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Molecular characterization of RIG-I, STAT-1 and IFN-beta in the horseshoe bat



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ABSTRACT

Wild Chinese horseshoe bats have been proven to be natural reservoirs of SARS-like coronaviruses. However, the molecular characterization of key proteins in bats still needs to be explored further. In this study, we used cloning and bioinformatics to analyze the sequence of RIG-I, STAT-1 and IFN- β in the immortalized cell lines from *Rhinolophus affinis* and *Rhinolophus sinicus*. Then, we treated different bat cells, mouse embryonic fibroblasts (MEF) and splenocytes with polyinosinic–polycytidylic acid (polyI:C) and vesicular stomatitis virus (VSV) to assess and compare antiviral immune responses between bats and mice. Our results demonstrated that bat RIG-I, STAT-1 and IFN- β showed close homology with human, mouse, pig and rhesus monkey. RIG-I and STAT-1 were both highly expressed in bat spleen. Furthermore, IFN- β was induced by polyI:C and VSV in both bat and mouse cells. These findings have provided new insight into the potential characteristics of the bat innate immune system against viral infection.

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1. Introduction

Wildlife is believed to be responsible for approximately 40% of the emerging infectious diseases in humans (Quan et al., 2013). Among them, bats have been found to be natural reservoirs for many important zoonotic viruses, such as the Marburg virus (Towner et al., 2009), Ebola virus (Leroy et al., 2005), Hendra virus (Chan and Chan, 2013) and severe acute respiratory syndrome (SARS) coronavirus (2003) (Lau et al., 2005; Yuan et al., 2010). A series of characteristics have supported bats as a suitable reservoir host for viruses, including their long lifespan, high species diversity, unique immune systems, gregarious roosting behaviors, and high spatial mobility and population densities (Calisher et al., 2006). Along with these characteristics, the immune system of bats is also believed to be one reason why bats can act as a reservoir host for viruses. However, as the first defense against viral infection, the innate immune system of bats is still poorly understood.

Similar to TLR in the antiviral innate immune system, RIG-I can detect viral RNA in the cytoplasm and trigger antiviral immune responses by driving type 1 interferon (IFN) production to control viral infection (Loo and Gale, 2011). Then, the released interferon can activate STAT-1 to induce the transcription of interferon-stimulated genes (ISGs) and protect the cell from virus infection (Chen et al., 1998; Liao et al.,

2000). Thus, whether or not the RIG-I–IFN–STAT-1 signal pathway is intact will affect the anti-virus effect of the cell.

Bats host more zoonotic viruses per species than rodents (Luis et al., 2013). Unlike the murine immune system, the immune system of bats has not been well demonstrated. Previous studies have shown the molecular characteristics of the toll-like receptors and RIG-I-like helicases of the fruit bat by sequencing and bioinformatic analysis (Cowled et al., 2011, 2012). However, detailed information about these genes in horseshoe bats, which are natural reservoirs of SARS-like coronaviruses, remains unavailable (Lau et al., 2005; Li et al., 2005; Lau et al., 2010). In order to understand RIG-I mediated antiviral immune response in horseshoe bats, we sequenced and analyzed the functional domains of RIG-I, STAT-1 and IFN- β in both *R. sinicus* and *R. affinis* which are regarded as natural reservoirs for SARS-like coronavirus. We also compared the transcriptional IFN- β expression between bat and murine cells under VSV and polyI:C stimulation.

2. Materials and methods

2.1. Cell culture

Bats were captured with mist netting or hoop nets in caves at multiple sites in Yunnan province. All bats captured were apparently healthy. The species of each bat was identified by field biologists and recorded. Bat cells used in this study, including *Rhinolophus sinicus* splenocytes (BS) and *Rhinolophus affinis* embryonic fibroblasts (BEF) cells, were provided by Professor Yaping Zhang (Kunming Institute of Zoology, Chinese Academy of Sciences, China). All cells were maintained in DMEM supplemented with 10% FBS. Primary mouse embryonic fibroblasts

Abbreviations: STAT-1, Signal Transducer and Activator of Transcription 1; MEF, mouse embryonic fibroblasts; polyI:C, polyinosinic–polycytidylic acid; VSV, vesicular stomatitis virus; ISGs, interferon-stimulated genes; IFN, interferon; RACE, rapid amplification of cDNA ends; BEF, *Rhinolophus affinis* embryonic fibroblasts; hIFN- α , human IFN- α .

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(MEF) and primary mouse splenocytes were generated from C57/BL6 mice. MEF were prepared from embryos dissected from the same pregnant female on day 13.5. Splenocytes were prepared with nylon mesh followed by the lysis of red blood cells. The Animal Ethics Committee of East China Normal University approved all studies involving bats and mice.

2.2. Rapid amplification of cDNA ends (RACE), cloning and DNA sequencing

The BS and BEF bat cells were transfected with 10 µg/ml low molecular weight polyI:C (Invivogen, CA, USA) using Fugene HD (Roche, USA) following the manufacturer's instructions. Cells were harvested 6 h after transfection and total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA). The total RNA concentration and quality were checked using spectrophotometry and agarose-gel electrophoresis, respectively. Total RNA (1 µg) was reverse-transcribed using the PrimeScript™ 1st strand cDNA Synthesis kit (TaKaRa, Japan). In order to clone cDNA from the coding region of bat mRNA, PCR was performed using primer sets designed from the orthologous sequences of dogs, mice, humans, rats, pigs and cattle, which are available in the GenBank database. To determine the remaining 5'- and 3'-terminal gene sequences, both 5' and 3' methods of rapid amplification of cDNA ends (RACE) were performed using a 5'-Full RACE Core Set and 3'-Full RACE Core Set (Takara, Japan). RACE primers were designed based on the first decoded sequences. PCR and RACE primers are listed in Table 1.

