

# Characterization of inflammatory cytokine profiles in cerebrospinal fluid of hand, foot, and mouth disease children with enterovirus 71-related encephalitis in Hangzhou, Zhejiang, China

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## Abstract

Enterovirus 71 (EV71) is an important etiological agent of hand, foot, and mouth disease (HFMD), which can also lead to severe neurological complications (eg, encephalitis) in young children. Although a series of reports on EV71 infection have been published, the pathogenic mechanism of EV71 infection is still not fully understood.

We evaluated the cerebrospinal fluid (CSF) levels of the inflammatory cytokines interleukin (IL)-8, IL-1 $\beta$ , IL-6, IL-10, tumor necrosis factor (TNF)- $\alpha$ , and IL-12p70 in 88 children with EV71-related encephalitis and 19 children with febrile convulsion (FC) with the use of commercial cytometric bead array kits.

The levels of IL-8, IL-1 $\beta$ , IL-6, and IL-10 in CSF were significantly higher in encephalitis group when compared with those observed in FC group, while no significant changes were noted in the levels of TNF- $\alpha$  and IL-12p70. In addition, significant and positive correlations among CSF IL-8, IL-1 $\beta$ , IL-6, and IL-10 were observed in encephalitis group. Furthermore, receiver operator characteristic analysis determined a cut-off value of 10.62 pg/mL for IL-6 to discriminate encephalitis patients from FCs with the sensitivity and specificity of 89.8% and 84.2%, respectively. Moreover, logistic regression analyses revealed that IL-6 was an independent predictor of EV71-related encephalitis (odds ratio = 23.241,  $P < .001$ ).

Our results indicate that 4 inflammatory cytokines (IL-8, IL-1 $\beta$ , IL-6, and IL-10) play important roles in the pathogenesis of EV71 infection. IL-6 may be used for the evaluation of EV71-related encephalitis and as a potential therapy candidate for EV71 infection.

**Abbreviations:** ALT = alanine transaminase, AUC = area under the ROC curve, CBA = cytometric bead array, CK-MB = creatine kinase isoenzyme MB, CSF = cerebrospinal fluid, ERK = extracellular regulated protein kinase, EV71 = enterovirus 71, FACS = fluorescence activating cell sorter, FC = febrile convulsion, HFMD = hand, foot and mouth disease, IFN = interferon, IRF = interferon regulatory factor, MAPK = mitogen-activated protein kinase, NLR = nucleotide-binding site leucine-rich repeat, qPCR = quantificational real-time polymerase chain reaction, RIG-I = retinoic-acid inducible gene I, ROC = receiver operator characteristic, RT-PCR = reverse transcription-polymerase chain reaction, TLR = toll-like receptor.

**Keywords:** cerebrospinal fluid, encephalitis, enterovirus 71, foot and mouth disease, hand, interleukin-6

## 1. Introduction

Hand, foot, and mouth disease (HFMD), typically characterized by mucocutaneous herpetic rashes on hand, feet, mouth, and buttocks, is a common transmissible infectious disease caused by

a myriad of human intestinal viruses. Enterovirus 71 (EV71), as a neurotropic virus, is particularly associated with this disease. Although HFMD is generally mild and self-limited, EV71-related cases have a higher incidence of neurological complications,

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including aseptic meningitis, encephalitis, meningoencephalitis, poliomyelitis-like paralysis, transverse myelitis, brain stem encephalitis.<sup>[1]</sup> Due to its potentially serious complications, EV71 infection attracts a raising concern. Unfortunately, to date, no established therapies are available for severe EV71 infection. Although an inactivated EV71 whole virus vaccine is commercially available recently, its clinical efficacy is not yet known.<sup>[2]</sup> Likewise, some studies recommended glucocorticoids, and intravenous immunoglobulin to treat severe EV71 infection, but sufficient evidence for their effectiveness is still lacking.<sup>[3,4]</sup> Thus, it is essential to identify potential effective antiviral targets.

