Caspase-1 Deficient Mice Are More Susceptible to Influenza A Virus Infection With PA Variation

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Background. Reassortment within polymerase genes causes changes in the pathogenicity of influenza A viruses. We previously reported that the 2009 pH1N1 PA enhanced the pathogenicity of seasonal H1N1. We examined the effects of the PA gene from the HPAI H5N1 following its introduction into currently circulating seasonal influenza viruses.

Methods. To evaluate the role of H5N1 PA in altering the virulence of seasonal influenza viruses, we generated a recombinant seasonal H3N2 (3446) that expressed the H5N1 PA protein (VPA) and evaluated the RNP activity, growth kinetics, and pathogenicity of the reassortant virus in mice.

Results. Compared with the wild-type 3446 virus, the substitution of the H5N1 PA gene into the 3446 virus (VPA/3446) resulted in increased RNP activity and an increased replication rate in A549 cells. The recombinant VPA/3446 virus also caused more severe pneumonia in (VPA/3446) resulted in increased RNP activity and an increased replication rate in A549 cells. The recombinant VPA/3446 virus also caused more severe pneumonia in A549 cells. The recombinant VPA/3446 virus also caused more severe pneumonia in A549 cells. The recombinant VPA/3446 virus also caused more severe pneumonia in A549 cells. The recombinant VPA/3446 virus also caused more severe pneumonia in A549 cells. The recombinant VPA/3446 virus also caused more severe pneumonia in A549 cells.

Conclusions. Although the PA from H5N1 is incidentally compatible with a seasonal H3N2 backbone, the H5N1 PA affected the virulence of seasonal H3N2, particularly in inflammasome-related innate immunity deficient mice. These findings highlight the importance of monitoring PA reassortment in seasonal flu, and confirm the role of the Caspase-1 gene in influenza pathogenesis.

Keywords. influenza reassortant; caspase-1; acidic polymerase; pathogenesis.

Influenza A viruses cause a contagious respiratory illness in humans. The public health threats that are posed by influenza viruses are categorized as seasonal, zoonotic, and pandemic [1]. Although seasonal flu infection is a common disease, seasonal influenza A viruses can cause severe illness and even death. From 1976 to 2006, annual seasonal influenza-associated patient deaths ranging from 3000 to 49 000 have been estimated in the United States, mostly affecting the elderly (aged ≥65 years) [2]. Because influenza A viruses also infect many different animal species, the risk of zoonotic transmission to humans is an ongoing threat, such as highly pathogenic avian influenza (HPAI) H5N1 that previously resulted in a mortality rate of approximately 60% in humans [3]. High morbidity and mortality occurred in influenza A pandemics in 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), 1977 (H1N1), and 2009 (H1N1). The potential emergence of new pandemics is due in part to the diversity of influenza virus that allows it to overcome species barriers through genetic mutations and reassortment. The pathogenicity of influenza viruses is influenced by multiple factors. The influenza viral proteins HA and RNP complex (PA, PB1, PB2, and NP) also play important roles in interspecies transmission [4].

Many molecular determinants of influenza virus pathogenicity have been identified. The E627 K alteration of PB2 is the most well-known molecular factor of viral adaption and pathogenesis [5–10]. Recent studies have reported that PA is a virulence factor in animal model. A T97I substitution in PA increased the virulence of low-pathogenic avian influenza (LPAI) H5N2 virus in mice [11]. The substitution of amino
acids S224P and N383D in PA contributed to the highly virulent H5N1 phenotype in duck [12]. The PA A36 T mutant showed enhanced the virulence of 2009 pandemic H1N1 influenza virus in mice [13]. The H2N1 virus retained the human-signature PA residue S552 resulted in faster replication kinetics in culture and increased pathogenicity in mice [14]. The substitutions in PA at position 349 and 605 enhanced virulence of LPAI H9N2 in mice [15]. Recently, the novel viral protein PA-X, which diminishes viral pathogenicity in mice, was found to be encoded by a second open reading frame (X-ORF) in the PA gene [16]. We previously reported that single PA gene of 2009 pandemic H1N1 virus reassortment can change seasonal H1N1 influenza virus replication and pathogenicity in mice [17]. However, the mechanism by which this occurs remains unclear.

