001.docx). Fig. S1 to S7, Tables S1 to S5, and supplemental methods.

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