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The NS1 protein of H5N6 feline influenza virus inhibits feline beta interferon response by preventing NF-kB and IRF3 activation

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Despite the apparent lack of a feline influenza virus lineage, cats are susceptible to infection by influenza A viruses. Here, we characterized in vitro A/feline/Guangdong/1/2015, an H5N6 avian influenza virus recently isolated from cats. A/feline/Guangdong/1/2015 replicated to high titers and caused CPE in feline kidney cells. We determined that infection with A/feline/Guangdong/1/2015 did not activate the IFN-β promoter, but inhibited it by blocking the activation of NF-kB and IRF3. We also determined that the viral NS1 protein mediated the block, and that the dsRNA binding domain of NS1 was essential to perform this function. In contrast to treatment after infection, cells pretreated with IFN-β suppressed viral replication. Our findings provide an example of an H5N6 influenza virus suppressing IFN production, which might be associated with interspecies transmission of avian influenza viruses to cats.

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1. Introduction

Influenza A viruses (IAVs) cause respiratory disease in humans and animals. They belong to the Orthomyxoviridae and are negative sense, single-stranded RNA viruses. Wild birds are probably the most important natural reservoir host of IAVs as they maintain a vast viral gene pool and pose a continuous threat to humans and other mammalian species (Yoon et al., 2014). Agricultural practices and ecological systems in China and other eastern Asian countries have provided ample opportunities for avian influenza viruses (IAVs) to cross species barriers and cause sporadic infections in mammals (Su et al., 2014a, 2014b, 2015a, 2015b). An AIV of the H5N6 subtype was responsible for a recent outbreak amongst birds in Laos, Vietnam, and China that resulted in the culling of ~100,000 chickens (Bi et al., 2015; Guillaume Belot et al., 2014; Qi et al., 2014; Shen et al., 2015; Wong et al., 2015; Wu et al., 2015a). H5N6 AIVs also pose a public health threat as four human infections with H5N6 were reported in China in 2014 (three of which were lethal) (He and Duan, 2015; Pan et al., 2016; Takai et al., 1990; Yang et al., 2015).

Companion animals (cats and dogs) have been considered potential “intermediate hosts” for the interspecies transmission of zoonotic pathogens (Parrish et al., 2015; Su et al., 2014c). In some locations, domestic cats live in an environment where waterfowl are present and thus can establish close contact with humans, poultry and wild birds. Serological studies have suggested that they may act as vectors of influenza transmission within households (McCullers et al., 2011; Su et al., 2013; Zhao et al., 2014). Cats are susceptible to AIVs as natural infections of cats with H3N2 (Jeon et al., 2013), H5N1 (Songserm et al., 2006; Yingst et al., 2006), and H5N6 have been reported (Yu et al., 2015). Recently, we have isolated two H5N6 AIVs from stray cats in Guangdong, China (Data not shown).

Type I interferons (IFNs) are cytokines involved in the activation of host antiviral mechanisms (Su et al., 2016a; Wu et al., 2015b).
IAVs have evolved multiple strategies to overcome the type I IFN signaling pathway by encoding antagonists (Haller et al., 2006). Rei-Lin Kuo et al., reported that infections of seasonal H3N2 and H2N2 viruses with H1N1, H5N1 viruses (Kuo et al., 2010) and H7N9 IAVs (Arlahti et al., 2014) blocked IFN activation in different cell types such as MDCK, A549 and monocyte-derived dendritic cells. However, the innate immunity in cats is less known, which limits the understanding of the effect of AIV infection on cat innate immune response. The construction of feline IFN-β promoter report system (Tian et al., 2015) would provide a good method to investigate it.

The non-structural protein 1 (NS1) of IAV is an IFN antagonist. It interacts with a diverse range of cellular factors to antagonize host antiviral defenses and promote viral replication (Garcia-Sastre, 2001). Activation of IRF-3 is blocked through NS1 specifically binding to RIG-I, which inhibits the expression of IFN-β (Mibayashi et al., 2007). It also suppresses RIG-I CARD ubiquitination mediated by TRIM25, which blocks the RIG-I signal transduction (Gack et al., 2009; Rajsbaum et al., 2012). Another mechanism by which the NS1 protein counteracts the host antiviral response is through the disruption of IKK (Gao et al., 2012). With regard to H5N1 viruses, some (but not all) NS1 proteins can effectively block IFN induction. For example, Peirong Jiao et al. reported that H5N1 avian influenza viruses isolated from ducks, A/Duck/Guangxi/12/2003 (DK/12) and A/Duck/Guangxi/27/2003 (DK/27), differed in their ability to induce/block IFN (Jiao et al., 2008) (i.e. DK12 inhibited IFN while DK27 did not), and this was due to a polymorphism at amino acid position 42 of NS1 (Jiao et al., 2008).