Avian influenza A viruses may cross species barriers to spread among humans. The recent emergence of a novel avian-origin reassortant H7N9 virus caused severe human infections in China [18, 19]. Thus, both the threat of H5N1 influenza viruses’ transmission in mammals and that posed by reassortment between HPAI and currently circulating seasonal influenza viruses represent serious public health concerns.

We therefore investigated whether HPAI H5N1 PA could alter virulence of currently circulating seasonal influenza viruses. We found that a reassortant seasonal H3N2 virus contains the PA gene of HPAI H5N1 exhibited efficient replication, but did not demonstrate significantly altered virulence in wild type C57BL/6 mice. Recently, the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome associated with caspase-1 was shown to contribute to anti-influenza host defenses by altering interleukin 1β (IL-1β) expression [20], indicating that IL-1β mediates acute pulmonary inflammatory pathology while enhancing survival during influenza infection [21]. We found that the H5N1 PA affected virulence of seasonal H3N2 influenza virus in inflammasome-related innate-immune deficiency mice. Our findings provide new insights into the PA-mediated pathogenesis of influenza infection in severe flu patients.

**MATERIALS AND METHODS**

**Cells and Viruses**

HEK293 T cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A549 cells were maintained in minimal essential medium (MEM) supplemented with 10% FBS. All cells were cultured at 37°C with 5% CO₂. The whole genome of seasonal H3N2 strain, A/TW/3446/02, was isolated in Chang Gung Memorial Hospital and the PA gene of H5N1 strain, A/VN/1194/04, were cloned into the pHW2000 vector as described elsewhere [17]. All experiments using recombinant viruses were performed under an enhanced animal biosafety level 3 (ABSL3+) at the National Defense Medical Center Institute of Preventive Medicine (IPM).

**Plasmid-based Reverse Genetics**

The wild-type virus and the reassortant H5N1 PA viruses were rescued using reverse genetics system reported by Hoffmann et al [22]. The rescued viral genomes were confirmed by sequencing. The stock viruses were propagated in MDCK cells followed by plaque assay titration as described [17].

**Chloramphenicol Acetyl Transferase Assay for Viral Polymerase Activities**

In total, 293 T cells were cotransfected with 0.5 μg of the pPolCAT-RT plasmid and 0.5 μg of each of the different combinations of PB2, PB1, PA, and NP protein expression plasmids. At 48 hours posttransfection, the cells were lysed in a 250 μl lysis buffer, and the cell extracts were analyzed for chloramphenicol acetyl transferase (CAT) expression using a CAT ELISA kit (Roche) according to the manufacturer’s instructions. All experiments were performed in triplicate.

**Mouse Experiments**

Animal experiments were performed in ABSL3+ laboratory at the National Defense Medical Center Institutes of Preventive Medicine (IPM) under an animal study protocol approved by IACUC of IPM. IL-1β−/− and Casp 1−/− mice (kindly provided by Dr Bernhard Ryffel, Centre National de la Recherche Scientifique) were maintained in SPF facilities at Chang Gung University. The wild-type C57BL/6 mice were purchased from BioLasco (Taiwan). Groups of 6-week-old mice were anesthetized intramuscularly with Zoletil 50 (25 mg/kg) and inoculated intranasally with 5 × 10⁶ plaque-forming units (PFU) of viruses in 50 μl phosphate-buffered saline (PBS). The infected mice were weighted for 14 days and observed daily for illness or death.

**Histopathologic and Immunohistochemical Analysis**

Mice (n = 3) were killed on 1 and 3 days postinfection (DPI). Lung tissue samples were collected at each time point from each experimental group and fixed in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. Histopathologic examination of hematoxylin and eosin (H&E) stained sequential sections were performed.

Semiquantitative assessment of influenza virus-associated inflammation in the lung was performed as reported elsewhere for alveolar and bronchiolar lesions [23]. We used the following scale to score the extent of damage: 0 = no damage; 1 = 1%–25%; 2 = 25%–50%; and 3 = 51%–100%. The severity of damage was scored as follows: 0, no inflammatory cells; 1, few inflammatory cells; 2, moderate numbers of inflammatory cells; and 3, many inflammatory cells. We also scored for presence of alveolar edema (no = 0, yes = 1) and alveolar hemorrhage (no = 0, yes = 1).
The cumulative scores for the extent and severity of inflammation comprised the total score per animal.