Although the pathogenesis of severe EV71 infection is not fully understood, host immune responses rather than EV71 itself or its genotype maybe 1 of vital determinants for the disease progression.<sup>[5,6]</sup> Previous studies demonstrated that the abnormally expressed inflammatory cytokines, and chemokines, and the imbalance of expression of pro- and anti-inflammatory cytokines contribute to the development and severity of EV71-associated disease.<sup>[7-10]</sup> However, data about EV71-caused HFMD complicated with encephalitis are limited, and key laboratory findings as potential indicators of EV71-related encephalitis are not well known. Therefore, further study uncovering the elusive mechanisms underlying host immune responses to EV71 infection, and identifying the important immune mediators will be crucial to antiviral treatment and early recognition of severe EV71 infection.

Hence, in the present study, we aimed to analyze

- (1) the cerebrospinal fluid (CSF) inflammatory cytokine profiles (interleukin [IL]-8, IL-1 $\beta$ , IL-6, IL-10, tumor necrosis factor [TNF]- $\alpha$ , and IL-12p70) of EV71-infected HFMD patients with encephalitis,
- (2) the correlation between CSF inflammatory cytokines and CSF parameters, and
- (3) the indicators for predicting encephalitis, which may contribute to an in-depth understanding of the immune response generated by EV71 infection, and the development of useful clinical biomarker/therapy target for EV71 infection.

## 2. Materials and methods

### 2.1. Study participants and sample collection

EV71-induced HFMD patients with encephalitis were enrolled during the HFMD epidemic season from April 2012 to September 2013. Febrile convulsion (FC) is a benign condition, triggered by fever (body temperature  $\geq 38^{\circ}\text{C}$ ) that is without any definitive cause, such as an intracranial infection, history of afebrile seizures, metabolic disorders, and acute electrolyte imbalance.<sup>[11]</sup> Of note, FC patients do not have a central nervous system (CNS) infection. Therefore, children with simple FC were included as a control group. Patients were recruited from Children's Hospital, Zhejiang University School of Medicine and Hangzhou Children's Hospital. The study protocol was approved by the Ethic Committees of both hospitals, and was in accordance with the Declaration of Helsinki. Written informed consent was obtained from the guardians of all study subjects.

HFMD was diagnosed when a patient presenting with oral ulcers and vesicular rash on the hands, feet, knees, or buttocks, with or without fever. Encephalitis was diagnosed when a patient had a disturbance in the level of consciousness, such as lethargy, drowsiness or coma, seizures or myoclonus, accompanied by CSF

pleocytosis. FC was defined as any seizure that resulting from fever in the absence of intracranial infection or metabolic disturbance. Simple FC was defined as generalized tonic-clonic seizures occurring in the first 48 hours of a febrile illness, and a single convulsion lasting less than 15 minutes in a 24 hours period followed by a brief postical period of drowsiness.<sup>[11]</sup>

Clinical samples included throat swabs, rectal swabs, and CSF from all patients in the acute phase of disease were collected within 24 hours of admission. All specimens were subsequently stored at  $-80^{\circ}\text{C}$  for further analysis. For each participants, data on demographic information, hematological parameters, CSF cytology, and CSF biochemical markers were collected by reviewing electronic medical records from both hospital.

EV71 infection was confirmed with a throat swab or rectal swab specimen using EV71-specific real-time reverse transcription-polymerase chain reaction as described previously.<sup>[12]</sup> Briefly, ribonucleic acids (RNAs) were extracted from specimens by Viral RNA Mini Kit (Qiagen, Hilden, Germany) and converted to complementary deoxyribose nucleic acid (PrimeScript RT reagent kit, Da An Gene, Guangzhou, China) according to the manufacturer's instructions, followed by real-time quantitative polymerase chain reaction reactions using a 7500 real-time RT-PCR system (Applied Biosystems, Foster, CA) based on TaqMan technology.

### 2.2. Determination of CSF inflammatory cytokine levels

The CSF concentrations of IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and IL-12p70 were quantified by cytometric bead array (CBA) assays. BD CBA human inflammatory cytokine kit (BD Biosciences, San Diego, CA) was used to determine cytokines by fluorescence activating cell sorter Calibur flow cytometer (Becton Dickinson, San Jose, CA) according to the manufacture's recommendations. All samples were measured in duplicate and the concentrations were calculated from the standard curve of duplicate standards.