Here, we first characterized the infection phenotype of a feline H5N6 influenza A virus in feline cells. We studied the ability of the virus to block the IFN response and identified the mechanism by which such suppression is achieved.

2. Results

2.1. H5N6 AIV can replicate efficiently and causes CPE in CRFK cells

No study was performed to assess the infection of H5N6 AIV in cat-origin cells. To better understand whether H5N6 AIV can replicate efficiently and lead to the CPE in CRFK cells, CRFK monolayers were inoculated with A/feline/Guangdong/1/2015 (MOI = 1 TCID50/cell). Cell supernatants were harvested at different timepoints and titrated on MDCK cells. CPE was observed at 48 h post-infection (hpi) (Fig. 1A), which coincided with the peak of virus growth (Fig. 1B). These results show that CRFK cells are susceptible and permissive to infection by A/feline/Guangdong/1/2015.

2.2. H5N6 AIV blocks activation of the feline IFN-β promoter

IFN synthesis is an early, host-mediated antiviral response. Previous studies showed that an H1N1 IAV can suppress the IFN-β response, and this suppression must play a key role in IAV pathogenesis (Talon et al., 2000; Wang et al., 2000). We speculated that suppression of the IFN-β response could be a key feature of H5N6 AIV in CRFK cells. To investigated whether H5N6 AIV could affect the feline IFN response, the feline IFN-β promoter luciferase reporter vectors were used.

CRFK cells were cotransfected with reporter plasmid IFN-33-Luc and control plasmid pRL-TK. Then, cells were challenged with an MOI of 0.1, 1 or 10 TCID50/cell of A/feline/Guangdong/1/2015 at 24 h post-transfection (hpt). The relative luciferase activity (RLA) was examined at 6, 12, and 24 hpi. As shown in Fig. 2A, infection with A/feline/Guangdong/1/2015 did not result in the activation of the feline IFN-β promoter at any of the MOIs used. We next investigated whether H5N6 AIV could inhibit activation of the...
Fig. 2. Evaluation of the IFN-β response during H5N6 virus infection. (A): CRFK cells were transfected with 0.5 μg/well of the reporter plasmid IFN+33-Luc and 0.02 μg/well of the pRL-TK plasmid. H5N6 virus with a MOI of 10, 1 or 0.1 TCID₅₀/cell were inoculated into the cells at 24 hpt. SeV infection was used as a positive control. Luciferase assays were performed at 6, 12 and 24 hpi, respectively. (B): CRFK cells were transfected with 0.5 μg/well of the reporter plasmid IFN+33-Luc and 0.02 μg/well of pRL-TK plasmid. H5N6 virus with a MOI of 10, 1 or 0.1 TCID₅₀/cell or DMEM (mock) were inoculated into the cells at 24 hpt. SeV was inoculated into each group at 6, 12 and 24 hpi. Luciferase assays were performed at 12 h after SeV inoculation. The expression of SeV N protein, H5N6 NS1 and GAPDH was evaluated by Western blotting. (C): CRFK cells were inoculated with H5N6 virus at a MOI of 1 TCID₅₀/cell. IFN-β expression was examined by Western blot at 3, 6, 9 and 12 hpi. (D, E, F): CRFK cells were transfected with 0.5 μg/well of reporter plasmids (3 × PRDII (D), 3 × PRDIII (E) or 6 × PRDIV (F)) and 0.02 μg/well of pRL-TK plasmid. H5N6 virus with a MOI of 1 TCID₅₀/cell or DMEM (mock) was inoculated into cells at 24 hpt. SeV was inoculated into each group at 12 hpi. Luciferase assays were performed at 12 h after SeV inoculation. The data shown represent the mean ± SD for three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared with mock infection.
feline IFN-β promoter. Sendai virus (SeV) was inoculated at 6, 12 and 24 hpt and the RLA was determined at 12 hpi. Infection of CRFK cells with A/feline/Guangdong/1/2015 (MOI of 1 or 10 TCID₅₀/cell) did inhibit SeV-mediated activation of IFN-β promoter at all time-points tested (Fig. 2B). In contrast, H5N6 infection at a lower MOI (0.1 TCID₅₀/cell) inhibited the induction of the IFN-β promoter by SeV stimulation only at 24 hpi (Fig. 2B). Examination of IFN-β expression induced by SeV by Western blot also showed a reduction in IFN-β in cells infected with A/feline/Guangdong/1/2015 (1 TCID₅₀/cell) at 6, 9, and 12 hpi (Fig. 2C).