The MultiVision Polymer Detection System (Thermo) was used for antigen staining in serial sections. The primary antibody used in the immunohistochemistry assay was a goat anti-influenza A antiserum (Chemicon AB1074, Millipore) that recognizes both the surface glycoproteins and internal proteins of the virus. Semiquantitative assessment of influenza virus-infected cells in the lungs was performed as reported elsewhere [24].

**Lung Viral Titers**

The lungs were harvested on 1 and 3 DPI. The right lung was homogenized in 1.0 mL of DMEM without FBS using a Precoll 24 Tissue Homogenizer (Bertin), and the homogenates were spun for 10 minutes at 1000 × g to remove cellular debris. The virus titers were determined by plaque assays in MDCK cells.

**Accession Numbers**

The viral nucleotide sequences described in this paper are available from Influenza Research Database (http://www.fludb.org/brc) under the following accession numbers: DQ415309 for the PA gene of A/TW3446/02 and AY651610 for A/VN/1194/04.

**RESULTS**

**H5N1 PA Subunit Altered the Activity of a Seasonal H3N2 Influenza Virus Polymerase**

We previously showed that the polymerase of a human isolated seasonal H1N1 with the PA subunit from 2009 pH1N1 virus demonstrated increased activity in human cells [17]. Thus, we investigated the effects of the reassortment of the H5N1 PA gene (VPA) on the polymerase activity in the seasonal H3N2 (A/TW3446/02). We analyzed polymerase activity in 293 T cells expressing the viral RNP components (PB2, PB1, PA, and NP) using a viral-like CAT reporter construct. The 3446 RNP was used as a backbone and the reassortant was generated by the replacement of the PA subunit with that from the avian H5N1 strain. The RNP complex of the reassortant virus with the H5N1 PA exhibited an activity that was approximately 6.4-fold higher in vitro than that of the wild-type 3446 virus (Figure 1), suggesting that the H5N1 PA subunit is more fit for the polymerase complex of 3446 strain.

**In Vitro Characterization of H5N1 PA Gene Reassortant Seasonal H3N2 Recombinant Virus**

The results of the RNP activity assays demonstrated the H5N1 PA made an important contribution to the activity of the 3446 viral RNP complex in human cells. To determine whether the replication of the reassortant virus was also increased, we constructed a reassortant virus (VPA/3446) that possessed the H5N1 PA gene in the genetic background of the 3446 virus by reverse genetics. We compared the growth kinetics of the reassortant virus with that of the wild-type 3446 virus in A549 cells.

**Figure 1.** Heterologous strain of influenza virus PA subunits enhance the activity of a seasonal H3N2 influenza virus polymerase. Polymerase activity assays were performed with human 293 T cells by transfection with vectors expressing the H3N2 (3446) polymerase subunits (PB2, PB1, PA, NP, and a vRNA based CAT reporter). The reassortant was generated by substitution of the H5N1 PA gene (VPA). The expression of RdRp and NP in the transfected cells was confirmed by Western blotting with using primary antibodies to detect PB2, PB1, PA, and NP proteins in the cell lysates. Lane 1: mock, 2: 3446, 3: VPA/3446. Data represent the mean ± SD. Similar results were obtained from 3 separate experiments. ***P<.001 (t-test). Abbreviation: CAT, chloramphenicol acetyl transferase.

**Figure 2.** Multicycle replication kinetics of recombinant viruses in infected A549 cells. A chimeric H3N2 virus containing H5N1 PA was rescued by reverse genetics. Multistep growth curve were used to monitor the viral growth kinetics of human A549 cell infected at an MOI of 0.001 and at the indicated post-infection time points. The titer was determined by plaque assay on MDCK cells. Abbreviations: MDCK, Madin-Darby canine kidney; MOI, multiplicity of infection.
In A549 cells, the VPA reassortant virus replicated well, reaching a maximum titer of 1.1 × 10⁶ PFU/mL at 48 hours postinfection (HPI), which was 10-fold higher than that of the wild-type 3446 virus (approximately 7 × 10⁴ PFU/mL at 36 HPI) (Figure 2), suggesting that the PA gene makes a critical contribution to the increased replication rate of VPA/3446 reassortant in human lung cells.

