Whole inactivated virus (WIV) vaccines are widely used in the swine industry to reduce clinical disease against homologous influenza A virus (IAV) infection. In pigs experimentally challenged with antigenically distinct heterologous IAV of the same hemagglutinin subtype, WIV vaccines have been shown to develop vaccine-associated enhanced respiratory disease (VAERD). We evaluated the impact of vaccine valency, age at vaccination, and duration between vaccination and challenge on the development of VAERD using vaccine containing 81-H1N2 and challenge with pandemic H1N1 (pH1N1) virus. Pigs were vaccinated with monovalent WIV MN08 (81-H1N2) and bivalent (81-H1N2–H3N2 or 81-H1N2–pH1N1) vaccines and then were challenged with pH1N1 at 3 weeks postboost (wpb). Another group was vaccinated with the same monovalent WIV and challenged at 6 wpb to determine if the time postvaccination plays a role in the development of VAERD. In a follow-up study, the impact of age at first WIV vaccination (at 4 versus 9 weeks of age) with a boost 3 weeks later (at 7 versus 12 weeks of age) was evaluated. A monovalent live-attenuated influenza virus (LAV) vaccine administered at 4 and 7 weeks of age was also included. All mismatched WIV groups had significantly higher lung lesions than the LAV vaccine, bivalent MN08–CA09, and control groups. Age of first vaccination or length of time between booster dose and subsequent challenge did not alter the development of VAERD in WIV-vaccinated pigs. Importantly, the mismatched component of the bivalent MN08–CA09 WIV did not override the protective effect of the matched vaccine component.

Influenza A virus (IAV) causes an acute respiratory disease in swine worldwide, which leads to significant economic losses to pork producers. Classical H1N1 (cH1N1) was the major cause of influenza in swine in North America until 1998 (1). Between 1997 and 1998, human seasonal H3N2 influenza viruses were introduced in the U.S. swine population in the form of double- and triple-reassortant viruses. The triple-reassortant viruses containing the H3, N2, and polymerase basic 1 (PB1) gene segments derived from human strains, the PB2 and polymerase acidic (PA) segments derived from avian strains, and the rest of the segments derived from the North American cH1N1 swine strains quickly spread and became endemic in U.S. swine populations. Eventually, the six-internal-gene constellation of the triple-reassortant viruses became dominant in swine influenza viruses in North America and was defined as the triple-reassortant internal gene (TRIG) cassette. The circulation of both H3N2 and cH1N1 viruses in the swine population led to additional reassortments that generated H1N2 viruses (2). This was closely followed by the introduction of human seasonal H1 around 2002, which further contributed to H1 IAV diversity in swine populations in the United States and resulted in multiple distinct phylogenetic clusters (α, β, γ, δ1, and δ2) (3–5). In 2009, the pandemic H1N1 virus (pH1N1) emerged from a reassortment of gene segments from North American cH1N1, TRIG, and Eurasian avian-origin H1N1 IAV lineages (6, 7) and was transmitted globally from humans to swine (8).

Commercial vaccines available for use in pigs in North America and Europe are primarily based on whole inactivated viruses (WIV) delivered in combination with proprietary adjuvants. WIV vaccines are effective at reducing clinical disease against homologous and antigenically related viruses by inducing an antibody response against the surface glycoprotein proteins hemagglutinin (HA) and neuraminidase (NA) (9, 10). However, vaccines with oil-in-water adjuvants have been associated with enhanced respiratory disease in swine following challenge with antigenically divergent heterologous IAV of the same HA subtype (11). A vaccine-associated enhanced respiratory disease (VAERD) model was previously reported, in which pigs vaccinated with a monovalent WIV 81-cluster (H1N2) vaccine and challenged with heterologous virus (pH1N1) demonstrated severe lung pathology in association with the absence of cross-neutralizing antibodies (10–13). The mechanisms that lead to VAERD are still unclear, but a lack of neutralizing antibodies and local cytokine dysregulation have been implicated (12, 13). Antibodies elicited following WIV vaccination were shown to bind to the HA2 domain of the heterologous challenge virus and are able to increase viral infectivity by enhancing fusion of the heterologous virus (14). The vaccination and challenge model described here is relevant to the current field situation in the United States and other regions with similar en-
demic virus lineages due to the prevalence of the δ1-H1 IAV lineages in swine in the United States (15) and the frequent spillover of pH1N1 from humans to swine (16, 17), as well as the use of these strains in monovalent or multivalent inactivated commercial or autogenous vaccines.

Our initial objective was to explore the impact of monovalent versus bivalent WIV and duration between vaccination and challenge on the development of VAERD. Based on results of study 1, we initiated a second study to evaluate the impact of age at WIV vaccination on the development of VAERD. Additionally, since experimental live-attenuated influenza virus (LAIV) vaccines can elicit protective immunity in pigs (18) and provide partial protection against heterologous challenge (19–21), a temperature-sensitive LAIV (tsLAIV) vaccine was included in the follow-up study to compare with the WIV platform with an 8-week duration between booster and challenge in a two-dose vaccination regimen. The results described here provide further understanding of the relevant vaccination and infection scenarios that can lead to VAERD.

