Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Virology 412 (2011) 9–18

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Differential host gene responses in mice infected with two highly pathogenic avian influenza viruses of subtype H5N1 isolated from wild birds in Thailand

Tsuyoshi Hayashi [a,b], Kridsada Chaichoune [c], Tuangthong Patchimasiri [d], Yasuaki Hiromoto [a,b], Yuri Kawasaki [a,b], Witthawat Wiriyarat [c], Warunya Chakritbudsabong [c], Natanan Prayoonwong [c], Natnapat Chaisilp [c], Sujira Parchariyanon [d], Parntep Ratanakorn [c], Yuko Uchida [a,b], Tomoyuki Tsuda [b], Takehiko Saito [a,b,*]

[a] Thailand-Japan Zoonotic Diseases Collaborating Center (ZDCC), Kasetklang, Chatuchak, Bangkok 10900, Thailand
[b] Research team for Zoonotic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Kannondai, Tsukuba, Ibaraki 305-0856, Japan
[c] The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals, Faculty of Veterinary Science, Mahidol University, Salaya, Phuttamonthon, Nakorn-pathom 73170, Thailand
[d] National Institute of Animal Health, Kasetklang, Chatuchak, Bangkok 10900, Thailand

ARTICLE INFO

Article history:
Received 17 September 2010
Returned to author for revision
21 October 2010
Accepted 21 December 2010
Available online 19 January 2011

Keywords:
Cytokine
Highly pathogenic avian influenza virus
Inflammation
Mice
Neutrophil
Wild bird

ABSTRACT

In Thailand, highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 had been isolated from various wild birds during the HPAI outbreak in poultries. In this study, we examined the pathogenicity of two wild bird isolates (A/Pigeon/Thailand/VSMU-7-NPT/2004; Pigeon04 and A/Tree sparrow/Ratchaburi/VSMU-16-RBR/2005; T.sparrow05) in mice. They showed similar replication in several organs and lethal outcome. However, on day 3 post-infection, Pigeon04 induced mRNA expression of proinflammatory cytokines (IL6 and TNFα) and MIP-2, neutrophil chemoattractant, in the lungs, resulting in severe pneumonia that was accompanied by neutrophil infiltration. In contrast, on day 7 post-infection, T.sparrow05 induced the expression of several cytokines to a greater extent than Pigeon04; it also potently induced mRNA expression of several cytokines in brains of the infected mice that triggered frequent inflammatory events. In sum, our study demonstrated that two HPAI viruses induced different host responses, despite having similar replications, resulting in lethal outcome in mice.

Introduction

In Thailand, there were three major outbreaks of highly pathogenic avian influenza (HPAI) subtype H5N1 in poultry during 2004–2005, followed by 4 sporadic ones up to 2007. During these outbreaks, H5N1 HPAI virus was isolated not only from poultry, but also from wild birds such as pigeon, tree sparrow and the open-bill stork (Siengsanan et al., 2009; Uchida et al., 2008). Wild bird surveillances of HPAI viruses during 2004–2007 in Thailand revealed that the HPAI viruses were detected in wild birds during the outbreaks and even after the goal of the eradication program in poultry had been accomplished in the area (Siengsanan et al., 2009).

Fatal HPAI viral infections have also been reported in mammalian species such as tigers, leopards, dogs, and cats (Amonsin et al., 2006; Keawcharoen et al., 2004; Songserm et al., 2006a; Songserm et al., 2006b). Such infections are thought to have occurred through ingestion of poultry meat or wild bird carcasses infected with the HPAI viruses (Amonsin et al., 2006; Keawcharoen et al., 2004; Songserm et al., 2006a,b). Human infections of the H5N1 HPAI virus in Thailand have also been reported (Chotpitayasunondh et al., 2005; Tiensin et al., 2005). In total, 25 human cases, 17 of which were fatal, have been reported during 2004–2006. Chotpitayasunondh et al. (2005) showed that most patients with HPAI had been exposed to infected poultry. These findings demonstrate sporadic interspecies transmission of the H5N1 HPAI viruses and imply that routine monitoring of not only poultries but also wild birds and domestic mammals is necessary for early detection as well as for preventing resurgence of the viruses.

Previous studies have shown that many patients infected with the H5N1 HPAI virus developed acute respiratory distress syndrome (ARDS), which is characterized by diffuse alveolar damage (DAD), lymphopenia, and multiple organ failure (MOF) (The Writing Committee of the World Health Organization Consultation on Human Influenza, 2005). Moreover, a typical finding in H5N1 infected patients is an aberrant level of proinflammatory cytokines and chemokines in the serum (de Jong et al., 2006; Ka-Fai et al., 2001). de Jong et al. (2006) reported that levels of proinflammatory

* Corresponding author. Research Team for Zoonotic Diseases, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan. Fax: +81 29 838 7914.
E-mail address: taksaito@affrc.go.jp (T. Saito).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.12.040

cytokines and chemokines, such as IP-10, MIG, IL-8, IL-10, IL-6 and IFNγ in the serum of H5N1 infected patients increased significantly compared to those of H3N2 or H1N1 human influenza patients or healthy controls. It is noteworthy that serum levels of most of these cytokines in the fatal cases of H5N1 were significantly higher than those in the survivors; these phenomena correlated with the high viral load in pharyngeal specimens (de Jong et al., 2006). A high level of TNFα expression in the pneumocyte of an H5N1-infected patient compared with a patient who died from a non-infective illness was reported (Peiris et al., 2004). Apoptosis was detected in alveolar epithelial cells or leukocytes in lung specimens of H5N1-infected patients (Uiprasertkul et al., 2007). In animal models, virulent H5N1 HPAI viral infection caused depletion of lymphocytes in the blood, lungs, and lymphoid tissues as well as apoptosis of lymphocytes in the lungs (Tumpey et al., 2000). Thus, apoptosis is thought to contribute to lymphopenia that is typically observed in H5N1 HPAI-infected patients (Korteweg and Gu, 2008; Tumpey et al., 2000).

Pathogenicity studies in animal models are useful tools for better understanding of the diseases caused by influenza viruses in humans. Previous studies have demonstrated that ARDS, high production of cytokine and apoptosis are found in lung tissue of mouse infected with the HPAI virus, suggesting that mouse is a suitable model for evaluating the pathogenesis of HPAI virus in humans (Perrone et al., 2008; Tumpey et al., 2000; Xu et al., 2006).

Wild waterfowls are thought to act as a natural reservoir of influenza A viruses, and these viruses do not usually cause fatalities in wild birds. However, in China, unexpected H5N1 HPAI outbreaks in wild migratory waterfowls occurred at Qinghai Lake in 2005, and thousands of migratory birds were found dead due to the viral infection, suggesting that H5N1 HPAI viruses could cause fatal infections in wild birds (Chen et al., 2005). Very importantly, Gilsdorf et al. (2006) suggested that HPAI viruses were transmitted to humans following close contact or the process of de-feathering infected wild swans. There is report that these wild bird isolates were pathogenic to not only chickens but also mice (Chen et al., 2006; Zhou et al., 2006). Songserm et al. (2006a) showed that a domestic cat was infected with an H5N1 HPAI virus due to eating a pigeon carcass infected with HPAI viruses in Thailand. HPAI viruses in wild birds have been suggested as possible potential threats not only to poultry but also to the public health of humans (Gilsdorf et al., 2006).

In this study, we examined the pathogenic characters of two H5N1 HPAI viruses isolated from wild birds during HPAI outbreaks among poultries in Thailand. One is a HPAI virus isolated from dead pigeon (Pigeon04), and the other is a HPAI virus isolated from live tree sparrow (T.sparrow05). Interestingly, these two HPAI viruses exhibited different levels of virulence *in vivo* in chickens and different growth properties *in vitro* in MDCK cells. In contrast, they showed similar replications *in vivo* in the lungs and brain of the infected mice, resulting in fatal outcome. Thus, to determine whether the differences of the replication profiles of two HPAI viruses between *in vitro* and *in vivo* were related to alterations of host gene responses in the lungs and brain infected with these two viruses, we compared host responses, particularly in host gene responses in the lungs and brain of mice infected with the two HPAI viruses.

