Intrinsic apoptosis and proinflammatory cytokines regulated in human astrocytes infected with enterovirus 71

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Enterovirus 71 (EV71) has emerged as a clinically important neurotropic virus following poliovirus eradication. However, the mechanism of EV71-induced neurological manifestation remains largely unclear. In this study, we showed that human astrocytes were susceptible to EV71 and viral RNA was first detected at 12 h post-infection (p.i.), whilst viral proteins were detected at 36 h p.i. EV71-infected astrocytes underwent apoptosis, in which cytochrome c was released from mitochondria to the cytosol and caspase-9 was activated. Interestingly, caspase-2 and -8 were not cleaved or activated during the infection, whilst a selective inhibitor of caspase-9, Z-LEHD-FMK, blocked the cleavage of caspase-3 and -7, indicating that only the mitochondria-mediated intrinsic apoptotic pathway was activated in EV71-infected astrocytes. EV71 infection also induced proinflammatory cytokines, including IL-6, IL-8, CCL5 and IFN-γ-inducible protein (IP)-10 in astrocytes, which may play a critical role in EV71-induced neuroinflammation and neurological complications. By using inhibitors of mitogen-activated protein kinases (MAPKs), we demonstrated that the induction of the cytokines was mainly regulated by the MAPK p38 signalling pathway as a significant reduction of the cytokines was observed when treated with p38 inhibitors. This study demonstrated that human astrocytes were susceptible to EV71, and the infection led to intrinsic apoptosis and induction of p38-regulated proinflammatory cytokines. These findings further our understanding of the neuropathogenesis in severe cases of EV71 infection.

INTRODUCTION

Enterovirus 71 (EV71) is a single-stranded, positive-sense RNA virus of the genus Enterovirus of the family Picornaviridae and is notable for its aetiological role in epemics of severe neurological diseases in children. The virus typically causes mild common-cold-like symptoms in patients and is one of the aetiological agents causing hand, foot and mouth disease. Although infection in most cases is self-limited, serious neurological diseases and complications manifesting as encephalitis, aseptic meningitis, brainstem encephalitis and motor neuron death can occur in severe cases (Ho, 2000). It was reported that a high percentage of patients died from fatal pulmonary oedema or cardiorespiratory collapse and the results from autopsy studies suggested that the cardiopulmonary disease is neurogenic (Lum et al., 1998), similar to findings in poliovirus infection (Baker, 1957). Recent studies showed that brainstem encephalitis and pulmonary oedema seemed to be strongly related to dysregulation of systemic inflammatory responses in the central nervous system (CNS), which has provided at least part of the basis for anti-inflammatory therapy with intravenous immunoglobulin and the approach appears to be effective (Lin et al., 2002a; Ooi et al., 2009; Wang et al., 2006). Understanding the viral neurotropism and neuropathogenesis in EV71 infection is of utmost importance in developing more effective clinical therapy for curing patients with severe symptoms.

Astrocytes, the most abundant glial cell population of the CNS, are of neuro-ectodermal origin, and are essential for brain homeostasis and neuronal function (Dong & Benveniste, 2001). Astrocytes perform many functions, including biochemical support of endothelial cells that form the blood–brain barrier (BBB), provision of nutrients to nervous tissues and maintenance of extracellular ion...
balance, and also play a role in the repair and scarring process of the brain and neurotransmitter buffering. Moreover, astrocytes are important in the initiation and modulation of immune responses triggered by a variety of insults, including virus infection. Following activation, astrocytes are endowed with the ability to secrete soluble mediators, such as CXCL10, CCL2, IL-1, TNF-\(\alpha\), IL-6 and granulocytic colony-stimulating factor, which were found to have an impact on both innate and adaptive immune responses (Aschner, 1998; Farina et al., 2007).

Clinically, patients infected with EV71 had high levels of inflammatory cytokines, such as IL-6, TNF-\(\alpha\) and IL-8, in the serum or cerebrospinal fluid (CSF) (Lin et al., 2002b; Wang et al., 2008a, 2012). The levels of IFN-\(\gamma\)-inducible protein (IP)-10 and IL-8 in CSF were significantly higher in patients complicated with both encephalitis and pulmonary oedema than in uninfected controls, suggesting that increased proinflammatory cytokines in the serum of EV71 patients might have originated in or at least be partially contributed from the infected foci of the CNS (Wang et al., 2008b). Little is known, however, about the type of cells that are susceptible to EV71 or are key contributors for virally induced cytokines in the CNS of severe EV71 patients.