MATERIALS AND METHODS

Study design, vaccination, and challenge. Pigs were obtained from a herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV). Upon arrival, pigs were treated prophylactically with ceftiofur (Zoetis, Florham Park, NJ), according to the label directions, to reduce the potential respiratory bacterial pathogens. Pigs were housed in biosafety level 2 (BSL2) containment during the vaccine and challenge phases of the study. The pigs were cared for in compliance with the Institutional Animal Care and Use Committee (IACUC) of the National Animal Disease Center (NADC), Ames, IA.

IAV strains A/California/04/2009 (pH1N1) (CA09), A/swine/Minnesota/02011/2008 (δ1-H1N2) (MN08), and A/swine/Texas/4199-2/1998 (H3N2) (wild-type TX98) were grown in Madin-Darby canine kidney (MDCK) cells with Opti-MEM (Life Technologies, Carlsbad, CA). Clariﬁed virus from infected culture was inactivated by UV irradiation, using the “sterilize” setting in a UV cross-linking chamber (GS Gene Linker; Bio-Rad, Hercules, CA). A commercial adjuvant (Emulsigen-D; MVP Laboratories, Inc., Ralston, NE) was mixed with inactivated virus at a 1:5 ratio (vol/vol) in a 2-ml dose delivered by the intramuscular route. Each dose of WIV contained approximately 128 hemagglutination units (HAU) of virus, as previously described (22).

In study 1, the WIV vaccines included were bivalent H1N2 (MN08)-H3N2 (TX98) WIV or bivalent H1N2 (MN08)-pH1N1 (CA09) WIV vaccine and monovalent H1N2 (MN08) WIV vaccine. In this study, a total of 40 3-week-old cross-bred pigs were randomly assigned to one of 5 groups. Pigs were vaccinated with either MN08-TX98 (n = 5) or MN08-CA09 (n = 5). Both groups were challenged at 3 weeks postboost (wpb) (at 9 weeks of age) with pH1N1, which is consistent with our previous studies (12). In addition, pigs (n = 14) were vaccinated with a monovalent WIV H1N2 (MN08) vaccine to evaluate the impact of the time between boost and challenge. Control pigs included nonvaccinated/nonchallenged group (NVC/N) (n = 8) and nonvaccinated/challenged group (NV/C) (n = 8). At 3 or 6 wpb, pigs were challenged with 2 ml of 10^6 50% tissue culture infectious dose (TCID50/ml) of pH1N1 intratracheally and 1 ml intranasally, as previously described (12).

Based on the results of the 3wpb-MN08 and 6wpb-MN08 groups, a further question arose regarding the impact of age at first vaccination in the context of a longer duration between vaccination and challenge in pigs older than those in our previously published model. To address this question, pigs were vaccinated with the monovalent MN08 WIV, as described above for study 1, or with a tsLAIV vaccine. The tsLAIV vaccine included in this study was generated by a reverse-genetics technique using the HA and NA gene segments from the MN08 strain cloned in combination with a swine-origin H3N2 backbone with modifications in the polymerase genes to render the strain temperature sensitive, as previously described (18, 23). Pigs were vaccinated at 4 weeks of age and boosted at 7 weeks of age with WIV (WIV-4/7) (n = 10) or LAIV (LAIV-4/7) (n = 10). One group of pigs was vaccinated with WIV at 9 weeks of age and boosted at 12 weeks of age (WIV-9/12) (n = 10). Control groups included nonvaccinated/challenged pigs (NV/C) (n = 10) and nonvaccinated/nonchallenged pigs (NV/NC) (n = 6). At 13 weeks of age, pigs were challenged with 2 ml of 10^6 TCID50/ml of pH1N1, as done in study 1 (described above). The experimental designs of studies 1 and 2 are summarized in Table 1.

For both studies, clinical signs were observed daily. Nasal swabs (Fisherbrand Dacron swabs; Fisher Scientific, Pittsburgh, PA) were taken at 0, 3, and 5 days postinfection (dpi) to evaluate nasal virus shedding. After serum collection, each pig was humanely euthanized with a lethal dose of pentobarbital (Fatal Plus; Vortech Pharmaceuticals, Dearborn, MI) at 5 dpi to evaluate lung and trachea lesions, cytokine concentrations in bronchoalveolar lavage fluid (BALF), and virus titers in the lungs.

Evaluation of macroscopic pneumonia. At 5 dpi, the percentage of the lung affected with purple-red consolidation typical of influenza virus in swine was visually estimated, as previously described (12, 24). Tissue samples from the trachea and right middle or affected lung lobe were fixed in 10% buffered formalin for histopathologic examination. Tissues were processed by routine histopathologic procedures and slides stained with hematoxylin and eosin (H&E) or stained by immunohistochemistry.