Transcriptional activation of the IFN-β gene upon viral infection requires the activation of several transcription factors such as IRF3, NF-κB or transcription factor activator protein-1 (AP-1). To analyze the activation of some of these transcription factors we transfected CRFK cells with reporter plasmids carrying multiple copies of various motifs (see methods). Cells were infected with a MOI of 1 TCID₅₀/cell of H5N6 virus at 24 hpt and further infected with SeV 12 h later. RLA was determined at 12 hpi with SeV. Fig. 2D, E and 2F show that H5N6 AIV infection blocked the activation of NF-κB (Fig. 2D) and IRF3 (Fig. 2E), but not of AP-1 (Fig. 2F). These results indicate that H5N6 AIV inhibited the feline IFN-β promoter activation via blocking activation of NF-κB and IRF3.

2.3. Activation of NF-κB and IRF-3 is prevented in CRFK cells by the NS1 protein of A/feline/Guangdong/1/2015

The expression of NS1 prevents the activation of the NF-κB and IRF3 pathways upon virus- and/or double stranded RNA (Talon et al., 2000; Wang et al., 2000). Phylogenetic analysis of NS1 sequences derived from H5N1 and H5N6 IAVs showed that the NS1 of A/feline/Guangdong/1/2015 (H5N6) clustered together with previously published H5N6 viruses but not with H5N1 viruses (Fig. S1). As the impact of H5N6 NS1 on the IFN response has not been previously determined, we wondered whether the NS1 protein of A/feline/Guangdong/1/2015 could block activation of IFN-β response. To this end, CRFK cells were cotransfected with IFN-λ3-Luc and pRL-TK, together with plasmid encoding the NS1 and further inoculated with SeV at 24 hpt. RLA was determined at 12 hpi. We observed that expression of H5N6 NS1 protein inhibited SeV-mediated activation of IFN-β promoter, which depended on a concentration of plasmid (Fig. 3A). We next examined whether NS1 could prevent or delay the activation of NF-κB and/or IRF3 by SeV. To this end, CRFK cells were cotransfected with plasmids 3 × PRDII, and 3 × PRDIII/I, together with pRL-TK and the NS1 expression plasmid. SeV was inoculated at 24 hpt and the RLA was determined at 12 hpi. Compared with the transfection with empty vector, the expression of the NS1 protein resulted in a 69.1% and 82.1% reduction in NF-κB and IRF3 activation induced by SeV, respectively (Fig. 3B). To further investigate if NS1 inhibited induction of the NF-κB and IRF3 pathways, CRFK cells pre-transfected with the NS1 expression plasmid or vector were inoculated with SeV at 24 hpt. At 0, 6, 9, and 12 hpi, nuclear proteins were prepared and subject to EMSA with a probe for NF-κB. The levels of activated NF-κB in the NS1 expression group were lower than in the control groups at 9 and 12 hpi (Fig. 3C). NF-κB contains p50 and p65 subunits. We also determined the levels of p65 by immunostaining. The levels of nuclear p65 from the cells transfected with the NS1 expression plasmid were lower than in the control groups at 6, 9 and 12 hpi (Fig. 3D). In the same experiment, cytoplasmic extracts were also obtained and subjected to immunostaining with specific antibodies for phosphorylated IRF3 and inactive IRF3. The result showed that NS1 reduced the levels of phosphorylated IRF3 at 6, 9 and 12 hpi with SeV (Fig. 3E). These results indicate that NS1 inhibits activation of the IFN-β response by blocking activation of the NF-κB and IRF3 pathways.

2.4. The NS1 domain responsible for binding to dsRNA is required for the suppression of IFN-β response

The NS1 protein consist of a dsRNA binding domain and an effector domain (Wang et al., 2000). Inhibition of host mRNA splicing, polyadenylation, and transport by NS1 is determined by the effector domain (Lu et al., 1994). The dsRNA binding domain plays an important role in impairing activation of IFN-β response. To test the minimum requirements of H5N6 NS1 for antagonizing the IFN-β response, plasmid expressing the minimal dsRNA binding domain of NS1 (1–73) and its mutant (R38A/K41A) were constructed. The double mutation would attenuate NS1 dsRNA binding ability. The minimal dsRNA binding domain of NS1 (1–73) led to a significant inhibition of IFN-β response upon SeV infection (Fig. 4A). In turn, such suppression was significantly reduced by double NS1 mutant (Fig. 4A). This result shows that the inhibition of the IFN-β response by NS1 is determined by the dsRNA binding domain.