Previous studies have reported that SF268 (human glioblastoma) and SK-N-MC (human neuroblastoma) could be infected with EV71 (Chang et al., 2004). Post-mortem studies revealed that viral antigens were found in the cytoplasm of neurons, neuronal processes and inflammatory cells. Astrocytes showed both hypertrophy and proliferation, which suggests that they are activated by EV71 infection (Lum et al., 1998; Yang et al., 2009). Previous studies have also shown that following EV71 infection, an increase of BBB permeability and injury was observed (Chen et al., 2007a). Recently, Hao-long et al. (2013) reported that vimentin phosphorylation and rearrangement may support virus replication, and that vimentin played a structural role in the formation of the replication factories. However, to date, there is little information about the susceptibility and host responses of astrocytes to EV71. In this study, we showed that human astrocytes (U251 cell line) were susceptible to EV71, which underwent cytochrome c-dependent intrinsic apoptosis. In response to EV71 infection, astrocytes secreted abundant proinflammatory cytokines, including IL-1\(\beta\), TNF-\(\alpha\), IL-6 and the chemokine CXCL10, and the response was mainly regulated by p38 mitogen-activated protein kinases (MAPKs). Our data demonstrate the significance of astrocytes in EV71 neuropathogenesis, which may be implicated in clinical prognosis of severe cases with EV71 infection.

**RESULTS**

**Replication of EV71 in human astrocytes**

To determine susceptibility of human astrocytes to EV71 infection, we inoculated the human astrocytic glioma cell line U251 with EV71 at m.o.i. 1 and the cells were examined following infection. The cytopathic effect (CPE) produced in U251 was monitored microscopically at 36, 48 and 72 h post-infection (p.i.). As shown in Fig. 1, the infected cells underwent morphological changes, including cell rounding, swelling and detachment from the surface, and eventually broke up into granules, devastating the cell monolayer after 48 h p.i. (Fig. 1a). Cell viability, assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay, decreased up to 50 % at 72 h p.i. in comparison with the control cells (Fig. 1b). An indirect immunofluorescence assay (IFA) was performed, with the cells stained by antibodies specific for the virus capsid protein (VP1). As shown in Fig. 1(c), VP1 was detected at 36 and 48 h p.i. in infected cells.

To demonstrate EV71 replication kinetics in U251 cells, we measured viral RNA copy numbers, which increased at 12 h p.i. up to 4.5 copies [per 10\(^5\) copies of glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] and reached a peak of 12 718 copies at 72 h p.i. (Fig. 2a). Culture supernatants were collected and subjected to TCID\(_{50}\) assay to titrate infectious virus (Reed & Muench, 1938). As shown in the viral replication curve (Fig. 2b), the EV71 yield increased throughout the infection up to 48 h p.i., when it peaked at 10\(^5\) TCID\(_{50}\) in infected cells. The viral yield decreased thereafter, probably due to extensive cell death. We also prepared total RNA from uninfected and infected U251 as well as RD and HT-29 cells for real-time reverse transcription (RT)-PCR to detect the expression of the scavenger receptor class B (member 2) (SCARB2) for EV71 (Jiao et al., 2014). Our data showed that the transcripts of SCARB2 were abundantly present in control cells (based on \(C_t\) values) and interestingly even upregulated or induced in response to EV71 infection in U251 cells, as in RD and HT-29 cells (Fig. 2c). Collectively, our data demonstrated that EV71 can infect and carry on a productive infection leading to cell death in human astrocytic culture.

