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Restored PB1-F2 in the 2009 Pandemic H1N1 Influenza Virus Has Minimal Effects in Swine

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PB1-F2 is an 87- to 90-amino-acid-long protein expressed by certain influenza A viruses. Previous studies have shown that PB1-F2 contributes to virulence in the mouse model; however, its role in natural hosts—pigs, humans, or birds—remains largely unknown. Outbreaks of domestic pigs infected with the 2009 pandemic H1N1 influenza virus (pH1N1) have been detected worldwide. Unlike previous pandemic strains, pH1N1 viruses do not encode a functional PB1-F2 due to the presence of three stop codons resulting in premature truncation after codon 11. However, pH1N1s have the potential to acquire the full-length form of PB1-F2 through mutation or reassortment. In this study, we assessed whether restoring the full-length PB1-F2 open reading frame (ORF) in the pH1N1 background would have an effect on virus replication and virulence in pigs. Restoring the PB1-F2 ORF resulted in upregulation of viral polymerase activity at early time points *in vitro* and enhanced virus yields in porcine respiratory explants and in the lungs of infected pigs. There was an increase in the severity of pneumonia in pigs infected with isogenic virus expressing PB1-F2 compared to the wild-type (WT) pH1N1. The extent of microscopic pneumonia correlated with increased pulmonary levels of alpha interferon and interleukin-1β in pigs infected with pH1N1 encoding a functional PB1-F2 but only early in the infection. Together, our results indicate that PB1-F2 in the context of pH1N1 moderately modulates viral replication, lung histopathology, and local cytokine response in pigs.

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae* and represent important pathogens of humans and animals. In the 20th century, humans experienced three IAV pandemics (1918, 1957, and 1968) that resulted in significant morbidity and mortality (62). These pandemic strains emerged through genetic reassortment between influenza viruses of avian origin and the circulating human strain. These events resulted in antigenic shift and the successful dissemination, in an immunologically naïve population, of a virus carrying a novel hemagglutinin (HA). In addition to the new HA, pandemic strains inherited the PB1 gene segment from the avian influenza donor virus (and the NA gene in 1957) (57). Like most avian influenza viruses, these previous pandemic strains expressed a full-length PB1-F2 gene. However, human influenza strains have invariably evolved to introduce truncations in the PB1-F2 open reading frame (ORF). It is tempting to speculate that PB1-F2 function is necessary for virus survival in birds but unnecessary or detrimental in humans. Its significance in pandemic strains remains obscure.

In the spring of 2009, a novel H1N1 IAV emerged in North America, causing acute respiratory disease in humans. The virus quickly spread throughout most regions of the world, prompting the World Health Organization (WHO) to declare an influenza pandemic on 11 June 2009 (13, 15). The new virus (pH1N1) resulted from genetic reassortment between swine influenza viruses (SIVs) circulating in North America and Eurasia. Specifically, six of its RNA segments (PB2, PB1, PA, HA, NP, and NS) are closely related to North American triple-reassortant (TR) H1N1 SIVs, whereas the NA and M gene segments are related to Eurasian H1N1 SIVs (15). This unique gene constellation has never been described among influenza isolates from anywhere in the world, and the precise evolutionary history of the 2009 pH1N1 is unknown (1, 13, 17, 57). Several outbreaks of pH1N1 virus infections in animals have been reported worldwide. These outbreaks were predominantly documented in pigs, but incidental infection in turkeys, cats, ferrets, dogs, and wild animals has been described (4, 14, 27, 37, 46, 50, 51, 58, 59, 61). In pigs, field outbreaks of pH1N1 have been reported in more than 20 countries, and epidemiological investigations have linked humans as the probable source (25, 27). Experimentally, pigs are susceptible to human pH1N1 viruses, and the virus is highly transmissible in swine (6, 29, 32, 70, 75). Pigs inoculated with the pH1N1 virus show clinical disease signs and pathology similar to those seen with infection with other SIVs (6, 29, 32, 70, 75).

Pigs are susceptible to human influenza viruses; however, and perhaps unlike humans, they appear susceptible to a wide range of avian influenza viruses. Experimental infection studies showed that pigs were susceptible to 13 different influenza virus subtypes (H1 to H3 and non-human-type HA types H4 to H13) (30). The mixing vessel attribute is consistent with the presence in the respiratory tract of pigs of the prototypical human-like and avian-like influenza receptors *N*-acetylneuraminic acid-α2,6-galactose (α2,6Gal) and *N*-acetylneuraminic acid-α2,3-galactose (α2,3Gal), respectively (28). Although subsequent studies have shown both types of receptors are also found in the respiratory tract of quail, pheasant, turkey, guinea fowl (31, 73), and humans (55), pigs have been commonly associated with two-way transmission of influenza viruses to and from humans (43, 45, 54, 71). Pigs were undoubtedly involved in the genesis of the 2009 pH1N1 (15). Since then, several reassortants between pH1N1 and circulating influenza A viruses have been isolated from pigs in

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several countries (26, 42, 51, 60, 69), raising great concerns about the potential acquisition of virulence markers by the pH1N1 virus upon reassortment with other strains in the swine host.

Among the virulence factors that could be acquired by pH1N1 is the nonstructural protein PB1-F2. In contrast to the three previous pandemic influenza viruses, pH1N1 does not encode a functional PB1-F2 due to the presence of three stop codons that causes a premature truncation (63). PB1-F2 is an 87- to 90-amino-acid (aa)-long protein encoded by an alternate (+1) open reading frame (ORF) within the PB1 gene. Translation of PB1-F2 mRNA is likely mediated by ribosomal scanning, and the protein is expressed early and transiently in infected cells (10). PB1-F2 is expressed mostly by IAVs of avian origin (78), and its presence is not required for viral replication in embryonated eggs, in tissue culture, or *in vivo* (11). However, PB1-F2 expression results in enhanced apoptotic-cell death in immune cells, viral pathogenicity in mice, and immunopathology (10, 12, 39, 40, 77). Additionally, the presence of PB1-F2 in both the 1918 and the mouse-adapted PR8 influenza A viruses enhances secondary bacterial pneumonia in the mouse model (40). Surprisingly, two recent studies investigating the role of PB1-F2 in the pathogenicity of clinically relevant human viruses and the pH1N1 concluded that the expression of PB1-F2 has minimal effects on the virulence of these viruses in murine and ferret models (19, 41). From these studies, it appears that the role of PB1-F2 in modulating influenza virus pathogenicity is cell type and virus strain specific and/or species dependent. The contribution of PB1-F2 to the virulence of influenza viruses in swine has yet to be determined.

