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Role of pattern recognition receptors and interferon-beta in protecting bat cell lines from encephalomyocarditis virus and Japanese encephalitis virus infection

Ronald Tarigan [a], Hiroshi Shimoda [b], Karla Cristine C. Doysabas [a], Maeda Ken [c], Atsuo Iida [a], Eiichi Hondo [a, *]

[a] Laboratory of Animal Morphology, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, Japan
[b] Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan
[c] Division of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan

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ABSTRACT

Bats are potential natural hosts of Encephalomyocarditis virus (EMCV) and Japanese encephalitis virus (JEV). Bats appear to have some unique features in their innate immune system that inhibit viral replication causing limited clinical symptoms, and thus, contributing to the virus spill over to humans. Here, kidney epithelial cell lines derived from four bat species (*Pteropus dasymallus*, *Rousettus leschenaultii*, *Rhinolophus ferrumequinum*, and *Miniopterus fuliginosus*) and two non-bat species (*Homo sapiens* and *Mesocricetus auratus*) were infected with EMCV and JEV. The replication of EMCV and JEV was lower in the bat cell lines derived from *R. leschenaultii*, *R. ferrumequinum*, and *M. fuliginosus* with a higher expression level of pattern recognition receptors (PRRs) (TLR3, RIG-I, and MDA5) and interferon-beta (IFN-β) than that in the non-bat cell lines and a bat cell line derived from *P. dasymallus*. The knockdown of TLR3, RIG-I, and MDA5 in Rhinolophus bat cell line using antisense RNA oligonucleotide led to decrease IFN-β expression and increased viral replication. These results suggest that TLR3, RIG-I, and MDA5 are important for antiviral response against EMCV and JEV in Rhinolophus bats.

1. Introduction

Bats are known as natural reservoirs of some deadly zoondotic viruses, which have a high impact on human health and include filoviruses (Marburg viruses), lyssaviruses (Rabies and ABLV), coronaviruses (SARS and MERS-CoV), and henipaviruses (Hendra and Nipah viruses) [1]. EMCV, a potential omnipresent zoonotic agent, has a broad host range with rodents as its natural reservoir [2]. As yet, there has been no report of EMCV isolation from bats; however, *Miniopterus fuliginosus* is supposed to be one of the natural hosts of EMCV due to the detection of a genome fragment of the virus in the bat's fecal guano [3]. JEV is a mosquito-transmitted flavivirus with humans as the definitive hosts and pigs as the amplification hosts [4]. JEV have been isolated from multiple species of fruits and insectivorous bats in several Asian countries [5]. Experimental infection of JEV in some insectivorous and fruit bats showed persistent viremia and viral replication in multiple organs without pathological symptoms or signs of encephalitis [6].

Bats remain asymptomatic after viral infection partly due to suppression of viral replication at an early stage of innate immune response in them [7]. Some bat species have "always on" interferon system because of higher and constitutive expression of interferon-alpha, persistent activity of ISGs, constitutive STAT1 phosphorylation, and broad tissue distribution of IRF7 in unstimulated bat primary cells derived from *Pteropus alecto,* and *Artibeus jamaicensis* [8]. Moreover, some bat species have a special mechanism to suppress excessive inflammation during MERS-CoV infection as identified in a bat cell line derived from *Eptesicus fuscus* and bat primary immune cells derived from *P. alecto* [9,10].

Innate immune response is initiated through recognition of viral pathogens by the PRRs and will converges at IRF3, which is followed by phosphorylation of IRF3 leading to initiation of antiviral responses such as activation of type I IFN, ISGs, and proinflammatory cytokines [11]. Previous studies have showed that TLR3, RIG-I, and MDA5 signaling is critical for IFN-β production and

* Corresponding author.
E-mail address: ehondo@agr.nagoya-u.ac.jp (E. Hondo).

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Abbreviations

ABLV	Australian bat-lyssavirus
AQP1	Aquaporin-1
CPE	Cytopathic effect
DMEM	Dulbecco's modified eagle medium
Dpi	Days post infection
EMCV	Encephalomyocarditis virus
FBS	Fetal bovine serum
IFN-β	Interferon-beta
IRF3	IFN regulatory factor 3
IRF7	IFN regulatory factor 7
ISGs	Interferon-stimulated genes
JEV	Japanese encephalitis virus
MERS-CoV	Middle East respiratory syndrome coronaviruses
MDA5	Melanoma differentiation associated protein 5
MOI	multiplicity of infection
MUC-1	Mucin-1
PRRs	Pattern recognition receptors
PEI	Polyethylenimine
RIG-I	Retinoic acid-inducible gene I
SARS	Severe acute respiratory syndrome
TLRs	Toll-like receptors