**EV71 induces apoptosis in human astrocytes**

To understand how cell death occurred in EV71-infected U251 cells, we examined caspase-3 activity in infected cells. By using a caspase-3 activity test kit, we found that the caspase-3 activity increased in a time-dependent manner in EV71-infected cells (Fig. 3a). We measured substrate cleavage and caspase activation by Western blot analysis, and detected that the cleavage of poly(ADP-ribose) polymerase (PARP), a hallmark feature of apoptosis, increased throughout the course of the infection, coinciding with the increase of VP1 expression (Fig. 3b). The cleaved forms of executioner caspases-7 and -3 were detected (Fig. 3c), indicating that cell death of astrocytes occurred throughout apoptosis. To examine the activation of specific caspses induced in EV71 infection, the cells were pretreated with a general caspase inhibitor (Z-VAD-FMK, 50 \(\mu\)M), a selective caspase-3 inhibitor (Z-DEVD-FMK, 100 \(\mu\)M) or a specific caspase-1 inhibitor (Ac-YVAD-CMK),...
followed by infection with EV71. After 72 h inoculation, cell lysates were collected and analysed by Western blot analysis. The results showed that inhibitors Z-VAD-FMK and Z-DEVD-FMK completely blocked EV71-induced cleavage of PARP (Fig. 3d). However, when treated with Ac-YVAD-CMK specific for caspase-1, no significant change of the cleaved PARP protein level was observed compared with the untreated control cells.

Intrinsic but not extrinsic pathway involved in EV71 infection-induced apoptosis

To identify upstream initiator caspases, we examined whether pro-caspase-8 or -9 was activated and involved in induced apoptosis. As shown in Fig. 4, cleaved/activated caspase-9 increased significantly after EV71 infection at 36 and 72 h p.i. (Fig. 4a). However, the levels of pro-caspase-8 were unchanged and the cleaved form of caspase-8 was not detected throughout EV71 infection (Fig. 4a), suggesting that the infection induced the activation of only the caspase-9-dependent intrinsic apoptotic pathway. This differential activation of caspases differed from that observed in HT-29 cells, a human intestinal epithelial cell line, which showed cleavage of both caspase-8 and -9 in response to EV71 (Fig. 4b). We also used a caspase activity test kit to measure activities of both caspase-8 and -9 in EV71-infected cells, and found that the activity of caspase-9 increased at 36 and 72 h p.i. (Fig. 4c), but no significant change was observed in the activity of caspase-8.

To further confirm that only caspase-9 was activated, the cells were pre-treated with Z-LEHD-FMK, a selective inhibitor of caspase-9, followed by EV71 infection. The results showed that the inhibitor effectively suppressed the cleavage of pro-caspase-3 and -7 (Fig. 4d), suggesting that caspase-9 was the upstream initiator caspase that cleaved and activated pro-caspase-3 and -7 in EV71-infected astrocytes.
Caspase-2 is also a well-established initiator caspase for the intrinsic apoptotic pathway (Boatright & Salvesen, 2003; Guo et al., 2002). From our observation as shown in Fig. 4(a), the level of pro-caspase-2 was not changed and cleaved caspase-2 was not observed, indicating that caspase-2 was not involved and caspase-9 activation was likely the only initiator in the activation of the intrinsic apoptotic pathway in EV71-infected astrocytes.

EV71 induced cytochrome c release, and regulation of Bax and survivin expression

We sought to identify the mechanism by which the intrinsic apoptosis was induced. In EV71-infected U251 cells, cytochrome c was released from the mitochondrial membrane into the cytosol. As shown in Fig. 5(a), decreased levels of cytochrome c were observed in the mitochondrial fraction, whilst the levels of cytochrome c increased in the cytosol at 48 and 72 h p.i. Along with this change, the expression of Bax was upregulated in both the cytosolic and mitochondrial fractions (Fig. 5a), suggesting that Bax may be the factor that was induced upon infection and subsequently promoted the cytochrome c release into the cytosol in EV71 infection.

Cytochrome c release can be activated by translocation of tBid, another pro-apoptotic Bcl-2 family member, onto the mitochondrial membrane. Our data showed that tBid, an active form of Bid, was present at a low level constitutively in the mitochondria of U251 cells without infection and its level remained unchanged throughout EV71 infection, suggesting that the cytochrome c release could be independent of tBid. tBid is processed from full-length Bid by activated caspase-8 or other proteinases. We have ruled out the involvement of caspase-8 in the cleavage of Bid, as caspase-8 was not activated in astrocytes, so there appeared to be no more tBid. This differed in intestinal epithelial cells, such as in HT-29 cells, where tBid was processed and translocated into the mitochondria in response to EV71 infection and caspase-8 activation (Fig. 5b). Indeed, we did not observe any obvious