Here, we restored the PB1-F2 ORF in pH1N1 and studied its effects on viral pathogenicity and host responses in pigs. Restoring PB1-F2 in the pH1N1 virus resulted in increased virus replication in swine respiratory explants. More importantly, in pigs, a pH1N1 virus expressing a complete PB1-F2 ORF increased virus replication in the lung and enhanced lung histopathology and higher pulmonary levels of alpha interferon (IFN-α) and interleukin-1β (IL-1β) than the wild-type (WT) virus but only at early times postinfection. Although these effects were minimal and observed only at early times postinfection, these findings suggest that PB1-F2 can modulate pH1N1 pathogenicity and cytokine responses in swine.

MATERIALS AND METHODS

Animal studies. Pig explants were prepared according to protocol in "Transmissibility of Influenza A Viruses in Swine," approved by the Institutional Animal Care and Use Committee, University of Maryland, College Park, MD. Swine pathogenicity studies were conducted in the high containment facilities at the National Animal Disease Center in Ames, IA, under protocol in "Influenza A Virus Pathogenesis and Host Response in Swine," approved by the USDA-ARS Animal Care and Use Committee. Animal studies adhered strictly to the U.S. Animal Welfare Act (AWA) laws and regulations.

Swine pathogenicity experiment. Three-week-old cross-bred pigs were obtained from a high-health herd free of SIV and porcine reproductive and respiratory syndrome virus. All pigs were treated with ceftiofur crystalline free acid (Pfizer Animal Health, New York, NY) to reduce bacterial contaminants prior to the start of the experiment. Twenty pigs were randomly divided into two groups ($n = 10$) and housed in separate isolation rooms. Pigs were infected intratracheally with 1×10^5 50% tissue culture infective doses ($TCID_{50}$) of either influenza virus A/California/04/09 (H1N1) (Ca/04) WT or an isogenic virus expressing PB1-F2 (Ca/04 KI) diluted in 2 ml of modified Eagle's medium (MEM) using previously described protocols (70). Following inoculation, pigs were monitored daily for clinical signs of disease, including fever, anorexia, inactivity, huddling, nasal discharge, conjunctivitis, coughing, and dyspnea. Nasal swabs were collected daily to measure viral shedding. Five pigs from each group were euthanized on day 1, and the remaining five animals were euthanized at 3 days postinfection (dpi) for evaluation of viral lung load, pathology, and host response to infection. Five additional pigs were inoculated with 2 ml MEM as described above and served as mock controls. Control pigs were euthanized at 3 dpi.

Cell lines and virus strains. Madin-Darby canine kidney (MDCK) cells were maintained in modified Eagle's medium (MEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), L-glutamine, and antibiotics. Human embryonic kidney cells (293T) were cultured in Opti-MEM I (Gibco, Grand Island, NY) containing 5% FBS and antibiotics. A/California/04/09 (H1N1) (Ca/04) was kindly provided by the Centers for Disease Control and Prevention (CDC), Atlanta, GA. Ca/04 wild type (WT) and the recombinants thereof were propagated in MDCK cells for 3 days at 35°C to produce viral stocks. The recombinant Ca/04 viruses used in this study were generated from cloned cDNAs and are described below.

Mutagenesis and rescue of recombinant influenza viruses. The eight gene segments of Ca/04 were amplified by reverse transcriptase PCR (RT-PCR) and cloned in the bidirectional reverse genetics (RG) plasmid derived from pHW2000 (24). The QuikChange II site-directed mutagenesis kit (Stratagene, Inc., La Jolla, CA) was used according to the manufacturer's protocols to introduce changes in the PB1-F2 open reading frame (ORF). The PB1-F2 ORF in the PB1 segment was restored by mutating the stop codons at position 12, to code for serine, and at positions 58 and 88, to code for tryptophan, as previously described (19). The mutations did not change the PB1 ORF. The recombinant viruses were generated by transfecting cocultured 293T and MDCK cells as previously described (24). In order to improve virus rescue and growth in tissue culture, the HA gene from the mouse-adapted Ca/04 (76) was used in these experiments. All RG plasmids and recovered recombinant viruses were fully sequenced to confirm their identity.

Minigenome assay to study polymerase activity. The minigenome assay was performed as described previously (49). Briefly, 1 μg of the plasmid containing the influenza virus-like NS viral RNA (vRNA) carrying the *Gaussia* luciferase (GLuc) reporter gene was transfected into 293T cells along with 1 μg of each of the RG plasmids encoding Ca/04 PB2, PB1, PA, and NP using the TransIT-LT1 (Mirus, Madison, WI) reagent following the manufacturer's recommendations. The Ca/04 PB1 plasmid contained either the WT PB1-F2 (truncated after codon position 11) or the artificially restored PB1-F2 ORF. The pCMV/SEAP (SEAP) plasmid, which expresses the secreted alkaline phosphatase, was cotransfected into cells to normalize the transfection efficiency. Alternatively, MDCK cells were transfected with GLuc reporter under the control of the canine polymerase I promoter (Pol I) for 12 h followed by infection with Ca/04 WT or Ca/04 KI virus at a multiplicity of infection (MOI) of 1 or 0.01. At the indicated time points, supernatant from transfected cells was harvested and assayed for both luciferase and secreted alkaline phosphatase activities using the BioLux *Gaussia* luciferase assay kit (NEB, Ipswich, MA) and the Phospha-Light secreted alkaline phosphatase reporter gene assay system (A&D, Foster City, CA) according to the manufacturers' instructions. Relative polymerase activity was calculated as the ratio of luciferase and SEAP luminescence from two independent experiments performed in quadruplicate.

Isolation, culture, and infection of porcine respiratory explants. Porcine nasal turbinate (NT), tracheal, and lung explants were prepared as described previously (65), with some modifications. All respiratory explants were cultured at an air-liquid interface at 37°C and 5% CO_2. NT and tracheal explants were cultured in 50% Dulbecco MEM (DMEM) (Gibco)-50% RPMI (Gibco) media supplemented with 100 U/ml penicillin (Gibco), 100 μg/ml of streptomycin (Gibco), 0.1 mg/ml gentamicin (Gibco), 25 μg/ml amphotericin B (Gibco), 0.3 mg/ml glutamine (BDH Biochemical), and nonessential amino acids (Sigma). Lung explant me-

