



## RESEARCH ARTICLE

# Cloning, expression, and antiviral activity of interferon $\beta$ from the Chinese microbat, *Myotis davidii*

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**Bats are natural reservoir hosts for many viruses that produce no clinical symptoms in bats. Therefore, bats may have evolved effective mechanisms to control viral replication. However, little information is available on bat immune responses to viral infection. Type I interferon (IFN) plays a key role in controlling viral infections. In this study, we report the cloning, expression, and biological activity of interferon  $\beta$  (IFN $\beta$ ) from the Chinese microbat species, *Myotis davidii*. We demonstrated the upregulation of *IFNB* and IFN-stimulated genes in a kidney cell line derived from *M. davidii* after treatment with polyI:C or infection with Sendai virus. Furthermore, the recombinant IFN $\beta$  inhibited vesicular stomatitis virus and bat adenovirus replication in cell lines from two bat species, *M. davidii* and *Rhinolophus sinicus*. We provide the first *in vitro* evidence of IFN $\beta$  antiviral activity in microbats, which has important implications for virus interactions with these hosts.**

**KEYWORDS** bat; interferon; IFN-stimulated genes; antiviral activity

## INTRODUCTION

Novel human pathogens continue to emerge, the majority of which originate in wildlife (Jones et al., 2008). Bats are the only flying mammals. They make up approximately 20% of extant mammalian diversity, representing the second largest order of mammals after rodents. Bats are found on all continents, except in polar regions and a few oceanic islands. The diversity of bat species and some of their unique biological features have allowed them to serve as natural reservoir hosts for many viruses (Shi, 2013). Some bat species carry emerging or reemerging infectious disease agents, including severe

acute respiratory syndrome coronaviruses (Lau et al., 2005; Li et al., 2005; Ge et al., 2013), henipaviruses (Halpin et al., 2000; Chua et al., 2002), and Ebola viruses (Pourrut et al., 2009). These viruses are pathogenic to humans and other mammals, but appear to cause no clinical disease in bats during natural or experimental infection (Williamson et al., 1998; Williamson et al., 2000; Middleton et al., 2007). The long co-evolutionary history of bats and viruses may have resulted in the adaptation of bats' immune systems to cope with viral infection. It is hypothesized that bat innate immunity allows bats to limit viral replication to very low levels, allowing persistent infections of viruses in bat populations (Papenfuss et al., 2012). However, because little information is currently available on any aspect of bat immunology and few bat-specific reagents exist, this hypothesis remains untested.

One of the earliest immune responses initiated after viral infection is the production of interferons (IFNs).

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IFNs provide the first line of defense against viral infection and play a pivotal role in complex cytokine networks (Randall and Goodbourn, 2008). Type I IFNs, some of the most important antiviral immune factors, are associated with the host's ability to reduce viral replication by inducing the expression of IFN-stimulated genes (ISGs).

To understand bat-virus interactions, the Australian black flying fox, *Pteropus alecto*, has been used as a model bat species (Cramer et al., 2009). This species is the reservoir of a variety of closely related viruses, including *rabies virus*, *Hendra virus*, and *Nipah virus* (Marsh and Wang, 2012). Some genes involved in the immune responses of *P. alecto* have been identified, and functional studies of these genes have been performed (Baker et al., 2010; Cowled et al., 2011; Virtue et al., 2011; Zhou et al., 2011a; Zhou et al., 2011b; Cowled et al., 2012). Type I IFNs were recently described in two other species of fruit bats, the Egyptian rousette, *Rousettus aegyptiacus*; the Malaysian flying fox, *Pteropus vampyrus* (Omatsu et al., 2008; Kepler et al., 2010); and two species of microbats, *Rhinolophus affinis* and *Rhinolophus sinicus* (Li et al., 2015). In this study, we analyzed the sequence and antiviral activity of IFN $\beta$  cloned from a Chinese microbat species, *Myotis davidii*. Our results demonstrate that IFN receptor (IFNR) binding site sequences of IFN $\beta$  are conserved and that *in vitro* antiviral activity is similar across many mammalian genera, including *Myotis*.

