The expression of scavenger receptor B2 in enterovirus 71-infected mice

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Abstract

Objectives: Scavenger receptor class B, member 2 (SCARB2) participates in early innate immune responses to infection, so our aim was to explore the expression and role of mouse SCARB2 (mSCARB2) in different tissues in EV71-infected mice.

Methods: ICR mice were inoculated intraperitoneally (i.p.) with EV71 0.1 ml $10^{7.5}$ TCID₅₀/ml. The control mice were injected i.p. with the same volume RD cell lysate. Mice were sacrificed by aether anesthesia at day 4, 8 and 12 post infection (p.i.), their brain, brainstem, spinal cord, cerebellum, lung and heart were dissected out for determining the number of copies of viral RNA by quantitative real-time PCR (qRT-PCR), detection of expression of mSCARB2 by immunohistochemistry, qRT-PCR and Western-blotting. Cytokines quantification by ELISA.

Results: The viral loads in central nervous system (CNS) were higher than in lung or/and heart. The expression of mSCARB2 increased in tissues of EV71-infected mice, however, the levels of mSCARB2 increased in CNS were higher than in lung or/and heart within a certain period of time, particularly in brainstem and brain. In addition, local TNF- α , IL-6 and IL-1 β levels of production were consistent with mSCARB2 levels of expression in tissues of EV71-infected mice. However, it presented a positive correlation between relative mSCARB2 mRNA level and TNF- α , IL-6 and IL-1 β levels in local tissues at day 4 and 8 p.i..

Conclusions: Our data revealed that the elevated local mSCARB2 may modulate pro-inflammatory cytokines induction in local tissues, particularly, in CNS of EV71-infected mice.

Keywords: Enterovirus 71, mice, mSCARB2, pro-inflammatory cytokines

Introduction

Enterovirus 71 (EV71), a neurotropic virus with undefined pathogenesis, has caused significant morbidity and mortality throughout the world, especially in the Asia-Pacific region since it was first detected in 1969 in the United State [1,2], including Singapore [3,4], South Korea [5], Malaysia [6], Japan [7], Vietnam [8], and China [9,10]. EV71, together with coxsackievirus A 16 (CVA16) infections are generally associated with hand, foot and mouth disease (HFMD), but EV71 infection occasionally progress to severe neurological disease, including aseptic meningitis, poliomyelitis-like paralysis, and possibly fatal encephalitis in neonates, especially brainstem encephalitis associated with pulmonary edema and cardiac insufficiency were the primary manifestations in patients with neurologic involvement [11,12]. Numerous animal models have been developed to study the pathogenesis of EV71 infection using the mouse-adapted strain of EV71 [13,14], innate immunodeficient mice [15]. The EV71 BrCr strain was demonstrated to induce neurological manifestation of tremor, ataxia, and brain edema in cynomolgus monkeys [16]. Moreover, EV71 BrCr infected mice also developed limbs paralysis and encephalitis [17].

SCARB2 (also known as Lysosomal Integral Membrane Protein II, LIMP II, LGP85 or CD36b like-2) is composed of 478 amino

acids and belongs to the CD36 family, which includes CD36 and scavenger receptor B, member 1 (SR-B I and its splicing variant SR-B II [18,19]. SCARB2 is one of the most abundant proteins in the lysosomal membrane and participates in membrane transportation and the reorganization of the endosomal/lysosomal compartment [19-21]. SCARB2 shuttles between these compartments and the plasma membrane [19]. SCARB2 is a type III double-transmembrane protein with a large extracellular domain (when it is present at the cell surface) and short cytoplasmic domains at the amino- and carboxy-terminus [18]. SCARB2 is expressed in a variety of tissues, including neurons in the CNS. SCARB2 deficiency in mice causes ureteric pelvic junction obstruction, deafness, and peripheral neuropathy, and SCARB2 deficiency in humans causes action myoclonus-renal failure syndrome (AMRF) [22,23]. The role of SCARB2 appears to be connected to the TNF- α -dependent and early activation of *Listeria* macrophages through internal signals linking the regulation of late trafficking events with the onset of the innate *Listeria* immune response [24].