susceptibility of cells against EMCV and JEV infection in humans and mice [12–16]. Only a few studies are available on PRRs and type I IFN signaling in bats during viral infection. Activation of TLR3, RIG-I, and MDA5 after poly(I:C) stimulation have been described in *P. alecto* kidney cell line, *Desmodus rotundus* fetal lung cell line, and *E. fuscus* kidney cell line; however, their role in stimulating type I IFN production has been clarified only in the *E. fuscus* kidney cell line [17–19]. Here, the influence of EMCV and JEV on PRRs–IFN–β signaling in bat cell lines derived from four bat species (*Pteropus dasymallus*, *Rousettus leschenaultii*, *Rhinolophus ferrumequinum*, and *Miniopterus fuliginosus*) was examined.

2. Material and methods

2.1. Cell lines and viruses

BHK-21 (Syrian hamster, kidney), HEK293T (human, kidney), FBKT1 (Ryukyu flying fox, *Pteropus dasymallus*, kidney), DEMKT1 (Leschenault's rousette, *Rousettus leschenaultii*, kidney), BKT1 (Greater horseshoe bat, *Rhinolophus ferrumequinum*, kidney), and YUBFKT1 (Eastern bent-wing bats, *Miniopterus fuliginosus*, kidney) cell lines were maintained in DMEM supplemented with 10% FBS, 2% L-Glutamine, 0.14% $NaHCO_3$, and 1% penicillin-streptomycin. The bat kidney cell lines were established as previously described [20,21] and were derived from proximal epithelial tubules cells based on the presence of AQP1 and MUC-1, specific markers for proximal and distal epithelial cells, respectively (Supplementary Fig. 1). Partial DNA sequence of AQP1 and MUC-1 from all cell lines can be found in Supplementary Fig. 2, and, 3; and Supplementary Table 1.

The EMCV strain used in this study, NIID-NU1, was provided by Dr. Kazuya Shirato (National Institute of Infectious Diseases, Japan). The genome sequence of NIID-NU1 strain of EMCV was determined (GenBank accession number LC508268). The EMCV strain NIID-NU1 was clustered into group 1A and most closely related to EMCV GS01 and PV21 strains, which were isolated from pigs (Supplementary Fig. 4; and Supplementary Table 2). The JEV strain, JEV/sw/Chiba/88/2002, was isolated from swine serum [22]. Both EMCV and JEV were propagated in BHK-21 cells and the virus titer was determined by plaque assay. All kidney cell lines were infected with EMCV and JEV at MOI of 1.0 and 0.01 in DMEM supplemented with 2% FBS medium inside biosafety level 2 laboratory.

2.2. Cell growth analysis

The cells were seeded at a concentration of 5×10^4 cells/well in a 24-well plate and were infected with EMCV and JEV at MOI of 1.0 and 0.01. Both mock and EMCV-JEV-infected cells were washed with PBS and were harvested using 0.25% Trypsin-EDTA at 1, 2, 3, 4, 5, 6, and 7 days post infection (dpi). Assessment of cell growth (cell viability and total live cell numbers) was carried out by trypan blue dye exclusion test. Unstained or live cells were counted using automated cell counter.

2.3. Nucleic acid extraction and qRT-PCR

Total RNA was collected from mock and EMCV and JEV-infected cells with ISOGEN II (Nippon Gene) and RNA cleanup were performed using RNeasy Mini kit (QIAGEN). The cDNA was synthesized using SuperScript IV First-Strand Synthesis System (Invitrogen). qRT-PCR was performed using Roche LightCycler 96 in conjunction with Thunderbird SYBR qPCR Mix (Toyobo) as per the manufacturer's instructions. Relative expression level of the target genes (TLR3, RIG-I, MDA5, and IFN-β) were normalized against GAPDH and is expressed as reciprocal of ΔCt. Fold change is represented as fold increase of expression level in infected cells over mock-infected cells. Viral genome copies was calculated using the EMCV and JEV standard curves that were created by serial dilution of a known number of EMCV and JEV PCR-amplified fragments.

2.4. Knockdown of PRRs in BKT1 cells

Phosphorothioate antisense RNA oligonucleotide (s-oligo) were synthesized by FASMAC (Japan) using the consensus sequences of TLR3, RIG-I, and MDA5 genes of three bat species (*R. ferrumequinum, M. fuliginosus,* and *R. leschenaultii)* (Supplementary Figs. 5, 6, and 7). BKT1 cells were transfected with the 120 pmol antisense RNA oligonucleotides against TLR3, RIG-I, and MDA5 (Supplementary Table 3) using PEI [23]. The knockdown was verified by measuring the expression level of TLR3, RIG-I, and MDA5 by qRT-PCR.