## MATERIALS AND METHODS

### Cell lines

Culture conditions for bat cell lines have been described previously (Li et al., 2010). The cell lines used in this study included an *M. davidii* primary kidney cell line (MdKi, previously called BK) and an immortalized *Rhinolophus sinicus* kidney cell line (RsKT). The MdKi cells were cultured with RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The RsKT cells were cultured in DMEM/F12-Hams medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. All cells were maintained at 37 °C in an incubator supplemented with 5% CO<sub>2</sub>.

### Viral culture

Bat adenovirus TJM (BtAdV-TJM) was grown in MdKi cells for 48 h in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C (Li et al., 2010). The virus-containing supernatant was collected and the 50% tissue culture infective dose (TCID<sub>50</sub>) was determined. *Vesicular stomatitis virus*-green fluorescent protein (VSV-GFP) (Fernandez, 2002) was grown in 293T cells for 48 h, the

virus-containing supernatant was collected, and the TCID<sub>50</sub> was determined. *Sendai virus* (SeV) was propagated in 10-day-old embryonated eggs, and the viral titer was determined by hemagglutination assays using chicken red blood cells.

### Stimulation of bat cells with poly I:C treatment and SeV infection

MdKi cells were seeded at  $1 \times 10^6$  cells/well in 6-well tissue culture plates. The cells were stimulated by transfection with 2  $\mu$ g/mL polyI:C (InvivoGen) using 5  $\mu$ L of Lipofectamine 2000 (InvivoGen) or by infection with SeV at 100 hemagglutinating units (HAU)/well. After stimulation, the cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The cells were harvested in RLT buffer (Qiagen) at 0, 6, 12, and 18 h post-infection (hpi) and stored at -80 °C until the RNA was extracted.

### PCR amplification of the *M. davidii* IFN $\beta$ gene (IFNB)

The complete *IFNB* gene was identified in the *M. davidii* genome (GenBank accession no: XM\_006764946). PCR and random amplification of cDNA ends (RACE) primers (Table 1) were designed based on the *M. davidii* genomic sequence. MdKi cells were infected with SeV and total RNA was extracted 6 hpi for use as a template for *IFNB* gene amplification. The samples were homogenized using QIAshredder (Qiagen). Total RNA was extracted using the RNeasy Mini Kit (Qiagen) with on-column DNase I treatment (Qiagen) to remove all traces of genomic DNA. The full-length sequence of *IFNB* was obtained using 5' and 3' RACE with the Full RACE Kit (Takara). The DNA amplification conditions included an initial denaturation step at 95 °C for 5 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 50 s; and a final extension at 72 °C for 10 min.

### Sequencing and phylogenetic analysis

The PCR and RACE products were cloned into the pGEM-T Easy Vector (Promega) for sequencing. The GenBank accession numbers of the sequences used in the phylogenetic analysis are as follows: NP\_001092910, *Equus caballus* (horse); NP\_002167, *Homo sapiens* (human); XP\_528553, *Pan troglodytes* (chimpanzee); NP\_034640, *Mus musculus* (mouse); NP\_062000, *Rattus norvegicus* (rat); NP\_001129259, *Canis lupus familiaris* (dog); AAA31056, *Sus scrofa* (pig); NP\_776775, *Bos taurus* (cattle); XP\_006765009, *Myotis davidii*; XP\_006098187, *Myotis lucifugus*; ELK06976, *P. alecto*; BAF37103, *Rousettus aegyptiacus*; NP\_001020007, *Gallus gallus* (chicken); XP\_002708014, *Oryctolagus cuniculus* (rabbit); KM056305, *Rhinolophus sinicus* (Chinese rufous horseshoe bat); and KM056306, *Rhino-*

*lophus affinis* (intermediate horseshoe bat). All sequence alignments were performed with the ClustalW program and visualized with GeneDoc software (Thompson et al., 1994). Signal peptides were identified with SignalP version 3.0. Protein domains were identified using the NCBI Conserved Domains search tool (Marchler-Bauer et al., 2011). The phylogenetic tree was generated in MEGA 6 using the neighbor-joining method with 1000 bootstrap replicates.