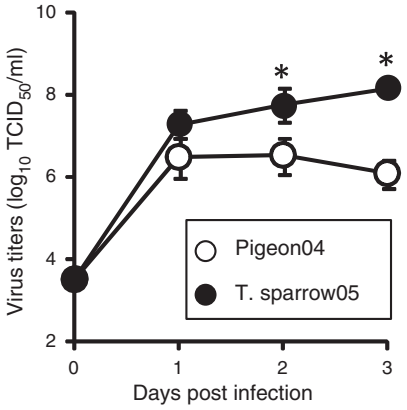

Fig. 1. Growth properties of two H5N1 HPAI viruses in MDCK cells. MDCK cells were infected with the HPAI viruses at a MOI of 0.01 $TCID_{50}$ (3.7 log_{10} $TCID_{50}$/ml). Supernatants were collected on days 1, 2 and 3 post-infection, and virus titers were determined on MDCK cells by $TCID_{50}$ assay. The data were indicated as mean virus titers ± standard deviations of three independent experiments. Asterisks indicate statistically significant differences (*$p < 0.05$ by Student t test).

Results

Characteristics of two H5N1 HPAI viruses isolated from wild birds

We isolated two HPAI viruses from wild birds during routine surveillance during the outbreak of HPAI viruses in Thailand in 2004–2005. A/Pigeon/Thailand/VSMU-7-NPT/2004 (Pigeon04) was isolated from a dead pigeon in Nakhon Pathom province in 2004, and A/Tree sparrow/Ratchaburi/VSMU-16-RBR/2005 (T.sparrow05) was from a clinically healthy tree sparrow in Ratchaburi province in 2005. We determined nucleotide sequences of protein-coding regions of all eight gene segments of Pigeon04 (AB576199-AB576206). All eight gene segments of T.sparrow05 were also sequenced in this study to find a mix population (A/G) at position 415 in NS1 compared to the sequenced done by Uchida et al. This was submitted to the Genbank (AB576207). Pigeon04 and T.sparrow05 had more than 99% and 97% identities in nucleotide and amino acid sequences, respectively. Phylogenetic analysis of the HA gene showed that these two viruses belong to clade 1 of the classification system (WHO/OIE/FAO H5N1 Evolution Working Group 2007) (Table 1, data not shown). The two viruses had similarly high infectivity titers in embryonated eggs and MDCK cells with titers, ranging from 7.6 to 7.9 $log_{10}EID_{50}$/mL and 7.1 to 7.2 log_{10}PFU/mL, respectively (Table 1). Interestingly, the plaque size of the two HPAI viruses varied. Pigeon04 produced mostly pinpoint plaques, whereas T.sparrow05 produced medium to large plaques, indicating different growth properties in the cultured cells *in vitro* between the two viruses (Table 1). Therefore, to assess the growth properties of two viruses, we examined viral growth kinetics of these viruses in MDCK cells. As shown in Fig. 1, T.sparrow05 robustly replicated in MDCK cells on days 2 and 3 post-infection, and the mean virus titers were 7.8 and 8.2 $log_{10}TCID_{50}$/mL, respectively. In

Table 1
Characteristics of highly pathogenic avian viruses used in this study.

Virus	Abbreviation	Date of specimen collection	Province	Status of host	H5 HA gene clade	Log_{10} EID_{50}/mL	Log_{10} PFU/ml in MDCK cells	Plaque size in MDCK cells[a]	MLD_{50} (log_{10} EID_{50})
A/Pigeon/Thailand/VSMU-7-NPT/2004	Pigeon04	13/2/2004	Nakornpatom	Dead	1	7.6	7.2	Pinpoint (76%) Medium (24%)	0.8
A/Tree sparrow/Ratchaburi/VSMU-16-RBR/2005	T.sparrow05	3/5/2005	Ratchaburi	Live	1	7.9	7.1	Pinpoint (6%) Medium (72%) Large (22%)	2.2

Pinpoint plaque, <1 mm in diameter; medium plaque, 1–2 mm in diameter; large plaque, >2 mm in diameter.
[a] Plaque size was measured in MDCK cells infected with HPAI viruses at 3 day post-infection.

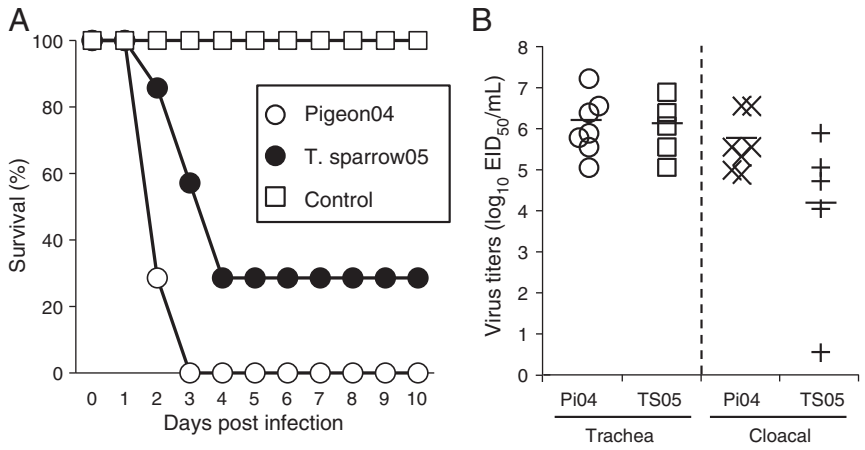

Fig. 2. Lethality and viral shedding of the two H5N1 HPAI viruses isolated from wild birds in chickens. (A) Survival rate of White Leghorn chickens after intranasal inoculation with Pigeon04 and T.sparrow05 at a dose of 10^6 EID_{50}. (B) Virus titers of trachea and cloacal swabs of dead chickens. The bars indicate the average for each group.

contrast, Pigeon04 replicated in MDCK cells to a less extent compared to T.sparrow05, and the titers were 6.5 and 6.1 log_{10}-$TCID_{50}$/mL, respectively (Fig. 1). Thus, T.sparrow05 efficiently replicated in MDCK cells, compared to Pigeon04. To compare the lethalities of the two HPAI viruses to chickens, we performed a survival experiment involving the exposure of White Leghorn chickens to the HPAI viruses. All chickens were killed due to Pigeon04 infection by day 3 post-infection (Fig. 2A). In contrast, 5 of 7 chickens were killed by T.sparrow05 infection by day 4 post-infection, but 2 survived until day 10 post-infection (Fig. 2A). The mean death times of the chickens were 47 and 70 h post-infection after Pigeon04 and T.sparrow05 inoculation, respectively. All the uninfected control chickens survived throughout the observation periods. Also, we measured virus titers in the trachea and cloacal