3. Results

3.1. EMCV and JEV replication in bat and non-bat cell lines

The bat and non-bat cell lines showed different replication levels of EMCV and JEV. EMCV and JEV replicated at a significantly lower level in three bat cell lines (DEMKT1, BKT1, and YUBFKT1) than in non-bat cell lines (BHK-21 and HEK293T cells) and FBKT1 cells (Fig. 1A and B). Higher replication level of EMCV and JEV in non-bat cell lines and FBKT1 cells resulted in massive cell death within 1 dpi for EMCV and 3 dpi for JEV (Fig. 1C and D). The CPE in non-bat cell lines and FBKT1 cells after EMCV and JEV infection with MOI of 1.0 and 0.01 appeared earlier than in three bat cell lines (DEMKT1, BKT1, and YUBFKT1). The CPE was observed in non-bat cell lines and FBKT1 cells within 1–2 dpi and 3–5 dpi after EMCV and JEV infection, respectively (Fig. 1C and D). Remarkably, in EMCV-infected YUBFKT1 and DEMKT1 cells; and in JEV-infected

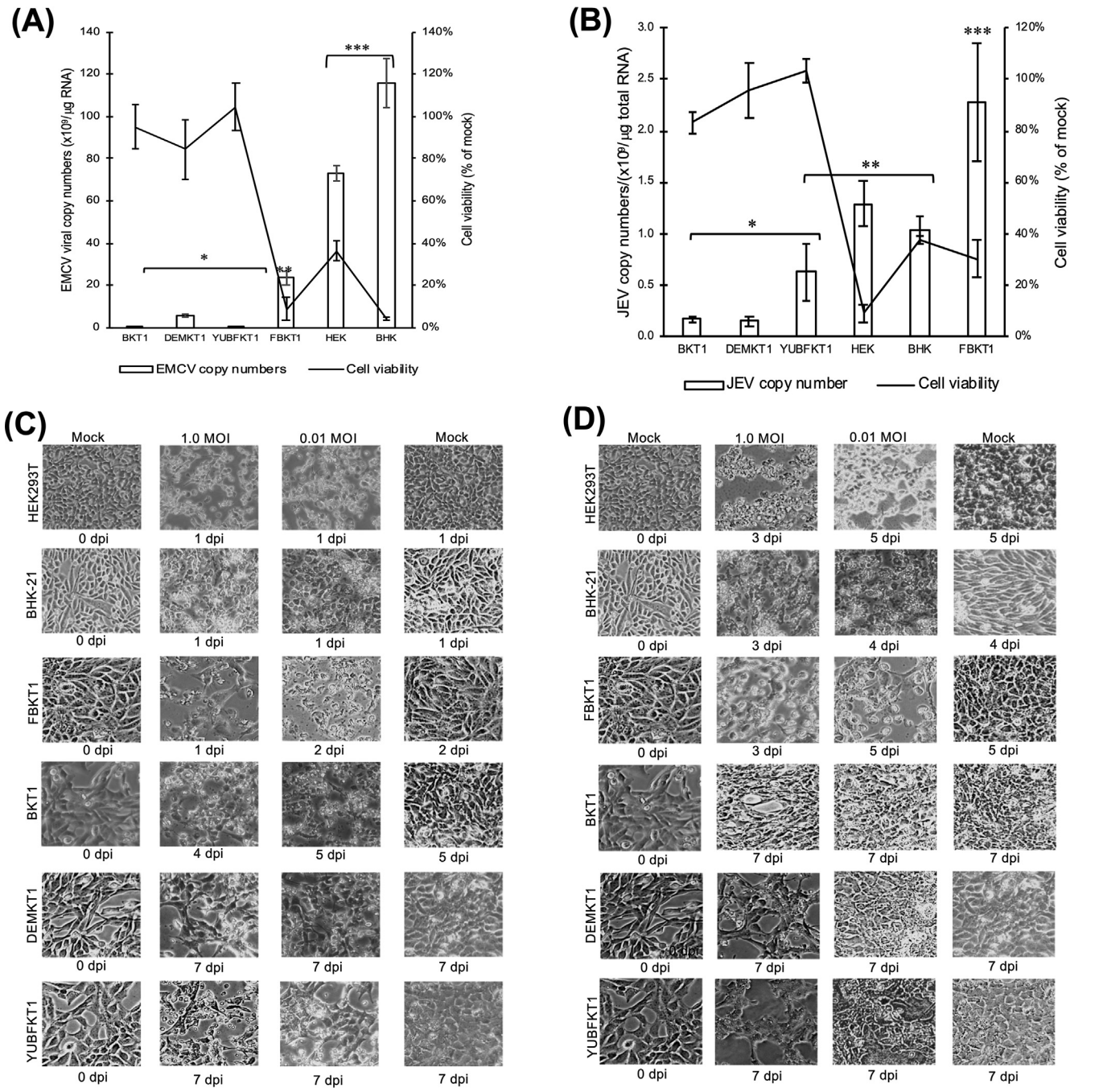

Fig. 1. EMCV and JEV replication was inhibited in some bat cell lines and showed delayed CPE. Viral copy numbers and cell viability in bat and non-bat cell lines 1 dpi; the cell lines were infected with EMCV at 1 dpi (A) and JEV at 3 dpi (B) with MOI of 1.0 (mean ± SD, n = 3). CPE in non-bat cell lines (HEK293T and BHK-21) and bat cell lines (BKT1, DEMKT1, and YUBFKT1 cells) that were infected with EMCV (C) and JEV (D) with MOI of 1.0 and 0.01. EMCV and JEV caused visible lytic CPE in non-bat cell lines. Statistical significance was calculated using one-way ANOVA, followed by Tukey's test. Values that do not share a common symbol are significantly different ($p < 0.05$).

BKT1, YUBFKT1, and DEMKT1 cells, the CPE was delayed to 7 dpi for MOI of 1.0 and 0.01 without complete lysis (Fig. 1C and D), but the total live cell numbers were still higher than other cells until 7 dpi (Supplementary Figs. 8E, 8F, 9D, 9E, and 9F).

3.2. The basal expression level of PRRs and IFN-β

Almost all bat cell lines had a higher basal expression level of TLR3, RIG-I, and MDA5 than non-bat cell lines (Fig. 2A). Among the bat cell lines, BKT1 and YUBFKT1 cell lines had the highest basal expression level of TLR3 and RIG-I. The basal expression of MDA5 was comparable among all bat cell lines. The pattern of basal expression level of IFN-β was different from that of PRRs. Only one bat cell line (YUBFKT1) had a significantly higher basal expression level of IFN-β than human cell line, while other bat cell lines did not show any difference in the expression level (Fig. 2A). The basal expression level of IFN-β in BHK-21 cells could not be measured because BHK-21 cell line is known to be deficient in IFN-α and β production [24].