### Expression of bat IFN $\beta$ in *Escherichia coli* and purification of the protein

The bat *IFNB* was cloned into the pET32a expression vector and was used to transform *Escherichia coli* strain DH10B. The clones were analyzed with restriction digestion to confirm the correct orientation of *IFNB*. Briefly, *Escherichia coli* strain BL21 (pET32-IFN $\beta$ ) was cultured, induced with isopropyl- $\beta$ -D-thiogalactoside, and then harvested by centrifugation. The sonicated cell lysates were treated with Triton X-100. The proteins were then immobilized on a metal affinity chromatography column and eluted with imidazole. Samples for analysis were collected at each purification step and separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Western blotting was performed with a His-tag-directed antibody as the primary antibody (1:1000) and an alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody (1:2000, Sino-American) as the secondary antibody.

### Antiviral activity assay of expressed recombinant bat IFN $\beta$ against VSV-GFP infection in bat cells

A VSV-GFP bioassay was performed to determine the antiviral activity of the expressed recombinant bat IFN $\beta$ . MdKi cells and RsKT cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well. After 24 h, the bat cells were left untreated or were treated for 24 h with five concentrations of bat IFN $\beta$  diluted in medium and then incubated with VSV-GFP at a multiplicity of infection of 1. After adsorption for 1.5 h at 37 °C in a 5% CO<sub>2</sub> incubator, the inoculum was removed and 100  $\mu$ L of medium was added to each well. GFP expression in the cells was visualized by fluorescence microscopy at 18 hpi. The number of fluorescent points was counted to evaluate the inhibitory effect of bat IFN $\beta$ .

### Antiviral activity assay of bat IFN $\beta$ against bat adenovirus in bat cells

Similar assays were performed in MdKi and RsKT cells using the bat adenovirus, BtAdV. The two bat cell lines were inoculated with BtAdV at a multiplicity of infection of 1 and incubated for 1 h at 37 °C. The virus-containing medium was then replaced with culture medium, and the cultures were incubated for 24 h in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. The culture super-

natant was collected and the TCID<sub>50</sub> was determined by observation of the cytopathic effect.

### Quantitative Real-time PCR

Quantitative Real-time PCR (qRT-PCR) was performed on the total RNA extracted from the cells. In brief, total RNA was used for the synthesis of cDNA with the PrimeScript<sup>®</sup> RT Reagent Kit with gDNA Eraser and SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II for real-time PCR (Takara). The relative gene expression after treatment was quantified with the StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems), and the comparative threshold cycle (Ct) method was used to calculate the fold change in gene expression. Relative gene expression was calculated using the mean values obtained from the arithmetic formula  $2^{-\Delta\Delta Ct}$  (Applied Biosystems). The cycling profile consisted of an initial denaturation at 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by a melting curve analysis. All data were normalized to the housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

## RESULTS

### Characterization of *M. davidii* IFN $\beta$

The expected DNA fragment of 561 bp was amplified from bat cells and sequenced. The deduced protein encoded by *IFNB* contained many features that are conserved in the IFN $\beta$  proteins of other species, including a signal-peptide cleavage site and several residues that are responsible for the binding of IFN $\beta$  to the type I IFNR (IFNAR) (Figure 1A).

Three-dimensional modeling of the bat IFN $\beta$  structure revealed five putative  $\alpha$ -helices (A–E) (Arnold et al., 2006). The *M. davidii* IFN $\beta$  sequence shares 43%–85% amino acid identity with IFN $\beta$  proteins of other mammalian species, sharing the highest similarity with IFN $\beta$  of another *Myotis* species. A phylogenetic analysis of bat IFN $\beta$  and other IFN $\beta$  sequences from a variety of vertebrates was performed. As previously described (Zhang et al., 2013), *M. davidii* IFN $\beta$  clustered with *M. lucifugus* IFN $\beta$  within the laurasiatherian mammalian sequences (Figure 1B).

