Biochemical and Biophysical Research Communications 447 (2014) 550–555

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential interferon pathway gene expression patterns in Rhabdomyosarcoma cells during Enterovirus 71 or Coxsackievirus A16 infection

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ARTICLE INFO

Article history:
Received 18 March 2014
Available online 13 April 2014

Keywords:
Enterovirus 71
Coxsackievirus A16
PCR array
JAK-STAT pathway
Interferon-stimulated genes

ABSTRACT

Exposure of cells to type I interferon (IFN) induces an antiviral state that prevents viral infection, but viruses can utilize multiple tactics to antagonize the host immune system. Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16) are two major pathogens that cause hand, foot, and mouth disease (HFMD), which is prevalent among children. We found that both EV71 and CA16 have different reactions to type I IFN pretreatment and induction patterns of type I IFN on Rhabdomyosarcoma (RD) cells. Further, a human-α and β IFN PCR array was employed to analyze the expressions of 84 genes related to the type I IFN pathway. We found significant up-regulation of multiple genes in the presence of type I IFN and differential regulation patterns during EV71 or CA16 infection in RD cells. For instance, EV71 infection repressed the JAK-STAT signaling pathway and interferon-stimulated gene (ISG) expression, whereas CA16 infection normally triggers the JAK-STAT pathway, leading to the expression of ISGs. Taken together, this study provides a comprehensive view of the differential impacts of EV71 and CA16 infection on 84 genes in the IFN pathway, shedding light on the different resistances of these viruses to type I IFN treatment and cytotoxic effects in RD cells.

1. Introduction

Hand, foot, and mouth disease (HFMD) is a serious epidemic disease that is mainly prevalent in the Asia Pacific region in children. The dominant causative agents are the nonenveloped viruses Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16), which belong to the *Picornaviridae* family [1,2]. The viral RNA genomes of EV71 and CA16 are both single positive strand RNAs about 7500 nt in length that are translated into four structural (VP1–VP4) and seven non-structural (2A–2C and 3A–3D) proteins. Both viruses utilize a cell lysis mechanism to release progeny virions for further infection [3,4]. HFMD due to EV71 and CA16 usually causes mild and self-limited infection. However, EV71 infection has a propensity to cause severe neurological disease, especially in acute infection cases, which is rarely observed in CA16 infections [5,6]. Molecular studies of the evolution of human enteroviruses show that EV71 and CA16 share a close genetic relationship, and the functions of their non-structural and structural proteins are relatively similar and conservative [5]. However, no effective vaccine or antiviral drug is yet available for HFMD [7].

Type I interferons (IFNs) are widely expressed cytokines that possess potent antiviral, antiproliferative, and immunomodulatory effects. These cytokines are the first line of defense against viral infections in cells [8]. As secreted factors, type I IFNs perform antiviral functions by binding to IFNAR, which results in the activation of Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) tyrosine phosphorylation, which in turn activates the signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2). The activated STAT complex translocates into the nucleus and binds to DNA regulatory sequences containing IFN-stimulated response elements to stimulate the transcription of hundreds of ISGs, leading to the generation of an antiviral response by the cell [9,10]. In contrast, viruses (e.g., Influenza A Virus, Hepatitis C Virus, Dengue Virus, and Respiratory syncytial virus) have adapted multiple strategies to evade specific host defense mechanisms induced by type I IFNs to establish infections [11–14]. EV71 also has the ability to antagonize the IFN pathway in multiple ways [15–17].

Abbreviations: HFMD, hand, foot, and mouth disease; IFN, interferon; EV71, Enterovirus 71; CA16, Coxsackievirus A16; CPE, cytopathic effect; moi, multiplicity of infection; ISGs, IFN-stimulated genes; IRF, interferon regulatory factor; RT-qPCR, real time quantitative polymerase chain reaction; WB, Western blotting.

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http://dx.doi.org/10.1016/j.bbrc.2014.04.021
0006-291X/

However, little is known about how CA16 infection influences the IFN pathway. Thus, it is important to comprehensively explore the interaction of these two related viruses with the type I IFN pathway.