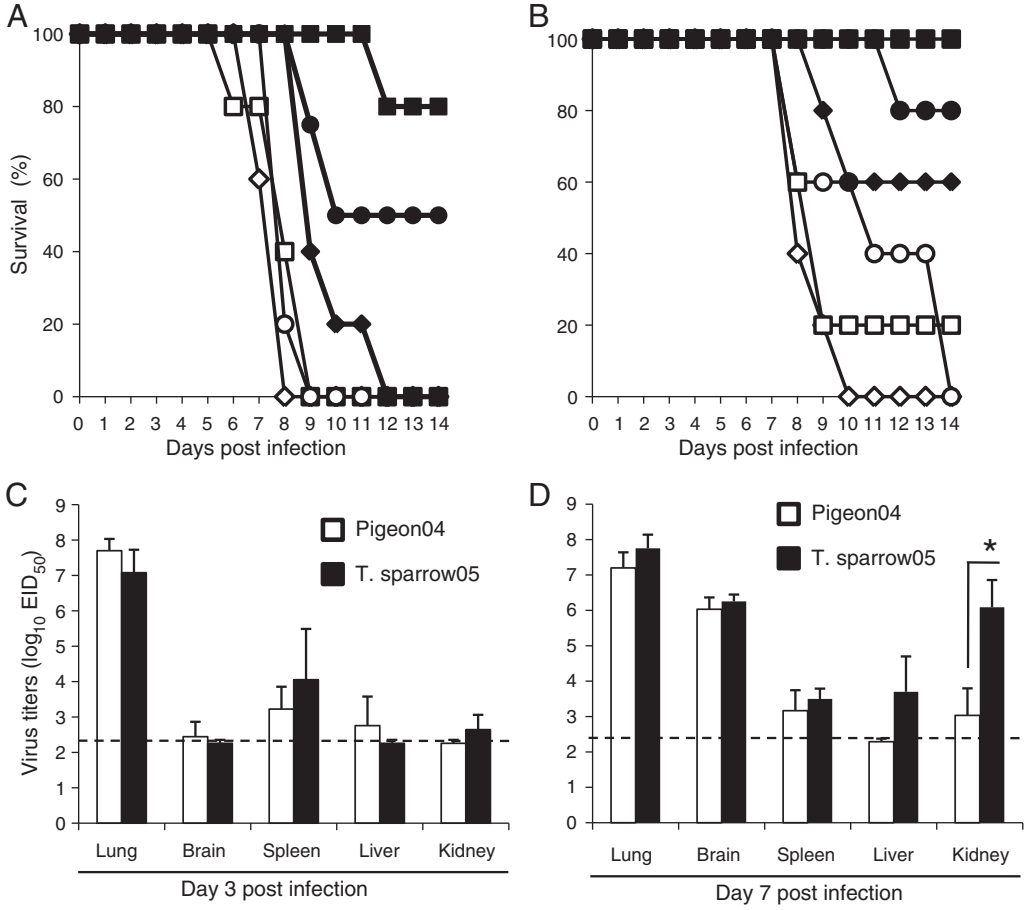

Fig. 3. Lethality and replication of the two H5N1 HPAI viruses isolated from wild birds in mice. (A and B) Survival rate of BALB/c mice infected with Pigeon04 (A) or T.sparrow05 (B) at the dose of 10^0 to 10^5 EID_{50} (10^0 EID_{50}, ■; 10^1 EID_{50}, ●; 10^2 EID_{50}, ◆; 10^3 EID_{50}, □; 10^4 EID_{50}, ○; 10^5 EID_{50}, ◊). (C and D) Viral distribution in mice infected with HPAI viruses. Mice were inoculated with Pigeon04 and T.sparrow05 at a dose of $10^{3.5}$ and 10^4 EID_{50}, respectively. Three mice in each group were sacrificed on days 3 and 7 post-inoculation for virus titration. Virus titers are presented as the mean values ± standard deviation. The dashed line indicates the detection limit of the virus titers. An asterisk indicates a statistically significant difference ($^*p < 0.05$ by Student *t* test).

swabs of dead chickens infected with HPAI viruses. As shown in Fig. 2B, dead chickens shed both viruses in these swabs at high virus titers, but Pigeon04 was shed more than T.sparrow05 in the cloacal swabs. Therefore, these data showed that Pigeon04 could cause more lethality than T.sparrow05 in chickens.

Lethality and viral distribution of mice infected with HPAI viruses

To compare virulence of the two wild bird isolates in mice, we firstly determined the 50% mouse lethal dose (MLD_{50}) of each virus. Pigeon04- or T.sparrow05-infected mice showed similar disease symptoms, such as ruffled fur, hunched posture and depression from day 6 post-infection. MLD_{50} of Pigeon04 and T.sparrow05 were $10^{0.8}$ and $10^{2.2}$ EID_{50}, respectively, indicating that both viruses were highly pathogenic to mice, and Pigeon04 were more virulent in mice than T.sparrow05 (Fig. 3A and B, Table 1). Next, to examine viral distributions of the two HPAI viruses in the host, we measured virus titers in lungs, brain, spleen, liver and kidney of the infected mice inoculated with Pigeon04 and T.sparrow05 at a dose of $10^{3.5}$ EID_{50} and 10^4 EID_{50}, respectively, on days 3 and 7 post-infection. On day 3 post-infection, both strains replicated very well in the lungs, and mean virus titers of Pigeon04 and T.sparrow05 were $10^{7.7}EID_{50}/g$ and $10^{7.1}EID_{50}/g$, respectively (Fig. 3C). Pigeon04 was also isolated from spleen, liver, and brain with the mean virus titers of $10^{3.2}EID_{50}/g$, $10^{2.7}EID_{50}/g$ and $10^{2.4}EID_{50}/g$, respectively, whereas T.sparrow05 was isolated from spleen and kidney with titers of $10^{4.1}EID_{50}/g$ and $10^{2.6}EID_{50}/g$, respectively (Fig. 3C). On day 7 post-infection, both viruses replicated robustly and similarly in the lungs, brain and spleen. The mean virus titers of Pigeon04 in lung, brain and spleen were $10^{7.2}EID_{50}/g$, $10^{6}EID_{50}/g$ and $10^{3.2}EID_{50}/g$, respectively, and those of T.sparrow05 in these organs were $10^{7.8}EID_{50}/g$, $10^{6.3}EID_{50}/g$ and $10^{3.5}EID_{50}/g$, respectively (Fig. 3D). Replication of the HPAI viruses in liver and kidney differed in mice infected with Pigeon04 or T.sparrow05. Pigeon04 was not isolated from liver, whereas T.sparrow05 was isolated with the mean virus titers of $10^{3.7}EID_{50}/g$ from liver (Fig. 3D). Also, Pigeon04 was isolated with the mean virus titer of $10^{3}EID_{50}/g$, whereas T.sparrow05 was isolated with mean virus titer of $10^{6.1}EID_{50}/g$ from kidney (Fig. 3D). These results clearly demonstrated that two HPAI viruses used in this study caused systemic and lethal infection to mice without adaptation. Thus, we compared the host responses in lungs and brains of mice infected with two viruses under conditions where both viruses replicated similarly in lungs and brains in the following study.

Histopathological changes of mice infected with two HPAI viruses isolated from wild birds

On day 3 post-infection, the lungs of mice infected with Pigeon04 and T.sparrow05 showed interstitial pneumonia accompanied with diffuse inflammation of the intra-alveolar septa (Fig. 4A and B). The inflammation in the lungs of Pigeon04-infected mice expanded to larger areas than that of T.sparrow05-infected mice. Moreover, the infiltrated cells present in the intra-alveolar septa were predominantly composed of mononuclear cells in the lungs of T.sparrow05 - infected mice, whereas they were composed of both neutrophils and mononuclear cells in the lungs of Pigeon04-infected mice (Fig. 4A and B). Viral antigens were detected mainly in bronchiolar epithelial cells in the lungs of infected mice (Fig. 4E and F). On day 7 post-infection, mild to severe interstitial pneumonia that was detected during the validation of the severity of inflammation was found in the lungs on Pigeon04 or T.sparrow05 infection (data not shown). Also, both viral antigens were detected in bronchiolar epithelial cells and alveolar macrophages (data not shown).

Encephalitis was observed in the brains of sacrificed or dead mice infected with HPAI viruses on days 7, 8 and 9 post-infection. However, T.sparrow05 induced it more frequently than Pigeon04 (Fig. 4C and D). Also, both viral antigens were detected in cells which were considered to be neuron and glial cells morphologically in the brains (Fig. 4G and H). No lesion was observed in the brains of the sacrificed mice infected with each virus on day 3 post-infection (data not shown). Moreover, no severe lesion was observed in other organs such as the spleen, heart, liver, kidney and intestine throughout the infection (data not shown). These results suggested that the two HPAI viruses used in this study induce different histopathological features in the lungs and brain tissues following infection.

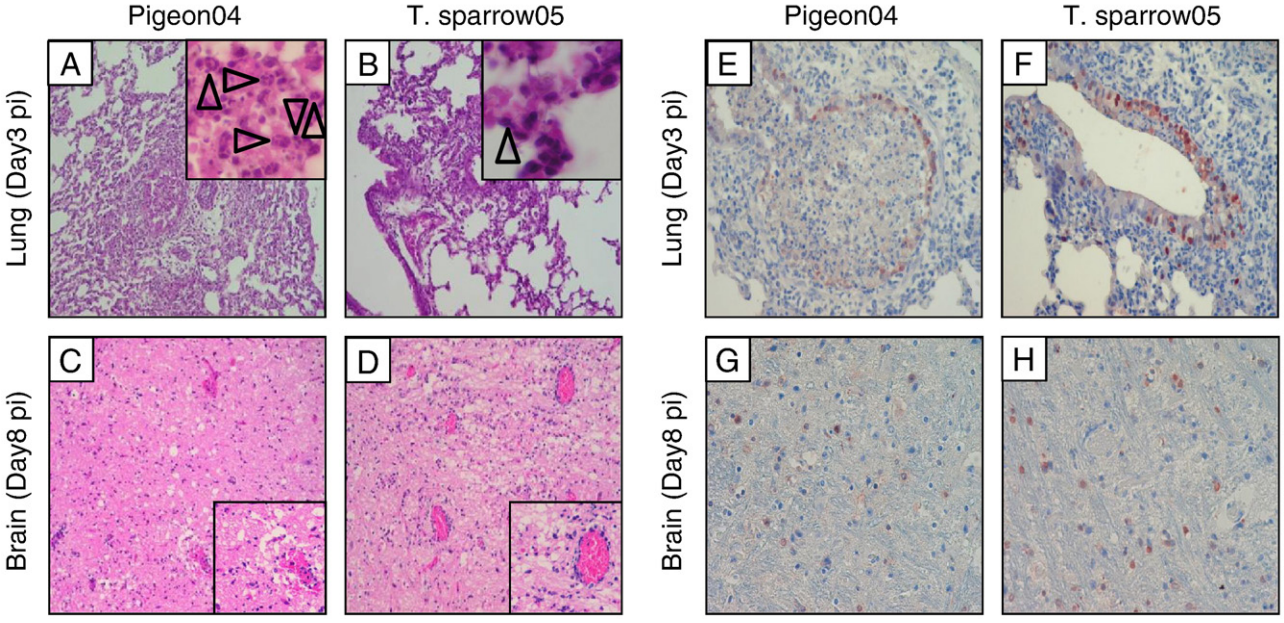

Fig. 4. Representative hematoxylin- and eosin-stained histopathological (A–D) and immunohistochemical (E–H) images of mice tissues infected with Pigeon04 (A, C, E and G) and T.sparrow05 (B, D, F and H). Lung sections of mice sacrificed on day 3 post-infection (A, B, E and F) and brain section of dead mice on day 8 post-infection (C, D, G and H) are shown. Immunohistochemistry detected viral antigen in the tissues. Arrowheads indicate neutrophil infiltrated into tissues. Original magnification: ×20 (A–D); ×40 (E–H).