### Bat IFNs and ISGs were highly upregulated in bat cells after poly I:C treatment and SeV infection

To examine the expression patterns of bat IFNs in response to polyI:C treatment or viral challenge, we used polyI:C and SeV to stimulate MdKi cells. The total RNA in the cells was isolated at different time points after infection. Successful infection by SeV was confirmed by RT-PCR (data not shown). The expression kinetics of *IFNB* and ISGs were determined by qRT-PCR. Following SeV infection, expressions of *IFNB* and ISGs were

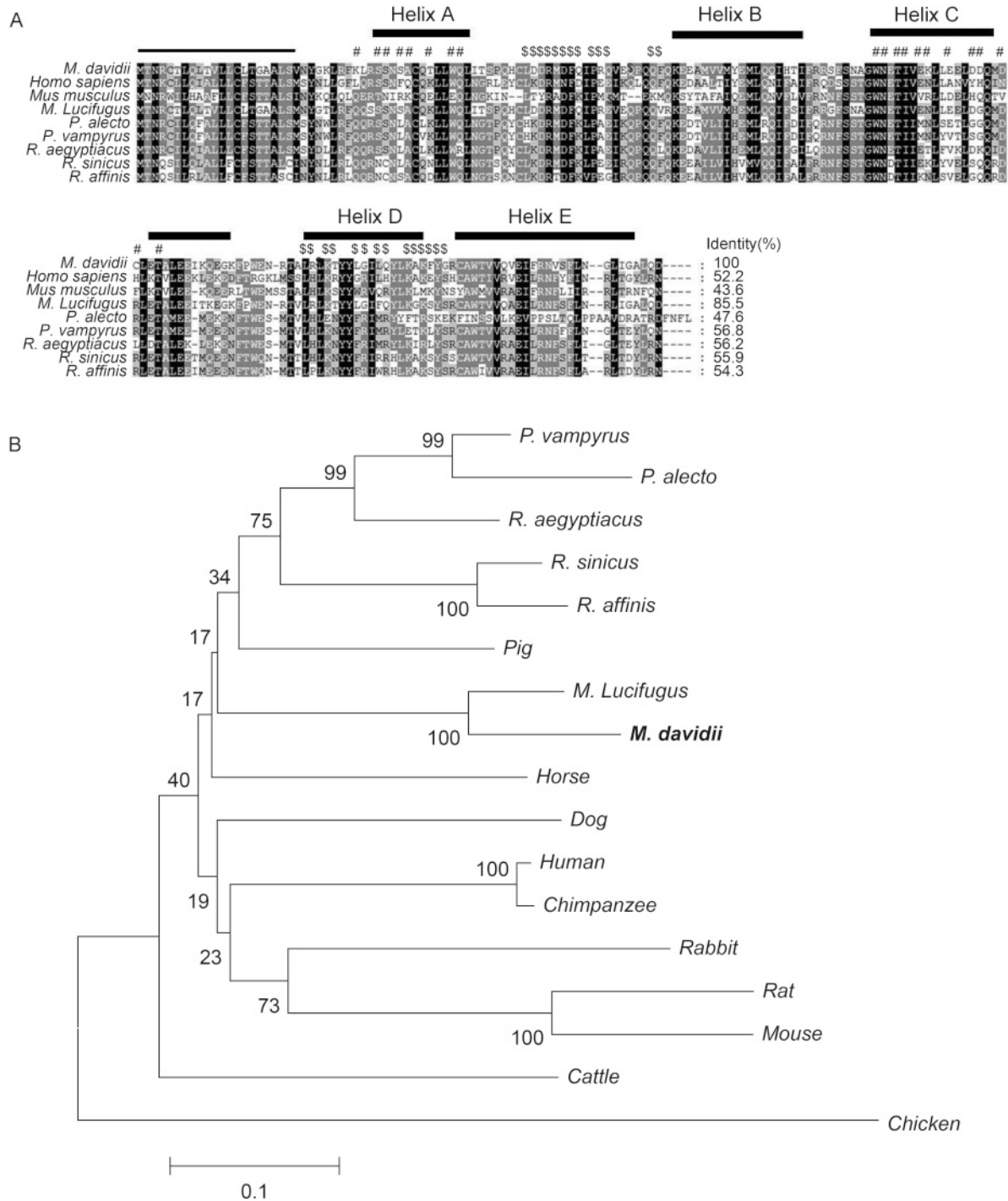


Figure 1. Sequence analysis of *Myotis davidii* IFN $\beta$ . (A) ClustalW-generated multiple amino acid sequence alignment of *M. davidii* and other bats, mice, and humans IFN $\beta$ . The predicted signal peptides are underlined. The putative IFNAR1-binding sites and IFNAR2-binding sites are indicated with “#” and “\$”, respectively. The secondary structure of IFN $\beta$  is shown above the alignment. Five helices, designated Helix A–E, are indicated with boxes. (B) Phylogenetic analysis based on an amino acid alignment of bat IFN $\beta$  with those of representative vertebrate species. Percentage branch support calculated with 1000 bootstrap replicates is indicated when it exceeds 60%. *M. davidii* IFN $\beta$  is highlighted in bold. The accession numbers of the IFN $\beta$  protein sequences used in this analysis are listed in the *Materials and Methods*.