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**Fig. 2.** EV71 replication in infected human astrocytes. (a) Detection of viral VP1 gene transcripts. Viral gene copies were quantified by real-time PCR. The number of gene copies was normalized to 10^5 copies of GAPDH. Each experiment was performed in triplicate. (b) Infectious viral titres in infected cells. Supernatants from infected cells were collected at designated time points post-inoculation of EV71. The infectious viral titres were measured by a standard TCID_{50} assay. (c) Presence of EV71 receptor SCARB2 on U251 cells. Total RNA was prepared from uninfected or infected U251 as well as HT-29 and RD cells for real-time RT-PCR with primers specific for SCARB2. C_{t} values for uninfected controls were between 18 and 19.
induction of Fas ligand (FasL), which could otherwise lead to stimulation of Fas and activate pro-caspase 8 (Fig. 5c). How tBid was processed constitutively in U251 cells remained unknown.

The activation of caspase signalling and apoptosis may also be enhanced by downregulation of anti-apoptotic factors. We examined the total cell lysates collected at various time points after infection, and found that anti-apoptotic Bcl-2 and survivin were downregulated at 48 and/or 72 h p.i. (Fig. 5d). In contrast, FLIP, an inhibitor of pro-caspase-8, remained unchanged. Reduced Bcl-2 may promote cytochrome c release from the mitochondria, and executioner caspase-3 and -7 were more active with the reduced levels of survivin in the cytosol.

**Induction of proinflammatory cytokines in EV71-infected astrocytes**

To illustrate host responses in EV71-infected astrocytes, we examined proinflammatory cytokine responses in U251 cells by real-time RT-PCR. The results showed that the level of IP-10 transcripts quickly increased up to 6.5-fold at 24 h and reached a peak of 29.5-fold at 48 h p.i. However, the levels of IL-6, IL-8 and CCL5 transcripts increased later in the infection at 48 and 72 h p.i., and the fold changes were milder, up to only 8- or 10-fold (Fig. 6).

To confirm the induction of the proinflammatory cytokines, we measured the protein levels in the culture medium with ELISA. The culture media from both infected...
and uninfected cells were collected at different time points after infection. Significant induction of IL-6, CCL5 and IP-10 was detected as shown in Fig. 7. An increase of IL-8 was also evident, although fold changes remained mild.

**Effect of MAPKs on the induction of inflammatory cytokines**

To evaluate the role of MAPKs in the induction of pro-inflammatory cytokines in EV71-infected astrocytes, we prepared total RNA from infected or uninfected cells pre-treated with inhibitors of extracellular signal-regulated kinases (ERKs) 1/2, c-Jun N-terminal kinases (JNKs) 1/2 and p38, respectively, and measured the induction of IL-6, IL-8, CCL5 and IP-10 with real-time RT-PCR. The results showed that in the presence of p38 and JNK inhibitors, the induction of IL-6, IL-8, CCL5 and IP-10 transcripts was evidently suppressed at 72 h.p.i. (Fig. S1, available in the online Supplementary Material), suggesting that the induction of these cytokines upon EV71 infection was dependent on p38 and JNK signalling in astrocytes. ERK1/2 could be involved in the induction of CCL5 and IP-10 (Fig. S1C, D), but its effect was much weaker than p38. The data collected at 48 h.p.i. were mainly inconclusive or negative, showing no involvement of MAPKs due to the low induction levels of all cytokines except IP-10 (Fig. S1D).
The above observation was corroborated and largely confirmed by ELISAs, as shown in Fig. 8. Compared with the cells pre-treated with inhibitors of ERK1/2 and JNK, the expression of cytokine genes in cells pre-treated with SB203580 was much lower at 72 h p.i. Collectively, p38 was the strongest in the induction of IL-6, IL-8, CCL5 and IP-10, and proinflammatory cytokines were dominantly regulated by the MAPK p38 signalling pathway in EV71-infected astrocytes.