Differential host gene responses in mice infected with the two HPAI viruses isolated from wild birds

Messenger RNA expression levels of genes categorized as (A) type I interferon, (B) Th1 type cytokine, (C) Th2 type cytokine, (D) proinflammatory cytokine, (E) chemokine, and (F) apoptosis were measured by real-time PCR in the lungs and brains from the infected mice (Figs. 5 and 6). TNFα were significantly up-regulated in Pigeon04-infected lungs compared to the T.sparrow05-infected or uninfected control lung on day 3 post-infection. In contrast, on day 7 post-infection, T.sparrow05 significantly induced TNFα expression in the lungs compared to the Pigeon04-infected or uninfected control (Fig. 5). Gene expression profiles of Interferon (IFNα and IFNβ), proinflammatory cytokine (IL6), chemokine (IP-10) showed similar tendencies on days 3 and 7 post-infection, suggesting that the timing of induction of the host gene response by viral infection differs between Pigeon04 and T.sparrow05. It should be noted that MIP-2, neutrophil chemoattractants, were highly induced by Pigeon04 infection on day 3 post-infection (Fig. 5). This result correlated with the histopathological changes between Pigeon04- and T.sparrow05-infected lung, in which the infiltration of neutrophils was observed in lungs only following Pigeon04 infection on day 3 post-infection (Fig. 4A and B).

In the brain, the levels of mRNA expression of any gene examined were not elevated upon viral infection on day 3 post-infection (Fig. 6). However, on day 7 post-infection, higher up-regulation of interferon (IFNα, IFNβ and IFNγ), proinflammatory cytokines (IL6 and TNFα), chemokines (MIP-2, CCL5 and IP-10) and apoptosis-related genes (TRAIL and FasL) were found following T.sparrow05 infection compared to Pigeon04 infection or the uninfected control (Fig. 6). It should be noted that both viruses were highly pathogenic to mice, and also replicated almost similarly in lung and brain on days 3 and 7 post-infection (Fig. 3). Therefore, these results suggest that the induction of host gene response by viral infection differs between Pigeon04 and T.sparrow05, but is not correlated with the lethality of the HPAI virus and viral load on organs.

Detection of cleaved forms of PARP and caspase-3 in the brains infected with the two HPAI viruses

To ascertain whether the up-regulations of mRNA levels of the apoptosis-related genes (TRAIL and FasL) were actually correlated with the induction of apoptosis, we assessed the levels of cleaved forms of caspase activated poly (ADP-ribose) polymerase (PARP) and caspase-3 in the brains infected with the HPAI viruses by Western blot analysis (Supplementary Fig. 1) However, we were not able to demonstrate the accumulation of the cleave forms of caspase-3 nor PARP in the brain homogenates of the infected mice.

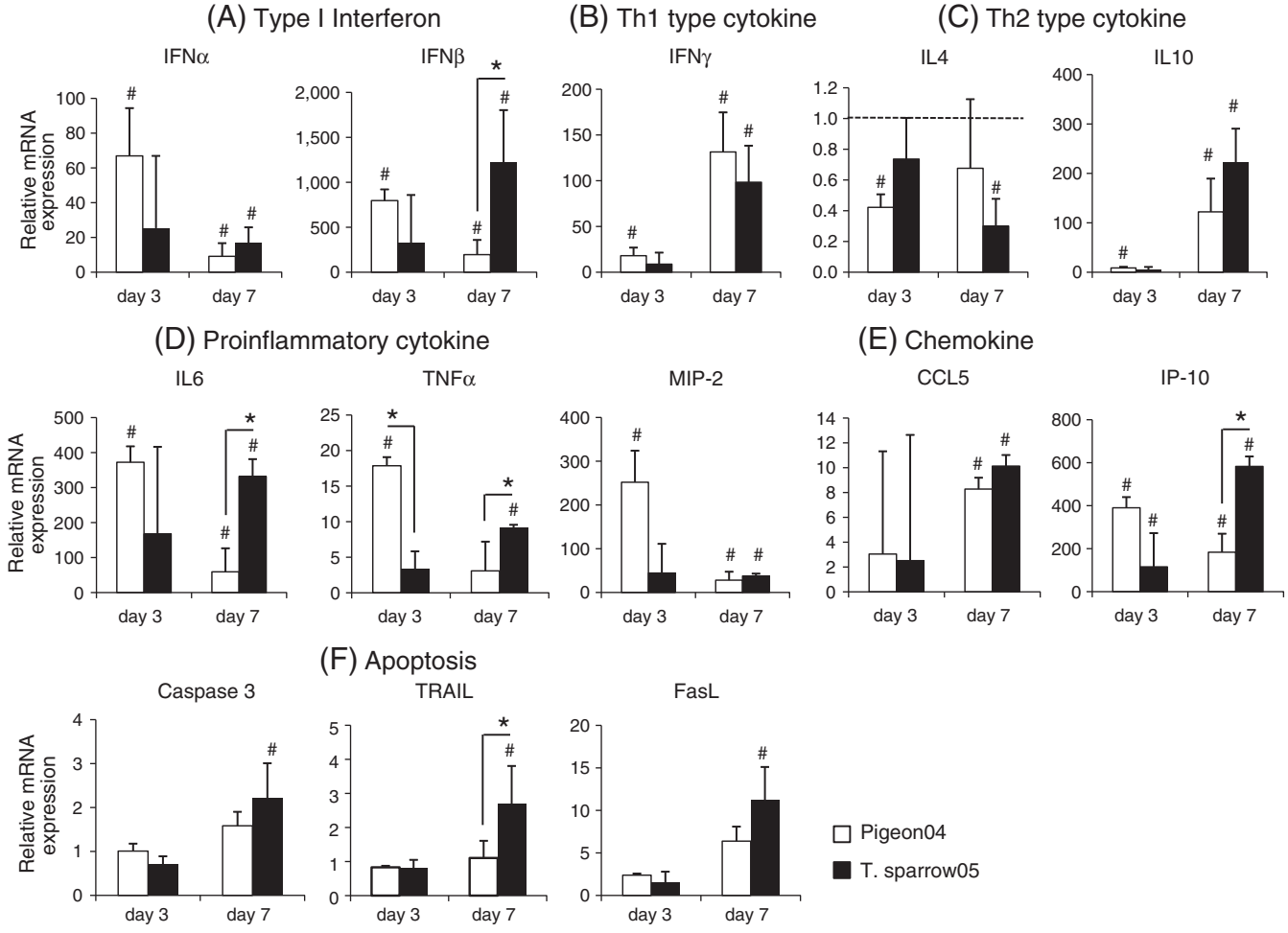

Fig. 5. Comparison of host gene responses of the lungs in mice infected with Pigeon04 and T.sparrow05. Mice were inoculated with Pigeon04 or T.sparrow05 at the dose of $10^{3.5}$ and 10^4 EID_{50}, respectively. At the indicated time, three mice in each group were sacrificed, and RNA was extracted, as mentioned in Materials and methods. The level of mRNA expression of each gene was examined by real-time PCR analysis using primers specific to the corresponding gene. mRNA levels indicate mean values ± standard deviations. Statistical analysis was performed for the Pigeon04-infected, T.sparrow05-infected and uninfected groups by ANOVA followed by Turkey analysis. The sharps indicate that the virus-infected group was significantly ($p < 0.05$) different from the uninfected group. Asterisks indicate that the Pigeon04-infected group was significantly ($p < 0.05$) different from the T.sparrow05-infected group.