dium consisted of M199 (Sigma) containing antibiotics (as above) and nonessential amino acids (Sigma), vitamin supplement at 10%, vol/vol (ATCC, Manassas, VA), 0.5 μg/ml hydrocortisone, and ITS (insulin, transferrin, selenium) supplement added at 10 ml/liter media. A 5-week-old swine donor obtained from a high-health-status farm whose animals are negative for IAV was used for this study. The animal was humanely euthanized with Beuthanasia-D (Intervet/Schering-Plough, Summit, NJ) at a dosage of 1 ml/4.5 kg of body weight, and the respiratory tissues were collected. NT and tracheal explants were dissected and washed 10 times with phosphate-buffered saline (PBS) containing antibiotics to remove bacterial contamination. Tissues were then cut into squares of 25 mm^2 each and placed with the epithelial surface upwards onto the filter membrane of the polyester tissue culture-treated inserts (Transwells; Corning, Lowell, MA) at an air-liquid interface in 12-well plates. The lower compartment was filled with 1 ml of explant media. The right apical lung lobe was expanded with a 1% type VII-A low-gelling-temperature agarose solution (Sigma, St. Louis, MO) that had been dissolved in lung explant media and cooled down to 37°C. The expanded lung was placed at 4°C for 10 min in a sterile container until the agarose solidified. The embedded lung tissue was then cut into 1-mm-thick slices using a microtome blade and hand microtome. Two sections of the lungs were obtained: the proximal lung (close to the start of the bronchial tree) and the distal lung (close to the lung alveoli). The procedure for culturing of lung explants was similar to the procedure used for the NT and tracheal explants. After 24 h, lung explants were washed with warm PBS to remove most of the agarose before infection. At 24 h of culture, explants were washed with PBS, and 10^6 $TCID_{50}$ of the recombinant viruses diluted in 500 μl of explant media was deposited in the upper compartment of Transwells for 1 h at 37°C. Subsequently, explants were washed three times with PBS, and the culture was replenished with 500 μl of explant media. One hundred microliters of upper compartment explant bathing medium was collected at 24, 48, and 72 h postinoculation to assess virus yields. Virus titers in respiratory explants were determined by the standard $TCID_{50}$ method in MDCK cells using an HA assay as the readout as described below.

Virus titration. Viral stocks and virus present in biological samples were titrated on MDCK cells, and the $TCID_{50}$/ml was determined by the method of Reed and Muench (52). Briefly, samples were serially diluted 10-fold in serum-free medium containing antibiotics and 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma), and 100 or 200 μl of the inoculum was overlaid onto confluent monolayers of MDCK cells seeded in 96-well plates. The cells with the sample were incubated for 3 days, and the endpoint viral titer was determined by an HA assay using 0.5% turkey red blood cells.

Pathological examination of swine lungs. At necropsy, lungs were removed *in toto* and evaluated to determine the percentage of the lung affected by purple-red, consolidated lesions that are typical of influenza virus infection in pigs. The percentage of the surface affected with pneumonia was visually estimated for each lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume as previously described (20). Each lung was then lavaged with 50 ml MEM to obtain bronchoalveolar lavage fluid (BALF). A veterinary pathologist scored all lungs and was blinded to the treatment groups.

Histopathology and immunohistochemistry. Tissue samples from the trachea and right middle lung lobe were taken and fixed in 10% buffered formalin for histopathological examination. Tissues were routinely processed and stained with hematoxylin and eosin. Microscopic lesions were evaluated by a board-certified veterinary pathologist blinded to treatment groups. Scoring of lesions was based on scales adapted from the work of Gauger et al. (16). In brief, individual scores were assigned to four parameters: bronchial and bronchiolar epithelial changes, bronchitis/bronchiolitis, peribronchiolar lymphocytic cuffing, and interstitial pneumonia. Trachea sections were scored similarly to the bronchi and bronchioles, based on epithelial changes and the degree of inflammation.

Influenza virus type A-specific antigen was detected in lung tissues using a previously described immunohistochemical (IHC) method with minor modification (72). Briefly, tissue sections were deparaffinized and hydrated in distilled water. Slides were quenched in 3% hydrogen peroxide for 10 min, rinsed three times in deionized water, and treated in 0.05% protease for 2 min. Slides were then rinsed three times in deionized water and once in Tris-buffered saline (TBS). Influenza A virus-specific monoclonal antibody (MAb) HB65 (ATCC, Manassas, VA), specific for the nucleoprotein (NP) of influenza A viruses, was applied at a 1:100 dilution, and slides were incubated at room temperature for 1 h. Bound MAbs were stained with peroxidase-labeled anti-mouse IgG followed by chromogen using the Dako LSAB2-horseradish peroxidase (HRP) detection system (Dako, Carpinteria, CA) according to the manufacturer's instructions. The slides were rinsed in deionized water and counterstained with Gill's hematoxylin. Antigen detection was given two scores: (i) airway epithelial labeling and (ii) alveolar/interstitial labeling. In airway epithelium, a 5-point scale was used: 0, none; 1, few cells with positive labeling; 2, mild scattered labeling; 3, moderate scattered labeling; 4, abundant scattered labeling (greater than 50% epithelium positive in affected airways). In the interstitium/alveoli, a 4-point scale was used: 0, none; 1, minimal focal signals; 2, mild multifocal signals; 3, abundant signals.

Quantification of cytokine/chemokine protein levels in bronchoalveolar lavage fluid. Levels of nine porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor alpha [TNF-α], and IFN-γ) in BALF were determined by multiplex enzyme-linked immunosorbent assay (ELISA) following the manufacturer's recommendations (SearchLight, Aushon Biosystems, Billerica, MA). Levels of IFN-α protein were measured by ELISA using F17 monoclonal antibody, K9 MAb, and recombinant porcine IFN-α (R&D Systems Inc., Minneapolis, MN) as previously described (5).

Statistical analysis. All statistical analyses were performed using GraphPad Prism software version 5.00 (GraphPad Software Inc., San Diego, CA). Comparison between two treatment means was achieved using a two-tailed Student *t* test, whereas multiple comparisons were carried out by two-way analysis of variance (ANOVA) considering time and virus as factors. The differences were considered statistically significant at a *P* value of <0.05.

RESULTS

Restoring PB1-F2 in the Ca/04 pH1N1 background upregulates early polymerase activity. It has been shown that PB1-F2 derived from the laboratory strain A/Puerto Rico/8/34 (H1N1) (PR8) interacts with the polymerase subunit PB1 and increases polymerase activity (38). We wanted to determine whether polymerase activity of a pH1N1 virus would be affected when the PB1-F2 ORF was restored. To this end, we first developed the entire reverse genetics system for the Ca/04 strain. The three stop codons present in the Ca/04 WT PB1-F2 were changed to code for serine (codon 12) and tryptophan (codons 58 and 88), thus allowing translation of the full-length PB1-F2 (Ca/04 knock-in or Ca/04 KI), as previously described (19, 47). These point mutations were silent in the PB1 ORF.