Animal models have been developed to detail the pathogenesis of EV71 infection. However, the majority of the research has been devoted to understanding the neurotropism and neuropathogenesis of EV71, whereas the immunopathogenesis

aspect of the viral infection has remained largely unknown. It was proposed that overwhelming virus replication combined with the induction of massive pro-inflammatory cytokines is responsible for the pathogenicity of EV71 [25-27]. Indeed, high levels of interleukin-1 β (IL-1 β), IL-6, IL-10, IL-13, gamma interferon (IFN- γ), and tumor necrosis factor alpha (TNF- α) in the serum and cerebral spinal fluid (CSF) from EV71-infected patients have been consistently reported [25,27,28]. In particular, CSF levels of IL-1 β , IL-6, and TNF- α were found significantly elevated in patients with pulmonary edema (PE) and encephalitis, demonstrating a strong correlation between pro-inflammatory cytokine production and clinical severity in EV71 infections [26,29], and in EV71-infected neonate mouse model sustained high levels of IL-6 [30]. SCARB2-deficient mice display a macrophage-related defect in *Listeria* innate immunity. They produce less acute phase pro-inflammatory cytokines/chemokines, MCP-1, TNF- α , and IL-6, but normal levels of IL-12, IL-10, and IFN- γ and 25-fold increase in susceptibility to *Listeria* infection [24].

In this study, we assessed the expression of SCARB2 and the production of pro-inflammatory cytokine during EV71 infection in the neonatal mouse. Our results indicate that EV71 infection leads to the expression of SCARB2 increased in different tissues, which correlated with the local elevated levels of pro-inflammatory cytokine induction, especially in CNS.

Materials and methods

Cells and viruses

Human Rhabdomyosarcoma (RD) cells (purchased from the Chinese Academy of Sciences Cell Bank, Shanghai, China.) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10-15% fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 100 IU of penicillin, and 100 μ g of streptomycin/ml at 37°C, 5%CO₂. Non-mouse-adapted EV71 strain BrCr (a kind gift from Institute of Medical Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Kunming, China.) was propagated in RD cells. Once the cells displayed cytopathic effect (CPE), they were harvested, and cellular debris was removed by centrifugation at 10,000 $\times$ g for 30 min. To prepare virus stocks, virus were propagated for one more passage in RD cells. The virus was purified by Amicon® Ultra 100 K device (Millipore) at 4,000 $\times$ g for 40 min. The 50% tissue culture infective dose (TCID₅₀) was determined in RD cells using the Reed and Muench formula [31], and working virus stocks at 10^{7.5}TCID₅₀ per ml.

Animals and treatments

ICR mice (purchased from Laboratory Animal & Animal Experiment Center, Qingdao, China). They were housed under specific-pathogen-free conditions, housing temperature at 23°C. All institution guidelines for animal care and use were strictly followed throughout the experiments. One-day-old ICR mice were inoculated i.p. with EV71 0.1 ml 10^{7.5}TCID₅₀/ml. The control mice were injected i.p. with the same volume RD

cell lysate and kept in separate cages. Their weight gain or loss and clinical signs, including ruffled fur, hunchback, wasting, limb weakness, limb paralysis, twitch, moribund and death were monitored daily up to 14 days after inoculation. The clinical score was graded as follows: 0, healthy; 1, weakness in hind limbs; 2, paralysis in a single limb; 3, paralysis in more than two limbs; 4, death [32]. In addition, mice per group were sacrificed by aether anesthesia at day 4, 8, and 12 post infection, respectively. After perfusion with PBS containing EDTA, their brain, cerebellum, brainstem, spinal cord, heart and lung were immediately dissected out for the extraction of RNA, for the extraction of protein or for immunohistochemical examinations, respectively. The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Science of Chinese Academy of Medical Sciences.

Virus detection in mice

For this study, quantitative real-time PCR (qRT-PCR) was used to determine the number of copies of viral RNA present in detected tissues. Total RNA was extracted from individual brain, cerebellum, brainstem, spinal cord, heart and lung using an RNAiso Plus Kit (Takara, Dalian, China) according to manufacturers' instruction. Next, total RNA was reverse-transcribed with random hexamers using a Reverse Transcription kit (Thermo Scientific). The cDNA was subjected to quantitative PCR in a 50- μ l reaction mixture (Thermo Scientific DyNAmo SYBR Green qRT-PCR Kit) with primers of EV71-S (5'-GCAGCCCCAAAGAAGCTTCAC-3') and EV71-A (5'-ATTTTCAGCAGCTTGGAGTGC-3') for EV71/BrCr of nucleotides 2372-2598 [14,33], and the conditions consisted of a denaturation step at 95°C for 15 min and 40 cycles of thermal cycling of 95°C for 10 s and 60°C for 60 s. The EV71 virus fragment of nucleotides 2372-2598 was used as real-time PCR standard by adjusting to a concentration gradient of 1 $\times$ 10⁷ copies/ μ l, 1 $\times$ 10⁶ copies/ μ l, 1 $\times$ 10⁵/ μ l, and 1 $\times$ 10⁴ copies/ μ l, and the DNA fragment with known copies was used as standard to calculate the copy number of virus RNA in the infected tissues. Quantitative real-time RT-PCR was performed using the Mxpro-Mx3000P system.