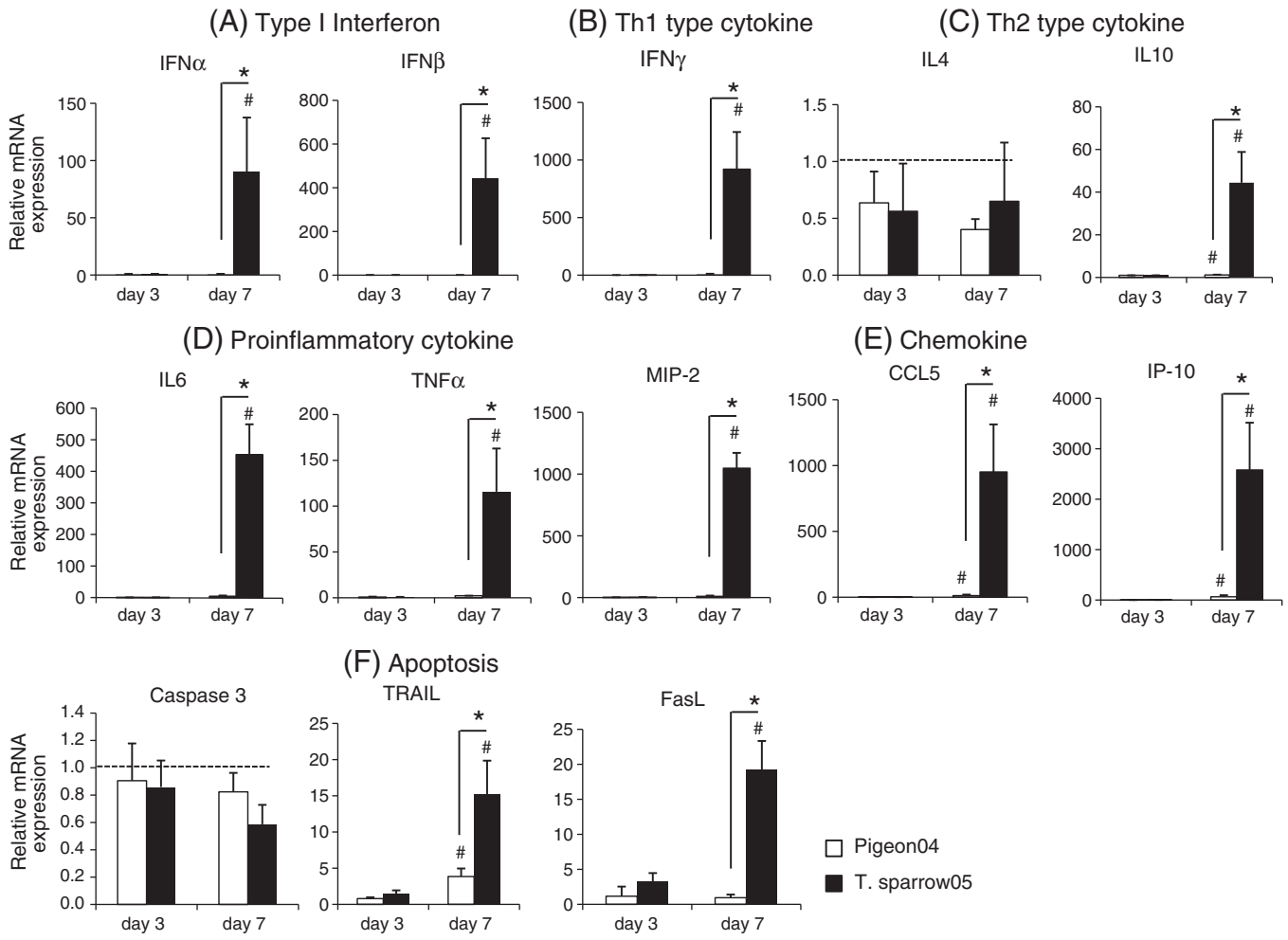

Fig. 6. Comparison of host gene responses of brains in mice infected with Pigeon04 and T.sparrow05. mRNA levels indicate mean values ± standard deviations. Sharps indicate that virus-infected group was significantly ($p < 0.05$) different from uninfected group. Asterisks indicated that Pigeon04-infected group was significantly ($p < 0.05$) different from T.sparrow05-infected group.

Molecular characteristics of HPAI viruses

Among the 11 proteins between Pigeon04 and T.sparrow05, there are 21 differences in amino acids (Table 2). There are several differences in amino acids in the ribonucleoprotein (RNP) complex, PB2, PB1, PA and NP, between the two viruses. Some of the changes in the amino acids are located in the functional domains of RNP that are essential for viral replication. Pigeon04 has residue 492-Ser in NP, whereas T.sparrow05 has residue 492-Asn. Recent studies suggest that C-terminal residues (490–496) of NP contributed to the regulation of NP oligomerization, which are involved with the transcriptional function of RNP (Ng et al., 2008). Also, Pigeon04 has the residues 315-Met and 451-Val in PB2, whereas T.sparrow05 has the residues 315-IIe and 451-IIe. Amino acid domains between residues 318–453 in PB2 are known as cap-binding domains which form a complex with m^7GTP, followed by initiation of transcription by RNP (Guilligay et al., 2008; Tarendeau et al., 2008). It was suggested that these amino acid differences may affect the viral transcriptional function of those viruses. Therefore, it would be necessary to determine amino acid residues affecting its function using the techniques such as mini genome assay and reverse genetics analysis in the future. Both viruses possess 627-Lys and 701-Asn in PB2 which are well known as virulence determinants in mice. Other amino acid differences between the two viruses showed no significant correlation with known viral functions.

Discussion

In this study, we examined the pathological characters of two Thai HPAI viruses isolated from wild birds in mice. The data showed that

Table 2
Amino acid differences between Pigeon04 and T.sparrow05.

Virus	Amino acid residue at position no.																				
	PB2			PB1	PB1-F2			PA	HA			NP	NA			M1	M2	NS1		NS2	
	206	315	451	96	33	65	74	275	152	418	492	492	54	59	312	168	89	130	197	45	61
Pigeon04	M	M	V	E	P	K	I	P	L	N	N	S	F	A	T	I	G	S	A	L	R
T.sparrow05	I	I	I	D	L	T	T	S	P	H	D	N	L	T	A	T	S	S/N	V	F	K

both Pigeon04 and T.sparrow05 caused lethal infection in mice and also replicated in multiple organs including the lungs and brain without adaptation (Fig. 3). However, host gene responses and lesions in the lungs and brains of the infected mice differed between the two viruses.

Our results raise several questions. First is the factor(s) that causes a difference in the responses of the host genes in Pigeon04 and T.sparrow05-infected lungs on day 3 post-infection. Viral distribution in lung tissue seems to be similar between the two viruses, suggesting that specific viral factors might contribute to the difference in host responses to the viruses (Fig. 3C). Specific amino acid substitutions of viral segments have been shown to be correlated with alterations in host cytokine responses after viral infection. A substitution of E627K in PB2 affects T-cell receptor activation of lungs in the early stages of viral infection, but it is not correlated with viral load (Fornek et al., 2009). Also, a substitution at P42S in NS1 has been shown to contribute to the antagonizing effect on antiviral cytokines, such as IFNα and IFNβ in HPAI virus infection (Jiao et al., 2008). However, both viruses possess Lys at residue 627 in PB2 and interestingly serine at 42 residue in NS1, implying that these substitutions in PB2 and NS1 are not significant viral determinants for the alterations in host cytokine responses after viral infection that were observed in this study. One could suggest that other amino acid substitution(s) not previously recognized might be involved in the different host responses in mice infected with Pigeon04 and T.sparrow05. Also, synergetic or redundant functions of each viral determinant would be implicated in the pathogenesis of the HPAI viruses in mice.

The second question is why host responses in the lungs on day 7 post-infection were inverted between Pigeon04 and T.sparrow05-infected mice. This was probably because Pigeon04 was unable to induce high levels of proinflammatory cytokines and chemokines in the lungs on day 7 post-infection because type I interferons (IFNα and IFNβ) that were induced on day 3 post-infection induced potent inhibition of these cytokines. In contrast, T.sparrow05 could likely induce proinflammatory cytokines and chemokines to a great extent in the lungs on day 7 post-infection as type I interferon was induced to a less extent in the lungs on day 3 post-infection. Smits et al. (2010) have demonstrated that type I interferon inhibited the induction of proinflammatory cytokines in SARS-CoV-infected aged macaques. They showed that low levels of induction of type I Interferon (IFNβ) opposed the high levels of induction of proinflammatory cytokines (IL6 and IL8) in the lungs of SARS-CoV-infected aged macaques compared with those of SARS-CoV-infected young adult macaques (Smits et al., 2010). In addition, it was shown that treatment with IFNα inhibited the induction of proinflammatory cytokines in the lungs of aged macaques infected with SARS-CoV (Smits et al., 2010).