### Table 1: Experimental designs of studies 1 and 2

| Group          | n  | Vaccine/valency       | Challenge virus | Age (wk) at: |
|----------------|----|-----------------------|----------------|--------------|
|                |    |                       |                | Priming  | Boost | Challenge |
| **Study 1**    |    |                       |                |          |       |           |
| MN08-TX98      | 5  | WIV bivalent          | MN08 (H1N2), TX98 (H3N2) | CA09 (pH1N1) | 4    | 7 | 10 |
| MN08-CA09      | 5  | WIV bivalent          | MN08 (H1N2), CA09 (pH1N1) | CA09 (pH1N1) | 4    | 7 | 10 |
| 3wpb-MN08      | 8  | WIV monovalent        | MN08 (H1N2)     | CA09 (pH1N1) | 4    | 7 | 10 |
| 6wpb-MN08      | 6  | WIV monovalent        | MN08 (H1N2)     | CA09 (pH1N1) | 4    | 7 | 13 |
| NV/C           | 8  | None                  | None            | CA09 (pH1N1) | ND*  | ND | 10 |
| NV/NC          | 8  | None                  | None            | ND          | ND | ND | ND |

**Study 2**

| WIV-4/7        | 10 | WIV monovalent        | MN08 (H1N2)     | CA09 (pH1N1) | 4    | 7 | 15 |
| WIV-9/12       | 10 | WIV monovalent        | MN08 (H1N2)     | CA09 (pH1N1) | 9    | 12 | 15 |
| LAIV-4/7       | 10 | LAIV monovalent       | MN08 (H1N2)     | CA09 (pH1N1) | 4    | 7 | 15 |
| NV/C           | 10 | None                  | None            | CA09 (pH1N1) | ND | ND | 15 |
| NV/NC          | 6  | None                  | None            | ND          | ND | ND | ND |

*ND*, not done.
(IHC) for IAV antigen (25). The slides were evaluated by a veterinary pathologist blinded to the treatment groups, and microscopic lesion severity was scored as previously described (12).

**Microbiological assays.** All pigs were screened for antibody against influenza A nucleoprotein (NP) by a commercial enzyme-linked immunosorbent assay (ELISA) (MultiELISA; Idexx, Westbrook, ME) upon arrival to ensure the absence of preexisting immunity from prior exposure or passively acquired maternal antibody. BALF samples were cultured for aerobic bacteria on blood agar and Casmin (NAD-enriched) plates to indicate the presence of concurrent bacterial pneumonia. PCR assays were conducted for porcine circovirus 2 (PCV2) (26). Commercial PCR assays for *Mycoplasma hyopneumoniae* and PRRSV were conducted with the VetMax *M. hyopneumoniae* reagents and the North American and European PRRSV-specific reverse transcription-PCR assay (Life Technologies, Carlsbad, CA), respectively, according to the manufacturer’s recommendations, to exclude other causes of pneumonia.

**Virus titration on lung and nasal samples.** Nasal swabs and BALF samples were collected and titrated on MDCK cells to evaluate virus shedding and replication in the nose and lungs, as previously described (12). MDCK-inoculated monolayers were evaluated for cytopathic effect (CPE) between 48 and 72 h postinfection, fixed with 4% phosphate-buffered formalin, and stained using immunocytochemistry (ICC) with anti-influenza A NP monoclonal antibody (27). A TCID50/ml per milliliter titer was calculated for each sample using a method described by Reed and Muench (28).

**Hemagglutination inhibition and whole virus ELISAs.** Serum samples from all pigs were collected prior to the first vaccination, prior to boost, prechallenge, and at 5 dpi. Sera from the WIV-4/7 and LAIV-4/7 groups were also collected at 4 weeks postboost. The sera were heat inactivated at 56°C for 30 min, and hemagglutination inhibition (HI) assays were conducted with 61-H1N2 (MN08) or pH1N1 (CA09) virus with antigen and turkey red blood cells (RBCs) as indicators using standard techniques, as previously described (29, 30). The reciprocal titers were divided by 10, log2-transformed, analyzed, and reported as the geometric mean.

IAV-specific IgG in serum samples and IAV-specific IgA and IgG in BALF samples were evaluated by enzyme-linked immunosorbent assays (ELISAs). Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with whole virus (MN08 or CA09) preparations at 100 HA units (50 μl/well), grown in serum-free Opti-MEM, and incubated at room temperature overnight, as previously described (13). Serum samples were diluted 1:2,000 in phosphate-buffered saline (PBS), and BALF samples were incubated at 37°C for 1 h with an equal volume of 10 mM dithiothreitol (DTT) to disrupt mucus. Then, BALF samples were diluted 1:4 in PBS. The assays were performed on each BALF or serum sample in duplicate. To compare the single-read results with endpoint titrations, serum was diluted 1:1,000, and BALF samples were diluted 1:2 in PBS, followed by 2-fold dilutions in PBS, according to the protocol described below. Endpoints were calculated as the dilution with an optical density (OD) of 2 times the standard deviation of the mean OD value for serum or BALF from negative-control pigs.

Plates were blocked for 1 h with 100 μl of 10% bovine serum albumin (BSA) in PBS and washed three times with 0.05% Tween 20 in PBS (PBS-T). The plates were incubated with samples (serum or BALF) at room temperature for 1 h, washed three times with PBS-T, and then incubated with peroxidase-labeled goat anti-swine IgA (Bethyl, Montgomery, TX) and IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) at 37°C for 1 h. The substrate 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS)-peroxide (Kirkegaard & Perry Laboratories) was added and incubated at 37°C for 8 min. To stop the color development, 50 μl of stop solution (Kirkegaard & Perry Laboratories) was added to each well, and the optical density (OD) at 405 nm was measured with an automated ELISA reader. Antibody levels were reported as the mean OD for each duplicate sample, and the mean ODs of each treatment group or geometric mean log2-transformed endpoint titer were compared.