2.3. Sequence and phylogenetic analysis

Full-length cDNA and deduced amino acid sequences were compared against sequences from other vertebrates reported in GenBank

using the BLAST program (<http://blast.ncbi.nlm.nih.gov>). The accession numbers of other mammalian species used for homology analysis are listed in Table 2. Protein domains were identified using the NCBI Conserved Domains search tool (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and SMART (<http://smart.embl-heidelberg.de/>). Multiple sequence alignment was performed using the ClustalW (version 1.83) Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>). Percentage identity was calculated using the FASTA program. Phylogenetic trees were generated in MEGA 6 using the neighbor joining method.

2.4. Tissue collection and Real-time qPCR

Three *R. affinis* bats were trapped in Yunnan, China, were humanely killed using ether, and organs removed. Tissues were stored at -80 °C in RNAlater solution (Life Technologies, USA). Total RNA was prepared from the spleen, liver, lung, heart, kidney, small intestine, brain, stomach, intestine, skeletal muscle and tongue. For each sample, 1 µg of total RNA was reverse-transcribed. The mRNA expression of RIG-I and STAT-1 was measured by Q-RT-PCR; the primers are listed in Table 1. Real-time qPCR was performed using the Eco Real-Time System (illumine, USA). Samples were run in triplicate and normalized to the control gene GAPDH. Real-time qPCR amplification reactions were carried out in a final volume of 25 µl, which contained 12.5 µl 2×SYBR Premix Ex Taq (TaKaRa, Japan), 0.5 µl (500 ng/µl) diluted cDNA template, 11.0 µl water, and 0.5 µl (10 µM) of each primer. PCR conditions were as follows, 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 15 s.

Table 1
Primers used in this study. A, primers for bats. B, primers for mice.

A					
Gene	Primer	Sequence 5'-3' (BS)	Sequence 5'-3' (BEF)	Application	
IFNβ	IFNβ-1F	ACATGACCACGCTGCACCTGA	ACATGACCACGCTGCACCTGA	RACE	
	IFNβ-1R	GCCACAGGAGGTTCTGACAAGCC	GCAGAGCGAGCCGAAGGATGC	RACE	
	IFNβ-2F	GCAGCTGTGCCTGGACAGTAGTC	ACATGACCACGCTGCCCTG	RACE	
	IFNβ-2R	ACAGGAGAGCGAGCTGAAGGATGC	GCCACAGGAGGCTCTGACAAGC	RACE	
	IFNβ-3F	TCGTCTGGAGACAGCCTTGGAGG	TCCTTCGGCTCGCTCTGCTCT	qRT-PCR	
	IFNβ-3R	TGGCTTTCAAGTGCCGCTGAT	GTCCGTCCTGTCCTTGAGGCAA	qRT-PCR	
	IFNβ-4F	ATGACCAACAGGTGCATCCT	ATGACCAACAGGTGCATCCT	Full-length amplification	
	IFNβ-4R	TCAGTTTCGGAGGTATTCTG	TCAGTTTCGGAGGTATTCTG	Full-length amplification	
	RIG-I	RIG-I-1F	TGCCAGAGAAAAGTGTACC	TGCCAGAGAAAAGTGTACC	RACE
		RIG-I-1R	GGTCCAGAATCTTCGTGACATAGT	GGTCCAGAATCTTCGTGACATAGT	RACE
RIG-I-2F		GCAACTGGACAACAGACACT	GCAACTGGACAACAGACACT	RACE	
RIG-I-2R		GTGACATAGTTCTGGAAAAGCGTGC	GTGACATAGTTCTGGAAAAGCGTGC	RACE	
RIG-I-3F		TGCTCTGCAGAAAAGTGCAAAGCTG	TGCTCTGCAGAAAAGTGCAAAGCTG	qRT-PCR	
RIG-I-3R		ACACCCAAAAGTCTTTGGTCTGGGG	ACACCCAAAAGTCTTTGGTCTGGGG	qRT-PCR	
RIG-I-4F		ATGACGCGCCGAGGAGCGG	ATGACGCGCCGAGGAGCGG	Full-length amplification	
RIG-I-4R		TCATTTGGRCATTTCTGCAG	TCATTTGGRCATTTCTGCAG	Full-length amplification	
STAT-1		STAT-1-1F	ACGGTCCCAGAGTGGAGGTGAA	ACGGTCCCAGAGTGGAGGTGAA	RACE
		STAT-1-1R	GGTCTGAAAACGGATGGTGGC	GGTCTGAAAACGGATGGTGGC	RACE
	STAT-1-2F	TTGATGGCCCTAAGGGAACCGGA	TTGATGGCCCTAAGGGAACCGGA	RACE	
	STAT-1-2R	GCCACTGTGCCAGTACTGTCTG	GCCACTGTGCCAGTACTGTCTG	RACE	
	STAT-1-3F	TGATGGCCCTAAGGGAACCGGA	TGATGGCCCTAAGGGAACCGGA	qRT-PCR	
	STAT-1-3R	AGGACCCAAAAGTCTCGAGACACC	AGGACCCAAAAGTCTCGAGACACC	qRT-PCR	
	STAT-1-4F	ATGTCCCAGTGGTATGAGCT	ATGTCCCAGTGGTATGAGCT	Full-length amplification	
	STAT-1-4R	ACAGTGCCCACTATCCGAGACACC	ACAGTGCCCACTATCCGAGACACC	Full-length amplification	
GAPDH	GAPDH-1F	TGCTGAGTATGTAGTGGAGT	TGCTGAGTATGTAGTGGAGT	qRT-PCR	
	GAPDH-1R	GTGCAGGAGGCATTGCTGAC	GTGCAGGAGGCATTGCTGAC	qRT-PCR	
B					
Gene	Primer	Sequence 5'-3'		Application	
IFNβ	IFNβ-F	CCTCCACGCTGCGTTCCTGC		qRT-PCR	
	IFNβ-R	AAGTCCGCCCTGTAGGTGAGGT		qRT-PCR	
VSV	VSV-F	ACGGCGTACTTCCAGATGG		qRT-PCR	
	VSV-R	CTCGGTTCAAGATCCAGGT		qRT-PCR	
GAPDH	GAPDH-F	AAGCTGTGGCGTATGGCCG		qRT-PCR	
	GAPDH-R	CCAGGCGGCACGTGATCC		qRT-PCR	

Table 2

A, accession numbers of bat sequences submitted to GenBank. B, accession numbers of protein sequences used in phylogenetic analysis. BEF: *Rhinolophus affinis* embryonic fibroblasts. BS: *Rhinolophus sinicus* splenocytes.