Immunohistochemical staining

The tissues from sacrificed mice were rinsed in 10% buffered formalin and then embedded in paraffin. Four micrometer sections were slid (Leica RM 2235) and placed on poly-L-lysine-coated glass slides before fixing with 3.7% paraformaldehyde. The sections were blocked by endogenous peroxidase for 10 min, nonspecific protein binding sites were also blocked for 10 min. The sections were incubated with mSCARB2 antibody (Abcam® *discover more*) 1:100 for 1 h, and then were incubated with secondary antibody IgG-Biotin and Streptavidin-HRP (Streptavidin-HRP Kit, CWbio.Co.Ltd, Beijing, China) for 10 min at room temperature, respectively. A red to brown peroxidase stain was developed using the

DAB Chromogenic Reagent kit (CWbio.Co.Ltd, Beijing, China), and the sections were examined with a light microscope after counterstaining with hematoxylin.

Detection of mSCARB2 gene expression

To examine mSCARB2 expression, total RNA from different tissues of EV71-infected mice and controls using an RNAiso Plus Kit (Takara, Dalian, China) following the manufacturer instructions were isolated. Total RNA was converted into cDNA by the reaction of reverse transcription (RT) using a Reverse Transcription kit (Thermo Scientific). The cDNA was subjected to quantitative PCR (Thermo Scientific DyNamo SYBR Green qRT-PCR Kit) with a Rotor-Gene RG-3000 System. The primers were mSCARB2-L1 (5'-TCTGCTGTCAACCAATAAGGC-3') and mSCARB2-R1 (5'-CCAGATCCACGACAGTCAAC-3'). The conditions consisted of a denaturation step at 95°C for 15 min and 40 cycles of thermal cycling of 95°C for 10 s and 60°C for 60 s. The GAPDH was used as an internal control. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ as described previously [34]. Each sample was run in triplicate.

Western blot analysis

For Western blot analysis of mSCARB2 in the various tissues, each sample was homogenized in ice-cold tissue extraction buffer (Invitrogen, Carlsbad, CA) containing 1% protease inhibitor cocktail. The homogenates were centrifuged at 11000×g for 30 min at 4°C. The BCA protein assay kit (PIERCE, UK) was used to assay the total protein of each sample. Samples with equal protein concentrations, were loaded onto an 8% SDS-PAGE. After electrophoresis, the proteins in the gels were transferred electrophoretically onto polyvinylidene fluoride membranes. Excess sites on the membrane were blocked by incubation for 2 h at room temperature, with 3% (wt/vol) nonfat dried skimmed milk in 20 mM Tris-HCl, PH 7.5, and 150 mM NaCl (Tris-buffered saline [TBS]). After a single washing with TBS, the membranes were incubated with anti-mSCARB2 antibodies (Abcam® *discover more*) 1:100 in TBS/0.05% Tween 20 by incubation overnight at 4°C. The membranes were then washed three times with TBS/0.05% Tween 20, and incubated with 0.3 µg/ml horseradish-peroxidase-labeled anti-rabbit IgG (Biosource) in 3% (wt/vol) nonfat dried skimmed milk in TBS for 1 h, at room temperature. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (ZSJQ Corp., Beijing, China) according to the manufacturer's instruction. The amounts of mSCARB2 proteins were expressed relative to those of the amount of GAPDH.

Cytokine quantification

Various tissues were harvested from sacrificed animals at indicated time point, weighed, and homogenized in 500 µl of 1×phosphate-buffered saline (PBS) immediately. The homogenates were centrifuged at 13,000×g for 10 min at 4°C, and the supernatant was collected and stored frozen at -80°C until further analysis. The levels of cytokines were

measured using a Solid Phase Sandwich ELISA kits (Mouse TNF-α, IL-6 and IL-1β Quantikine, R&D Systems), and following the manufacturer's instructions. Sensitivities of the TNF-α, IL-6 and IL-1β assays according to manufacturer protocol were 7.21 pg/ml, 1.8 pg/ml and 4.8 pg/ml, respectively. Intraassay and interassay coefficients of variation were: TNF-α: 3.9% and 6.2%; IL-6: 3.9% and 8.9%; IL-1β: 4.6% and 6.6%.