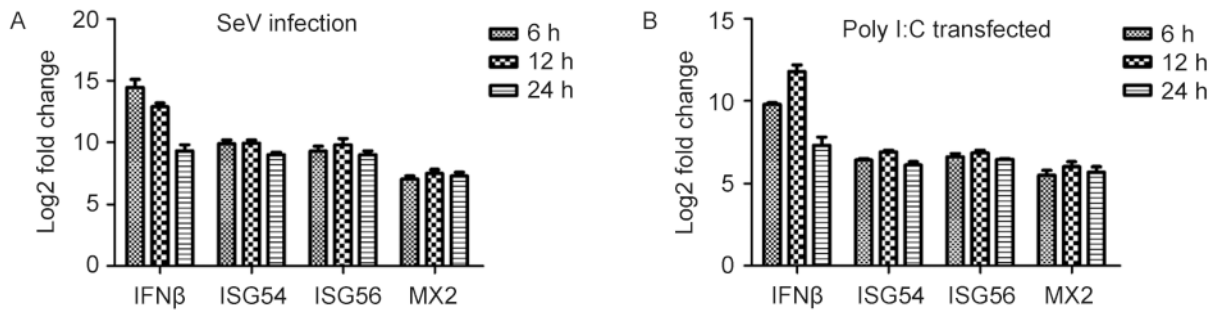


Figure 2. Analysis of bat *IFNB* and ISG expression in MdKi cells after treatment with polyI:C (A) or infection with SeV (B). Cells were transfected with polyI:C or infected with SeV and collected at the indicated time points. *IFNB* and ISG mRNAs were measured by qRT-PCR. The data were normalized to the housekeeping gene *GAPDH*. Data are the mean values of three separate experiments and the error bars represent standard errors.

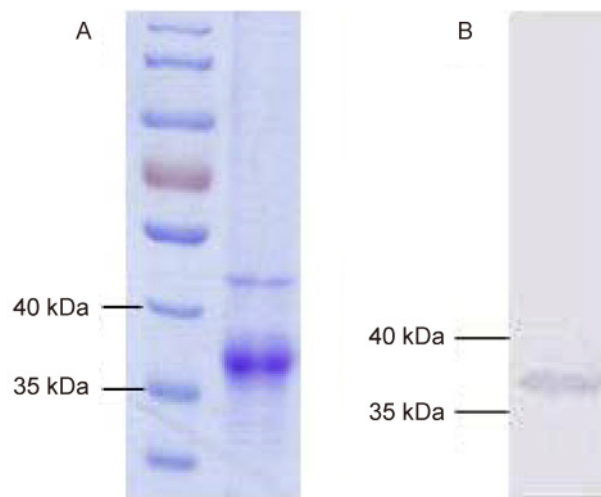


Figure 3. SDS-PAGE (A) and western blotting (B) analyses of recombinant *M. davidii* IFN $\beta$  expressed in *Escherichia coli*. (A) Purified recombinant *M. davidii* IFN $\beta$  protein was resolved with 12% SDS-PAGE and analyzed following Coomassie Brilliant Blue staining. (B) Purified recombinant *M. davidii* IFN $\beta$  was analyzed by western blotting, using an antibody directed against the His tag.

induced at 6 hpi and peaked at 12 hpi (Figure 2A). Similar to SeV infection, treatment with the double-stranded RNA analog polyI:C also induced *IFNB* and ISG expression (Figure 2B).