To elucidate the molecular basis of the host IFN pathway response to EV71 and CA16 infection, an IFN-α and β response PCR array was used to detect the impact of EV71 and CA16 infection on 84 genes associated with the IFN pathway, as well as to identify ISGs responsible for the potential anti-viral effect(s) regulated by virus infection.

2. Materials and methods

2.1. Cells and virus propagation

Human Rhabdomyosarcoma (RD) cells were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. The EV71 (BrCr Shenzhen) and CVA16 (Shenzhen05-1) strains were propagated on RD cells for 48 h, and supernatants were collected and filtered through a 0.22-μm filter. Viral titers were measured by absolute real time quantitative PCR (RT-qPCR) quantification combined with cytopathic effect (CPE) assays and expressed as the 50% tissue culture infective dose (TCID50/mL).

2.2. Reagents and antibodies

Rabbit anti-STAT1, STAT2, phosphorylated STAT1 (p-STAT1) and phosphorylated STAT2 (p-STAT2) were purchased from Cell Signaling Technology. Rabbit anti-IFNAR1 and IFNAR2 were purchased from Abcam. Rabbit anti-IFITM1/2/3, BST2, and ISG15 antibodies were purchased from Proteintech. Rabbit anti-GAPDH antibody was purchased from Sigma. IgG secondary horseradish peroxidase (HRP) conjugated anti-rabbit antibody was obtained from Santa Cruz Biotechnology. T4 DNA ligase and the pMD-19T cloning vector were purchased from TaKaRa. Bovine serum albumin (BSA) was obtained from Becton, Dickinson and Company.

2.3. RNA extraction and RT-qPCR

Cells were collected for RNA extraction using Trizol reagent (Life Technologies), and reverse transcription was performed using a GoScript Reverse Transcriptase Kit (Promega). RT-qPCRs were conducted using the Two-Step SYBR-Green method in an ABI 7300 real-time PCR system with the following amplification profile: one cycle at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The target fragment of the 5′ untranslated regions (UTR) was cloned into pMD-19T to generate pMD-19T-5UTR, which was serially diluted 10-fold serial in distilled water as a standard, and EV71 or CA16 virus RNA levels were assessed by absolute RT-qPCR. The mRNA expression levels of IFN-α and IFN-β were measured by relative RT-qPCR. The *gapdh* gene was used as a control. The primers used in this study are listed in Table 1.

2.4. MTS viability assay for virus CPE

To assess the cytotoxic effects of virus infection on RD cells, MTS assays were performed (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega). Briefly, cells were infected with EV71 or CA16 with multiplicity of infection (moi) of 0.1 for 6, 12, 24, 32 and 48 h and then incubated with the MTS labeling reagent for 4 h. The absorbance of soluble formazan produced by cellular reduction of MTS was measured at 490 nm using a 96-well plate reader.

2.5. Evaluation of IFN treatment and virus infection by PCR array

Confluent RD cells were divided into six groups (type I IFN-treated group, EV71-infected group, CA16-infected group, IFN pretreated and EV71-infected group, IFN pretreated and CA16-infected group, and the mock control group) and differentially treated with IFN-α2b and IFN-β (500 IU/mL) for 24 h, with or without EV71 or CA16 infection (moi = 0.1) for another 48 h. Cells without IFN addition and virus infection were used as mock controls. Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen) combined with potential genomic DNA elimination (RNase-Free DNase, Qiagen). RNA purity and concentration were measured using a NanoDrop ND-1000 (Thermo Scientific). Reverse transcription of 1 μg of total RNA was performed using an RT^2 First Strand Kit (Qiagen). Then, a Human IFN-α, β Response RT^2 profiler PCR array (SuperArray Bioscience Corporation, Qiagen) was probed on an ABI 7300 system (Applied Biosystems, CA) to analyze the relative expression of 84 genes involved in the IFN pathway. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with online SABiosciences Data Analysis Software. All tested gene expression was normalized to the expression of an internal control gene. The fold changes caused by various treatments were mutually compared as described in Table 1 in the Supplemental material.