To study viral polymerase activity in the absence or presence of PB1-F2, we used an influenza virus minigenome assay as previously described (49). Cotransfection of 293T cells was performed with plasmids containing the Ca/04 PB2, PB1 (with or without full-length PB1-F2), PA, and NP genes and the influenza replicon carrying GLuc. Transfection efficiency was normalized using a plasmid containing the SEAP reporter gene under the control of an RNA Pol II promoter. Polymerase activity is monitored by the Gluc/SEAP ratio. These experiments revealed that the presence of PB1-F2 led to higher polymerase activities than in its absence (Fig. 1A) but only at an early time point (12 hours posttransfection [hpt]). The minigenome assay does not reflect all of the regulatory

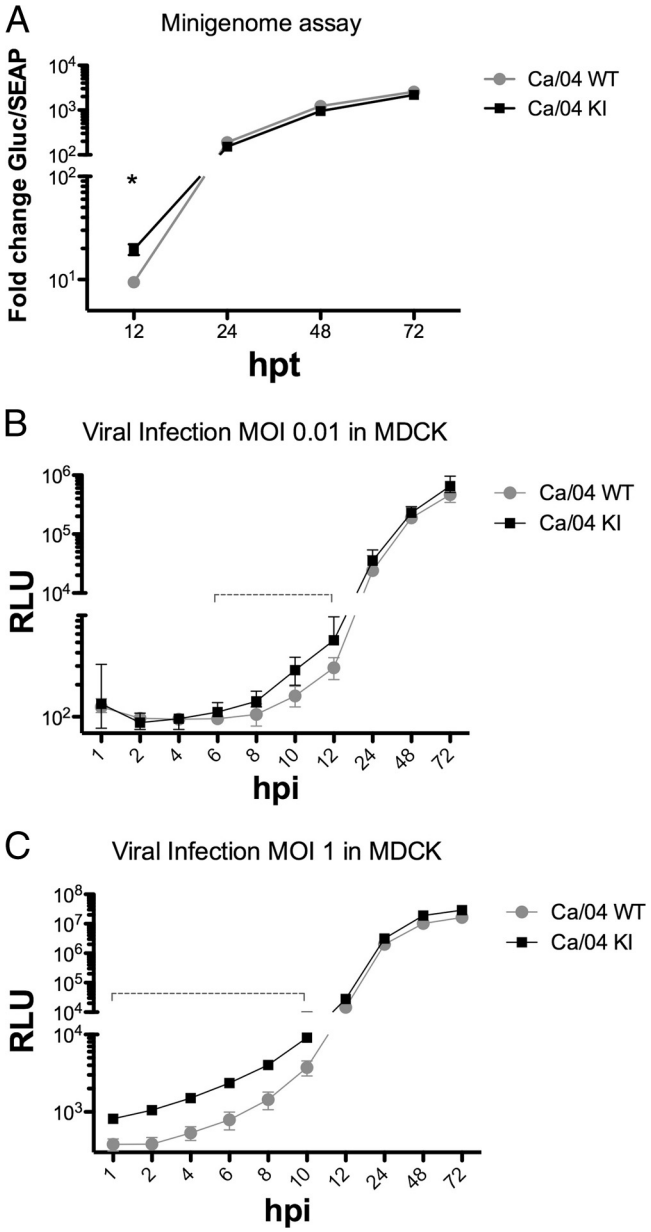

FIG 1 PB1-F2 upregulates early polymerase activity. (A) Minigenome assay. 293T cells were transfected with plasmids encoding the minimal components required for viral transcription and replication (PB2, PB1, and PA polymerase subunits, NP, and a vRNA influenza virus-driven luciferase reporter replicon expressing GLuc) and with pCMV/SEAP, a plasmid to normalize transfection efficiency, as previously described (49). At the indicated hours posttransfection (hpt), the supernatant was harvested and assayed for both luciferase and phosphatase activities. (B and C) Kinetics of polymerase activity after virus infection. MDCK cells were transfected with 1 μg of GLuc reporter under the control of the canine Pol I reporter, followed by infection with Ca/04 (WT or KI) virus at an MOI of 0.01 (B) or 1 (C). Gluc activity was determined as described in Materials and Methods at the indicated hours postinfection (hpi). Data are expressed as polymerase activities (mean ± standard error [SE]) determined from two independent experiments performed in quadruplicate. A two-tailed Student *t* test was used to determine significant differences between two treatment means for each data point. An asterisk and dashed brackets indicate statistically significant differences ($P < 0.05$).

events that occur during the course of infection since it involves only the minimal components required for viral transcription and replication (53). To overcome this drawback and study the effect of PB1-F2 on polymerase activity in the context of the viral life cycle, we transfected MDCK cells with GLuc reporter under the control of the canine Pol I reporter, followed by infection with Ca/04 (WT or KI) virus at an MOI of 0.01 or 1. Consistent with the results from the minigenome, the presence of PB1-F2 enhanced viral polymerase activity but only at early times postinfection. The enhancement in polymerase activity was statistically significant ($P < 0.01$) between 6 and 12 hpi when an MOI of 0.01 was employed (Fig. 1B) and between 1 and 10 hpi when an MOI of 1 was used (Fig. 1C). Together, these results imply that PB1-F2 promotes temporal regulation of pH1N1 polymerase activity.

PB1-F2 enhances Ca/04 replication in porcine respiratory explants. It has been demonstrated that expression of full-length wild-type PB1-F2 by pH1N1 has no effects on viral replication in human A549 cells or MDCK cells (19, 47). However, cell lines do not exhibit the natural physiological conditions and cellular complexity present in the respiratory tract. To address this limitation and study the impact of PB1-F2 expression in a relevant biological system, we developed an *ex vivo* organ culture model of the pig respiratory tract maintained at an air-liquid interface. These tissue explants retained their cytoarchitecture (data not shown) and supported productive replication of the recombinant Ca/04 influenza viruses (Fig. 2). Restoring PB1-F2 in Ca/04 improved virus replication in explants of nasal turbinates (Fig. 2A), trachea (Fig. 2B), and proximal lung (Fig. 2C). No differences were observed in explants of the distal lung (Fig. 2D). This enhancing effect of PB1-F2 in virus production was mainly seen at 24 hpi but not at 48 or 72 hpi. These data indicate that PB1-F2 modulates moderately early viral production in swine respiratory tissues infected *ex vivo*.

Restored PB1-F2 ORF increases virus replication in swine lungs. To evaluate whether the enhanced viral yields in porcine respiratory explants displayed by Ca/04 KI would correlate with increased viral replication *in vivo*, we inoculated groups of 3-week-old pigs ($n = 10$/group) with the Ca/04 WT or Ca/04 KI viruses. From each group, 5 pigs were euthanized at 1 and 3 dpi and viral titers in BALF were determined. Consistent with the explant data, restoring the PB1-F2 ORF resulted in statistically significant ($P < 0.05$) increases in viral loads in the lungs at either time point (Fig. 3A). Increased Ca/04 KI replication in the lungs was further corroborated by the more pronounced pulmonary expression of influenza NP antigen by IHC analysis (Fig. 3C). As expected, mock-inoculated pigs had neither detectable virus nor IHC influenza-positive cells in BALF or lung tissues (data not shown). Collectively, these results suggest that restoring the PB1-F2 ORF in the Ca/04 virus leads to increased viral replication *in vivo*.