### Expressed recombinant bat IFN $\beta$ inhibited virus replication in bat cells

The expressed recombinant IFN $\beta$  of the *Myotis* bat was analyzed with SDS-PAGE and confirmed with western blotting using an antibody directed against the His tag (Figure 3). To assess the antiviral activity of the recombinant bat IFN $\beta$ , MdKi and RsKT cells were pretreated with bat IFN $\beta$ . As shown in Figure 4, the treatment of MdKi and RsKT cells with IFN $\beta$  before VSV-GFP infection caused a significant dose-dependent reduction in the number of VSV-GFP-positive cells compared to those in the mock-treated cells. A similar inhibitory effect was observed during the infection of

these two bat cell lines with BtAdV (Figure 5). These results demonstrate that recombinant bat IFN $\beta$  mitigated viral infection in bat cells *in vitro*.

## DISCUSSION

In this study, we cloned and characterized IFN $\beta$  from a Chinese microbat, *M. davidii*. Compared with IFN $\beta$  sequences from other mammals, the *M. davidii* IFN $\beta$  sequence shares highest amino acid identity with that of *M. lucifugus* (85%). A number of conserved features were identified in the bat IFN $\beta$  sequence, which is consistent with its functional equivalence to the IFN $\beta$  proteins of other species. To examine the expression pattern of bat IFN $\beta$  in response to polyI:C treatment or viral challenge, we used polyI:C and SeV to stimulate MdKi cells. The qRT-PCR results demonstrated that bat *IFNB* and ISGs

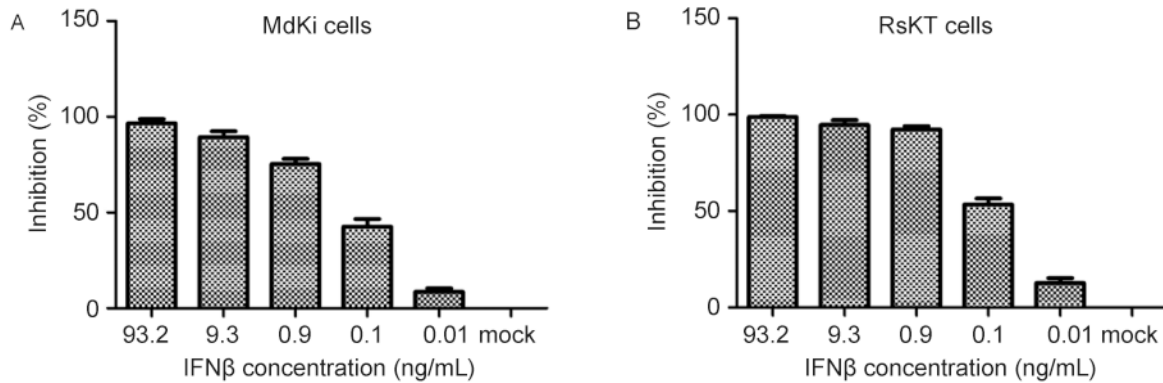


Figure 4. Antiviral activity assay of IFN $\beta$  against VSV-GFP in RsKT cells (A) and MdKi cells (B). Cells were treated for 24 h with five serial dilutions of IFN $\beta$  in medium and incubated with VSV-GFP. GFP expression in the cells was visualized and measured at 18 hpi.

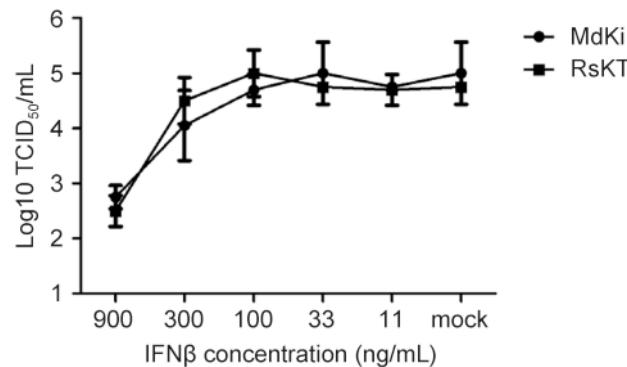


Figure 5. Antiviral activity assay of bat IFN $\beta$  against bat adenovirus (BtAdV) in MdKi and RsKT cells. Cells were treated with serial dilutions of IFN $\beta$  or mock medium for 24 h and infected with BtAdV for another 24 h. The culture supernatant was collected and the TCID<sub>50</sub> was determined by observation of the cytopathic effect.