2.6. Protein extraction and Western blotting (WB) analysis

Proteins were extracted from cells using RIPA lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 0.5% Triton X-100 and Complete Protease Inhibitor Cocktail Tablets, Roche) on ice for 30 min. Supernatants were collected after centrifugation and mixed with 5× SDS loading buffer. After boiling for 10 min, samples were separated on 12% polyacrylamide gels and transferred onto PVDF membranes (GE) for WB analysis. Membranes were incubated with 5% skim milk for 1 h at room temperature and incubated with the indicated primary antibody at different dilutions at 4 °C overnight. After three washes, membranes were incubated with 1:5000 HRP-conjugated secondary antibody for 1 h at room temperature. GAPDH was used as a control. Signals on the membranes were detected by the ELC (Millipore) method.

2.7. Statistical analysis

For statistical significance tests, data were compared using Student's *t*-test. Differences in virus titers among the groups are

Table 1
Primers sequence used in this study.

Gene	Forward primer	Reverse primer
Virus 5′-UTR	5′ CCGTGTAGATCAGGTCGATGAG 3′	5′ GGGCCGGAGGACTACTAACTAG 3′
Human IFN-α	5′ GAAATACAGCCCTTGTGCCTG 3′	5′ GAAAGCGTGACCTGGTGTATGA 3′
Human IFN-β	5′ CTGCATTACCTGAAGGCCAAG 3′	5′ GAAGCAATTGTCCAGTCCCAG 3′

expressed as the means ± standard deviations. Values were considered significantly different at $P < 0.05$.

3. Results

3.1. EV71 and CA16 infection induces the CPE and IFN production in RD cells

To investigate the impact of EV71 and CA16 infection on RD cells, cell viability and type I IFN production were measured. The viability of RD cells after EV71 or CA16 infection did not exhibit obvious differences at 6 h post-infection (p.i.). At 12 h p.i., the viable cell count slightly decreased in EV71-infected cells, but CA16 infection had no significant viability effect. When virus infection was extended to 24 and 32 h, the RD cells displayed a distinct viability decrease in the EV71-infected group, and CA16 infection also induced a loss in viability though less than that caused by EV71. At 48 h p.i., cell viability declined even further in both the EV71- and CA16-infected groups (Fig. 1A).

Next, the level of IFN-α and IFN-β production stimulated by EV71 and CA16 infection was measured by RT-qPCR at the same time points examined above. Neither virus caused an obvious difference of IFN-α and IFN-β expression at 6 h p.i. From 12 to 32 h p.i., IFN-α and IFN-β levels began rising in both EV71- and CA16-infected cells. The IFN mRNA levels stimulated by EV71 were relatively lower than those stimulated by CA16 at the same time-points. EV71 infection tended to stimulate the production of IFN-β, whereas IFN-α expression was more enhanced in CA16-infected cells (Fig. 1B and C). RT-qPCR detection of EV71 and CA16 viral loads revealed that EV71 viral RNA levels were higher than CA16 (Fig. 1D). These results demonstrate that both EV71 and CA16 infection produced the CPE in RD cells. EV71 infection has more robustly cytotoxic, with a higher replication rate than CA16, which is consistent with the relatively weak type I IFN stimulation caused by EV71 in RD cells.