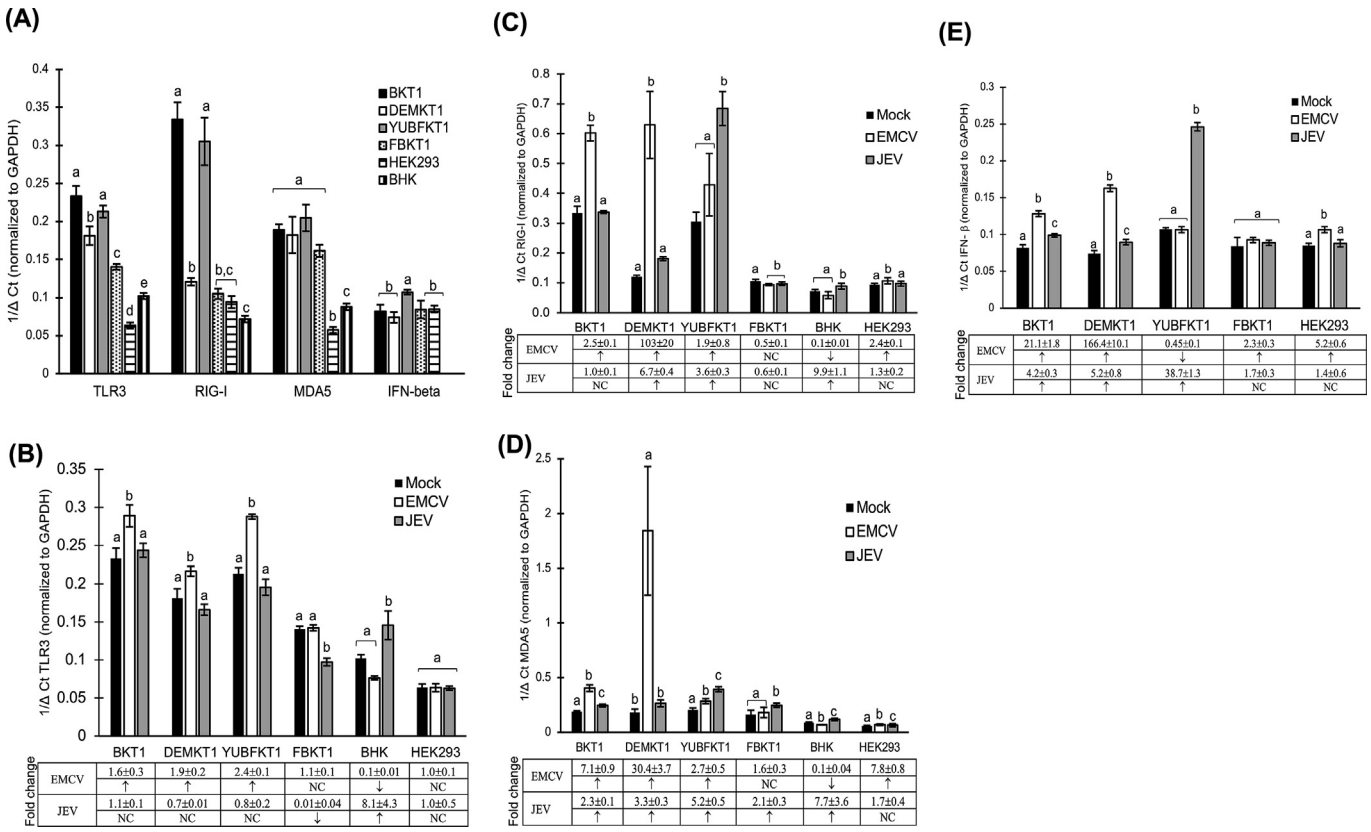

Fig. 2. Some bat cell lines have a higher basal expression level of PRRs and increased expression level of PRRs and IFN-β in some bat cell lines after EMCV and JEV infection. Basal expression level of TLR3, RIG-I, MDA5, and IFN-β in bat and non-bat cells (A). Expression level and fold change of TLR3 **(B)**, RIG-I **(C)**, MDA5 **(D)**, and IFN-β **(E)** after EMCV and JEV infection at MOI of 1.0 in bat and non-bat cell lines 1 dpi (mean ± SD, n = 3). Differences between treatments were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($p < 0.05$).

3.3. PRRs and IFN-β expression level with EMCV- and JEV- infected cell lines

EMCV and JEV infection resulted in a higher expression level of PRRs in bat cell lines with a lower viral replication level (DEMKT1, BKT1, and YUBFKT1) (Fig. 2B, C, and 2D). Up-regulation of TLR3, RIG-I, and MDA5 after EMCV infection was observed in those cell lines, with a high up-regulation of RIG-I and MDA5 in DEMKT1 cells (Fig. 2C–D). RIG-I and MDA5 were also up-regulated in HEK293T cells but their expression level was still lower than that in the bat cell lines. Down-regulation of all PRRs and RIG-I was observed in BHK-21 cells and FBKT1 cells, respectively (Fig. 2B, C, and 2D). Among JEV-infected cells, RIG-I and MDA5 were up-regulated in all cell lines, except FBKT1 cells (Fig. 2C). Down-regulation of TLR3 was observed only in FBKT1 cells (Fig. 2B). IFN-β was highly up-regulated in all bat cell lines that showed resistance against EMCV and JEV infection, except in YUBFKT1 cells after EMCV infection (Fig. 2E). HEK293T and FBKT1 cells also showed up-regulation of IFN-β with a lower fold change (Fig. 2E).