**Cytokine assays.** Aliquots of BALF from studies 1 and 2 were analyzed for a panel of cytokine proteins. Study 2 BALF aliquots were centrifuged at 400 x g for 15 min at 4°C to pellet cellular debris, and cell-free lung lavage (CF-LL) was used in the analysis; however, it was not possible to retrospectively run the study 1 BALF in this manner. Cytokine concentrations for interleukin-1 beta (IL-1β), IL-2, IL-4, IL-6, IL-8, IL-10, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) were determined by multiplex ELISA performed according to the manufacturer’s recommendations (SearchLight; Aushon Biosystems, Billerica, MA), as previously described (12). Cytokine concentrations were reported as the mean of the results from duplicate samples for statistical analysis.

**Statistical analysis.** Lung lesion scores, log10-transformed virus titers, log2-transformed HI reciprocal titers, mean OD ELISA of IgA or IgG levels, and cytokine concentrations were analyzed using analysis of variance (ANOVA), with a P value of ≤0.05 considered significant (GraphPad Prism software version 6.00; San Diego, CA). Response variables shown to have a significant effect by treatment group were subjected to pairwise comparisons using the Tukey-Kramer test.

**RESULTS**

**Clinical disease.** All pigs were negative for IAV NP-specific antibodies prior to vaccination, and at 5 dpi, all pigs were negative for PCV2, *M. hyopneumoniae*, PRRSV, and nucleic acids in BALF. In addition, aerobic bacterial pathogens were not detected in the BALF samples at 5 dpi in either study. Pigs were monitored daily following challenge. Pigs in the WIV-vaccinated and challenged groups demonstrated clinical signs such as coughing or respiratory distress, which were consistent with previous descriptions of VAERD (11, 12). In contrast, the LAIV group demonstrated mild symptoms of respiratory distress, as previously observed (23), during postchallenge. All challenged groups demonstrated febrile responses at 1 dpi, defined as two standard deviations above the mean rectal temperature prior to challenge (≥39.8°C), that were significantly higher than those for the NV/NC group. By 2 through 4 dpi, only the WIV groups displayed a febrile response. At 5 dpi, all vaccinated/challenged groups recovered from the febrile status.

**Lung pathology.** In both studies, macroscopic lesions consistent with VAERD were observed in WIV groups at 5 dpi; these consisted of purple-colored and multifocal-to-coalescing consolidation of the cranioventral regions of the lung typical of IAV infection in swine (representative lungs shown in Fig. 1A to E). Microscopic lesions in the WIV groups consisted of necrotic or irregular, stacked, and proliferative epithelium in affected bronchioles at 5 dpi, with luminal neutrophils and occasional intraepithelial exocytosis (representative micrographs shown in Fig. 2A to E). Large numbers of lymphocytes expand the bronchiolar lamina propria, displacing the affected epithelium. Lymphocytes form dense peribronchiolar cuffs in VAERD–affected lungs and alveolar septal thickening, with large numbers of widespread lymphocytes and macrophages. Multifocal alveolar lumina contain varied numbers of neutrophils and macrophages or abundant edema with occasional hemorrhage.

In the bivalent WIV groups of study 1, MN08-TX08 pigs had significantly higher percentages of macroscopic lung lesions (12.1%), whereas, in contrast, bivalent MN08-CA09-vaccinated pigs did not display VAERD. The presence of the mismatched H1N2 strain in the WIV vaccine did not interfere with the protective response provided by the homologous vaccine antigen, yet the presence of the H3N2 in the mismatched bivalent vaccine did not alter the VAERD resulting from the MN08-mismatched H1 component of the WIV vaccine (Fig. 1F). The differences in micro-
scopic lesion scores between groups were consistent with the macroscopic lesions (Fig. 2F). The 3- versus 6-week period between boost and challenge did not alter the VAERD outcome in the monovalent WIV groups, as both the 3wpb-MN08 and 6wpb-MN08 groups had significantly higher percentages of macroscopic pneumonia (17.7% and 15.4%, respectively; Fig. 1F) and microscopic lung lesion scores (15.7 and 13.8, respectively; Fig. 2F) than the NV/C control group (mean percentage of macroscopic lesions of 6.1% and mean microscopic score of 6.0) and were not statistically different from each other.

To determine if age at the time of WIV vaccination has an effect on the development of VAERD and to further define the impact of timing between boost and challenge for a WIV vaccine in the VAERD model, study 2 was conducted. Similar to the mismatched WIV groups above, both the WIV-4/7 and WIV-9/12 groups had significantly higher percentages of macroscopic lung lesions (20.2% and 15%, respectively) than the NV/C control (3.7%; Fig. 1G). In contrast, pigs in the LAIV-4/7 group did not demonstrate a magnitude of macroscopic pneumonia (6.2%) consistent with VAERD. The microscopic lesion scores in the WIV-4/7 (15) and WIV-9/12 (12.6) groups were significantly higher than those in the LAIV-4/7 (5.3) and NV/C control (3.9) groups (Fig. 2G). These data indicate that the animal’s age at the time of vaccination (4 or 9 weeks of age) did not alter the development or pathological aspects of VAERD.