A			
Gene	BS	BE	
IFN β	KM056305	KM056306	
RIG-I	KM056307	KM056308	
STAT-1	KM056309	KM056310	
B			
Species	RIG-I	STAT-1	IFN β
<i>Homo sapiens</i> (human)	NP_055129.2	ADA59516.1	NP_002167.1
<i>Mus musculus</i> (mouse)	NP_766277.3	NP_001192242.1	NP_034640.1
<i>Rattus norvegicus</i> (rat)		AAF20200.1	NP_034640.1
<i>Bos taurus</i> (cattle)		AAF20200.1	NP_776775.1
<i>Canis lupus familiaris</i> (dog)			NP_001129259.1
<i>Equus caballus</i> (horse)			NP_001092910.1
<i>Sus scrofa</i> (pig)	NP_998969.2	AAF20200.1	NP_001003923.1
<i>Salmo salar</i> (salmon)	NP_001157171.1		
<i>Rousettus aegyptiacus</i>		BAI43911.1	BAF37103.1
<i>Pteropus vampyrus</i>			ADK11364.1
<i>Macaca mulatta</i> (Rhesus monkey)	ABG67971.1		
<i>Cricetulus griseus</i> (hamster)		EGW08591	
<i>Cervus elaphus</i> (deer)		ADO17542	
<i>Pteropus alecto</i>	NP_001277087.1	ELK04796.1	

2.5. Poly(I:C), VSV-induced IFN- β mRNA expression

The bat cells and mouse cells were transfected and stimulated using Fugene HD (Roche, USA) with 1 μ g/ml poly(I:C), vesicular stomatitis virus (VSV) at MOI 1. Total RNA (1 μ g) was reverse-transcribed. Primers for real-time qPCR are listed in Table 1.

2.6. Statistical analysis

Data are expressed in terms of mean \pm SD. Variables between groups were compared using one-way ANOVA, followed by Student – Newman – Keuls procedure when the assumption of equal variances did not hold. Two-tailed p values of <0.05 were considered statistically significant. The experimental data represent the results obtained from at least three independent experiments.

Table 3

Similarity of *Rhinolophus* to human, mouse and other genes. The numbers in the upper line indicate nucleotide identity; numbers in the lower line in the bracket represent amino acid identity.

A		Human	Mouse	Pig	Rhesus monkey	<i>Pteropus alecto</i>	<i>Rhinolophus sinicus</i>				
RIG-I	<i>Rhinolophus sinicus</i>	87.34 (82.28)	77.54 (73.58)	85.81 (80.66)	86.44 (80.24)	90.97 (85.68)	(–)				
	<i>Rhinolophus affinis</i>	87.48 (82.95)	77.72 (73.04)	85.91 (80.45)	86.52 (79.91)	90.90 (85.58)	98.03 (96.45)				
B		Cattle	Human	Mouse	Pig	Rat	<i>Rousettus aegyptiacus</i>	<i>Rhinolophus sinicus</i>			
STAT-1	<i>Rhinolophus sinicus</i>	86.91 (90.67)	91.57 (95.47)	85.78 (91.53)	90.99 (94.45)	87.13 (92.53)	93.92 (96.80)	(–)			
	<i>Rhinolophus affinis</i>	86.87 (90.93)	91.53 (95.73)	85.52 (92.06)	90.95 (94.72)	86.87 (92.80)	93.83 (97.07)	98.98 (99.47)			
C		Cattle	Dog	Horse	Human	Mouse	Pig	Rat	<i>Rousettus aegyptiacus</i>	<i>Pteropus vampyrus</i>	<i>Rhinolophus sinicus</i>
IFN- β	<i>Rhinolophus sinicus</i>	54.86 (51.61)	75.04 (55.38)	78.79 (63.44)	75.71 (58.82)	64.72 (49.73)	78.79 (65.05)	66.67 (49.20)	83.42 (68.82)	83.78 (69.35)	(–)
	<i>Rhinolophus affinis</i>	53.26 (54.30)	73.44 (58.06)	78.79 (61.83)	74.11 (60.96)	64.18 (51.87)	77.54 (66.67)	65.96 (50.80)	81.28 (72.04)	81.28 (73.66)	95.37 (89.78)

3. Results

3.1. Cloning and sequence analysis of RIG-I, STAT-1, and IFN- β

Information on the whole genome and an accurate gene sequence of two horseshoe bats *R. sinicus* and *R. affinis* is still incomplete. In order to sequence the complete gene structures of RIG-I, STAT-1 and IFN- β , PCR was performed in *R. sinicus* splenocytes (BS) and *R. affinis* embryonic fibroblasts (BEF) using the primers shown in Table 1. The open reading frames of RIG-I, STAT-1 and IFN- β were 2790 bp, 2253 bp and 561 bp, respectively, in both *R. sinicus* and *R. affinis* cells. The detailed sequences of those genes have been submitted to GenBank (Accession Nos. KM056305–KM056310, Table 2A).

The accession numbers of other mammalian species used for homology analysis are described in Table 2B. The homology of the nucleotide and amino acid sequences between bats and other mammals was compared. The *Rhinolophus* RIG-I sequence shared 78–91% nucleotide and 73–86% amino acid identity to RIG-I genes from other species, sharing the highest similarity with the fruit bat *Pteropus alecto* (Table 3). The *Rhinolophus* STAT-1 sequence was highly conserved, sharing 86–94% nucleotide and 90–97% amino acid identity to STAT-1 genes from other mammalian species, sharing the highest similarity with the fruit bat, *Rousettus aegyptiacus* (Table 3). The *Rhinolophus* IFN- β sequence showed 53–84% nucleotide and 49–74% amino acid identity to IFN- β genes from other mammalian species, with the highest similarity reported for the fruit bat *Pteropus vampyrus* (Table 3).