3.4. Knockdown of pattern recognition receptors in BKT1 cells

The knockdown of TLR3, RIG-I, and MDA5 was confirmed by qRT-PCR in which the only the expression level of PRRs was significantly reduced in BKT1 cells (Fig. 3A). The expression level of IFN-β was also decreased in BKT1 cells but it was not as intensive as that of PRRs (Fig. 3B). Knockdown of PRRs in other bat cell lines (DEMKT1 and YUBFKT1 cells) was not successful, as the expression level of PRRs was not decreased in those cell lines.

Knockdown of TLR3, RIG-I, and MDA5 led to reduced expression level of IFN-β after EMCV and JEV infection (Fig. 3C–D), dramatic CPE at 2 and 5 dpi, respectively (Fig. 4C), and a significantly lower number of live cells than uninfected knocked down cells since days 2 and 3 of EMCV and JEV infection, respectively (Supplementary Fig. 10). After EMCV infection, the TLR3 knocked down cells showed the lowest expression of IFN-β (Fig. 3C); however, unpredictably, they did not demonstrate the highest EMCV replication level (Fig. 4A). The cells knockdown for MDA5 exhibited the highest EMCV replication level (Fig. 4A). After JEV infection, all cells knocked down for either of the three PRRs showed a comparable IFN-β expression level (Fig. 3D), while the cells knockdown for MDA5 exhibited a comparatively higher JEV replication level (Fig. 4D).