Fig. 5. Involvement of apoptosis-related proteins. (a) Cleavage of Bax and release of cytochrome c (‘Cyto c’). Cytosolic (‘Cyto’) and mitochondrial (‘Mito’) fractions of the cell lysates were prepared from uninfected (‘Ctl’) and infected cells at 24, 48 and 72 h p.i. The levels of Bax and cytochrome c were determined by Western blot analyses. The assays were repeated at least twice. (b) Increased tBid in mitochondria of EV71-infected HT-29 cells. Cell lysates were prepared from EV71-infected HT-29 cells and subjected to Western blot analyses with antibodies for Bid or tBid. (c) No induction of FasL in infected U251 cells. Total RNA was prepared from infected U251 cells at various time points for real-time RT-PCR with primers for FasL to measure its fold change in response to infection. (d) Downregulation of Bcl-2 and survivin and upregulation of Bax in EV71-infected U251 cells. Cells were infected with EV71 and total cell lysates were collected and analysed by Western blot analyses.
The role of astrocytes in the neuropathogenesis of severe EV71 patients has not been studied extensively. Astrocytes are the most abundant glial cell population of the CNS and participate in local innate immune responses triggered by invading micro-organisms. Cytokines and chemokines can be produced in large quantity in astrocytes upon activation and could mediate regulatory influence on the blood/CNS interface or target neighbouring cells and promote leukocyte recruitment, resulting in the local amplification of inflammatory responses (Sofroniew & Vinters, 2010; Wang et al., 2008a). Here, we provide evidence that astrocytes are susceptible to EV71 infection, leading to cell death through the intrinsic apoptotic pathway. In addition, proinflammatory cytokines such as IP-10 and IL-6 were induced in EV71-infected astrocytes, and could be key elements in exacerbating neurological symptoms in severe cases.

EV71 infection can cause a massive neuronal dysfunction or destruction in severe clinical cases, and the mechanism by which the virus causes neurological symptoms is not fully understood. Some studies have identified viral antigens from brain tissues of patients with encephalitis (Hsueh et al., 2000; Lum et al., 1998; Shieh et al., 2001; Yang et al., 2009), indicating that viral infection of the neuronal cells or cell-mediated immunity may be involved in pathogenesis. Haolong et al. (2013) have reported that EV71 VP1 activated calcium/calmodulin-dependent protein kinase II (CaMKII), resulting in the rearrangement of vimentin in astrocyte cell line U251, whereas cytokine storm has been considered to be the main cause of severe cardiopulmonary manifestations (Lin et al., 2003; Wang et al., 2012). However, few studies have been carried out with regard to the role of major immune cells, such as astrocytes or microglia, in the pathogenesis of EV71 infection. To the best of our knowledge, our report demonstrates for the first time that in EV71-infected U251 cells, intrinsic, but not extrinsic, apoptosis was triggered, leading to cell death, and proinflammatory cytokines were significantly induced. We therefore hypothesize that EV71 infection in astrocytes and the resultant consequences may critically contribute to the pathogenesis of the illness, especially for those severe cases with encephalitis.

In certain viral infections, apoptosis could be a defensive mechanism for the host to suppress viral infection by inhibiting the generation and spread of viral progeny with aborted infection. Different pathways are employed in cells in response to apoptotic stimuli. Previous studies showed that EV71 proteases 2A and 3C induced apoptosis in fibroblasts and neural cells (Kuo et al., 2002; Li et al., 2002). Chen et al. (2006) found that increased FasL expression may account for Jurkat T cell apoptosis, whilst EV71 triggered neuronal apoptosis through activation of Abl–Cdk5 signalling (Chen et al., 2007b). Here, we demonstrate that EV71-induced apoptosis in human astrocytic cells depends on the activation of caspase-9, but not caspase-8 or -2. This unique mechanism for only mitochondria-mediated intrinsic apoptosis to be activated in astrocytes differs from the process in lymphocytes infected with EV71.

Finally we evaluated the role of NFκB signalling in the regulation of cytokines in U251 cells. U251 cells were pre-treated with inhibitors of NFκB and IκB kinase (IKK) prior to infection with EV71. At various time points, total RNA was prepared, and the induction of IL-6 and IP-10 transcripts was measured by real-time PCR. Treatment with the inhibitors showed little effect on the induction compared with the controls, suggesting that NFκB signalling may be dispensable for the induction of IL-6 and IP-10 (Fig. S2A). We also monitored the degradation of IκBz, the inhibitor of NFκB. The results showed that no evident downregulation of IκBz was observed throughout the infection, whilst IκBz degradation could be easily detected when the cells were treated with TNF-α as a control (Fig. S2B). Taken together, we conclude that p38 of MAPKs, but not NFκB signalling, played a key role in the regulation of proinflammatory cytokines in human astrocytes infected with EV71.