The predicted amino acid sequences were analyzed by conserved domain analysis to identify putative domains. The bat RIG-I contained two N-terminal CARD domains, a DEXD/H-box RNA helicase domain and a C-terminal regulatory domain, which were all typical domains for RIG-I (Fig. 1). The ATP binding site, the Mg²⁺ binding site in the DEXD domain and the nucleotide binding region in the helicase domain were necessary for the activation of RIG-I (Fig. 1). The highly conserved domain organization indicated a similar function with other mammalian species. The deduced protein sequence of bat STAT-1 shared high similarity when aligned with cattle, deer, hamsters, humans, mice, rats, pigs and fruit bats (Fig. 2). The bat STAT-1 contained a STAT-int domain, a STAT-alpha domain, a STAT-bind domain (DNA binding domain), an SH2 domain and a STAT1-TAZ2 bind domain. The phosphorylation binding rocket and the hydrophobic binding rocket in the SH2 domain are important for phosphorylation and nuclear translocation (Fig. 2). The alignment of the deduced bat IFN- β protein sequences with cattle, horses, pigs, humans, mice, rats, dogs and fruit bats showed that bat IFN- β contained the transmembrane domain and the IFab

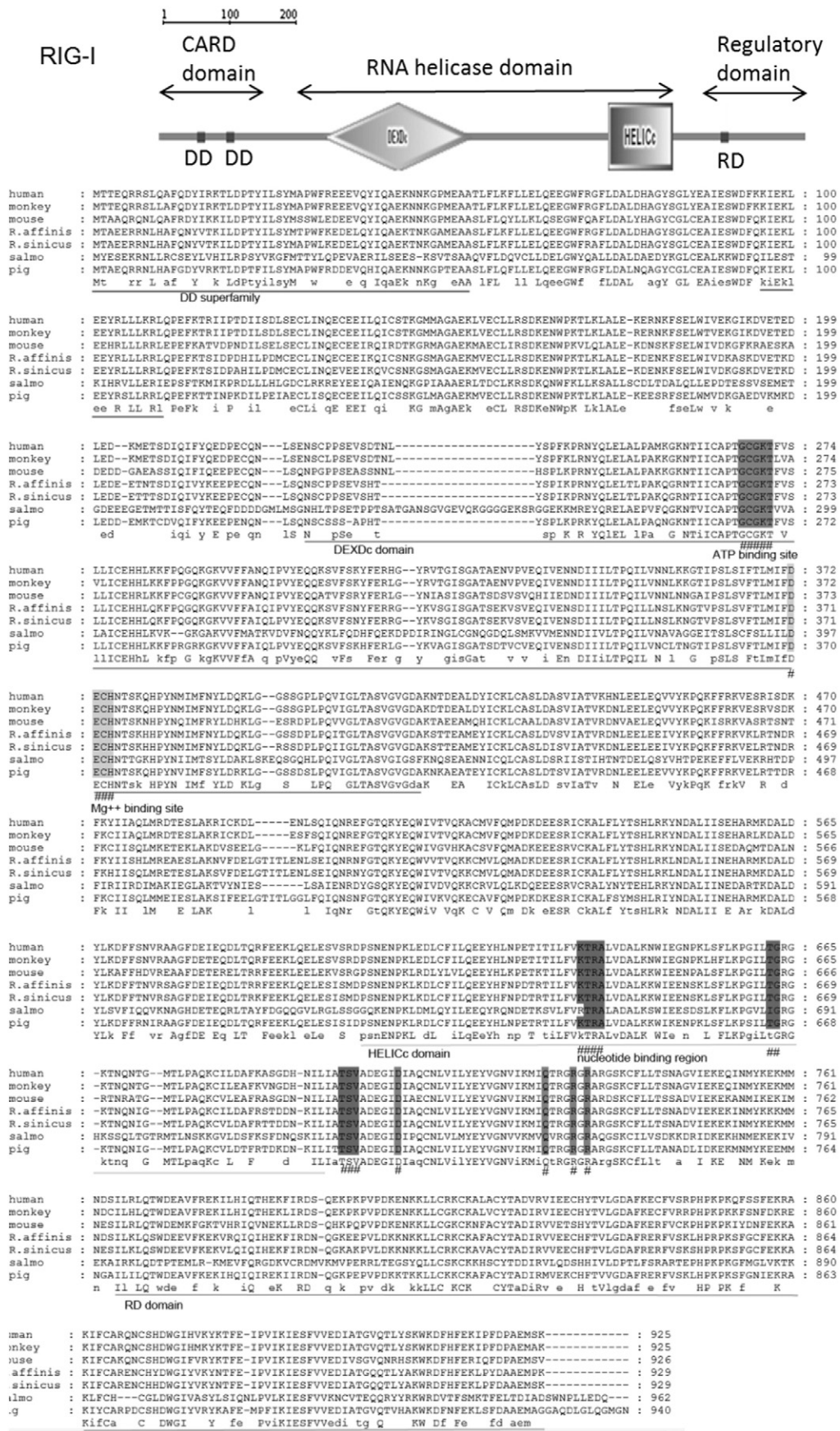


Fig. 1. Predicted protein domain architecture of bat RIG-I and alignment of the deduced amino acid sequence of bat RIG-I genes with other species. Conserved domain architecture analysis was performed for *R. sinicus* and *R. affinis* RIG-I protein. Abbreviations: DD: DEATH domain superfamily; DEXDc: DEAD-like helicases superfamily, HELICc: helicase superfamily c-terminal domain. RD: regulatory domain.