4. Discussion

In this study, the bat cell lines derived from *R. leschenaultii* (DEMKT1), *R. ferrumequinum* (BKT1), and *M. fuliginosus* (YUBFKT1) showed resistance against EMCV and JEV infection while massive cell death was observed in bat cell line derived from *P. dasymallus* (FBKT1). This is the first report on resistance against EMCV in bat cells since the lung cells from *Tadarida brasilensis* (Tb1.Lu cells) did not show resistance against EMCV infection [25]. On the other hand, the resistance of bat cell lines against JEV infection has been reported in a lung cell line derived from *T. brasilensis* and primary kidney cells derived from *R. leschenaultii*, which did not develop CPE until 7 dpi [26].

In this study, the DEMKT1, YUBFKT1, and BKT1 cell lines showed limited CPE, lower replication level of EMCV and JEV, and higher

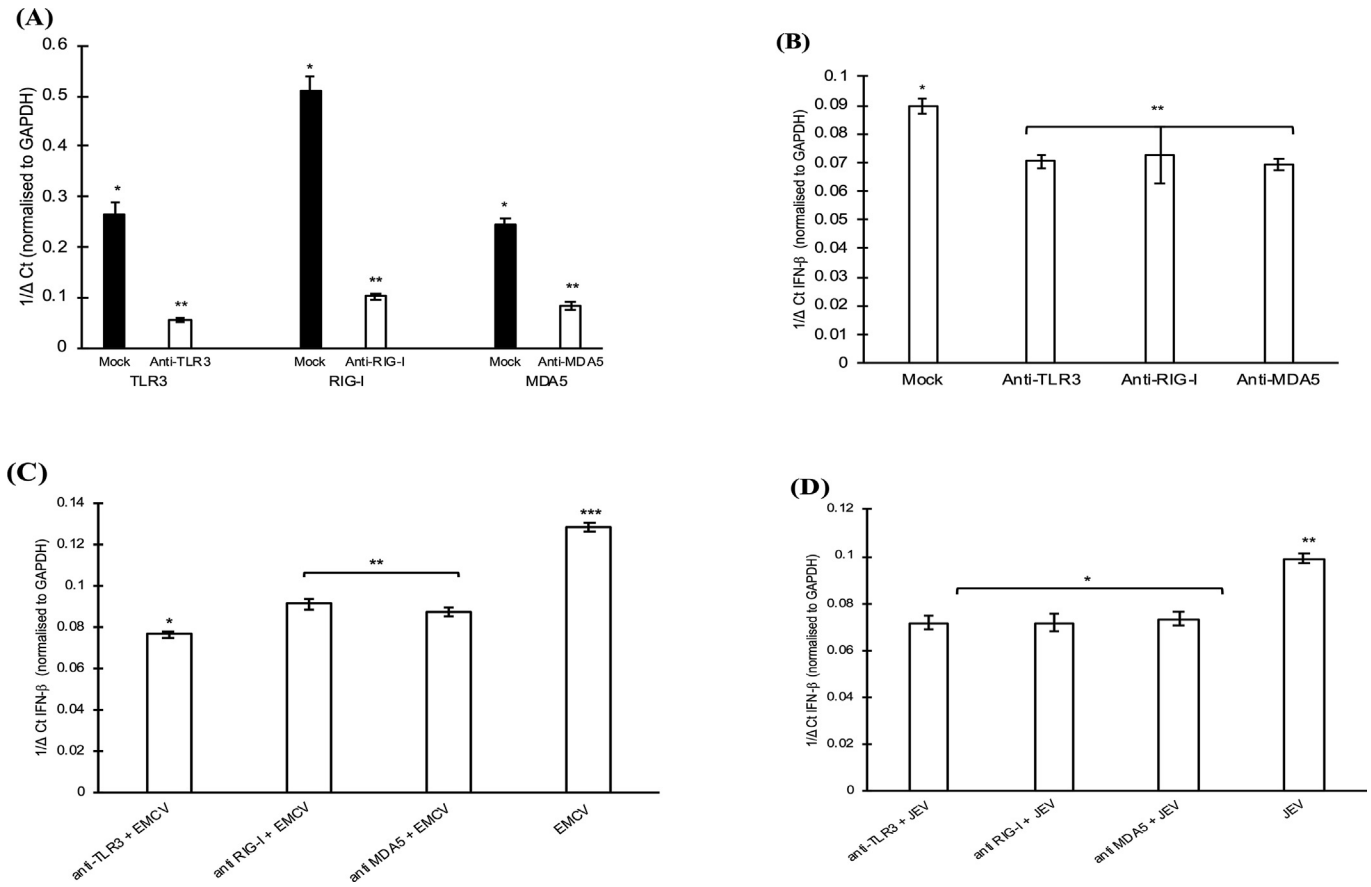

Fig. 3. Knockdown of TLR3, RIG-I, and MDA5 reduced expression level of IFN-β in BKT1 cells. Expression level of TLR3, RIG-I, MDA5 **(A)** and IFN-β **(B)** after knockdown of TLR3, RIG-I, and MDA5 in BKT1 cells. Expression level of IFN-β, TLR3 **(C),** RIG-I **(D)**, and MDA5 **(E)** in BKT1 cells knocked down for TLR3, RIG-1, or MDA5 1 dpi of EMCV and JEV infection at MOI 1.0 (mean ± SD, n = 3). Differences between cells were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common symbol are significantly different ($p < 0.05$).

basal expression level of PRRs than other cell lines. The high basal expression level of PRRs (TLR3, RIG-I, and MDA5) has been observed in several bat species, including *R. leschenaultii, Rhinolophus affinis,* and *Desmodus rotundus* [19,27,28]. This high basal expression level of PRRs in the above three species of bats enables quick elimination of EMCV and JEV through stimulation of type I IFN production. Conversely, the FBKT1 cells derived from *P. dasymallus* showed a low basal expression of PRRs. This is consistent with previous study wherein the expression level of TLR3, RIG-I, and MDA5 in *P. alecto* kidney was very low compared to that in other tissues; however, the kidneys of *R. leschenaultii* and *R. affinis* had an expression level of TLR3, RIG-I and MDA5 comparable to other tissues [18,28–30]. There seems to be high species dependency of bats in innate immune system [19].