PB1-F2 aggravates microscopic pneumonia despite no differences in nasal virus shedding or macroscopic lesions in pigs. To determine if PB1-F2 affects the kinetics of viral shedding in pigs, nasal swabs collected from each pig from 1 to 3 dpi were titrated in MDCK cells. Virus shedding in nasal secretions was detected only at 3 dpi, and there was no difference between the Ca/04 WT and Ca/04 KI viruses (Fig. 4A). Infected pigs did not show any overt clinical signs of disease regardless of the virus used. At necropsy, pigs in both the Ca/04 WT and Ca/04 KI groups had cranioventral lung consolidation, with 5% to 10% of lung involvement at 3 dpi (Fig. 4B). There were no differences between the two

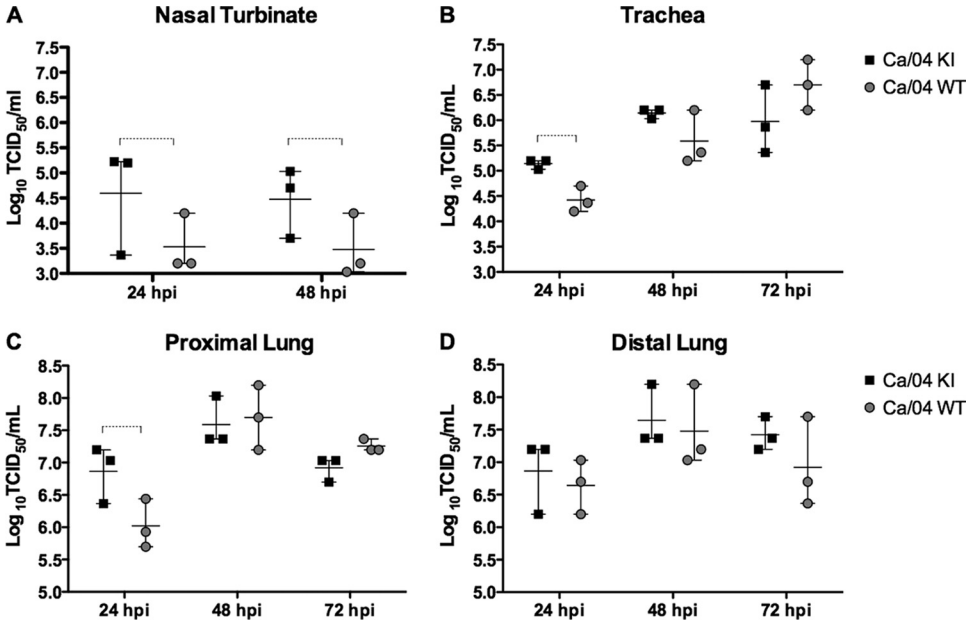

FIG 2 Replication of PB1-F2 recombinant viruses in porcine respiratory explants. Explants from nasal turbinates (A), trachea (B), and proximal (C) and distal (D) lung were infected with 10^6 $TCID_{50}$ of either Ca/04 WT or KI. The bathing medium was collected at the indicated time points and titrated by the $TCID_{50}$ method in MDCK cells. Values shown are the mean and range of virus titers (log_{10} $TCID_{50}$/ml) obtained from triplicate explant preparations. A two-tailed Student *t* test was used to determine significant differences between two treatment means for each data point. Dashed brackets indicate statistically significant differences ($P < 0.05$).

virus groups, indicating that PB1-F2 has no effect on the macroscopic lung pathology caused by Ca/04 in swine. However, differences in lung inflammation were detected at the microscopic level. Quantitative histopathological analyses revealed that pulmonary inflammation was enhanced in pigs in the Ca/04 KI group compared to the Ca/04 WT group (Fig. 5A and C). Unlike the mock-inoculated animals, which had normal histological features, the lesions in virus-infected pigs were typical of influenza virus pneumonia in pigs and were characterized by multifocal to widespread necrotizing bronchitis and bronchiolitis, light peribronchiolar lymphocytic cuffing, and multifocal interstitial pneumonia. Both viruses induced similar levels of tracheitis and cilium loss in the tracheal epithelium (Fig. 5B).

PB1-F2 exacerbates the pulmonary levels of IFN-α and IL-1β in swine. Cytokine production after influenza virus infection has been implicated in the pathogenesis of influenza in mammals, including pigs (66). To further characterize the effect of PB1-F2 in the pathological manifestations of pH1N in swine, we measured the protein levels of 10 porcine cytokines/chemokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α, IFN-α, and IFN-γ) in BALF collected at 1- and 3-dpi necropsies. Pulmonary levels of IFN-α and IL-1β were significantly higher at 1 dpi in pigs infected with Ca/04 KI than in those infected with Ca/04 WT (Fig. 6A and B). The production levels of other cytokines/chemokines (IL-6, IL-8, and IFN-γ), however, were similar between the two groups infected with the PB1-F2 recombinant viruses (Fig. 6C to E). IL-2, IL-4, IL-10, IL-12p70, and TNF-α did not reach detectable levels in this experimental infection (data not shown). Collectively, these results indicate that PB1-F2 induces higher expression levels of IFN-α and IL-1β early during pH1N1 infection in pigs and that this heightened production of proinflammatory mediators may have contributed to the more severe microscopic pneumonia developed by Ca/04 KI-infected animals.

DISCUSSION

Outbreaks of influenza A viruses (IAVs) are among the most significant causes of respiratory disease in both humans and animals. Occasionally, pandemic influenza viruses emerge when a virus acquires a new HA molecule to which the majority of the population is immunologically naïve, resulting in devastating levels of morbidity and mortality (64). Virulence of influenza viruses is considered a polygenic trait. Molecular studies in different animal models have determined that the gene constellation of a particular influenza virus strain within a specific host plays a pivotal role in disease outcome (3, 9, 22). Several proteins in the viral genome have clearly identified roles in virulence: the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are responsible for viral binding, entry, and release (48); the polymerase protein complex formed by PB2, PB1, and PA proteins, which drives genome transcription and translation (18, 74); and the nonstructural protein NS1, which disarms the interferon-based defense system of the host and also modulates other important aspects of the virus replication cycle (21).