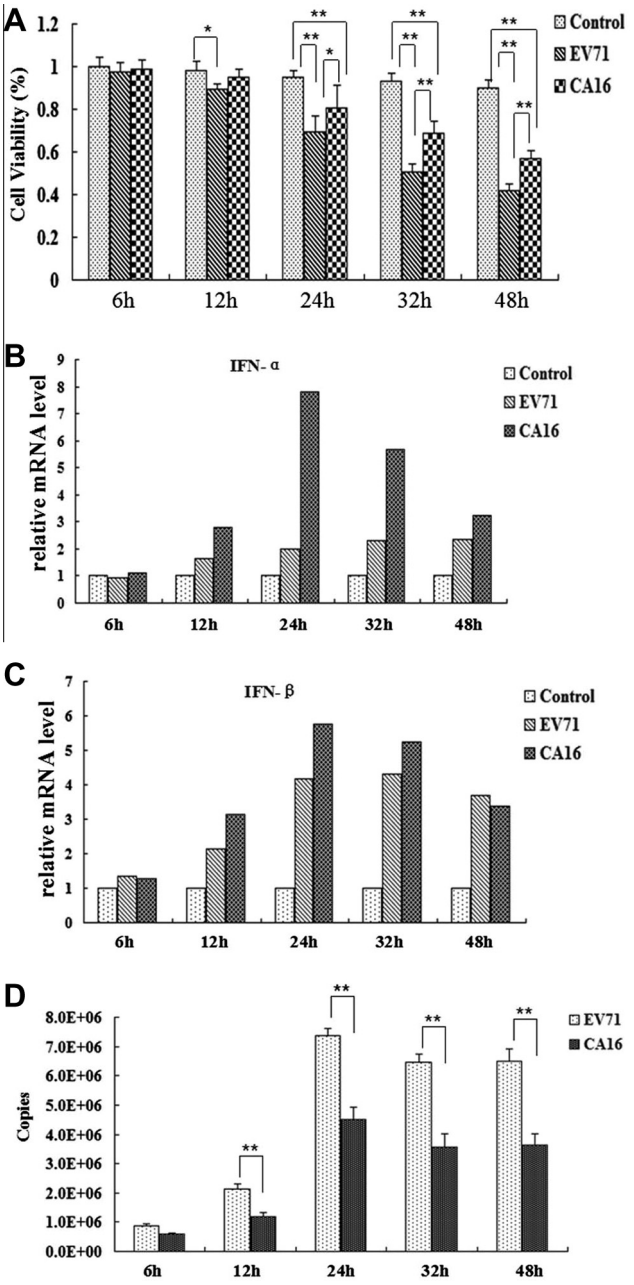

Fig. 1. RD cell viability and type I IFN production upon EV71 or CA16 infection. RD cells were infected with EV71 or CA16 with a MOI = 0.1 for 6, 12, 24, 36, and 48 h. Cell viability was assessed by MTS assay (A), and the expression of IFN-α (B) or IFN-β (C) at different time points was measured by relative RT-qPCR. Gapdh was use as control to normalize the expression of each gene. Cellular viral loads were quantified by absolute RT-qPCR at the indicated time point p.i. Uninfected cells were used as controls (D). $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test, $n = 3$).

3.2. Type I IFN treatment inhibits EV71 and CA16 replication

Nest, the effects of type I IFNs on EV71 and CA16 replication were explored. As shown in Fig. 2A and B, both low doses of IFN-α2b and IFN-β did not significantly affect intracellular EV71 replication, except high levels of IFN-α2b (2000 IU/ml) and IFN-β (1000 and 2000 IU/ml), which displayed an obvious repressive effect on EV71 in RD cells. However, CA16 was more sensitive to IFN