were highly upregulated in the bat cells after polyI:C treatment or SeV infection. Furthermore, the expressed recombinant bat IFN $\beta$  protein exerted antiviral activity against human VSV and BtAdV. To our knowledge, this is the first study to report the antiviral activity of IFN $\beta$  in a microbat.

Previous studies have reported that recombinant type III IFN and type II IFN from the megabat *P. alecto* inhibited the replication of Pulau virus, Semliki Forest virus, and Hendra virus (Zhou et al., 2011a; Janardhana et al., 2012). Another research group characterized IFN $\kappa$  and IFN $\omega$  from the European serotine bat, *Eptesicus serotinus*. Both type I IFNs have been shown to inhibit the replication of different lyssaviruses, to different extents, in susceptible bat cells (He et al., 2014). However, the antiviral activity of microbat IFN $\beta$  is less studied. Here, our results indicate that the *M. davidii* IFN $\beta$  protein has antiviral activity, consistent with type I IFNs of other bat species. Our results also indicate that bat IFN has antiviral activity similar to that of other mammalian IFNs.

As in other mammals, the bat immune system comprises both innate and adaptive elements (Baker et al., 2013; Brook and Dobson, 2015). Recent studies have demonstrated that type I IFN $\alpha$  and IFN $\beta$  from *Rousettus aegyptiacus* and type II IFN $\gamma$  and type III IFN $\lambda$  from *P. alecto* play crucial roles in virus interactions with these hosts (Zhou et al., 2011b; Janardhana et al., 2012). In addition to IFN sequence characterization, the induction of IFNs, their signaling pathways, and their antiviral activities following viral infection have also been investigated in cell lines derived from *Eidolon helvum* and *P. alecto* (Biesold et al., 2011; Virtue et al., 2011). These results provide evidence that the signaling molecules downstream of the IFN response in bats are probably similar to those in other mammals. In addition to the innate immune response, studies have examined the adaptive immune response in bats, demonstrating that bats do mount antibody responses to various viral infections, including lyssaviruses, filoviruses, and henipaviruses (Hayman et al., 2013). However, it seems that their humoral adaptive immunity is less effective at controlling viral trans-

mission (Plowright et al., 2008; Turmelle et al., 2010; Brook, 2015). From the limited number of bat immune studies available, it seems that the bat has immunological features similar to those of other mammals.

Recently, the whole-genome sequences of two distantly related species, the fruit bat *P. alecto* and the insectivorous bat *M. davidii*, became available (Zhang et al., 2013). Comparative analyses indicated unexpected concentrations of positively selected genes in the DNA damage checkpoint and nuclear factor  $\kappa$ B pathways, which might play a role in bat immunity. Because the DNA damage response plays an important role in host defenses and is a known target of virus interaction, a link between the metabolism and immunity of bats may explain the tolerance to viruses observed in bats (Wang et al., 2011; O'Shea et al., 2014). These issues warrant further investigation to determine whether these unique characteristics are associated with the asymptomatic nature of viral infections in bats.

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## COMPLIANCE WITH ETHICS GUIDELINES

All experiments were conducted under the guidelines of the Animal Ethics Committee in Wuhan Institute of Virology, Chinese Academy of Sciences. All the authors declare that they have no competing interest.

## AUTHOR CONTRIBUTIONS

Y.L. and Z.S. conceived and designed the experiments; Y.L., L.W., and P.Z. performed the experiments; Y.L., L.W., Q.Z., and M.W. analyzed the data; X.Y. and X.G. provided the reagents and materials essential for the research; Y.L. wrote the paper.

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