Third is why host responses in brains differed between Pigeon04 and T.sparrow05-infection on day 7 post-infection. The viruses did not induce mRNA expression of cytokines on day 3 post-infection due to inadequate viral replication in the brains (Fig. 3C). Moreover, the potent induction of type I interferon in the lungs of Pigeon04-infected mice on day 3 post-infection may have inhibited cytokine induction in the brain as well as the lungs on day 7 post-infection. Our hypothesis would be supported by a previous study where IFNβ treatment inhibited the induction of proinflammatory cytokines in the brain by inhibiting the proliferation and migration of Th1 cells to the central nervous system (CNS) though the blood–brain barrier in patients with multiple sclerosis (Satoh, 2006). In addition, the avian H5N1 HPAI virus was reported to likely induce proinflammatory cytokines including IL6, IL1β and TNFα in mice primary glial cells such as microglia and astrocytes, and that the release of proinflammatory cytokines in CNS is involved with encephalitis (Wang et al., 2008), showing that other mechanisms could also be involved. As shown in Fig. 4G and H, both viruses were detected in cells which were considered as glial cells morphologically. The induction of type I interferon in the lungs of the infected mice on day 3 post-infection may have contributed to the inhibition of the proinflammatory cytokines in glial cells. Indeed, Aaron et al. reported that serum neutralizing antibodies (NAbs) against IFNβ in multiple sclerosis inhibited the production of a proinflammatory cytokine (IL6) and IP-10 in human astrocytes, suggesting that the serum level of IFNβ affects cytokine production within the CNS (Shapiro et al., 2006). Monteerarat et al. (2010) reported strain-dependent induction of TNFα in human macrophages by HPAI virus infections. Moreover, host response towards viral infection has been suggested to vary according to the type of cells infected with the virus. Further analysis of host cell- and strain-dependency for cytokine inductions by the HPAI viruses is essential.

Fourth is the implication of the varying host responses to the pathobiological outcome of the infection. Salomon et al. (2007) demonstrated that pre-treatment with glucocorticoids suppressed host cytokine response but did not significantly alter lethality of HPAI viruses in infected mice, even though host cytokine responses after viral infection were suppressed by the pre-treatment. This suggested that the exaggeration of inflammatory lesion caused by excessive cytokine inductions was not essential for a fatal outcome. Efficient viral replication at the early stage of the infection appeared crucial for the pathogenicity of the viruses. The presence of a particular cytokine (s), that is yet to be elucidated, at a certain level as well as pathological damage to the lungs caused by efficient viral replication in the lungs seem to be enough for viral infections to cause fatalities in mice. Host responses such as cytokine induction and pathological damage in T.sparrow05-infected mice were relatively delayed compared to Pigeon04-infected mice in the earlier phase of infection. The more severe inflammation observed in the brains of T.sparrow05-infected mice later in the infection likely enhanced virulence of the virus resulting in lethal outcome for mice infected with either virus. These findings suggest that T.sparrow05 is more neurovirulent than Pigeon04; however, further comparative investigation on neurovirulence of the two isolates is necessary.

In this study, we showed that two viruses induced different expression of several cytokines examined in the lungs and brain of the infected mice at the gene transcriptional level. However, we did not determine cells or cell types that were associated with the host gene responses observed in the lungs and brains of the mice infected with the HPAI viruses, since tissue homogenates were used for the analysis. Further studies with *in situ* hybridization and/or immunohistochemistry methods targeting the genes or their products identified in this study would reveal the association between the host gene responses and the viral infection in an individual cell level of the lungs and brains of the infected mice in detail. Quantification of cytokine productions and a comprehensive gene expression analysis by microarray would also help better understanding of the pathogenesis of the HPAI viruses. Also, we could not demonstrate the association of caspase-3 nor PARP cleavage with the up-regulation of TRAIL and FasL in the brain homogenates. Because the induction of apoptosis in the brain upon viral infection may not be induced ubiquitously in the brains of the infected mice, *in situ* relationship between the up-regulation of the apoptosis-related genes and cells involved in apoptosis in the brains also need to be scrutinized.

In conclusion, we demonstrated that two HPAI viruses isolated from wild birds in Thailand induce different host responses in mice, despite similar replications in lungs and brain, resulting in lethal outcome in mice. The lethality of HPAI viruses to mice could not be determined based solely on host gene response or severity of lesions in the lungs and brain infected with the viruses. Of note, these two viruses differed in their lethalities to chicken, suggesting that they possess different virulence determinant(s) to poultry also (Fig. 2). Detailed analysis of the pathogenic characteristics of both viruses used in this study in chickens and in their natural hosts, pigeon and tree sparrow, would provide further enlightenment on the virulence mechanism of HPAI viruses.

Materials and methods

Viruses

Viruses used in this study are shown in Table 1. Virus stocks were propagated in Madin–Darby canine kidney (MDCK) cells, and stocked at -80 °C before use. The 50% egg infectious dose (EID_{50}) titers were determined by serial titration of viruses in 10- or 11-day-old embryonated eggs, and were calculated by the method of Reed and Muench (1938). All experiments with the H5N1 HPAI viruses were performed in a biosafety level 3 containment laboratory at Mahidol University, Thailand. Animal experiments were conducted under the guidelines of Animal Care and Use Protocol on the approval of The Faculty of Veterinary Science Animal Care and Use Committee, Mahidol University.

Genomic sequencing and phylogenetic analysis

Total viral RNA was extracted from culture fluid in MDCK cells using the RNeasy mini kit (Qiagen, Hilden, Germany), and was reverse transcribed to cDNA. Then, the coding regions of the viral gene segments were amplified by polymerase chain reaction (PCR). PCR products were purified, and sequenced directly using the Big Dye Terminator sequencing kit, version 3.1 (Applied Biosystems, Foster City, CA, USA) on ABI Prism 3100 genetic analyzer (Applied Biosystems). Sequences of the primers using PCR and sequence reactions are available upon request. The sequences obtained were aligned with Bioedit software version 7.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and processed to generate a phylogenetic tree with the neighbor-joining method by MEGA software version 4.0 (Kumar et al., 2008).

Viral growth kinetics in MDCK cells

Confluent MDCK cells were infected with Pigeon04 and T.sparrow05 at a multiplicity of infection of 0.01 $TCID_{50}$ and incubated at 37 °C. On days 1, 2 and 3 post-infection, the supernatant was collected and titrated in MDCK cells, calculated by the method of Reed and Muench (1938), and expressed as $TCID_{50}$/mL.

Chicken experiments

Specific pathogen-free (SPF) embryonated eggs of White Leghorn (*Gallus gallus domesticus*) chickens were purchased from Nisseiken (Kobuchisawa, Yamanashi, Japan), and were imported to Thailand under an import permit from the Department of Livestock Development, Thailand. Eggs were hatched and raised for 5 weeks in a conditioned room in the Faculty of Veterinary Science, Mahidol University. Groups of seven 5-week-old White Leghorn chickens were inoculated intranasally with 10^6 EID_{50} of the HPAI viruses or mock infected with medium at a volume of 0.1 mL, and were observed daily for clinical signs or death up to day 10 post-infection. Trachea and cloacal swab samples were collected from the dead chickens in a 2 mL volume of freezing medium, and titrated as described above. Viral shedding in the trachea and cloacal swabs was determined by calculating the EID_{50}/mL of these samples.

Mice experiments

Viral inoculation

To determine the 50% mouse lethal dose (MLD_{50}) of the HPAI viruses, 7-week-old female SPF BALB/c mice (National Laboratory Animal Center, Nakhon Pathom, Thailand) were anesthetized intraperitoneally with nembutal (30 mg/kg of body weight), and were inoculated intranasally in groups of 4–5 mice with HPAI viruses in 10-fold serial dilutions containing 10^0 to 10^5 EID_{50} in a 0.01 mL volume. The mice were observed daily for clinical signs or death up to day 14 post-infection. The MLD_{50} scores of each virus were calculated by the method of Reed and Muench (1938). To examine viral distribution, pathology, and host gene response of the mice infected with the HPAI viruses, groups of 24 mice were inoculated as described above with Pigeon04 and T.sparrow05 at a dose of $10^{3.5}$ and 10^4 EID_{50}, respectively. We set up viral inoculation dose of Pigeon04 and T.sparrow05 at $10^{3.5}$ EID_{50} and 10^4 EID_{50}, respectively, since the viruses replicated similarly in the lungs and brains when inoculated with those doses, leading to fetal outcome. Then, on days 3 and 7 post-inoculation, the 6 live mice in each group were euthanized, and dissected the appropriated organs. To determine the profiles of cleaved forms of PARP and Caspase3 in the mice infected with the HPAI viruses, groups of 6 mice were inoculated with Pigeon04 and T.sparrow05 as described above. On days 3 and 7 post-infection, the 3 mice in each group were sacrificed and the brains were subjected to Western blot analysis.