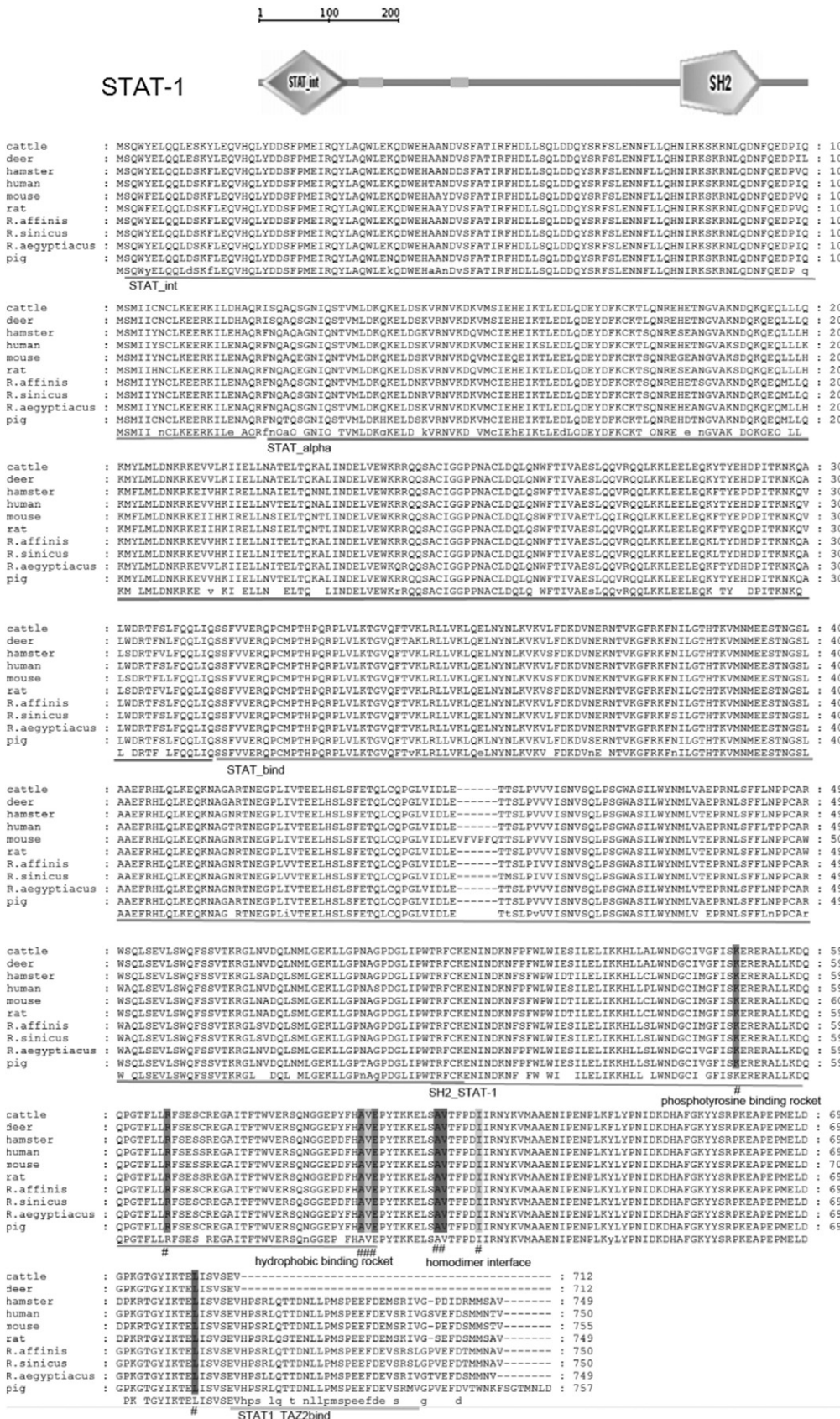


Fig. 2. Predicted protein domain architecture of bat STAT-1 and alignment of the deduced amino acid sequence of bat STAT-1 genes with other species. Conserved domain architecture analysis was performed for R. sinicus and R. affinis STAT-1 protein. Abbreviations: STAT_int: STAT protein-protein interaction domain, SH2: Src homology 2 domains.

domain, which are responsible for the cytokine release and antiviral functions seen in other mammals. The IFNAR binding sites are critical for IFN- β receptor recognition and biological activity. The N-glycosylation site is a posttranslational modification site, which plays a role in regulating protein solubility and stability of IFN- β (Fig. 3).

Phylogenetic trees of RIG-I, STAT-1 and IFN- β were constructed based on the amino acid sequences from *R. sinicus*, *R. affinis* and other species. *Rhinolophus* STAT-1 and IFN- β were the most closely related to human genes, and RIG-I was the most closely related to pigs (Fig. 4).

3.2. Transcriptional analysis of RIG-I and STAT-1 in *R. affinis*

The basic transcriptional levels of RIG-I and STAT-1 were determined by qRT-PCR in 11 tissues collected from *R. affinis* (Fig. 5); both RIG-I and STAT-1 were ubiquitously expressed in these 11 tissues. Also, RIG-I and STAT-1 have similar expression profiles that are both strongly expressed in spleen, which was the most important immune organ.

3.3. Induction of IFN- β in bat and mouse cells

RIG-I is essential for the activation of immune responses to viruses by recognizing RNA viruses, and poly(I:C), a synthetic dsRNA analogue (Kato et al., 2006; Pichlmair et al., 2006). The bat and mouse cells were transfected with 1 μ g/ml poly(I:C) to induce IFN- β mRNA expression. The IFN- β induction was gradually increased and reached its peak at 24 h (BEF) and 9 h (BS) in bat cells while the mRNA expression of IFN- β kept on increasing before 9 h (MEF) and 6 h (MS) in mouse cells. Furthermore, the poly(I:C) induced IFN- β mRNA fold change was much lower in mouse cells when compared with bat cells. The splenocytes showed less IFN- β induction compared with embryonic fibroblast cells under poly(I:C) transfection (Fig. 6). Furthermore, we also

infected the bat embryonic fibroblast cells with VSV at MOI 1 to check the invading VSV and IFN- β induction by qRT-PCR. Interestingly, the VSV mRNA level in BEF cells (more than 9000 folds) was increased much more quickly than in MEF cells (less than 2000 fold) in the first 6 h. Then the expansion of VSV was restrained obviously (only 3 times increased) in the next 18 h in the BEF cells. Whereas, the VSV mRNA at 24 h was increased more than 70 times than 6 h in MEF cells. Meanwhile, the VSV induced IFN- β expression in BEF cells was also much higher than in MEF cells which could partially explain the restrained VSV expansion in BEF cells (Fig. 7).