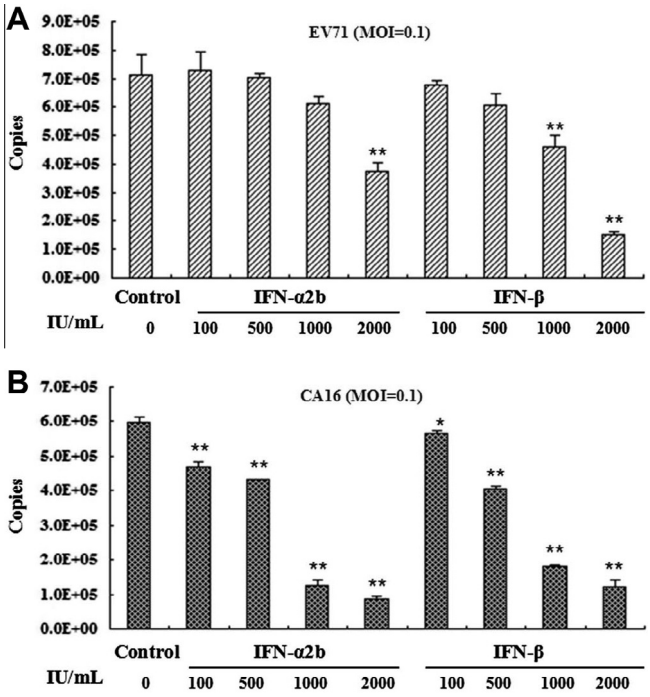

Fig. 2. Different inhibitory patterns of type I IFN treatment on EV71 and CA16 replication. RD cells were pretreated with increasing concentrations of IFN-α2b or IFN-β (100, 500, 1000, or 2000 IU/mL) for 24 h followed by EV71 or CA16 (MOI = 0.1) infection for 48 h. Cells were collected for RNA extraction, and EV71 (A) and CA16 (B) RNA levels were measured using absolute quantitative RT-qPCR. Cells without type I IFN addition were used as controls. Copies represent the load of virus in 100 μg RNA. $^{*}P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test, $n = 3$).

pretreatments, with IFN-α2b (100 IU/mL) and IFN-β (500 IU/mL) displaying significant repression effects. A more obvious inhibitory effect was observed with high doses of IFNs on CA16 infection. However, it should be noted that the viral load of EV71 was approximately 2–4folds higher than CA16 under the same IFN treatments as demonstrated by RT-qPCR analysis. These results indicate that the replication of EV71 was more resistant than CA16 to type I IFN pretreatment, which is consistent with the different replication rates and CPEs displayed by EV71 and CA16 in RD cells.

3.3. Different reactions of EV71 and CA16 to the Type I IFN pathway

Based on previous results, a human IFN alpha, beta response RT^2 profiler PCR microarray was utilized to obtain a comprehensive view of the expression level changes of 84 genes caused by type I IFN treatment and EV71 or CA16 infection in RD cells. All array data are listed in Table 1 in the Supplemental material. Overall, the expression of multiple genes increased, including JAK-STAT pathway-related genes and multiple ISGs with known antiviral functions, indicating that the RD cells established a viral defense post-IFN treatment (Fig. 3A). IFN pathway genes displayed significantly different reactions to EV71 or CA16 infection, with only a small number of genes being up-regulated upon EV71 infection whereas the majority of genes, including many ISGs, displayed significant down-regulation. In contrast, only a small number of genes were down-regulated by CA16 infection whereas multiple genes displayed obvious up-regulation (Fig. 3B). When IFN-pretreated virus-infected cells were compared with IFN-treated mock-infected RD cells, almost all 84 genes were inhibited by EV71 infection with different degrees. On the contrary, CA16 infection yielded a completely different regulation pattern, which was characterized by strong up-regulation of IFNs, JAK-STAT pathway genes, and ISGs (Fig. 3C). These data indicate that EV71 infection partially repressed gene expression in the type I IFN pathway, whereas CA16 infection has IFN pathway stimulation effect.