Pathogenesis Experiments in Wild Type C57BL/6 Mice

We examined the virulence of the VPA/3446 reassortant and wild-type 3446 viruses in mice. Wild-type C57BL/6 mice were inoculated intranasally with 5 × 10⁵ PFU of each virus, and morbidity and mortality were monitored daily for 2 weeks. We found that neither the 3446 virus nor the VPA/3446 reassortant cause any significant disease symptoms in wild-type B6 mice (Figure 3A). However, there was a difference in virus replication in the lungs during the first 72 hours of infection, the lung viral titer of the mice infected with the VPA/3446 reassortant was 2.2 × 10⁴ PFU/mL on 1 DPI, and no virus was detected on 3 DPI, whereas no 3446 virus was detected on 1 DPI in the lungs of mice infected with the wild-type virus (Figure 3B). These results confirmed that, unlike avian H5 influenza viruses, human H3 influenza A viruses are not virulent in mice without prior adaption [25, 26].

Pathogenesis Experiments in Inflammasome-related Innate Immunity Deficient C57BL/6 Mice

Recent studies have demonstrated that C57BL/6 mice are more resistant to influenza infection than other mouse strains [27, 28]. The NLRP3 inflammasome induces caspase-1 activation, resulting in the production of the cytokines, IL-1β and interleukin 18 (IL-18), which make important contributions to anti-influenza immune responses. To obtain a more detailed understanding of the impact of the reassortment of the H5N1 PA gene on the virulence of seasonal H3N2 viruses, we used mouse knockout models to determine the contribution of the reassortant to pathogenicity in innate immune deficient mice. Mice lacking IL-1β that were challenged with the VPA/3446 reassortant exhibited slight weight loss (<5%) as early as 4 DPI. The wild-type 3446 virus caused no symptoms in the IL-1β−/− mice. Both viruses were detected in the lungs of IL-1β−/− mice on 3 DPI. However, neither was detected on 5 DPI (Figure 3B). The average lung viral load of the VPA/3446 reassortant was 1.3 × 10⁵ PFU/mL on 1 DPI, but a 2.6-log reduction in titer was observed on 3 DPI. In comparison, the viral titers in the lungs of IL-1β−/− mice were significantly different between the reassortant and wild-type viruses, with the titer of the VPA/3446 reassortant 10-fold higher (1-log) than that of the 3446 virus on 1 and 3 DPI. The Caspase-1 knockout mice infected with
Figure 4. Histopathology and immunohistochemistry in lungs of wild-type and knockout mice inoculated with 3446 and VPA/3446 viruses. The left lung of (A) wild-type B6, (B) IL1β knockout, and (C) Casp-1 knockout mice infected with 3446 and VPA/3446 H3N2 viruses (5 × 10⁵ PFU/mouse) was collected on 1 and 3 DPU using a standard protocol. Tissue samples were embedded in paraffin and cut into 5-μm sections using for antigen detection using IHC staining and pathological examination using HE staining. D, Histopathological scoring of the lung sections. Wild-type and knockout mice (n = 3) were
the VPA/3446 reassortant exhibited weight loss within 7 DPI (up to 19% on 2 DPI), before slowly recovering. The 3446 virus did not cause significant weight change in Casp 1−/− mice. The lung titers of the VPA/3446 reassortant was significantly higher (2-log) in Casp 1−/− mice than that of the 3446 virus on 1 and 3 DPI (Figure 3B), and neither virus were detected on 5 DPI. In addition, the average lung titers of the VPA/3446 reassortant were significantly higher in Casp 1−/− mice than those in IL-1β−/− mice.