2.5. Over-expression of feline IFN-β prior to infection inhibits H5N6 AIV replication

As infection with A/feline/Guangdong/1/2015 inhibited IFN-β activation, we wondered whether the virus was able to replicate in the presence of IFN-β. We therefore analyzed the effect of the feline IFN-β on virus production. At 12 hpt, CRFK cells pre-transfected with a plasmid encoding the IFN-β for 12 h were infected with a MOI of 1 TCID₅₀/cell A/feline/Guangdong/1/2015 and virus titers in the supernatant were determined 24 hpi. As shown in Fig. 5A, over-expression of feline IFN-β resulted in a reduction in virus yield by 1.2 ± 0.20 log₁₀TCID₅₀. However, the over-expression of IFN-β at 12 hpi could not significantly inhibit virus replication (Fig. 5B). These results indicated that the over-expression of IFN-β before challenge could inhibit H5N6 virus replication, but its over-expression after challenge could not.

3. Discussion

Influenza viruses that are similar with Coronaviruses, are RNA zoonotic viruses which have highly susceptible to recombination and cross species transmission (Su et al., 2016b). It has been previously established that cats are susceptible to AIVs as natural infections in cats with H3N2 (Jeoung et al., 2013), H5N1 (Songserm et al., 2006; Yingst et al., 2006) and H5N6 (Yu et al., 2015) AIVs have been reported. Innate immunity is the first line of defense against viral infection, which critically depends on a successful type I interferon response (Rodriguez-Calvo et al., 2014). While many viruses have evolved mechanisms to subvert the IFN-β pathway (Didcock et al., 1999; Fensterl et al., 2005; Wang et al., 2011, 2010, 2000), it was less known how avian influenza virus circumvent this innate protective response in cats. In this study, we found that an H5N6 avian influenza virus isolated from cats could replicate well and led to the CPE in CRFK cells. Using dual luciferase report gene systems driven by the feline IFN-β-responsive promoter, we found that virus infection inhibited the induction of IFN-β response by blocking the NF-κB and IRF3 pathways. Further, we found that NS1 protein, a general inhibitor of the interferon signaling pathway, could inhibit feline IFN response by reducing the entry of NF-κB into nuclei and the phosphorylation of IRF3, which depended on the dsRNA binding domain. Additionally, over-expression of IFN-β before infection could inhibit H5N6 virus replication. This study helps us to understand how an H5N6 avian influenza virus can disrupt the cat innate immune response and then successfully infect cats.

During influenza virus infection, interactions between the viral haemagglutinin (HA) and cell oligosaccharides containing sialic
acid (SA) residues contributes to the virus entry to cells. Avian-origin and human-origin viruses chiefly recognize alpha-2, 3 and alpha-2, 6 sialic acid-linked receptors, respectively. A report had indicated that both receptors were distributed broadly in the spleen, pancreas, lung, bronchus, kidney, trachea and gastrointestinal tract in the cat (Wang et al., 2013), which increased the risk of influenza virus infection.

IAVs are classified in multiple subtypes and can evolve quickly by mutation and gene segment reassortment. Not all the isolates like the well-characterized laboratory strain A/PR/8/34 can block the production of IFN-β, and infection with different viruses results in moderate IFN-beta stimulation (Hayman et al., 2006). Based on the conclusion that the amino acid S42 of NS1 is responsible for the H5N1 influenza virus to inhibit the IFN response (Jiao et al., 2008), the amino acid S42 of NS1 in H5N6 used in this study may contribute to the blockage of IFN response. Thus, we thought it was necessary to investigate whether the avian influenza virus isolated from cats could affect the IFN-β response. While several studies reported that influenza virus isolated from birds or humans can block the IFN-β response (Gack et al., 2009; Gao et al., 2012; Hale et al., 2008; Jiao et al., 2008; Kuo et al., 2010; Mibayashi et al., 2007), no study has investigated whether cat isolates can also inhibit this pathway.