Statistical analysis

All statistical analyses were done with GraphPad Prism, version 5.0 (GraphPad 4 Software, San Diego, CA), for Mac. Kaplan-Meier survival curves were analyzed by a log rank test. Clinical score curves were analyzed by the Kruskal Wallis test. Other experiments were analyzed by Student's *t* test or by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Pearson's correlation was used to analyze the relation between pro-inflammatory cytokines and mSCARB2. A P-value of <0.05 was considered as statistically significant.

Results

EV71 Infection in mice

The mice infected with virus were monitored daily for 14 days after inoculation with virus. In this study, infected mice developed severe symptoms. Fatigue in the hind limbs occurred at day 1-2 p.i., followed by paralysis in a single limb or/and paralysis in more than two limbs at days 3-7 p.i., or showed other signs of encephalitis such as hunched posture, lethargy, or ataxia, and death occurred at 2-7 p.i.. The healthy mice in the cell lysate control group was no a single mouse dead. Among the observed three groups (A, B, C), the survival curves were no significantly different (**Figure 1A**). But 7-8 days later, the survivors' symptoms gradually restored. In the three groups (A, B, C), the clinical scores were no significantly different (**Figure 1B**). However, their body weights appeared to grow slowly.

EV71 Strain BrCr Displays Neurotropism in ICR mice

At day 1 p.i., viral RNA were only detected in spinal cord, but were not detected in brainstem, cerebellum, brain, heart and lung. The number of copies of EV71 RNA detected at day 4 p.i. were in lung ($3.99 \pm 0.13 \log_{10}$ copies/mg tissue), heart ($3.11 \pm 0.12 \log_{10}$ copies/mg tissue), brain ($5.31 \pm 0.30 \log_{10}$ copies/mg tissue), brainstem ($6.17 \pm 0.18 \log_{10}$ copies/mg tissue), spinal cord ($5.59 \pm 0.12 \log_{10}$ copies/mg tissue), and cerebellum ($4.51 \pm 0.26 \log_{10}$ copies/mg tissue), however, the virus was gradually eliminated (**Figures 2A-2F**). A histopathological examination of the infected mice in different time was carried out. Marked lesions and/or obvious signs of inflammation were observed for the brain, brainstem, spinal cord, cerebellum, but heart and lung showed less lesions and/or signs of inflammation (data not shown).

Different Tissues of EV71-infected Mice Express mSCARB2
Expectedly, mSCARB2 immuno-reactivity was not only

Figure 1. One-day-old ICR Mice Were Inoculated i.p. with EV71. The survival rates (A) and clinical scores (B) of the infected mice (a group, n=6) were monitored over a 14-day period. Results are representative of 3 independent experiments.

observed in lung, heart, brain, brainstem, spinal cord and cerebellum cells, but also the obvious immuno-reactivity was observed at day 4 p.i. compared to controls, and gradually decreased in later days (Figure 3). These results suggested that expression of mSCARB2 increased in these tissues after mice with EV71 infection.

In this study, we found that the mSCARB2 mRNA levels elevated in all selected tissues at day 4 p.i., but the mSCARB2 mRNA levels were higher in brainstem ($P<0.001$), brain ($P<0.01$), spinal cord ($P<0.01$) and cerebellum ($P<0.05$) than in lung or/and heart. However, at day 8 p.i., the mSCARB2 mRNA levels obviously decreased, still in brainstem ($P<0.001$), brain ($P<0.05$) were higher compared to lung or/and heart. At day 12 p.i., only the mSCARB2 mRNA level in brainstem were observed higher than in lung or/and heart ($P<0.05$) (Figure 4A). Figure 4A also showed that the mSCARB2 mRNA levels in

Figure 2. Virus Loads in Tissues from ICR Mice Infected with EV71 via The i.p. Route. One-day-old mice were inoculated i.p. with EV71. At day 2, 4, 6, 8 and 12 p.i., animals (n=6) were euthanized, and virus titers in the lung (A), heart (B), brain (C), brainstem (D), spinal cord (E), and cerebellum (F) were determined by qRT-PCR. Results are expressed as Log viral RNA copies per milligram of tissue and values are means±SEM of triplicate experiments.

brainstem and brain were higher than in spinal cord and cerebellum. These results suggest that the expression of mSCARB2 increased obviously in CNS of EV71-infected mice, especially, in brainstem and brain (Figure 4A).