After EMCV and JEV infection, RIG-I and MDA5 were highly up-regulated in DEMKT1 and BKT1 cells (Fig. 2C–D), which possibly led to up-regulation of IFN-β to reach antiviral state (Fig. 2E), and consequently resulted in limited EMCV and JEV replication (Fig. 1A and B). Inability of FBKT1 and HEK293T cells to limit EMCV replication might be due to their inability to increase the expression of PRRs and IFN-β as high as that in DEMKT1 and BKT1 cells (Fig. 2B, C, 2D, and 2E). Similar to other flaviviruses, JEV also can antagonizes type I IFN production to evade antiviral immunity and benefit viral replication through suppression of RIG-I and MDA5 to inhibit type I IFN production [31,32]. The suppression of RIG-I and TLR3 observed in FBKT1 after JEV infection should have caused limited increase in IFN-β production (Fig. 2B, C, and 2E). In contrast, JEV failed to suppress the PRRs in BKT1, DEMKT1, and YUBFKT1 cells.

Knockdown of TLR3, RIG-I, and MDA5 resulted in decreased expression level of IFN-β, suggesting that these PRRs are important for stimulating IFN-β production in Rhinolophus bat cells (BKT1 cells). Increased replication level of EMCV and JEV after knockdown of PRRs indicates that TLR3, RIG-I, and MDA5 are responsible for suppressing EMCV and JEV replication in BKT1 cells through IFN-β production. IFN-β is predominantly induced by TLR3 after EMCV infection but comparable IFN-β expression level was shown in all cells knocked down for PRRs after JEV infection. In a previous study, knockdown of PRRs in *E. fuscus* kidney cells also showed that IFN-β is predominantly induced by TLR3 than RIG-I and MDA5 after poly(I:C) transfection [17]. Even though the TLR3 knockdown cells had the lowest IFN-β expression level, knockdown of TLR3 did not result in the highest EMCV replication level. Knockdown of MDA5 resulted in the highest EMCV and JEV replication level among all cells knocked down for PRRs (Fig. 4A and 4B). Previous studies have showed that MDA5 is the dominant mediator of type I IFN and cytokine response during EMCV infection in mice because of RIG I degradation by EMCV 3C protease [12,16]. MDA5 seemed to have a greater role in stimulating antiviral pathway in Rhinolophus bat cells because it was highly up-regulated as compared to other PRRs after EMCV infection of BKT1 cells and was the only up-regulated PRRs after JEV infection (Fig. 3B, C, and 3D). It is possible that MDA5 stimulates antiviral pathways other than IFN-β during EMCV and JEV infection of BKT1 cells.