DISCUSSION

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We sought the mechanism by which intrinsic apoptosis was induced, and examined the induction of Bcl-2 and IAP (inhibitors of apoptosis) family members in EV71 infection. Our results showed that anti-apoptotic Bcl-2 and survivin were both downregulated, but the expression of pro-apoptotic Bax increased. In cells, Bax could undergo conformational changes, oligomerize and insert into the mitochondrial outer membrane, causing the alteration of
its permeabilization and the release of cytochrome c, which subsequently activates the intrinsic apoptotic pathway (Franklin, 2011; Hsu et al., 1997). However, even though FasL (Fig. 5c) and TNF-α (data not shown) were slightly upregulated, caspase-8 was not cleaved and the extrinsic apoptotic pathway was not activated in EV71-infected U251 cells. We are not conclusive about how the receptor-mediated extrinsic apoptotic pathway was suppressed in EV71-infected astrocytes. However, our data showed that Bid (Bcl-2 inhibitory BH3-domain protein), a substrate of caspase-8, was not cleaved, indicating that caspase-8 was indeed not active and FLIP, an inhibitor of caspase-8 activation, was present in U251 cells. In fact, the finding that extrinsic apoptosis was not activated in EV71-infected human astrocytes is consistent with an earlier report showing that human astrocytes are resistant to FasL-induced apoptosis. According to that study, CaMKII was constitutively phosphorylated and activated in human astrocytes, which mediated the expression and phosphorylation of FLIP, and subsequently inhibited procaspase-8 cleavage (Song et al., 2006).

Enterovirus infection can cause severe neuronal symptoms, which may not be caused directly by viral infection. Evidence has indicated that the pathogenicity could be the outcome of excessively induced harmful proinflammatory responses (Rhoades et al., 2011). Inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, have been reported to have neurotoxic effect on brain cells (Camelo et al., 2000; Chaparro-Huerta et al., 2002; Kimura, 2006; Lucas et al., 2006; Yoneda et al., 2001). Moreover, mediators, such as IL-1β, TNF-α and IL-6, released by astrocytes, can alter BBB permeability and attract immune cells from the blood circulation into the neural tissue, thus facilitating an adaptive immunity in addition to proinflammatory innate response, both detrimental to brain tissues (de Vries et al., 1996; Fiala et al., 1997; Mayhan, 2002). Previous studies showed that following EV71 infection, an

**Fig. 7.** Cytokine and chemokine production from EV71-infected astrocytes as measured by ELISA. The culture media of U251 cells infected with EV71 were collected at various time points after infection for ELISA measurement of IL-6, IL-8, CCL5 and IP-10. Values represent mean ± SD from triplicate cultures from three independent experiments.
increase of BBB permeability and its injury were observed (Chen et al., 2007a) due to direct consequence of viral infection and cell death or increased concentration of certain cytokines, such as TNF-α (Fiala et al., 1997). It was reported that the level of IL-6 in CSF was significantly higher in severe cases with neurological complications and the IL-6 concentration in CSF has been considered to be correlated with disease severity in EV71 infection (Lin et al., 2002b, 2003; Wang et al., 2003). In fact, EV71 patients with encephalitis and pulmonary oedema presented with higher levels of the proinflammatory cytokines in CSF than the blood (Wang et al., 2012). Consistent with this observation in human EV71 patients, a significant elevation of the IL-6 level was also observed in an EV71-infected neonate mouse model. Administration of anti-IL-6 neutralizing antibodies after the onset of the clinical symptoms successfully improved survival rates and clinical scores of the patients. This suggests that IL-6, with its levels strongly correlated with disease severity, may play a major role in EV71-induced immunopathogenesis (Khong et al., 2011).