4. Discussion

The innate immune system is the first defense against invading pathogens. Understanding the virus-associated pattern recognition receptor and signaling pathway may be important for exploring the characteristics of the bat immune system. For this reason, RIG-I like helicase genes have been identified in the black flying fox *P. alecto* (Cowled et al., 2012). STAT1 has been shown to be phosphorylatable and to translocate to the nucleus when stimulated with human IFN- α (hIFN- α) in *R. aegyptiacus* (Fujii et al., 2010). Type I interferon has been found increased by poly(I:C), exogenous bat type I IFNs and alphavirus in the Rousette bat primary kidney cells (BPKCs) and African fruit bat cells (Omatsu et al., 2008; Biesold et al., 2011). Cells from *P. alecto* have been shown to produce IFN- λ after stimulation with the poly(I:C) transfection and after infection with the bat paramyxovirus, Tioman virus (Zhou et al., 2011). Conversely, the Tioman virus and paramyxovirus Hendra virus did not activate type I IFN expression and interferon signaling (Virtue et al., 2011). However, due to a lack of fundamental research, the antiviral immunity of the bat is still poorly understood. Thus, we sequenced and analyzed three genes (RIG-I, STAT-1 and IFN- β),

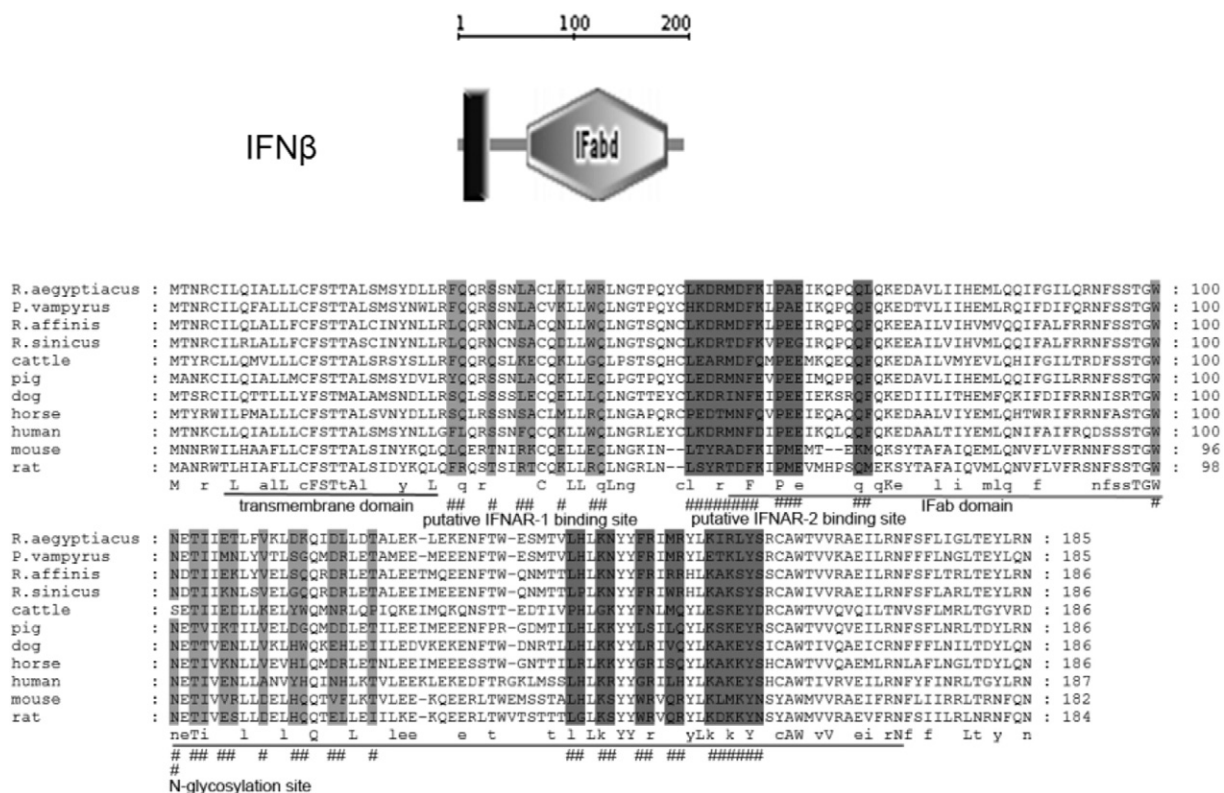


Fig. 3. Predicted protein domain architecture of bat IFN- β and alignment of the deduced amino acid sequence of bat IFN- β genes with other species. Conserved domain architecture analysis was performed for *R. sinicus* and *R. affinis* IFN- β protein. Abbreviations: IFab: interferon alpha, beta and delta.