A recently added member to the family of IAV proteins with a role in virulence is PB1-F2. Unlike other influenza viruses that have become adapted to humans, the 2009 pH1N1 virus lacks most of the known hallmarks of influenza virus virulence, including PB1-F2 (63). However, with the continued circulation of pH1N1 in humans and animals, especially in swine, there are opportunities for acquisition of PB1-F2 through mutations or genetic reassortment with cocirculating animal influenza viruses. We examined the functional significance of PB1-F2 acquisition by pH1N1 polymerase activity *in vitro* in mammalian cells (human, canine, and porcine tissue explants) as well as viral replication, pathogenicity, and host response to infection in its natural host,

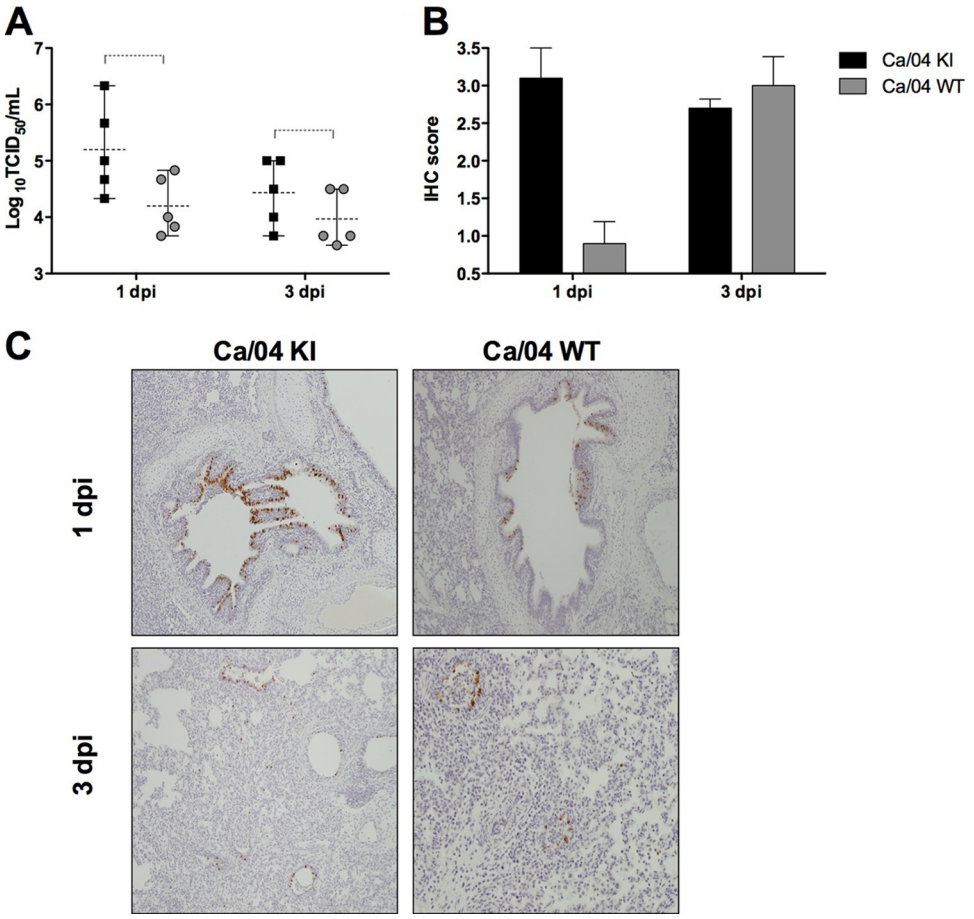

FIG 3 Replication and immunohistochemical analysis of PB1-F2 recombinant viruses in swine lungs. Groups of pigs ($n = 10$) were infected with 10^5 $TCID_{50}$ of PB1-F2 recombinant viruses, and five animals from each group were euthanized either at 1 or 3 dpi. The lungs were collected and processed for virus titration and immunohistochemical analysis. (A) Pulmonary replication of PB1-F2 isogenic viruses in pigs. Values are means and ranges of virus titers (log_{10} $TCID_{50}$/ml) in bronchoalveolar lavage fluid (BALF) collected at the indicated time points. Two-way ANOVA was used to determine significant differences between two treatment groups. Dashed brackets indicate statistically significant differences ($P < 0.05$). (B) Immunohistochemical staining against influenza A virus nucleoprotein (NP) in the lungs of infected pigs. Values given are the mean ± the standard error of the mean IHC scores based on the percentage of influenza virus-positive cells in the airway and lung interstitium. (C) Representative IHC slides depicting viral antigen primarily in airway epithelium at 1 and 3 dpi. Scattered labeling in the interstitium at 3 dpi is present.

the pig. The data demonstrated that the full-length PB1-F2 in a pH1N1 virus impacts polymerase activity, viral replication efficiency both *ex vivo* and *in vivo*, lung inflammation, and local cytokine responses to infection. However, these differences are only minor, which suggests that acquisition of the PB1-F2 ORF may not produce significant phenotypic changes in the virulence of pH1N1 viruses for pigs. These observations are consistent with the report by Hai et al. (19), which shows minimal effects of PB1-F2 in

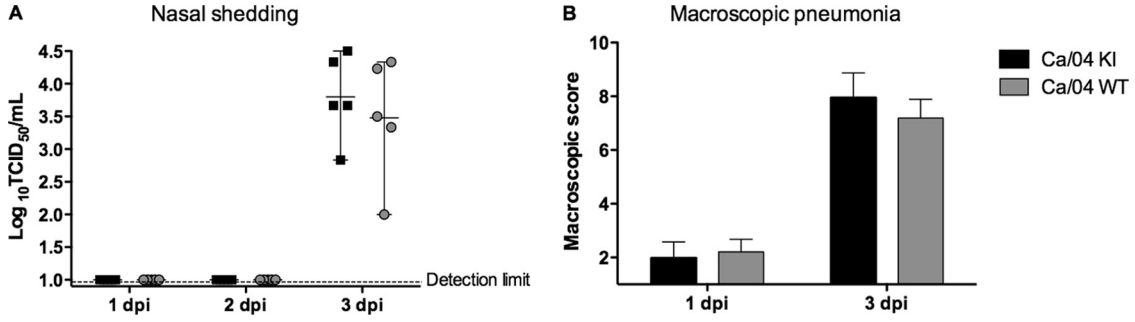

FIG 4 Viral shedding and macroscopic pneumonia are unaltered by PB1-F2 expression in swine. Groups of pigs ($n = 10$) were infected with 10^5 $TCID_{50}$ of PB1-F2 recombinant viruses, and nasal swabs were collected from 1 to 3 dpi for measuring virus shedding. At 1 and 3 dpi, five animals from each group were euthanized, and the lungs were scored for gross pneumonia. (A) Viral shedding in nasal secretions of pigs. Viral titers in nasal swabs were determined by $TCID_{50}$ on MDCK cells. Values are shown as the mean and range and expressed as $log_{10}TCID_{50}$/ml. (B) Percentage of macroscopic lung lesions. Values are shown as the mean ± the standard error of the mean. The differences are not statistically significant (two-way ANOVA).