Identification of astrocytes as a source of cell types for proinflammatory IL-6, CCL5 and IP-10 in CNS is significant for understanding viral pathogenesis in EV71 infection. Our previous studies have shown that MAPKs, including ERK1/2 and JNK1/2, but not p38 MAPK, were activated in EV71-infected HT-29 cells (Wang et al., 2015). We also observed an activation of ERK1/2 in EV71-infected Vero cells, but no evident increase in the

![Fig. 8. Protein levels of proinflammatory cytokines regulated by MAPKs. U251 cells were pre-treated with inhibitors for ERK1/2 (U0126), JNK1/2 (JNKi) or p38 (SB203580) at 1 h prior to EV71 infection. The culture media were collected at 48 and 72 h p.i. for ELISAs to measure the levels of (a) IL-6, (b) IL-8, (c) CCL5 and (d) IP-10. The experiments were repeated at least three times (t-test; *P<0.05, **P<0.01).](http://jgv.microbiologyresearch.org)
phosphorylation of other MAPKs was detected throughout the time-course of EV71 infection (data not shown), which was consistent with the findings of Tung et al. (2007). However, phosphorylation of p38 and JNK was indeed observed in SK-N-SH cells (Tung et al., 2010), suggesting that MAPK phosphorylation and activation may depend on specific cell types. Apparently, p38 was activated in astrocytes as well, which may exacerbate pathogenicity by promoting proinflammatory cytokine induction. Further studies should be undertaken to characterize the underlying mechanism by which p38 is activated in EV71 infection, which may help develop novel clinical therapeutics for severe EV71 patients.

### Methods

#### Cells and reagents.

The human astrocyte cell line U251 and African green monkey kidney epithelial Vero cells were purchased from the Cell Bank of Chinese Academy Sciences (Shanghai, China). The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Invitrogen), supplemented with 10% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine, non-essential amino acids and sodium pyruvate (Invitrogen). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO2.

Rabbit anti-EV71 structural protein VP1 antibody was purchased from Beijing Biosynthesis Biotechnology. Rabbit anti-cytokrome c, goat anti-pro-caspase-3, mouse anti-PARP and mouse anti-β-actin antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-cleaved caspase-7 and rabbit anti-cleaved caspase-9 antibodies were obtained from Cell Signaling Technology. Rabbit anti-caspase-2 antibody and rabbit anti-caspase-8 antibody were purchased from R&D Systems. HRP-conjugated anti-rabbit and anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology. MTT was purchased from Sigma-Aldrich. Super Signal ECL reagent kits were purchased from Thermo Fisher. Caspase-3, -8 and -9 activity detection kits were purchased from Desenbio Company. The general caspase inhibitor Z-VAD-FMK and selective inhibitors of caspase-1, -3 and -9 were purchased from Calbiochem. Chemicals purchased from EMD Chemicals included inhibitors for MAPKs, U0126 (ERK1/2), SB203580 (p38) and InSolution JKNI/2 Inhibitor II (JNK1/2), and the inhibitors for NFκB and IKK [6-amino-4-(4-phenoxypyphenethylamino)quinazoline and wedelolactone, respectively].

#### Virus culture and titration.

EV71 Fuyang strain belongs to the C4a cluster of the C4 subgenotype as verified through sequence analysis of the VP1 region (Zhang et al., 2010) and was kindly provided by Dr Wu Bin (Jiangsu Provincial Centers for Disease Control and Prevention). EV71 was propagated in Vero cells. Virus titres were determined in the cells by measuring TCID50, and the virus stocks were diluted viruses from 10^−2 to 10^−9 in DMEM were inoculated to Vero cells in 96-well plates, and the cells were incubated for 7 days at 37 °C before titres were calculated by counting the wells with CPE. EV71 Fuyang strain as well as EV71-infected Vero cells were harvested at 24 h, 48 h and 72 h post-infection (h.p.i.) for another 4 h. The survival rate of cells was expressed as the ratio of OD570 of infected cells to OD570 of uninfected cells. The assay was performed in triplicate for each concentration.