To examine the lung pathology of infected mice, the lungs were collected at 1 and 3 DPI and serial sectioned. Sections were stained with H&E or analyzed for the presence of viral antigen by immunohistochemical (IHC) staining. The VPA/3446 virus infected Casp 1−/− mice severe diffuse alveolar damage. Mild alveolar damage was observed in the VPA/3446 virus-infected IL-1β−/− mice and wild-type mice, whereas 3446 virus infection caused no damage in any of the mouse strains (Figure 4A–C). The inflammation resulting from VPA/3446 virus infection the thickening of the alveolar septa, epithelial necrosis, RBC extravasation, and inflammatory cells infiltration (Figure 5).

The bronchiolar epithelium of the VPA/3446 virus group had multifocal necrosis with moderate peribronchiolar infiltration of inflammatory cells. In the bronchiolar lumen, small amounts of cellular debris mixed with fibrin and edema fluid were present. In contrast, the 3446 virus infection caused no lesions in the bronchioles of wild-type or either strain of knockout mice. The semiquantitative histological scoring showed that the VPA/3446 infection caused more severe damage than the 3446 infection, with the most severe lung lesions occurring in the VPA/3446-infected Casp 1−/− mice (Figure 4D).

The IHC staining indicated the presence of influenza A virus antigen. Different levels of influenza virus antigen expression were observed near the focal inflammation sites in lung sections. In the VPA/3446 virus group, antigen expression occurred predominantly in the bronchiolar walls of the wild-type and knockout mice on 1 DPI. However, the extent of antigen expression was greater in the bronchiolar epithelium of infected knockout mice than in the wild-type mice. On 3 DPI, the lung sections of the IL-1β−/− and Casp 1−/− mice showed decreased viral antigen expression in the bronchiolar epithelium, whereas no viral antigen was observed in the lung sections of the wild-type mice. Only the VPA/3446-infected Casp 1−/− mice had widespread expression of viral antigen in alveolar epithelium. No viral antigen expression was detected in alveolar epithelium of the wild-type mice infected with virus, and very little antigen expression was detected in the alveolar epithelium of the 3446-infected knockout mice (Figure 4A–C). The semiquantitative IHC scoring showed the highest antigen expression in the bronchioles and alveoli of the Casp 1−/− mice infected with the VPA/3446 reassortant virus. The viral antigen expression in the VPA/3446-infected IL-1β−/− mice was most prominent in the bronchioles, with diffuse expression occurring in a small number of alveolar epithelium cells, whereas low IHC scores were recorded for the wild-type and knockout mice infected with the 3446 virus (Figure 4E and 4F).

**DISCUSSION**

Reassortment is a critical mechanism in the generation of novel pandemic strains of influenza viruses, such as that which caused the 2009 H1N1 influenza pandemic. The 2009 H1N1 pandemic virus contained a unique combination of RNP genes, which included the avian-like PB2, PA, human-like PB1, and classic swine-like NP [29].

The transmission of the HPAI H5N1 virus among humans as a result of reassortment with circulating human influenza viruses is a major global public health concern. Recent studies have suggested that the PA gene plays an important role in the pathogenicity of the H5N1 virus [12, 30, 31]. Our previous study showed that the PA from the 2009 H1N1 pandemic virus altered pathogenicity of a seasonal H1N1 virus. Sun et al [32] reported that the PA gene of 2009 H1N1 pandemic virus increased the virulence of avian H9N2 reassortants. Moreover, Zhang and colleagues [33] recently found that the PA gene of