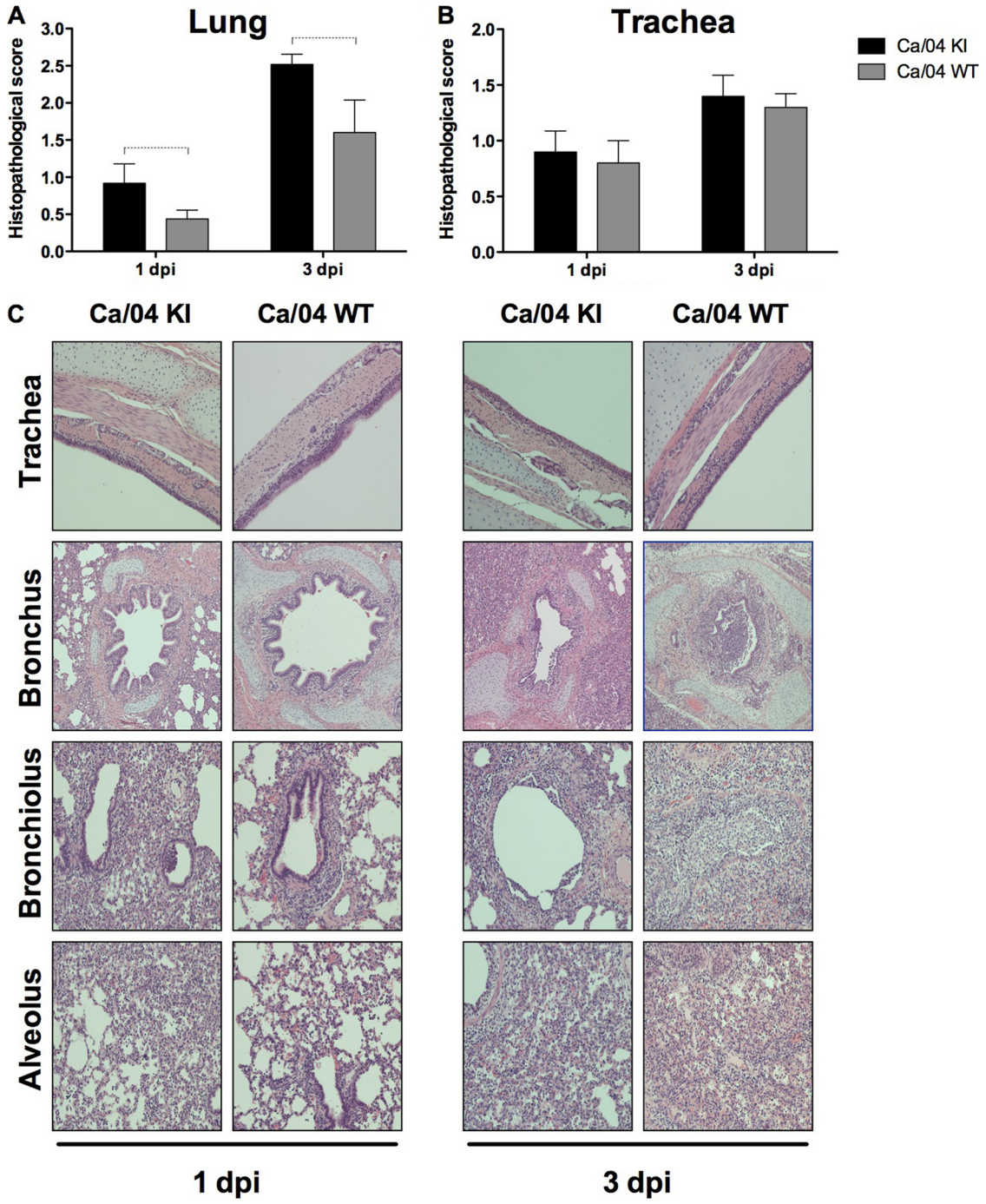

FIG 5 PB1-F2 exacerbates microscopic pneumonia in swine. Groups of pigs ($n = 10$) were infected with 10^5 $TCID_{50}$ of PB1-F2 recombinant viruses. At 1 and 3 dpi, five animals from each group were euthanized, and the histopathological changes in the lower respiratory tract were evaluated. (A) Histopathologic scores in the lungs. The differences are statistically significant (two-way ANOVA, $P < 0.05$). (B) Histopathologic scores of trachea. The differences are not statistically significant (two-way ANOVA, $P > 0.05$). (C) Photomicrographs representing microscopic pneumonia in Ca/04 KI and Ca/04 WT virus. Dashed brackets indicate statistically significant differences ($P < 0.05$).

the background of a pH1N1 in animal models. Evolutionarily, the full-length PB1-F2 was lost in some human and swine influenza viruses through incorporation of premature stop codons. This is in contrast to avian influenza viruses, in which the entire PB1-F2 ORF is highly conserved (78). Thus, PB1-F2 may have comparable functional roles in humans and pigs. Our pathogenicity studies with pigs offer the possibility of translating the findings to humans, given the outbred nature of swine and the comparable distributions of sialic acid receptors and similarities of influenza clinical signs, pathology, and cytokine response between the two species (2, 44, 68).

In an attempt to characterize the molecular mechanisms by which PB1-F2 might affect viral replication, we studied the effects

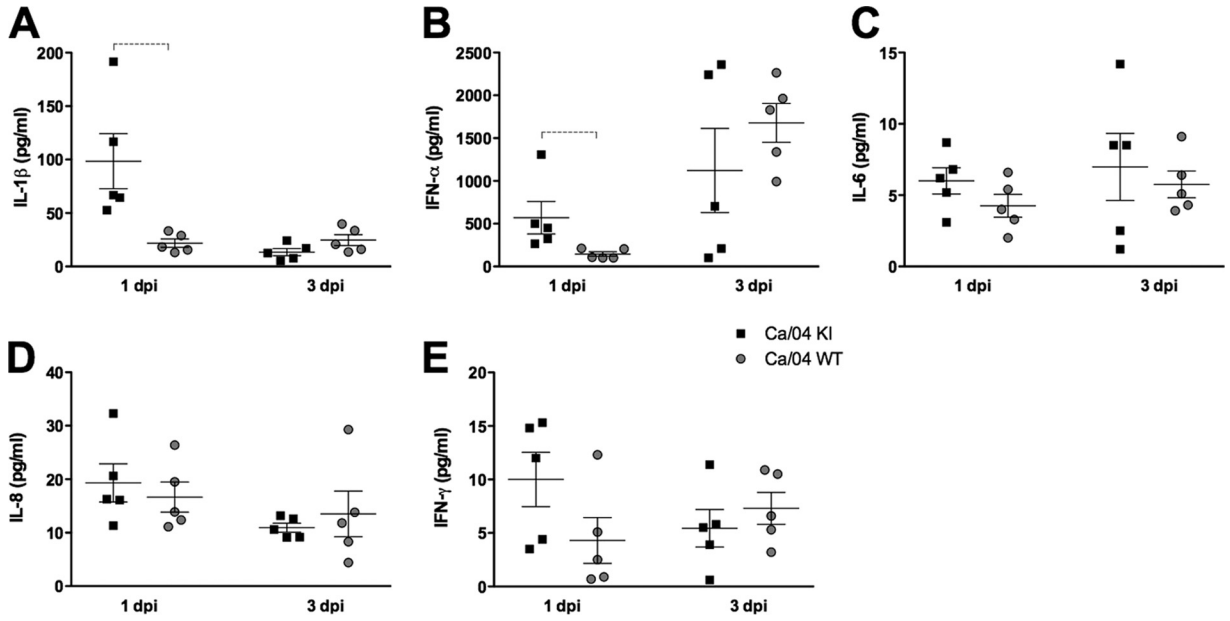

FIG 6 PB1-F2 exacerbates the pulmonary levels of IFN-α and IL-1β in swine. Groups of pigs ($n = 10$) were infected intratracheally with 10^5 $TCID_{50}$ of PB1-F2 recombinant viruses. On days 1 and 3 postinfection, animals from each group were euthanized, and the cytokine/chemokine levels in BALF were determined by ELISA. Data are shown as the mean ± the standard error of the mean for five animals in each challenge group. Two-way ANOVA was used to determine significant differences between groups. When the interaction between factors (time and virus) was significant, a two-tailed Student *t* test was used, considering time and virus, to determine significant differences between two treatment means for each data point. Dashed brackets indicate statistically significant differences ($P < 0.05$).