Microscopic lung lesions were more severe in the VAERD-affected groups WIV-4/7 (Fig. 2A) and WIV-9/12 (Fig. 2B) than in NV/C controls (Fig. 2D). The LAIV-4/7 and NV/C groups had
microscopic lesions at 5 dpi of lesser severity, with mild bronchiolar epithelial changes, light infiltrates forming peribronchiolar cuffs, and mild alveolar septal thickening in proximity to the affected bronchioles (Fig. 2C and D, respectively). The NV/NC groups did not demonstrate microscopic lesions consistent with IAV pneumonia (group mean microscopic scores are summarized in Fig. 2G).

**Virus levels in nasal secretions and in lungs.** All pigs were negative for IAV in nasal swabs at 0 dpi. In study 1, virus was recovered at 3 and 5 dpi in nasal swabs and in BALF (5 dpi) from nasal swabs collected from the MN08-TX08 and 3wpb- or 6wpb-MN08 groups. However, MN08-CA09-vaccinated pigs did not shed virus, and BALF samples were negative for virus, again demonstrating the absence of interference by the mismatched heterologous virus vaccine antigen with the matched homologous response in the bivalent WIV vaccine (Fig. 3A to C).

However, the virus titers in vaccinated pigs from study 2 were significantly lower in nasal swabs and BALF at 5 dpi than those in the NV/C group (Fig. 3D to F). Despite the few pigs with detectable virus at 10^2 TCID_{50}/ml at 3 dpi in the WIV-9/12 group (1/9 pigs) and at 10^3 TCID_{50}/ml at 5 dpi in the WIV-4/7 group (3/10 pigs), the presence of challenge virus in the lungs was confirmed by IAV-specific IHC (Fig. 3G). Virus was not detected at any time in nasal swabs or BALF samples in the NV/NC control group.

**HI antibody responses.** In study 1, HI antibodies against TX98 and CA09 were detected only in the MN08-TX98 and MN08-CA09 groups prior to challenge, respectively (Fig. 4A and B). All vaccinated groups had significant HI titers against MN08 compared to those of the control groups (NV), and the MN08-CA09 group had the lowest HI titers against MN08 (Fig. 4C).

In study 2, the WIV-4/7 and LAIV-4/7 groups demonstrated significantly higher HI titers against MN08 virus from the pre-boost until the necropsy day (15 weeks of age) than the control groups. Although the WIV-9/12 group had significantly higher HI titers than the nonvaccinated controls, this group had significantly lower HI titers than the WIV-4/7 group at all equivalent postvaccination time points evaluated (Fig. 4D). The NV/C and NV/NC group HI mean titers remained below the detection threshold (HI titer, <40). None of the groups had cross-reacting HI antibodies to the heterologous challenge virus (CA09) at 0 dpi.

**Systemic IgG levels against whole MN08 and CA09 virus.** The IAV-specific antibody response following vaccination was further explored by measuring IgG antibody titers in sera to the priming (MN08) and challenge (CA09) viruses using whole-virus ELISA. In general, the antibody responses to the different vaccine compositions and regimens were consistent with previous VAERD studies with monovalent vaccines given at 4 and 7 weeks of age. In study 1, MN08-TX98, MN08-CA09, and 3wpb- or 6wpb-MN08

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**FIG 2** Representative microscopic lung lesions. In study 2, at 5 dpi, pigs vaccinated with WIV-4/7 (A) and WIV-9/12 (B) demonstrated significantly higher microscopic lung lesion scores, consisting of widespread alveolar septal thickening, necrotic-to-proliferative epithelial bronchiolitis, marked peribronchial lymphocytic cuffing, suppurative bronchiolitis, and alveolar edema, than pigs vaccinated with the LAIV-4/7 (C) and NV/C (D) groups; results for the latter two groups were not significantly different from results for the NV/NC negative-control group (E). Group mean microscopic scores are shown for studies 1 (F) and 2 (G). Statistically significant differences between treatment group means are identified by different lowercase letters (P ≤ 0.05). The error bars represent the standard errors of the means.
groups showed significantly higher titers of anti-MN08 and anti-CA09 IgG in serum prior to challenge (dpi 0) (Fig. 5A and C) and postchallenge (dpi 5) (Fig. 5B and D) than the controls. In study 2, both WIV groups had significantly higher anti-MN08 and cross-reactive anti-CA09 IgG titers in serum than the LAIV-4/7 group and control groups prior to challenge (0 dpi) (Fig. 5E and G) and postchallenge (5 dpi) (Fig. 5F and H). Following challenge, the LAIV-4/7 group had significantly higher titers of anti-MN08 and anti-CA09 IgG in serum than the control groups, but they had lower titers than the WIV groups (Fig. 5F and H). The geometric mean titers from serum samples demonstrated a pattern similar to that of the mean OD single-sample results, without a clear connection to the clinical outcome (see Fig. S1 in the supplemental material), with the exception of the relatively reduced peripheral IgG response in the LAIV-vaccinated pigs.