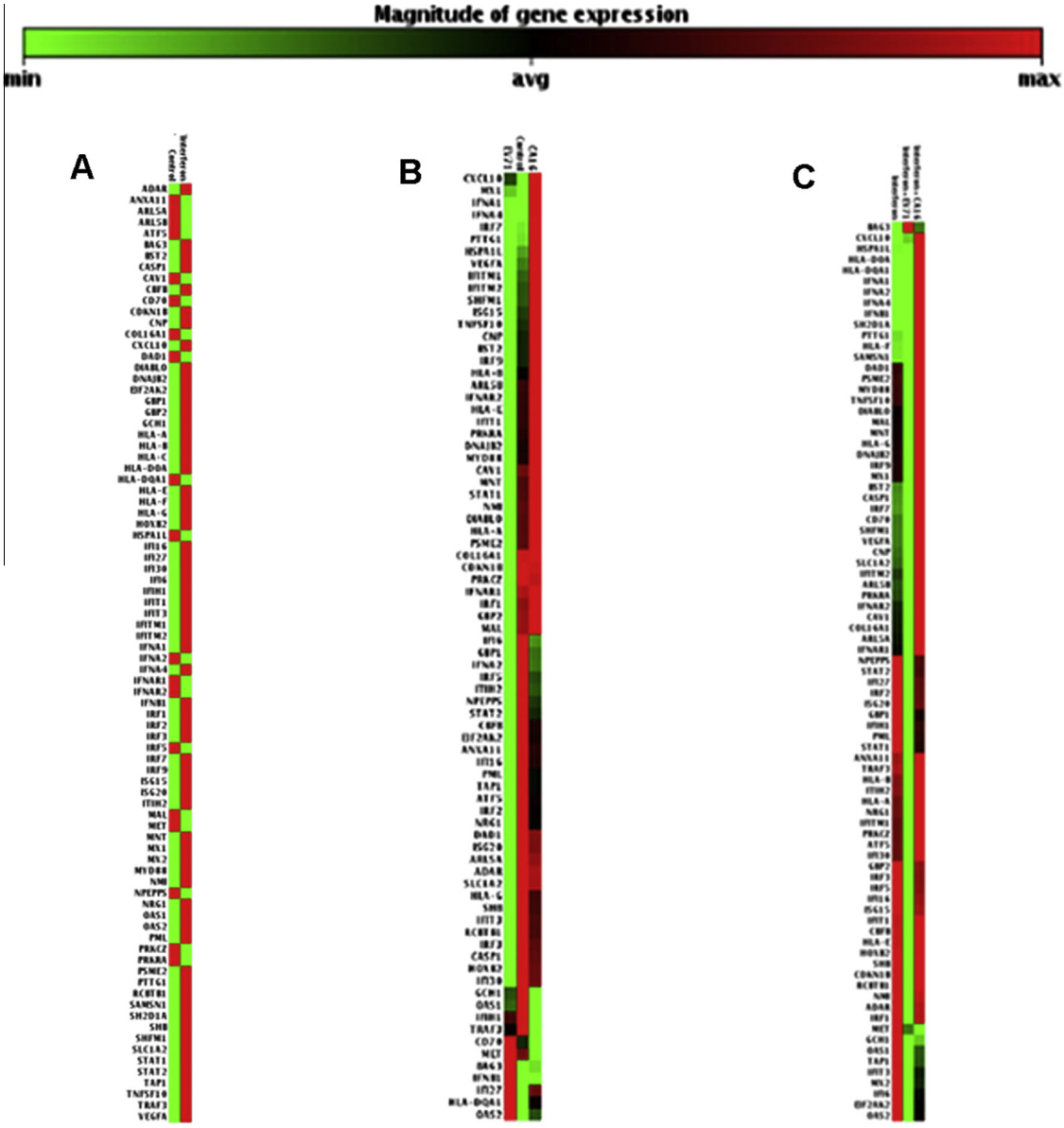

Fig. 3. Effects of IFN treatment and virus infection on IFN pathway gene expression. RD cells were divided into six groups as described in the materials and methods. Cells were collected for RNA extraction, and the expression of 84 genes was assessed using an IFN PCR array based on the relative RT-qPCR method. Fold-changes of each gene were compared between two groups. IFN treated group versus control group (A); virus-infected groups versus control group (B); IFN pre-treated virus-infected groups versus IFN treated group (C).

3.4. Effects of EV71 and CA16 infection on JAK-STAT pathway and ISG expression

To verify the influence of EV71 and CA16 infection on the JAK-STAT pathway and ISG expression, proteins collected from IFN treated and virus-infected RD cells were analyzed by WB. As shown in Fig. 4A, the levels of IFNAR1, IFNAR2, STAT1 and STAT2 were reduced at different degrees by EV71 infection. However, CA16 infection had no inhibitory effect on these proteins. Besides, the levels of multiple ISGs were measured upon virus infection. All ISGs stimulated by IFN were down-regulated in EV71-infected cells, whereas the expression of these ISGs was enhanced by CA16 infection (Fig. 4B). Next, the activation of JAK-STAT pathway influenced by virus infection was investigated. IFN treatment induced STAT1 and STAT2 phosphorylation in the mock-infected cells, which indicates that the JAK-STAT pathway was activated. However, the phosphorylated STAT1 and STAT2 were significantly reduced in the EV71-infected cells compared with CA16-infected cells (Fig. 4C). These results indicate that EV71 infection suppressed the activation of JAK-STAT pathway, which further repressed the expression of ISGs. However, CA16 infection enhanced the expression of multiple ISGs.