The expression of mSCARB2 protein showed moderate signals (~70~85 KDa band) at day 4 p.i. in brainstem, brain, spinal cord, cerebellum, lung and heart, weaken band at day 8 and 12 p.i., and controls. These results further conformed the mSCARB2 gene expression tested by qRT-PCR. The protein expression of mSCARB2 had the similar trend with gene expression (Figures 4B-4E).

Figure 4. Detection of mSCARB2 in Different Tissues by qRT-PCR and Western blot. One-day-old ICR mice were inoculated i.p. with EV71. (A) mSCARB2 gene expression, the animals (n=6, at each time points) were sacrificed at day 4, 8 and 12 p.i.. Values are means $\pm$ SEM of triplicate experiments. The results were normalized to GAPDH. *** p<0.001, ** p<0.01, * p<0.05 vs. lung or/and heart. (B) mSCARB2 protein expression in controls. (C) mSCARB2 protein expression at day 4 p.i.. (D) mSCARB2 protein expression at day 8 p.i.. (E) mSCARB2 protein expression at day 12 p.i.. 1, Lung. 2, Brainstem. 3, Spinal cord. 4, Cerebellum. 5, Brain. 6, Heart.

Local Levels of Pro-inflammatory cytokines were elevated in EV71-infected mice

Enhanced cytokine production has been proposed to contribute to EV71 pathogenesis in both humans and mice [26,28,30]. Local TNF- α , IL-6 and IL-1 β levels were significantly higher in the various tissues homogenates prepared from EV71-infected animals at day 4 p.i. than in those from age-matched noninfected controls. Meanwhile, TNF- α , IL-6 and IL-1 β levels were significantly higher in CNS (brain, brainstem, spinal cord and cerebellum) than in lung or/and heart (Figures 5A-5C). At day 8 p.i., these pro-inflammatory cytokines levels decreased in all tested tissues, but still higher in CNS than in lung or/and heart (Figures 5A-5C), and at day 12 p.i., these pro-inflammatory cytokines further declined, however, IL-6 and IL-1 β levels in brainstem and brain presented higher compared to in lung or/and heart (Figures 5A-5C).

In this study, we found that local mSCARB2 expression were consistent with TNF- α , IL-6 and IL-1 β production in the brain, brainstem, spinal cord, cerebellum, lung and heart

from EV71-infected mice. Surprisingly, it presented a positive correlation between relative mSCARB2 mRNA level and TNF- α , IL-6 and IL-1 β levels in local tissues at day 4 p.i. and at day 8 p.i., till at day 12 p.i., it showed no correlation (Table 1). These results suggested that the elevated pro-inflammatory cytokines in a certain range in local tissues induced higher expression of mSCARB2.

Discussion

We have demonstrated that one-day-old ICR mice were infected by the EV71 BrCr strain *in vivo*, and we used these models to assess the expression of mSCARB2 in CNS, lung and heart. The survival rates and clinical scores of infected mice were used to measure clinical symptoms or activities. After infection with EV71, virus was detected within various tissues by qRT-PCR. Our results indicated that one-day-old ICR mice are susceptible to EV71 infection and develop into CNS infection, as observed for humans. Upon infection via the peritoneal route, ICR mice consistently displayed hunchback,

Figure 5. TNF- α , IL-6 and IL-1 β Levels in Different Tissues from EV71-infected Mouse. One-day-old ICR mice were inoculated i.p. with EV71. At day 4, 8, and 12 p.i., six mice per time point were sacrificed, and various tissues were harvested and homogenized. The levels of TNF- α (A), IL-6 (B) and IL-1 β (C) in different tissues homogenates from EV71-infected animals and controls were quantified individually by ELISA. The results are expressed as the means $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. lung or/and heart. Data are representative of three independent experiments.

Table 1. Correlations of local levels of pro-inflammatory cytokines and expression of mSCARB2 in EV71-infected mice.