In this study, we found that an H5N6 avian-like virus isolated...
from a cat had acquired the ability to antagonize the IFN-β activation, suggesting a role in virus pathogenesis in this host. Critical transcription factors for regulating IFN-β expression include IRF-3/7, NF-κB and AP1. Some of the proteins involved in NF-κB and IRF3 pathways are responsible for the amplification of intracellular signals from upstream stimuli. MAVS is one of such proteins and its inhibition contributes to the blockade of the IFN signal pathway of NF-κB and IRF3. A previous study showed that the influenza virus polymerase regulated the host anti-viral response through binding to MAVS and thus inhibiting IFN production (Iwai et al., 2010). AP-1 is a downstream effector of the MAPK signaling pathway that regulates the production of pro-inflammatory cytokines upon TLR4 activation (Adler et al., 2010). The MAPK signaling pathway differs from the NF-κB and IRF3 pathways. We have shown that infection with A/feline/Guangdong/1/2015 inhibited the activation of IRF3 and NF-κB and did not affect the AP1 activation, which was consistent with other reports.

NS1 protein is a product of the smallest RNA segment and acts as a virulence factor by inhibiting host immune responses. Mibayashi et al. first reported that the NS1 of IAV prevents the transcriptional induction of IFN-β by binding to RIG-I and inhibiting downstream activation of IRF3 (Mibayashi et al., 2007). It has also been shown that NS1 specifically inhibits TRIM25-mediated RIG-I CARD ubiquitination, which blocks RIG-I signal transduction (Gack et al., 2009; Rajsbaum et al., 2012). A new mechanism by which IAV NS1 counteracts the host NF-κB-mediated antiviral response is through the disruption of IKK pathway (Gao et al., 2012). Besides suppressing the IFN-β response, NS1 can also shut off host gene expression (Khaperskyy and McCormick, 2015). In this study, we showed that the NS1 of A/feline/Guangdong/1/2015 suppressed SeV-mediated induction of IFN-β promoter via reducing the levels of nuclear p65 and phosphorylated IRF3 in feline cells. Both NF-κB and IRF3 pathways were inhibited by NS1 expression, which was determined by the dsRNA-binding domain of NS1. NS1 protein can bind to influenza virus RNA and also nonspecifically to dsRNA in vitro (Talon et al., 2000). It was speculated that NS1 may block the formation of dsRNA, which is necessary for the activation of IRF3 and NF-κB. However, the exact molecular mechanism(s) of such inhibition should be investigated further. Our findings also provide an example of a H5N6 virus from cats suppressing IFN production.

4. Materials and methods

4.1. Viruses and cells

Crandell-Reese feline kidney (CRFK) cells (ATCC) were grown in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin and 100 μg/ml of penicillin.
of streptomycin. A/feline/Guangdong/1/2015 (H5N6) was isolated in MDCK cells from a swab obtained from a stray cat. Virus isolation and subtyping were performed as previously described (Li et al., 2010) and stored at −80 °C. All experiments with H5N6 virus were performed under biosafety level 3 (ABSL-3) conditions.

Sequences of the novel feline strain are available in GenBank (accession numbers: KT762444-KT762451 and KT762452-KT762459). Phylogenetic analyses of the novel strain show that it is closely related to avian H5N6 viruses that have been circulating in southern China (Data not shown).

4.2. Virus infections

CRFK cells were infected at various doses at 37 °C. One hour after incubation, virus was removed, and cells were incubated in DMEM with 1% FBS, 100 μl of penicillin and 100 μg/ml of streptomycin. Cytopathic effect (CPE) at 48 h post-infection was assessed. To assess virus growth kinetics, supernatant of infected CRFK cells were harvested at 12, 24, 36, 48 and 72 hpi and titrated in MDCK cells.

4.3. Virus titration

Briefly, 10-fold dilutions of viruses were prepared, and 0.1 ml of diluted virus was added to the 96-wells. After 1 h of viral adsorption in MDCK cells, the fresh DMEM containing 1% FBS and 1% antibiotics was added. The viral replication were determined via the Enzyme-linked-immunosorbent assay (ELISA) at 72 hpi (Zhang et al., 2015) and were expressed as the median log_{10} (TCID_{50}/ml) according to the method of Reed and Muench (Reed and Muench, 1938).