Local IgA and IgG levels against whole MN08 and CA09 virus. To evaluate the local immune response against vaccine antigen(s) and heterologous challenge antigen, IgA and IgG antibody titers in the lungs (BALF) in response to different WIV formulations were assessed. In study 1, the titers of anti-MN08 and cross-
reactive anti-CA09 IgA were significantly higher in all vaccinated groups than in the controls (Fig. 6A and B). All vaccinated groups had significantly higher titers of anti-MN08 and cross-reactive anti-CA09 IgG than the controls (Fig. 6C and D). In study 2, the WIV and LAIV groups had significantly higher titers of anti-MN08 and cross-reactive anti-CA09 IgA (Fig. 6E and F) and IgG (Fig. 6G and H) than the NV controls. The geometric mean titers from BALF samples demonstrated a pattern similar to that of the mean OD single-sample results (see Fig. S2 in the supplemental material), with the exception of the 6wpb-MN08 group, showing titers that were relatively higher than those of the mean OD single-sample results and more consistent with those of the other MN08-vaccinated groups.

**Cytokine concentrations in lungs.** The concentrations of cytokines in the lungs were evaluated for association with immunopathology, a characteristic feature of VAERD-affected pigs (12, 13). In study 1, the concentrations of cytokines in BALF samples were only significantly higher than those in the NV/C control group in the monoclonal 6wpb-MN08 group challenged at 6 wpb, which was at 13 weeks of age. This group showed levels (mean) of IL-4 (16.7 pg/ml), TNF-α (13.5 pg/ml), IL-2 (121.8 pg/ml), IL-1β (1243.5 pg/ml), and IFN-γ (142.7 pg/ml) (Fig. 7A to E) that were significantly elevated compared to those of the NV/C and NV/NC controls. In addition, the 6wpb-MN08 group had an increased level of anti-inflammatory IL-10 (133.4 pg/ml) (Fig. 7F). In study 2, the cytokine concentrations in cell-free BALF samples were measured. The WIV-4/7 group demonstrated significantly higher cytokine protein levels of IL-1β (299.7 pg/ml) and IFN-γ (75.9 pg/ml) and the anti-inflammatory cytokine IL-10 (174.3 pg/ml) (Fig. 7G to I) than the NV/C and NV/NC controls.

**DISCUSSION**

Contemporary IAV in swine is rapidly evolving and increasing in antigenic diversity, so the need for improved vaccines that induce cross-protection against heterologous viruses is important to reduce rates of infection in swine and transmission between species (8, 31). The efficacy of WIV vaccines can be limited when pigs are challenged with heterologous viruses (9, 10, 32, 33), as demonstrated by the VAERD model. When pigs were vaccinated with a WIV containing a 1-cluster H1N2 (MN08) virus and challenged with a mismatched virus of the same HA subtype, pH1N1, enhanced lung pathology was demonstrated (11, 12).

The VAERD model traditionally used prior to these studies was based on naive pigs receiving the first dose of WIV vaccine at approximately 4 weeks of age, a boost at 7 weeks of age, and challenge at 10 weeks of age. The objectives of the current studies were to determine if age at the time of WIV vaccination, WIV valency
FIG 5 Systemic IgG response following bivalent and monovalent WIV and LAIV vaccination. (A to D) Study 1. Serum anti-MN08 IgG titers prior to challenge (A) and postchallenge (B) and cross-reactive serum anti-CA09 IgG titers prior to challenge (C) and postchallenge (D) are shown. (E to H) Study 2. Serum anti-MN08 IgG titers prior to challenge (E) and postchallenge (F) and cross-reactive serum anti-CA09 IgG titers prior to challenge (G) and postchallenge (H) are shown. Statistically significant differences between treatment group means are identified by different lowercase letters (P ≤ 0.05). The error bars represent the standard errors of the means.
Mucosal IAV-specific IgA and IgG titers in pigs vaccinated with bivalent and monovalent WIV and LAIV. Pigs were vaccinated and challenged as described in Materials and Methods, and BALF was collected at 5 dpi to evaluate IgA and IgG specific to the indicated virus (MN08 vaccine virus or CA09 challenge virus).

(A to D) Study 1. Titers of anti-MN08 IgA (A), anti-CA09 IgA (B), anti-MN08 IgG (C), and anti-CA09 IgG (D) are shown. (E to H) Study 2. Titers of anti-MN08 IgA (E), anti-CA09 IgA (F), anti-MN08 IgG (G), and anti-CA09 IgG (H) are shown. Statistically significant differences between treatment group means are identified by different lowercase letters ($P \leq 0.05$). The error bars represent the standard errors of the means.
formulation, or time between vaccination and challenge would alter the development of VAERD.