Viral distributions in mice organs

Extracted tissue samples including lung, brain, spleen, liver and kidney were homogenized to make suspensions of 10% in MEM containing antibiotics. The homogenates were titrated in the eggs from the initial dilution of 1:10 (lungs, brain, liver and kidney) or 1:20 (spleen). Virus titers were calculated by the method of Reed and Muench (1938), and expressed as EID_{50}/g of tissue. The limits of virus detection were $10^{2.2}$ EID_{50}/g for lungs, brain, liver and kidney, and $10^{2.5}$ EID_{50}/g for spleen.

Histopathology

Lung, brain, spleen, heart, liver, kidney and intestine from the infected animals were fixed in 10% neutral phosphate buffered formalin. Fixed samples were embedded in paraffin, sectioned, then stained with hematoxylin and eosin (HE), and observed microscopically. For viral antigen staining, lung and brain sections were processed for immunostaining. Goat anti-influenza A virus polyclonal antibody (OBT1551, AbD Serotec) and horseradish peroxidase anti-goat Ig conjugate (Histofine Simple Stain, Nichirei Inc.) were used for the primary and secondary antibodies, respectively.

RNA extraction and real-time PCR

Part of the lungs or brains were preserved in RNA later solution (AM7021, Ambion), and stocked at -80 °C before isolation of RNA. Total RNA was extracted from these samples using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, purified in isopropyl alcohol, and diluted in RNase-free water. Total RNA was treated with DNase I (1 unit per 1 μg RNA) (Promega, Madison, WI, USA) to remove the residual genomic DNA at 37 °C for 1 h, followed by inactivation of DNase I at 65 °C for 10 min. Then, the DNase-treated RNA samples were re-purified by RNA mini kit. cDNA was synthesized from mRNA with $oligo(dT)_{20}$ primers using SuperScript™ III First-strand Synthesis System For RT-PCR (18080-051, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA samples were diluted (1:10) and used as template. PCR reactions were performed equal amounts of cDNA samples with previously published primers specific for target genes (IFNα, IFNβ, IFNγ, IL4, IL10, IL6, TNFα, MIP-2, CCL5, IP-10, Caspase3, TRAIL, FasL) and β-actin (Cheeran et al., 2007; Ghoshal et al., 2001; Guo et al., 2005; Inoue et al., 2009; Jiankuo et al., 2003; Monrad et al., 2008; Terrazzino et al., 2002; Zaheer et al., 2007; Zhang et al., 2008) (Supplementary Table 1) and SYBR® Premix Ex Taq™ II (RR081A, Perfect Real Time, TAKARA), as illustrated by the manufacturer. Quantitative real-time PCR analysis was run in triplicate with Cromo4 (Bio-lad laboratories) by following cycle parameters: 1 cycle at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Differences in gene expressions were calculated by the $2^{-\Delta\Delta}$Ct method and expressed as fold change in gene expression (Livak and

Schmittgen, 2001). β-Actin was used as endogenous control to normalize quantification of the target gene. Average results ± standard deviations were expressed as fold change compared to the untreated mice.

Western blot analysis

Brain tissue samples were homogenized with 5 volumes of suspension buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl) containing protein inhibitor cocktail (complete, Roche). Then, the samples were mixed with 2×sample buffer (EzApply, AE-1430, ATTO), incubated at 95 °C for 5 min, and electrophoresed on 12.5–15% SDS–polyacrylamide gels. Proteins were transferred to PVDF membrane in Bio-Lad wet transfer unit, and the membranes were blocked in 5% skim milk for 1 h at room temperature. Then, the membranes were incubated overnight at 4 °C with primary antibodies as follows: caspase-3 antibody (1:1000, #9662, Cell Signaling, Beverly, MA), PARP antibody (1:1000, #9542, Cell Signaling), cleaved PARP (Asp214) antibody (mouse specific) (1:1000, #9544, Cell Signaling), β-actin (13E5) rabbit mAb (1:1000, #4970, Cell Signaling). After three washes, the membranes were incubated for 1 h at room temperature with secondary antibodies (anti-rabbit IgG, HRP-linked antibody, 1:2000, #7074, Cell Signaling). The membranes were additionally washed three times, target proteins were visualized using ECL Plus Western Blotting Detection Reagents (RPN2132, GE Healthcare).

Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.12.040.

Acknowledgments

This work was supported by the program of the Founding Research Center for Emerging and Reemerging Infectious Diseases launched by a project commissioned by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

References

Amonsin, A., Payungporn, S., Theamboonlers, A., Thanawongnuwech, R., Suradhat, S., Pariyothorn, N., Tantilertcharoen, R., Damrongwantanapokin, S., Buranathai, C., Chaisingh, A., Songserm, T., Poovorawan, Y., 2006. Genetic characterization of H5N1 influenza A viruses isolated from zoo tigers in Thailand. Virology 344, 480–491.

Cheeran, M.C.J., Hu, S., Palmquist, J.M., Bakken, T., Gekker, G., Lokensgard, J.R., 2007. Dysregulated interferon-gamma responses during lethal cytomegalovirus brain infection of IL-10-deficient mice. Virus Res. 130, 96.

Chen, H., Li, Y., Li, Z., Shi, J., Shinya, K., Deng, G., Qi, Q., Tian, G., Fan, S., Zhao, H., Sun, Y., Kawaoka, Y., 2006. Properties and Dissemination of H5N1 Viruses Isolated during an Influenza Outbreak in Migratory Waterfowl in Western China. J. Virol. 80, 5976–5983.

Chen, H., Smith, G.J.D., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., Webster, R.G., Peiris, J.S.M., Guan, Y., 2005. Avian flu H5N1 virus outbreak in migratory waterfowl. Nature 436, 191.

Chotpitayasunondh, T., Ungchusak, K., Hanshaoworakul, W., Chunsuthiwat, S., Sawanpanyalert, P., Kijphati, R., Lochindarat, S., Srisan, P., Suwan, P., Osotthanakorn, Y., Anantasetagoon, T., Kanjanawasri, S., Tanupattarachai, S., Weerakul, J., Chaiwirattana, R., Maneerattanaporn, M., Poolsavathitikool, R., Chokephaibulkit, K., Apisarnthanarak, A., Dowell, S., 2005. Human disease from influenza A (H5N1), Thailand, 2004. Emerg. Infect. Dis. 11, 201–209.

de Jong, M.D., Simmons, C.P., Thanh, T.T., Hien, V.M., Smith, G.J.D., Chau, T.N.B., Hoang, D.M., Van Vinh Chau, N., Khanh, T.H., Dong, V.C., Qui, P.T., Van Cam, B., Ha, D.Q., Guan, Y., Peiris, J.S.M., Chinh, N.T., Hien, T.T., Farrar, J., 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat. Med. 12, 1203–1207.

Fornek, J.L., Gillim-Ross, L., Santos, C., Carter, V., Ward, J.M., Cheng, L.I., Proll, S., Katze, M.G., Subbarao, K., 2009. A single-amino-acid substitution in a polymerase protein of an H5N1 influenza virus is associated with systemic infection and impaired T-cell activation in mice. J. Virol. 83, 11102–11115.

Ghoshal, K., Majumder, S., Zhu, Q., Hunzeker, J., Datta, J., Shah, M., Sheridan, J.F., Jacob, S.T., 2001. Influenza virus infection induces metallothionein gene expression in the mouse liver and lung by overlapping but distinct molecular mechanisms. Mol. Cell. Biol. 21, 8301–8317.

Gilsdorf, A., Boxall, N., Gasimov, V., Agayev, I., Mammadzade, F., Ursu, P., Gasimov, E., Brown, C., Mardel, S., Jankovic, D., Pimentel, G., Ayoub, I., Elassal, E., Salvi, C., Legros, D., Silva, C.P.D., Hay, A., Andraghetti, R., Rodier, G., Ganter, B., 2006. Two clusters of human infection with influenza A/H5N1 virus in the Republic of Azerbaijan, February-March 2006. Euro Surveill. 11, 122–126.

Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R.W.H., Ortin, J., Hart, D.J., Cusack, S., 2008. The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat. Struct. Mol. Biol. 15, 500–506.

Guo, L., Hu-Li, J., Paul, W.E., 2005. Probabilistic regulation in TH2 cells accounts for monoallelic expression of IL-4 and IL-13. Immunity 23, 89–99.

Inoue, K., Shiota, S., Yamada, K., Gotoh, K., Suganuma, M., Fujioka, T., Ahmed, K., Iha, H., Nishizono, A., 2009. Evaluation of a new tumor necrosis factor-alpha-inducing membrane protein of Helicobacter pylori as a prophylactic vaccine antigen. Helicobacter 14, 135–143.