4.4. Plasmids

To clone the NS1 of A/feline/Guangdong/1/2015, eggs were inoculated with the aforementioned virus and total RNA from the allantoic fluid was prepared through the Virus DNA/RNA Mini Kit (Axygen) according to the instruction. A reverse-transcription kit (Takara) for producing cDNA was used. Inoculation motif (from plasmid and 0.02 μg plasmid pRL-TK (Promega). Where indicated, the cells were also mock infected or infected with H5N6 virus with a MOI of 0.1–10 TCID_{50} at 24 hpi. SeV (100 hemagglutinating activity units/well) was inoculated into the cells at H5N6 infection 6, 12, or 24 hpi to stimulate an IFN-β response. Cells were lysed at 12 hpi, and firefly and Renilla luciferase activities were determined with the Dual-Luciferase reporter assay system (Promega), according to the manufacturer’s instructions. RLA in each sample was determined using the ratio between the activities of firefly and Renilla luciferases (Tian et al., 2015). The data are shown as the mean RLA with standard deviation from three independent experiments.

4.6. Western blot analysis

Cell monolayers were washed with PBS and lysed with Radio Immunoprecipitation Assay Lysis (RIAL) Buffer (Beyotime, P0013C). Lysates were harvested and further cleared by centrifugation at 12,000 g for 10 min at 4 °C. The concentration for the total protein content was determined with a BCA protein assay kit (Beyotime). Total of 30 μg protein lysate was resolved by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with 5% skim milk for 2 h at room temperature, then incubated overnight at room temperature for 2 h with specific rabbit anti-IFN-β antibody (ab140211, Abcam), rabbit anti-flag antibody (ab1162, Abcam), rabbit anti-GADPH antibody (ab22555, Abcam), rabbit anti-IRF3 antibody (ab68481, Abcam), rabbit anti-PRD (phosphor S386) antibody (ab76493, Abcam), rabbit anti-NF-κB p65 antibody (ab32536, Abcam) and mouse anti-NP antibody (MAB3326, Abnova). After three rinses, membranes were incubated at room temperature for 1 h with IRDye 680DX conjugated anti-rabbit/mouse IgG (1:8000; Rockland Immunochemicals). The membranes were analyzed with an Odyssey infrared imaging system (LI-COR Biosciences).

4.7. EMSA

Electrophoretic mobility shift assay (EMSA) was performed to determine the activation of NF-κB. Briefly, nuclear extracts were made with Nuclear and Cytoplasmic Extraction Reagents (78833, Thermo Fisher), according to the manufacturer’s protocol. Nuclear extracts with 2 μg were analyzed in EMSA. A DNA probe labeled with IRDye 700 infrared dye containing a NF-κB binding site (5′-AGTGAGGAGCTTCCAGG-3′) (8297–07924, LI-COR) was used in these assays. A binding reaction was performed at room temperature for 30′ and 1 μl (25 fmol) probe was used for each assay. 1 μg Poly (dl:dc) (20148E, Thermo Fisher) was added to prevent non-specific binding of proteins to the NF-κB oligonucleotide probe. After pre-electrophoreses the gel (4% polyacrylamide gel in 0.5 × TBE) for 30 min, total 25 μl sample with 5 × Loading Buffer was loaded and the running condition was 100 V for an 8 × 8 × 0.1 cm gel for 40 min. After the running, the gel was analyzed with an Odyssey infrared imaging system (LI-COR Biosciences).

4.8. Analysis of antiviral effect of IFN-β

The feline IFN-β gene was cloned into p3 × Flag-CMV-10, the expression of which was determined by Western blot. CRFK cells (2 × 10^4/well) were transfected with 1 μg of plasmid expressing the IFN-β or empty plasmid (mock) for 24 h. Then the medium was removed, and a MOI of 1 TCID_{50}/cell of virus was inoculated into the cells. At 24 hpi, the cell supernatant from each infection group was collected for analysis of viral titers. In another experiment, 12 h after infection with a MOI of 1 TCID_{50}/cell of virus, cells were transfected with expressing plasmid or empty plasmid. The virus yields of cell supernatants were determined at 24 hpi. Total protein from each group cells was extracted and the expression of IFN-β and GAPDH was determined by Western blotting.
4.9. Phylogenetic analysis

The phylogenetic tree was constructed with MEGA software (version 4.0) by using the neighbor-joining algorithm. The bootstrap replications were set as 1000.

4.10. Statistic

The significance was determined with an unpaired t-test and a one-way ANOVA using the Prism 5.0 software (GraphPad Software). The value of $P < 0.05$ was considered as a significance.

Author contributions

Lifang Wang and Jin Tian conceived the study and wrote the paper. Xiniang Fu, Yun Zheng, Pei Zhou, Bo Fang, San Huang, Xin Zhang, Jidang Chen and Zongxi Cao performed and analyzed all the experiments. Guihong Zhang revised the manuscript. Shoujun Li designed the study and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jdci.2017.04.003.

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