of Ca/04 PB1-F2 on polymerase activity using an IAV untranslated region (UTR)-driven GLuc reporter gene. We found that PB1-F2 upregulates early (12-hpt) viral polymerase activity but does not do so at later time points (24 to 72 hpt). These results contrast with those reported by Chen et al., which indicated that Ca/04 PB1-F2 enhanced polymerase activity at 48 hpt (8). The discrepancy between this and the previous study with regard to polymerase function may be due to differences in the reporter gene used as the polymerase activity readout and the length of the restored PB1-F2 ORF. Chen et al. used a chloramphenicol acetyltransferase (CAT) reporter system and mutated only 2 stop codons in the PB1-F2 ORF, generating an 87-aa product instead of a 90-aa peptide product (19). It must be noted, however, that polymerase activity in our assay was evaluated in human and canine cells; however, it appears to correlate well with the replication of these viruses in *ex vivo* swine respiratory explants and *in vivo* in swine lungs.

As the presence of PB1-F2 augmented the replication of some viral strains in tissue culture (41, 56), we studied the growth kinetics of PB1-F2 Ca/04 recombinant viruses in porcine respiratory explants. Respiratory explants have proved to be a valuable system to study influenza-host interaction in a scalable, well-defined, and controlled experimental setting (7, 65). Our results are in harmony with the hypothesis that PB1-F2 modulates viral replication, since higher viral yields were observed in explants derived from both the upper and lower respiratory tracts (Fig. 2). Interestingly, this effect of PB1-F2 in virus production (and polymerase activity) (Fig. 1) was mainly observed at early time points, consistent with the early and transient expression of PB1-F2 in the course of infection (10, 33, 40). Furthermore, the increased *ex vivo* viral replication paralleled the lung viral load in pigs infected with Ca/04 KI (Fig. 4). In contrast, viral shedding in nasal secretions was not affected by PB1-F2. This may be explained by the route of inoculation used in our *in vivo* studies. Pigs were infected intratracheally, a route that deposits the virus in the lower respiratory tract and more consistently reproduces lung pathology (36). Shedding was detected only at 3 dpi, which could be a time point that makes it difficult to distinguish modest differences in shedding. However, we cannot rule out the possibility that PB1-F2 could affect viral shedding beyond 3 dpi and/or in intranasally infected animals. These studies are beyond the scope of this work and warrant further investigation.

Even though PB1-F2 had no effect on the gross lung pathology or clinical disease, Ca/04 KI-infected pigs developed more severe microscopic pneumonia as early as 1 dpi. This finding fit with previous reports that demonstrated that PB1-F2 increases the severity of pulmonary lesions in mouse models, either in the context of viral infection (40) or simply by administration of peptides (39). Moreover, our results are in agreement with ferret studies by Hai et al. that reported no changes in clinical outcome with a PB1-F2-expressing Ca/04 compared to WT controls, although the authors did not assess lung pathology (19). Thus, it could be postulated that during primary viral infection the microscopic pulmonary changes induced by PB1-F2 are not large enough to be manifested clinically or detected macroscopically. However, the effects of PB1-F2 enhancement of virulence might be seen under field conditions, where immunosuppressive conditions and secondary bacterial infections can aggravate the outcome of influenza virus infections. In contrast to previous reports that showed significant clinical signs in pigs infected with various strains of pH1N1, infection of pigs with the Ca/04 WT and Ca/04 KI viruses in our studies resulted in mild clinical signs (32, 35). There are several factors that could account for this difference. In previous studies, 10-fold more virus was used to inoculate pigs, and the

clinical manifestation of the disease became obvious after 3 dpi. Our animal studies were terminated at 3 dpi, and therefore we did not allow the clinical signs to develop. In addition, previous studies used virus isolates that were not previously cloned. We have previously shown that that pH1N1 viruses may have contained a mixed population of similar strains with different effects on virulence (76). In order to distinguish differences attributed to a single factor, we generated reverse genetics clones of Ca/04, and the only difference between Ca/04 WT and the Ca/04 KI is the presence of a full-length PB1-F2 in the latter.

The proinflammatory cytokine response is critical for recruiting effector cells to the site of an infection. However, elevated or prolonged cytokine production can also contribute to the pathological changes observed during infection. To better understand the mechanism of lesion severity in Ca/04 KI-infected pigs, we measured the pulmonary concentration of 10 cytokines/chemokines during the acute stage of infection. Increased levels of IFN-α and IL-1β proteins were found in the lungs of pigs infected with Ca/04 KI compared to what was seen for the Ca/04 WT (Fig. 6). This exacerbation of the host innate response was observed at 24 hpi but not at 72 hpi, adding to the notion that changes mediated by PB1-F2 occur shortly after infection. To our knowledge, this is the first report showing that PB1-F2 enhances type I IFN *in vivo*. Our results are consistent with a recent study which demonstrated that the WSN PB1-F2 exacerbates type I IFN expression in human respiratory epithelial cells (33). In both pigs and humans, there is a strong positive correlation between local IFN-α levels and viral titers in nasal secretions and clinical disease (23, 68). In addition, increased levels of IL-1β have also been linked to neutrophil infiltration in the lungs and to the severity of pneumonia in pigs (34, 67). The exacerbated inflammatory response in pigs could be a direct effect of PB1-F2 early during infection. Thus, it is possible that enhanced expression levels of IFN-α and IL-1β are not the cause of respiratory disease but markers of the extent of viral replication. The modulation of the porcine immune system by PB1-F2 and its interaction with host factors warrant further investigation and should shed light into the strain- and host-dependent molecular mechanisms of PB1-F2. It remains to be determined whether the phenotypic differences observed with these two isogenic pH1N1 viruses are common features of other swine influenza viruses.

In summary, we have shown that PB1-F2 modulates several aspects of pH1N1 influenza virus interaction with swine, both *ex vivo* and *in vivo*. The present study fills a critical gap regarding the virulence determinants for influenza virus in swine. Our studies provide important insights into the impact that genetic changes may have on the virulence of pH1N1 for mammalian species, including humans.

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