4. Discussion

During virus infection, host cells utilize multiple mechanisms to combat the infection. Similarly, viruses have also evolved multiple ways to inhibit host immune responses [9,10]. To explore the pathophysiological differences between EV71 and CA16 infections on host and cells, we focused on the type I IFN pathway and uncovered evidence supporting the different effects of EV71 and CA16 infection on type I IFN pathway.

The replication rates of viruses reflect their ability to infect certain types of cells and further contribute to the CPE [18]. The production of type I IFN stimulated by virus infection has an inhibitory effect on the virus [11]. The differences in CPE, replication rate, and IFN production that we observed, combined with the IFN pretreatment experiments, demonstrated that EV71 is more resistant to type I IFN inhibitory effects than CA16 in RD cells. The stimulation patterns and selective inhibitory effect of IFN-α and IFN-β on these two viruses also indicated different regulation patterns of the type I IFN pathway in EV71- and CA16-infected cells.

Our array results revealed elevated expression of multiple ISGs in RD cells upon IFN treatment, indicating that a strong anti-viral state was established. However, EV71 infection could overcome such a barrier by down-regulating the expression of multiple genes, including those involved in the JAK-STAT pathway (IFNAR1/2 and STAT1/2), interferon regulatory factors (IRFs), and ISGs (IFITM1, ISG15, and BST2). The down-regulation of IFNA2 and MyD88 indicates that other inhibitory mechanisms are also used by EV71 to repress the IFN pathway [15–17]. In contrast, CA16 infection stimulated the IFN response pathway, especially repressing GCH1 and OAS1 expression. One possible mechanism of CA16 inhibition is via its non-structural proteins, which possess DNA binding activity to direct inhibit the transcription of certain ISGs during CA16 infection, because the JAK-STAT pathway was not inhibited by CA16 infection. EV71 and CA16 have similar genomes and proteins functions [5]. However, the structural differences between non-structural proteins that possess protease activity (2A and 3C) and those with DNA binding activity may contribute to the profound differences exerted on the IFN pathway by