In conclusion, TLR3, RIG-I, and MDA5 play an important role in antiviral response against EMCV and JEV infections, especially in Rhinolophus bats. Based on the results of this study and

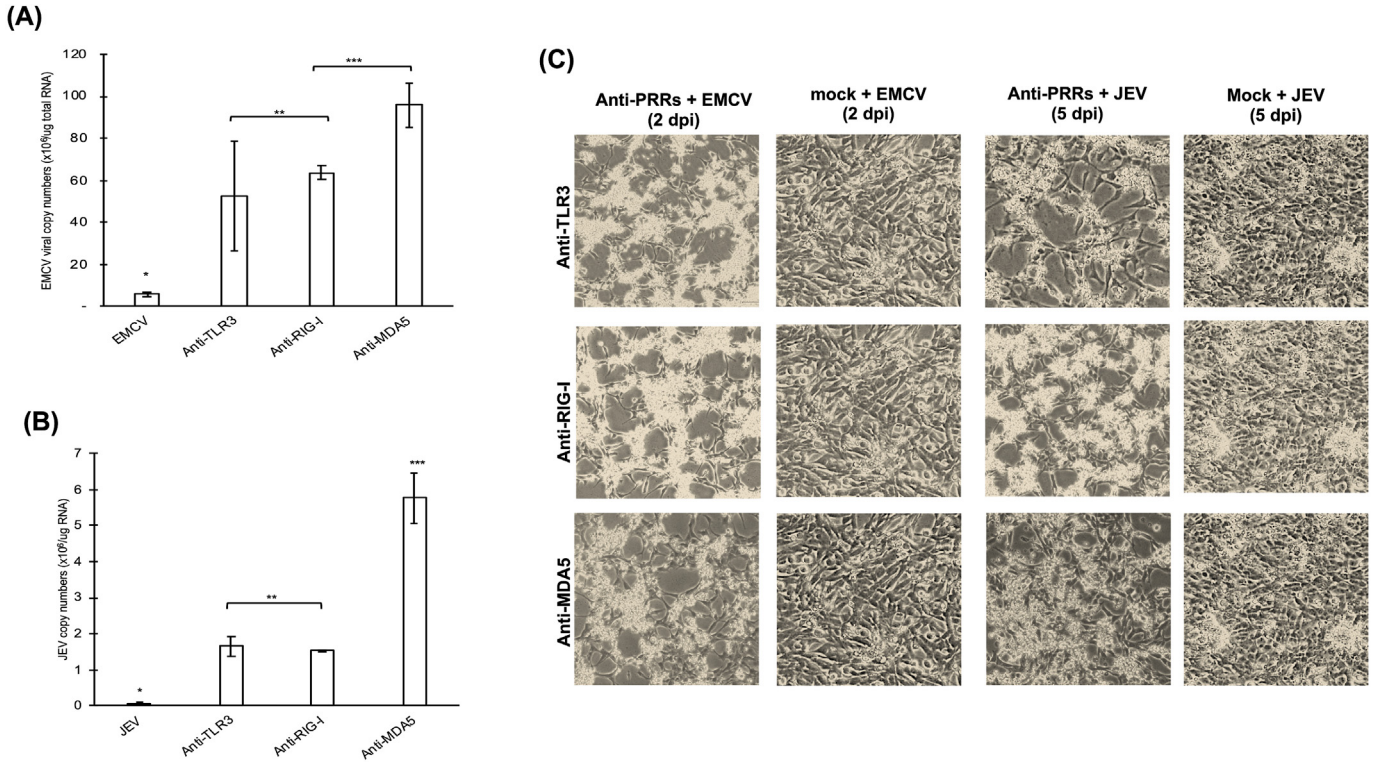

Fig. 4. Knockdown of TLR3, RIG-I, and MDA5 led to increased replication level of EMCV and JEV in BKT1 cells. EMCV **(A)** and JEV **(B)** viral copy numbers in TLR3, RIG-I, and MDA5 knockdown cells 1 dpi with MOI of 1.0. **(C)** CPE observed in TLR3, RIG-I, and MDA5 knockdown cells 2 dpi for EMCV and 5 dpi for JEV. Statistical significance was calculated using one-way ANOVA, followed by Tukey's test. Values that do not share a common symbol are significantly different ($p < 0.05$).

considering the wide diversity of bats worldwide (over 1100 species), the importance of PRRs in eliciting antiviral response might be variable among bat species [33].

Declaration of competing interest

The authors have no financial or commercial conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.04.060.

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