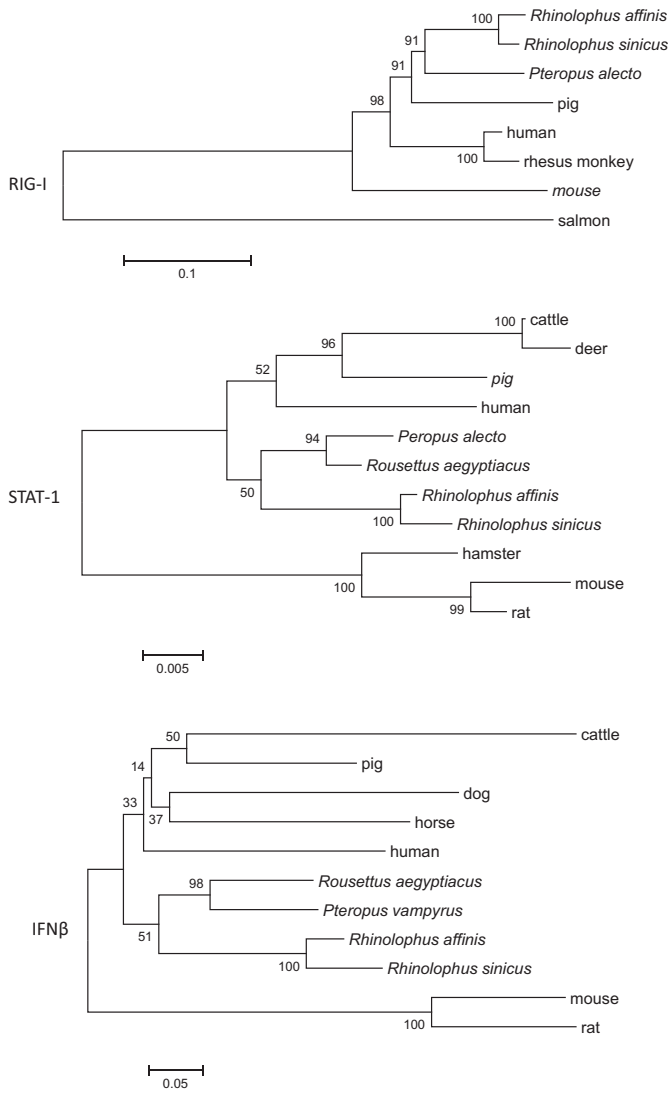


Fig. 4. Phylogenetic analysis based on amino acid alignments.

which are all key genes involved in innate immune responses. Our data suggested that bat RIG-I, STAT-1 and IFN-β share a high degree of similarity with other mammalian species.

We detected the constitutive expression of RIG-I and STAT-1 in the horseshoe bat and found that they are highly expressed in the spleen, lung and intestine (Fig. 5). The spleen is a well-known immune tissue; therefore, it is reasonable that RIG-I and STAT-1 are most highly expressed in the spleen. Interestingly, RIG-I and STAT-1 were also highly expressed in the lung and intestine, which are both highly sensitive organs to respiratory viruses and enteroviruses. This provides circumstantial evidence for RIG-I and STAT-1 being key genes for bats in the fight against viral infection.

In order to explore the differences in IFN induction between horseshoe bat cells and mouse cells, we transfected bat and mouse cells with poly(I:C) to induce IFN-β. As shown in Fig. 6, the mRNA level of IFN-β was highly induced by poly(I:C) in BEF cells, with a more than 30,000-fold change. Only several hundred-fold changes were seen in MEF cells, and similar results were also observed in bat and mouse splenocytes. The massive IFN-β production in bat cells can serve to control viruses effectively, and this character of the bat immune system contributes to bats as a natural host of a variety of viruses. Furthermore, when we infected the bat and murine cells with live VSV, the replication of VSV was restrained obviously in bat cells. Whereas, the mRNA level of IFN-β in bat cells was induced to a much higher level than mouse cells which could be a main reason for reduced VSV replication in bat cells. It has been shown that bats host more zoonotic virus species than rodents; the total number of zoonotic viruses identified in bats was lower than in rodents, as there is approximately twice the number of rodent species as bat species (Luis et al., 2013). We can postulate that the highly expressed IFN-β in bat cells is beneficial in preventing bats from developing certain virus infections. Despite the fact that unique features of bat's immune responses in anti-viral infection cannot be fully demonstrated, the basic characterization of bat immune genes and signaling remains necessary and will lead to a better understanding of the bat immune system.

5. Conclusions

We found the genetic sequence of RIG-I, STAT-1 and IFN-β in Chinese horseshoe bats to have high homology with those of humans, mice, pigs and rhesus monkeys. The expression pattern of these genes in bats was similar to their homologous genes in mice. The RIG-I ligands poly(I:C) and VSV were used to treat bat and murine cells. In particular, the poly(I:C) and VSV-induced IFN-β mRNA expression in bat cells was stronger than in murine cells, suggesting that the bat's innate immune responses can fight against the virus. Consistently, the long-term expansion of VSV was restrained obviously in bat cells than mouse cells.

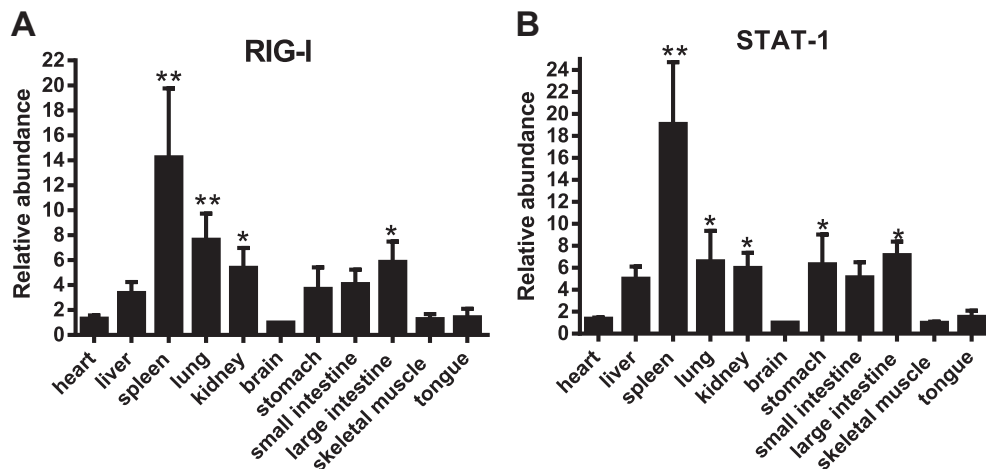


Fig. 5. Quantitative baseline mRNA expression analysis of RIG-I and STAT-1 in *Rhinolophus affinis* tissues. Tissue mRNA expression levels of RIG-I (A) and STAT-1 (B) were determined by qRT-PCR and normalized relative to GAPDH. N = 3 individual apparently healthy wild-caught bats. Error bars represent standard deviation.