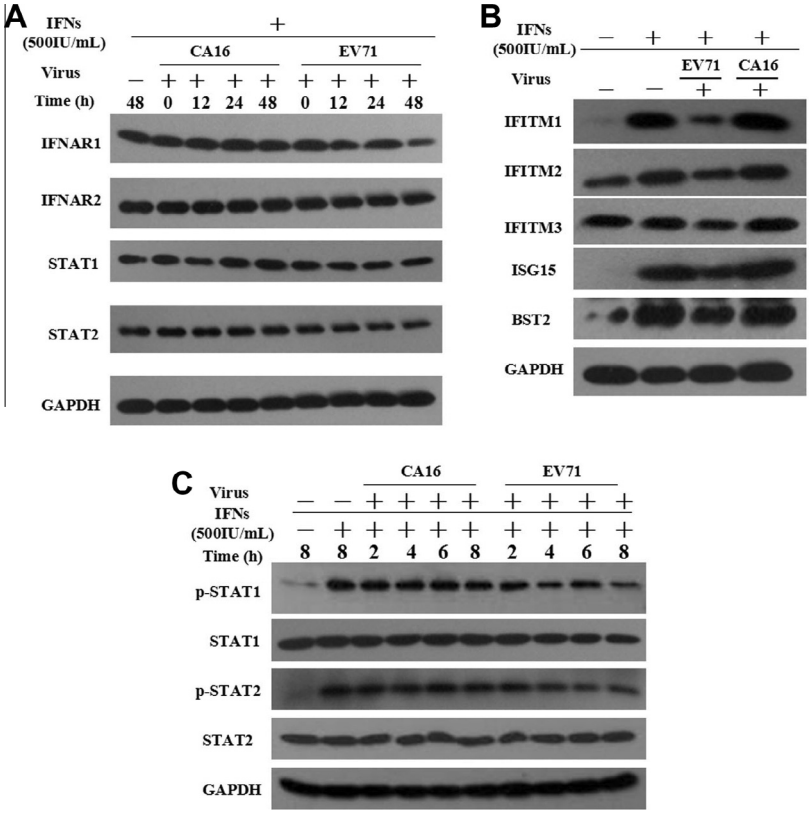

Fig. 4. Effects of EV71 and CA16 infection on JAK-STAT pathway and ISG expression. RD cells were pretreated with 500 IU/mL IFN-α2b plusing IFN-β for 24 h followed by EV71 or CA16 (MOI = 0.1) infection for 0, 12, 24 and 48 h. Levels of IFNAR1, IFNAR2, STAT1, STAT2 in each experimental group were analyzed by WB (A). WB analysis was conducted to detect changes in ISG levels in RD cells at 48 h p.i. (B). RD cells were infected with EV71 or CA16 (MOI = 10) for 24 h followed by 500 IU/mL IFN-α2b plusing IFN-β stimulation for 2, 4, 6 and 8 h. The level of STAT1/2 and p-STAT1/2 were detected by WB analysis (C). GAPDH was used as an internal control for normalization.

EV71 and CA16. Indeed, these proteins perform multiple roles during virus infection, further influencing their pathogenicity [17,19,20]. Future work will explore the reason(s) for these different effects on the IFN pathway and search for ISGs that possess anti-viral potential because high doses of IFN treatment still have a strong inhibitory effect on EV71 and CA16. Collectively, EV71 evolved the ability to antagonize the innate IFN immune response, mainly through weakening the activation of the JAK-STAT pathway, whereas CA16 had no such inhibitory effect but stimulated the JAK-STAT pathway and specially reduced the expression of a few genes.

Besides, the up-regulation of HLA-DQA1 by both EV71 and CA16 infection may reflect the susceptibility of individuals to HFMD infection caused by EV71 or CA16, which can partly explain the different prevalence and severity of symptoms in HFMD patients [21]. CXCL10 is used as a predictor during early treatment of HCV by IFN/ribavirin therapy and is highly elevated in HCV-infected IHH cells [22,23]. The up-regulation of CXCL10 by EV71 and CA16 can be used as a molecular marker during HFMD disease diagnosis. SLC1A2 clears the excitatory neurotransmitter glutamate from the extracellular space at synapses in the central nervous system to prevent neuronal damage from excessive activation [24,25]. Combined with the rare neurological symptoms caused by CA16 infection, CA16 infection had no significant impact on SLC1A2 expression, but EV71 infection resulted in strong down-regulation of SLC1A2. Thus, we speculate that the neurological symptoms caused by EV71 infection may be associated with the down-regulation of SLC1A2, though these conjectures require further study for confirmation.

The results of our screen utilizing the PCR array and subsequent verification experiments demonstrate that EV71 and CA16 infections result in different reactions to the type I IFN pathway and ISG stimulation in RD cells, despite EV71 and CA16 being very similar at the genome and structural levels. Understanding the differences between these two viruses will help to uncover ISGs with potential inhibitory effects and lead to targeted therapy for HFMD caused by EV71 or CA16 virus infection.

Author contributions

Po Tien and Wei Zhang designed the study and wrote the manuscript; Wei Zhang, Lei Zhang and Zhiyong Wu performed the experiments and data analysis.

Supportive foundations

This work was supported by Grants from the National Basic Research Program of China (973 Program) (Nos. 2011CB504703, 2010CB530102) and the National Natural Science Foundation of China (NSFC, Grant Nos. 81321063, 31270211, 31370201).

Acknowledgments

We are grateful to Prof. Paul Chu of guest professor of Institute of Microbiology, Chinese Academy of Sciences, for critically reading the manuscript and providing constructive comments and suggestions, and for Weihua Zhuang and Fulian Liao for their kind help with the experiments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.021.

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