Jiankuo, M., Xingbing, W., Baojun, H., Xiongwin, W., Zhuoya, L., Ping, X., Yong, X., Anting, L., Chunsong, H., Feili, G., Jinquan, T., 2003. Peptide nucleic acid antisense prolongs skin allograft survival by means of blockade of CXCR3 expression directing T cells into graft. J. Immunol. 170, 1556–1565.

Jiao, P., Tian, G., Li, Y., Deng, G., Jiang, Y., Liu, C., Liu, W., Bu, Z., Kawaoka, Y., Chen, H., 2008. A Single-Amino-Acid Substitution in the NS1 Protein Changes the Pathogenicity of H5N1 Avian Influenza Viruses in Mice. J. Virol. 82, 1146–1154.

Ka-Fai, T., Paul, K.S.C., Kui-Fat, C., Wai-Ki, L., Woon-Yee, L., Kit-Fai, W., Nelson, L.S.T., Dominic, N.C.T., Rita, Y.T.S., Thomas, A.B., John, S.T., Augustine, F.C., 2001. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J. Med. Virol. 63, 242–246.

Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R.A.M., Amonsin, A., Payungporn, S., Noppornpanth, S., Wattanodorn, S., Theamboonlers, A., Tantilertcharoen, R., Pattanarangsan, R., Arya, N., Ratanakorn, P., Osterhaus, A.D.M.E., Poovorawan, Y., 2004. Avian influenza H5N1 in tigers and leopards. Emerg. Infect. Dis. 10, 2189–2191.

Korteweg, C., Gu, J., 2008. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. Am. J. Pathol. 172, 1155–1170.

Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9, 299–306.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$CT method. Methods 25, 402.

Monrad, S., Killen, P., Anderson, M., Bradke, A., Kaplan, M., 2008. The role of aldosterone blockade in murine lupus nephritis. Arthritis Res. Ther. 10, R5.

Monteerarat, Y., Sakabe, S., Ngamurulert, S., Srichatraphimuk, S., Jiamtom, W., Chaichuen, K., Thitithanyanont, A., Permpikul, P., Songserm, T., Puthavathana, P., Nidom, C., Mai, L., Iwatsuki-Horimoto, K., Kawaoka, Y., Auewarakul, P., 2010. Induction of TNF-alpha in human macrophages by avian and human influenza viruses. Arch. Virol. 155, 1273–1279.

Ng, A.K.-L., Zhang, H., Tan, K., Li, Z., Liu, J.-H., Chan, P.K.-S., Li, S.-M., Chan, W.-Y., Au, S.W.-N., Joachimiak, A., Walz, T., Wang, J.-H., Shaw, P.-C., 2008. Structure of the influenza virus A H5N1 nucleoprotein: implications for RNA binding, oligomerization, and vaccine design. FASEB J. 22, 3638–3647.

Peiris, J., Yu, W., Leung, C., Cheung, C., Ng, W., Nicholls, J., Ng, T., Chan, K., Lai, S., Lim, W., Yuen, K., Guan, Y., 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 21, 617–619.

Perrone, L.A., Plowden, J.K., García-Sastre, A., Katz, J.M., Tumpey, T.M., 2008. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog. 4, e1000115.

Reed, L., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.

Salomon, R., Hoffmann, E., Webster, R.G., 2007. Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc. Natl Acad. Sci. USA 104, 12479–12481.

Satoh, J., 2006. Interferon -beta therapy in multiple sclerosis. Nippon Rinsho 64, 1297–1309.

Shapiro, A.M., Jack, C.S., Lapierre, Y., Arbour, N., Bar-Or, A., Antel, J.P., 2006. Potential for interferon beta-induced serum antibodies in multiple sclerosis to inhibit endogenous interferon-regulated chemokine/cytokine responses within the central nervous system. Arch. Neurol. 63, 1296–1299.

Siengsanan, J., Chaichoune, K., Phonakngnuen, R., Sariya, L., Prompiram, P., Kocharin, W., Tangsudjai, S., Suwanpukdee, S., Wiriyarat, W., Pattanarangsan, R., Robertson, I., Blacksell, S.D., Ratanakorn, P., 2009. Comparison of outbreaks of H5N1 highly pathogenic avian influenza in wild birds and poultry in Thailand. J. Wildl. Dis. 45, 740–747.

Smits, S.L., de Lang, A., van den Brand, J.M.A., Leijten, L.M., van Ijcken, W.F., Eijkemans, M.J.C., van Amerongen, G., Kuiken, T., Andeweg, A.C., Osterhaus, A.D.M.E., Haagmans, B.L., 2010. Exacerbated innate host response to SARS-CoV in aged non-human primates. PLoS Pathog. 6, e1000756.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Meemak, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., Poovorawan, Y., 2006a. Avian influenza H5N1 in naturally infected domestic cat. Emerg. Infect. Dis. 12, 681–683.

Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A., Chutinimitkul, S., Thanawongnuwech, R., Poovorawan, Y., 2006b. Fatal avian influenza A H5N1 in a dog. Emerg. Infect. Dis. 12, 1744–1747.

Tarendeau, F., Crepin, T., Guilligay, D., Ruigrok, R.W.H., Cusack, S., Hart, D.J., 2008. Host determinant residue lysine 627 lies on the surface of a discrete, folded domain of influenza virus polymerase PB2 subunit. PLoS Pathog. 4, e1000136.

Terrazzino, S., Bauleo, A., Baldan, A., Leon, A., 2002. Peripheral LPS administrations up-regulate Fas and FasL on brain microglial cells: a brain protective or pathogenic event? J. Neuroimmunol. 124, 45–53.

The Writing Committee of the World Health Organization Consultation on Human Influenza, 2005. Avian Influenza A (H5N1) Infection in Humans. N. Engl. J. Med. 353, 1374–1385.

Tiensin, T., Chaitaweesub, P., Songserm, T., Chaisingh, A., Hoonsuwan, W., Buranathai, C., Parakamawongsa, T., Premashthira, S., Amonsin, A., Gilbert, M., Nielen, M., Stegeman, A., 2005. Highly pathogenic avian influenza H5N1, Thailand, 2004. Emerg. Infect. Dis. 11, 1664–1672.

Tumpey, T.M., Lu, X., Morken, T., Zaki, S.R., Katz, J.M., 2000. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. J. Virol. 74, 6105–6116.

Uchida, Y., Chaichoune, K., Wiriyarat, W., Watanabe, C., Hayashi, T., Patchimasiri, T., Nuansrichay, B., Parchariyanon, S., Okamatsu, M., Tsukamoto, K., Takemae, N., Ratanakorn, P., Yamaguchi, S., Saito, T., 2008. Molecular epidemiological analysis of highly pathogenic avian influenza H5N1 subtype isolated from poultry and wild bird in Thailand. Virus Res. 138, 70–80.

Uiprasertkul, M., Kitphati, R., Puthavathana, P., Kriwong, R., Kongchanagul, A., Ungchusak, K., Angkasekwinai, S., Chokephaibulkit, K., Srisook, K., Vanprapar, N., Auewarakul, P., 2007. Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. Emerg. Infect. Dis. 13, 708–712.

Wang, G., Zhang, J., Li, W., Xin, G., Su, Y., Gao, Y., Zhang, H., Lin, G., Jiao, X., Li, K., 2008. Apoptosis and proinflammatory cytokine responses of primary mouse microglia and astrocytes induced by human H1N1 and avian H5N1 influenza viruses. Cell. Mol. Immunol. 5, 113.

Xu, T., Qiao, J., Zhao, L., Wang, G., He, G., Li, K., Tian, Y., Gao, M., Wang, J., Wang, H., Dong, C., 2006. Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. Am. J. Respir. Crit. Care Med. 174, 1011–1017.

Zaheer, A., Sahu, S.K., Wu, Y., Zaheer, A., Haas, J., Lee, K., Yang, B., 2007. Diminished cytokine and chemokine expression in the central nervous system of GMF-deficient mice with experimental autoimmune encephalomyelitis. Brain Res. 1144, 239–247.

Zhang, J., Li, Y., Zheng, X., Gao, Q., Liu, Z., Qu, R., Borneman, J., Elias, S.B., Chopp, M., 2008. Bone marrow stromal cells protect oligodendrocytes from oxygen-glucose deprivation injury. J. Neurosci. Res. 86, 1501–1510.

Zhou, J.-Y., Shen, H.-G., Chen, H.-X., Tong, G.-Z., Liao, M., Yang, H.-C., Liu, J.-X., 2006. Characterization of a highly pathogenic H5N1 influenza virus derived from bar-headed geese in China. J. Gen. Virol. 87, 1823–1833.