Other vaccine platforms, such as LAIV, have been approved and broadly used in humans (34, 35) but are not currently licensed for use in swine. Experimental LAIV vaccines have been shown to induce a broadly cross-protective immune response against antigenically different IAV in pigs (19, 22, 36, 37). In addition, LAIV or natural infection can induce more efficient cross-protective response due to the activation of antibody- and cell-mediated immunity (38). A tsLAIV using a contemporary swine-origin IAV strain with the TRIG backbone developed by reverse genetics (18) demonstrated efficacious induction of cell-mediated and humoral responses in pigs against a homologous virus (39). Another vaccine study using tsLAIV containing pH1N1 and challenged with heterologous virus, a 81-H1N2 IAV (the reverse order of the study here) (21), demonstrated a cross-protective immune response similar to that shown in the present study. However, when the reverse order is used with a WIV vaccine (WIV containing pH1N1) and challenge (81-H1N2 IAV), VAERD still occurred (40).

Pigs were previously shown to develop VAERD in the course of

FIG 7 (A to F) Study 1. Cytokine protein concentrations (in picograms per milliliter) in BALF (5 dpi) of pigs vaccinated with bivalent (MN-TX98 and MN08-CA09) and monovalent 3wpb-MN08 or 6wpb-MN08, challenged with pH1N1. IL-4 (A), TNF-α (B), IL-2 (C), IL-1β (D), IFN-γ (E), and IL-10 (F) concentrations are shown. (G to I) Study 2. IL-1β (G), IFN-γ (H), and IL-10 (I) protein concentrations (in picograms per milliliter) in cell-free BALF (5 dpi) of vaccinated and pH1N1-challenged groups and control groups (NV/C and NV/NC) are shown. The data are presented as box plots demonstrating the median cytokine concentration (in picograms per milliliter) per group, with the 5th and 95th percentile and standard deviation. Statistically significant differences (P < 0.05) of vaccinated groups compared to controls are shown by asterisks.
IAV infection as early as 24 h postinfection (12). Microscopic lung lesions in VAERD are characterized by widespread interlobular edema with hemorrhage, as observed in pigs coinfected with PCV2 and PRRSV (11, 24); however, the VAERD–affected pigs in our experimental model are free of these additional viral pathogens. In the current study, regardless of the age of vaccination or duration between boost and challenge, pigs vaccinated with WIV formulated with the MN08 virus demonstrated significant macro- and microscopic pneumonia, consistent with VAERD (12, 13, 23). The only exception was the bivalent MN08-CA09 group, which was formulated with the homologous challenge strain as well. In addition, mismatched monovalent and bivalent WIV vaccines did not protect pigs from shedding virus and virus replication in lungs, as observed at 3 and 5 dpi in study 1. Despite the low virus titers in nasal swabs and lungs of pigs in the WIV groups in study 2, virus was detected in lungs by IHC. Limited pH1N1 virus shedding at 3 and 5 dpi with the WIV-H1N2-vaccinated pigs was similarly observed when challenged at 10 weeks of age (23); however, another study using the reverse order of viruses with a subunit vaccine (pH1N1) and challenge with δ1-H1N2 IAV at 15 weeks of age demonstrated high virus titers in nasal swabs and lungs (21). Therefore, the age of challenge (15 weeks of age) may be combined with variation between virus strains and variation between outbred pigs in an interaction that contributed to the limited virus shedding at 3 and 5 dpi in WIV-vaccinated groups from study 2. In contrast to WIV vaccines, virus antigen was not detected in the lungs of pigs in the LAIV group by IHC, suggesting cross-protection against pH1N1 even at 8 weeks following boost, similar to what was observed previously for the inverse combination of CA09 LAIV and MN08 challenge (21). This study confirmed that the LAIV vaccine induced a protective immune response against heterologous infection longer than that observed in previous studies with pigs challenged at 3 wpb (10 weeks of age) (23).

Study 2 demonstrated that vaccinating naive pigs with MN08-WIV at 4 weeks of age (WIV-4/7), in direct comparison with administering the priming dose at 9 weeks of age (WIV-9/12), elicited an HI antibody response increased in magnitude, and this was a consistent trend with other immune parameters discussed below. However, this increased HI response was not appreciable in study 1 in either monovalent WIV group (3wpb-MN08 and 6wpb-MN08) that received the first dose at 4 weeks of age, similar to study 2. Both of these groups had HI antibody responses similar to our previous studies at the prechallenge time point (12, 13). With the exception of the WIV MN08-CA09 group, none of the vaccinated groups developed detectable HI antibodies against the heterologous challenge virus.

All WIV-vaccinated and challenged pigs demonstrated high levels of anti-MN08 and cross-reactive anti-CA09 IgG in serum and BALF, irrespective of the age of vaccination, time of infection, or vaccine valency (monovalent or bivalent WIV vaccine), which is a consistent immune parameter in WIV-vaccinated pigs that develop VAERD. In contrast, LAIV vaccination followed by challenge resulted in minimal cross-reactive serum IgG antibodies postchallenge (BALF). This lower level of cross-reactive IgG response observed in the LAIV group than in the WIV groups may be correlated with the absence of enhanced lung lesions, corroborating previous findings that compared WIV and LAIV platforms with a heterologous challenge at 10 weeks of age (23). Although the precise contribution of these cross-reactive nonneutralizing antibodies to the VAERD outcome remains unclear, it is hypothesized that they may contribute to antibody-dependent cell-mediated cytotoxicity (ADCC) (41) or activate the complement cascade (42). Moreover, it was found that the nonneutralizing cross-reactive IgG elicited following WIV vaccination and associated with the development of VAERD upon challenge was targeted to the conserved HA2 stalk domain of the challenge virus (14). These cross-reactive anti-HA2 antibodies also increased pH1N1 virus infectivity in vitro in MDCK cells, likely by promoting virus fusion (14). Although the MN08-WIV vaccine induces high levels of cross-reacting IgG anti-pH1N1 directed to the HA2 domain, LAIV-vaccinated pigs did not show an HA2 domain binding preference (23).