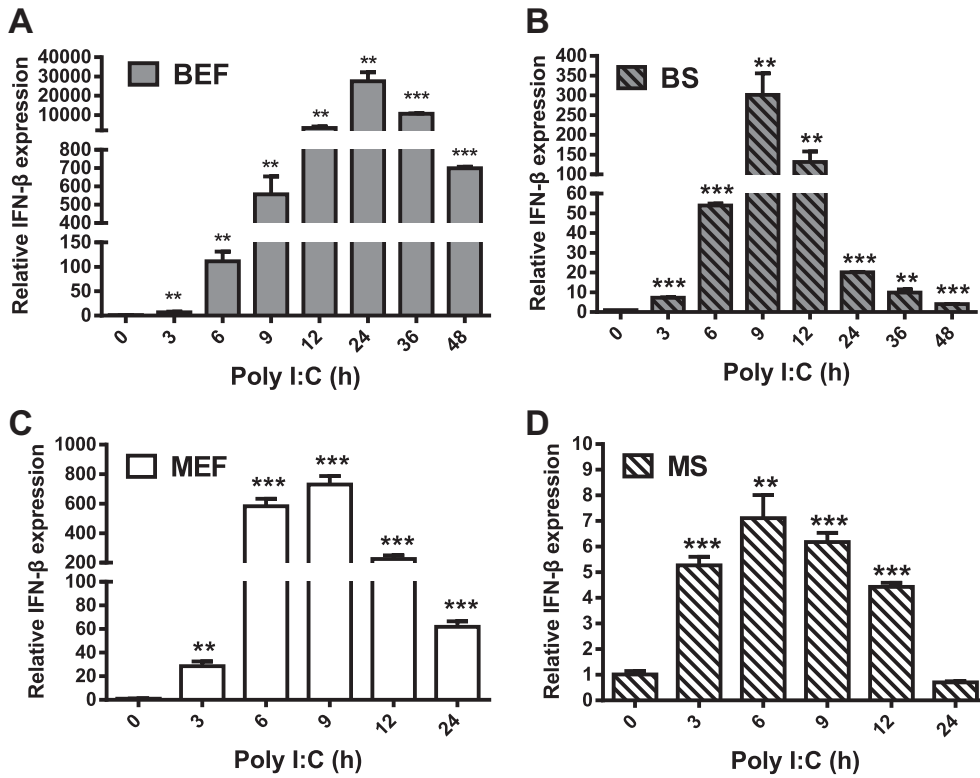


Fig. 6. Expression of IFN-β under polyI:C transfection in bat and mouse cells. All cells were transfected with 1 μg/ml polyI:C for the indicated amount of time. Results are given as fold-change relative to mock transfection. The data were normalized against the house-keeping gene GAPDH. N = 4 replicate cultures. Error bars represent standard deviation. BEF: *Rhinolophus affinis* embryonic fibroblasts (A). BS: *Rhinolophus sinicus* splenocytes (B). MEF: mouse embryonic fibroblasts (C). MS: mouse splenocytes (D).

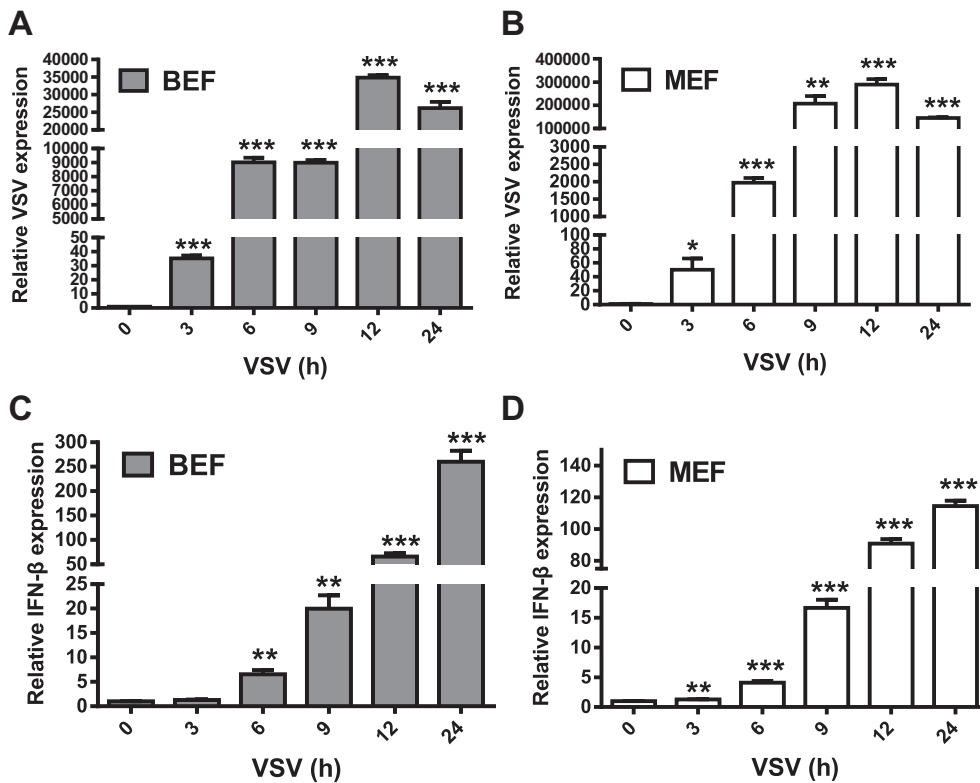


Fig. 7. Expression of IFN-β against VSV infection in bat and mouse cells. All cells were treated with VSV at MOI 1. Results are given as fold-change relative to mock transfection, respectively. The data were normalized against the house-keeping gene GAPDH. N = 4 replicate cultures. Error bars represent standard deviation. BEF: *Rhinolophus affinis* embryonic fibroblasts (A & C). MEF: mouse embryonic fibroblasts (B & D).

Conflict of interest

We declare that we have no conflict of interest.

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