Time	Cytokines/ tissues	mSCARB2 Pearson correlation/Significance (two-tailed)					
		Brainstem	Brain	Spinal cord	Cerebellum	Heart	Lung
Day 4 p.i.	TNF- α (pg/mL)	0.933 0.007	0.829 0.041	0.899 0.015	0.825 0.043	0.989 <0.01 ^a	0.913 0.011
	IL-6 (pg/mL)	0.958 0.003	0.693 0.127	0.861 0.028	0.903 0.014	0.989 <0.01 ^a	0.920 0.009
	IL-1 β (pg/mL)	0.955 0.003	0.830 0.041	0.918 0.010	0.910 0.012	0.959 0.003	0.920 0.009
Day 8 p.i.	TNF- α (pg/mL)	0.983 <0.01 ^a	0.839 0.037	0.915 0.011	0.938 0.006	0.973 0.001	0.962 0.002
	IL-6 (pg/mL)	0.968 0.002	0.820 0.046	0.981 0.001	0.960 0.002	0.982 <0.01 ^a	0.922 0.009
	IL-1 β (pg/mL)	0.978 0.001	0.828 0.042	0.980 0.001	0.959 0.002	0.992 <0.01 ^a	0.935 0.006
Day 12 p.i.	TNF- α (pg/mL)	-0.518 0.293	0.396 0.473	0.665 0.149	-0.154 0.771	0.586 0.222	-0.501 0.311
	IL-6 (pg/mL)	-0.784 0.065	-0.625 0.184	-0.623 0.187	-0.702 0.120	-0.583 0.224	-0.459 0.359
	IL-1 β (pg/mL)	-0.580 0.227	-0.726 0.102	-0.493 0.323	-0.684 0.134	-0.423 0.403	-0.641 0.170

^a Correlation is significant at the 0.01 level (2-tailed)

limb weakness, and limb paralysis prior to death. Similar to human manifestations of EV71 encephalomyelitis [11], the virus exhibited a strong tropism for the CNS of ICR mice, with the numbers of viral RNA copies in CNS (brainstem, brain, spinal cord and cerebellum) were higher than in lung or/and heart, especially, in brainstem and brain higher than other tissues coinciding with the severity or even death of the animals. In addition, all sick mice exhibited massive neuronal damage, increased levels of cytokines, as reported previously for severe cases of human EV71 disease [35].

In this study, we found that the expression of mSCARB2 moderately increased in CNS, lung and heart in EV71 (BrCr)-infected mice, and the expression of mSCARB2 was higher in CNS than in lung or/and heart at day 4 p.i., especially, in brainstem and brain, and at day 8 and 12, the expression of mSCARB2 decreased. The TNF- α , IL-6 and IL-1 β production significantly increased in the CNS of EV71 infected mice in comparison with lung and heart at day 4 p.i.. At day 8 and 12 p.i., the levels of TNF- α , IL-6 and IL-1 β production decreased. Interestingly, the expression of mSCARB2 in various tissues of EV71-infected mice has the similar trend to the production of TNF- α , IL-6 and IL-1 β . Surprisingly, our data revealed a positive correlation between relative mSCARB2 mRNA level and TNF- α , IL-6 and IL-1 β levels in local tissues at day 4 p.i. and at day 8 p.i., but at day 12 p.i., it showed no correlation.

Carrasco-Marín E et al., presented evidence for the specific role of LIMP-2/SCARB2 in the innate immune response to *Listeria monocytogenes* and in phagocytosis. LIMP-2 tightly controls the number of cytosolic LM and the induction of acute phase pro-inflammatory cytokines such as MCP-1, TNF- α , and IL-6. However, the production of late pro-inflammatory

cytokines, such as INF- γ and IL10, was not regulated by LIMP-2/SCARB2 [24]. In infection, two cytokines involved in macrophages (M ϕ) activation: TNF- α and INF- γ . TNF- α acts as an early signal in innate immunity, INF- γ is a late signal. It has been claimed that exogenous action of TNF- α promotes an early activating state in M ϕ s that triggers the cytosolic microbicidal mechanisms [36-38]. In EV71 infection, SCARB2 may also participates in exogenous M ϕ activation, the early signals modulated by TNF- α .

Taken together, we assumed that in EV71 infected mice, the elevated local mSCARB2 may regulate the early innate immune response to EV71, or even modulate pro-inflammatory cytokines induction; mSCARB2 may also act as the invasive receptor for the enterovirus 71 although no experimental evidence has ever been provided support this hypotheses, because human SCARB2 (hSCARB2) have been identified as cellular receptors for EV71, and mSCARB2 exhibited 85.8% amino acid identity and 99.9% similarity to hSCARB2 [39,40]; the elevated expression of mSCARB2 in EV71-infected mice may play other roles, which are not clear now.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	JL	ZC
Research concept and design	✓	✓
Collection and/or assembly of data	✓	--
Data analysis and interpretation	✓	✓
Writing the article	✓	--
Critical revision of the article	✓	✓
Final approval of article	--	✓
Statistical analysis	✓	--

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