Robust IAV-specific IgG responses in the lungs are not consistently observed following experimental WIV vaccination and heterologous challenge; however, MN08-specific IgG in the BALF samples from the WIV-vaccinated and challenged groups was detected in study 1. MN08-specific IgA in BALF was detected mainly in challenged pigs vaccinated with the bivalent MN08-TX98 and monovalent MN08 vaccines when challenged at 3 wpb. This may be explained by a rapid booster response postchallenge related to priming with these vaccines. In contrast, pigs vaccinated with MN08-CA09 did not demonstrate marked levels of IgA response in BALF, likely due to the consumption of the IgA directed against homologous CA09 challenge virus. Intranasal LAIV vaccines consistently prime mucosal IgA and IgG responses associated with cross-protection (18, 21–23). In our study 2, LAIV vaccination and challenge resulted in levels similar to those of the WIV platform of IAV-specific IgA in BALF against both strains, yet the LAIV vaccine provided significant cross-protection even 8 wpb, similar to other studies that demonstrated that LAIV provided a cross-protective immune response over the WIV platform without the risk of VAERD at 3 or 8 wpb (21, 23).

Cytokines play a role in the regulation of the lymphocyte activation, T and B cell proliferation, and differentiation during IAV infection in pigs (43, 44). Elevated pulmonary proinflammatory cytokines are associated with excessive inflammation (45, 46) and likely play a role in lung damage in VAERD-affected pigs (13). Despite the severe lung pathology observed in all WIV-MN08-mismatched vaccinated groups, only the monovalent MN08-WIV group challenged at 6 wpb showed markedly increased levels of the IL-2, IL-1β, IFN-γ, and IL-10 cytokines in BALF. In contrast, although TNF-α and IL-4 were found at significant levels in BALF, the increase was not as marked. Although an increased cytokine profile was not found in all WIV groups that developed VAERD in study 1, such cytokine dysregulation, as previously observed (13, 23), may have a role in the neutrophil recruitment and exacerbation of the inflammatory response that may be linked with the immunopathology of the lungs (44, 45). Likewise, in study 2, the WIV group primed at 4 weeks of age, boosted at 7 weeks of age, and challenged at 8 wpb demonstrated a cytokine profile of increased IL-1β, IFN-γ, and IL-10 in BALF. The difference in the magnitude of cytokine protein levels between studies 1 and 2 may be due to the switch to using cell-free BALF in study 2, giving lower but less-variable results by targeting protein released into the interstitium and reducing the detection of intracellular proteins. However, the 6-wpb pigs from study 1 demonstrated higher virus titers in BALF that may also be related to the higher cytokine profile in these pigs.

Age at first vaccination seemed to impact WIV immunogenicity as well as the cytokine responses in a comparison of the WIV-
4/7 and WIV-9/12 groups. Thus, although the age of vaccination did not appear to significantly influence the development of VAERD following WIV vaccination, there was a trend of increased severity of lung pathology in the pigs first vaccinated at a younger age. This potentially interesting trend should be further explored in the context of basic neonatal immunity in relation to commercial vaccine application, albeit with the knowledge that most piglets in farm settings would have some degree of passive maternal immunity at this age due to vaccine usage or natural exposure in their dams, and very few would likely be naive as in this experimental model.

In conclusion, these data indicate that the age at first vaccination or length of time between booster dose and subsequent heterologous challenge did not alter the development of VAERD in WIV-vaccinated pigs. In addition, the inclusion of an H3N2 virus in a bivalent WIV vaccine with MN08 did not alter the induction of VAERD, whereas the inclusion of the mismatched MN08 strain with the matched pH1N1 in a bivalent WIV vaccine did not abrogate the protective response to the homologous virus. This is consistent with previous findings in which antisera from WIV-pH1N1-vaccinated pigs blocked pH1N1-MDCK infectivity compared to the increased infectivity of pH1N1 in the presence of antisera from WIV-MN08-vaccinated pigs (14).

Although WIV vaccines induce a robust peripheral immune response and are efficacious against homologous virus, vaccine antigen/challenge virus mismatch can lead to a vaccine failure ranging from loss of cross-protection to severe lung pathology (VAERD) that is difficult to diagnose in the field. Therefore, it is necessary to understand the benefits versus risk of inactivated vaccines used by the swine industry due to the potential mismatch of circulating IAV strains and to determine if the immune response can be improved by other vaccine platforms that induce balanced cross-protective antibody and cellular immune responses against IAV.

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