![Figure 5](https://academic.oup.com/jid/article-abstract/208/11/1898/852576)  
**Figure 5.** Representative pictures of H&E stained lung sections showing diffuse alveolar damage in Casp 1−/− mice infected with VPA/3446 reassortant virus. The alveolar architecture is obliterated by thickening of the alveolar septa. The alveolar lumina are with alveolar macrophages, neutrophils, and erythrocytes in a mixture of fibrin, edema fluid, and cellular debris. Magnification: 640x.
the 2009 H1N1 pandemic virus promoted H5N1 virus transmission in guinea pigs. Recent World Health Organization (WHO) surveillance data showed that H3N2 virus is the predominant circulating seasonal flu virus in many countries. Although the PA from H5N1 is incidentally compatible with a seasonal H3N2 backbone, which is a different lineage from that of seasonal H1N1 and avian H5N1, we showed that a reassortant RNP of seasonal H3N2 RNP containing the H5N1 PA subunit exhibited significantly increased activity, which is consistent with the findings of a previous study of the reassortment between the seasonal H3N2 and HPAI H5N1 viruses [34]. However, Li et al [35] reported that a LPAI H5N1 PA reassortment decreased seasonal H3N2 RNP activity. We also showed that a H5N1 PA reassortant virus replicated faster than the parental strains in human lung epithelial cells. These results are consistent with the findings from our polymerase activity assays. However, the increased of RNP activity and replication in culture did not lead to increased virulence in mice. Both the PA reassortant and the parental H3N2 viruses caused no significant clinical signs or overt weight changes in C57BL/6 mice after intranasal inoculation. Previous studies indicated that seasonal human H3N2 viruses lacked pathogenicity in mice and ferrets, including DBA.2 mice, which are highly susceptible to influenza A viruses [1, 26]. Our data showed that PA gene reassortment did not increase the virulence of a seasonal H3N2 virus in mice. However, in contrast to the parental H3N2 virus, we showed that a H5N1 PA reassortant virus was capable of replication in the lungs of C57BL/6 mice on 1 DPI.

The differences in pathogenicity between the H5N1 PA reassortant and the parental H3N2 viruses may not be easily discriminated in wild type C57BL/6 mice with normal immunity. Recently, the NLRP3 inflammasome was shown to be an important component in the host defenses against influenza infection [36–38]. Previous studies reported that mice lacking functional NLRP3 inflammasome-related genes were more susceptible to influenza infection [20]. Caspase-1 was recently reported to play a key role in mediating influenza-related pathogenesis in asthma patients [39]. Previous studies of PR8-infected interleukin 1β (IL-1β) or caspase-1 deficient mice also reported that enhanced mortality, decreased lung immunopathology, and less weight loss and lower lung viral titers [21, 36], but reduced in the tracheal epithelial cells from Casp 1−/− mice [39]. However, IL-1R deficient mice infected with low pathogenic HK486 strain lost significantly more weight in late stage of infection [40]. We observed that Casp 1−/− mice infected with the VPA/3446 reassortant virus revealed more severe pneumonia, greater body weight loss, and higher lung viral titers during the early stage of infection than the VPA/3446-infected IL-1β−/− mice. However, no mortality occurred in H5N1 PA reassortant H3N2 virus-infected animals. The inconsistencies between our findings of previous studies may have been caused by the use of different influenza A virus strains. The PR8 virus is the well-known mouse-adapted laboratory strain that can replicate efficiently in mouse lungs to result in viral pneumonia and frequently death. In contrast, seasonal human H3N2 viruses are not mouse-adapted strains and therefore replicate poorly in the lungs of wild-type mice, resulting in the clearance of the virus early during infection. However, it was recently reported that swine-origin H3N2v viruses can replicate more efficiently than seasonal human H3N2 viruses in a ferret model [41]. Seasonal influenza virus infection has been reported to be associated with significant mortality in various patient populations. Thus, reassortants between circulating human and animal influenza viruses represent the greatest influenza-related public health risk.

Avian influenza A virus may cross species barriers to infect humans. The recent avian H7N9 virus in China, a novel avian-origin reassortant virus containing genes from H7N3 (H7), H7N9 (N9), H9N2 (the remaining of 6 internal genes) viruses [18, 19], and H5N1 are examples. Therefore, reassortment between H5N1 and human H3N2 may occur. We found that PA derived from H5N1 enhances the pathogenicity of the mild seasonal H3N2 virus. Enhanced virulence was clearly observed in Casp1−/− mice, suggesting that host-harboring deficiency in caspase-1 related pathways may be more vulnerable to the reassorted strain.

In conclusion, we found that the acquisition of the HPAI H5N1 PA gene by seasonal H3N2 strains may change their pathogenicity in NLRP3 inflammasome pathway impaired mice. Our findings have clinical implications for determining whether CASP1 genetic variation may be involved in affecting the susceptibility and severity of influenza infection in different human populations.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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