#### Quantitative real-time PCR.

Total RNA (500 ng) extracted from uninfected and infected cells using a RNeasy kit (Qiagen) were used for RT with a PrimeScript RT reagent kit (TaKaRa) following the manufacturer’s manual. Real-time PCR was performed with 1 μl cDNA in a total volume of 10 μl with SYBR Premix Ex Taq II (TaKaRa) according to the manufacturer’s instructions. Relative gene expression levels were normalized by a GAPDH control. Fold change of each gene expression level was calculated following the formula: 2^ΔCt of gene–ΔCt of GAPDH. Viral gene copies were quantified on the basis of a TaqMan-based real-time PCR with labelled probes specific for each gene. Viral gene copy numbers were expressed as the number of target gene copies per 10^5 copies of GAPDH.

#### Subcellular protein extraction and Western blot.

To extract subcellular proteins, cells were treated with pre-cooled 1% NP-40 lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 2 mM PMSF, 2 mM NaF, 1 mM Na3VO4, 1 μg aprotinin ml−1 and 1 μg leupeptin ml−1 on ice for 20 min. After low-speed centrifugation (500 g, 5 min), the supernatant was harvested as a cytosolic fraction. The pellet was further treated with pre-cooled 25% (v/v) glycercol lysis buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 2 mM PMSF, 2 mM NaF, 1 mM Na3VO4, 1 μg aprotinin ml−1 and 1 μg leupeptin ml−1 on ice for 30 min. After high-speed centrifugation (12 000 g, 5 min), the supernatant was taken as a nuclear fraction. The mitochondrial fraction, we performed preparation using a Kits as mitochondrial protein extraction kit (Keygenotec) following the manufacturer’s instructions. After measurement with a bichinonic acid (BCA) protein assay kit (Pierce), cell lysates were subjected to SDS-PAGE and transferred to an Immunoblot PVDF membrane (Millipore), followed by incubation with primary antibodies. After overnight incubation and washes, the membrane was incubated with a HRP-conjugated secondary antibody. Images were captured using a FluoroChem FC2 Imaging System (Alpha Innotech) and developed manually.

#### Caspase activity analysis.

Cytosolic lysates of infected and control cells were prepared and normalized by the BCA assay as described earlier. Caspase-3, -8 and -9 activities were detected by using a caspase activity assay kit (Desenbio) performed according to the kit instructions. Ac-DEVD-pNA, IETD-pNA and LEHD-pNA were used as the substrates for caspase-3, -8 and -9, respectively. A0 was measured in an ELISA reader and the data were expressed as fold relative to the control.

#### ELISA for cytokines.

Cell culture supernatants were harvested at various time points from infected or control U251 cells by centrifugation at 1000 g for 15 min at 4 °C. Quantitative measurements of IL-6, IL-8, CCL5, IP-10 and TNF-α were performed in 96-well plates using solid-phase sandwich-type ELISA kits (Biosource International) following the manufacturer’s instructions. After incubation for 3 h and six 15–30 s soakings in a wash solution, each well was further incubated in the presence of a biotinylated antibody specific to a cytokine. The plates were then incubated for 45 min before undergoing six washes. HRP-conjugated streptavidin was added to each well for 45 min for colorimetric development. The reaction was stopped by the addition of an acidic stop solution (0.2 M sulfuric acid) and A492 was measured. Serial dilutions of the standard controls were prepared and used to plot a standard curve of absorbance utilizing linear regression analysis. The plates were read using a SpectraMax 340PC microplate reader (Molecular Devices) at 450 nm and the assay was repeated three times.
Immunofluorescence analysis. EV71-infected and non-infected U251 cells were fixed with 4% paraformaldehyde at 4 °C overnight and permeabilized with 0.1% Triton X-100 on ice for 10 min, followed by three washes with 1× PBS, then blocked with 5% BSA at 37 °C for 2 h. The cells were incubated with rabbit anti-EV71 structural protein (VP1) antibody at 1:200 diluted in PBS/Tween (PBST) containing 1% BSA at 4 °C overnight. After being washed with PBST three times, the samples were incubated with an FITC-conjugated anti-rabbit antibody at a 1:200 dilution for another 1 h at 37 °C. The cells were then washed and incubated with 1 μg DAPI ml−1 in PBS for 10 min. After three washes the cells were covered with one droplet of anti-fade reagent (Sigma-Aldrich) and observed under an Olympus confocal laser scanning microscope.

Statistical analysis. Two-tailed Student’s t-test was used to evaluate the data. The data shown are the mean ± SEM of three independent experiments. P ≤ 0.05 was considered statistically significant.

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