### 2.3. Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 23.0 software (SPSS, Chicago, IL) was used for data analysis. Normally distributed data were expressed as mean  $\pm$  standard deviation, and Student *t* test was used for between group comparisons accordingly. Meanwhile, nonparametric distributed data were expressed as medians and interquartile range, and Mann-Whitney *U* test was used for between group comparisons accordingly. Correlations between the selected variables were examined by Spearman rank correlation analysis. Receiver operator characteristic (ROC) curves were performed to determine the optimal cut-off value of inflammatory cytokines. Logistic regression model was performed to estimate the relationship between EV71 related-encephalitis and cytokines. For all tests, a difference with *P*-value less than .05 was considered to be significant.

## 3. Results

### 3.1. Demographic data and clinical characteristics

A total of 107 patients were enrolled in this study and were divided into 2 groups, namely, HFMD children with EV71-related encephalitis, ( $n=88$ ) and patients with simple FC ( $n=19$ ). Demographics, clinical characteristics, and laboratory tests of all

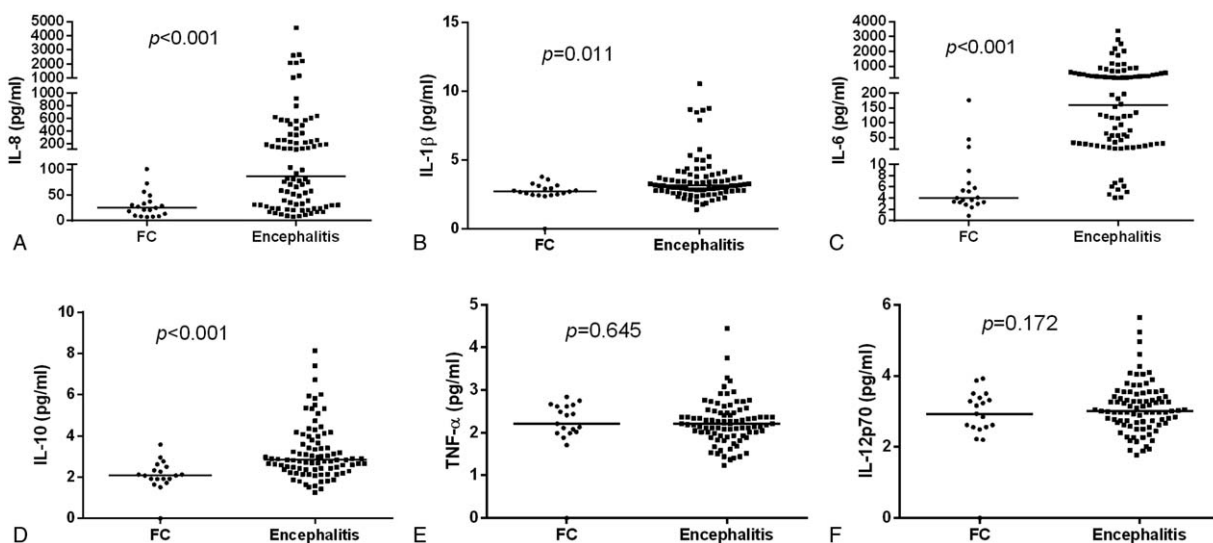
**Table 1**  
**Demographic and clinical characteristics of the study participants.**

Characteristics	Febrile convulsion (n = 19)	Encephalitis (n = 88)	P
Gender (male/female)	11/8	53/35	.851
Age, mo	21.00 (15.00–32.00)	29.50 (19.25–38.25)	.110
Hospital stay, d	6.00 (5.00–7.00)	9.00 (9.00–10.00)	<.001
fever duration before admission, d	1.00 (1.00–3.00)	2.00 (1.00–3.00)	.018
fever duration during admission, d	1.60 (1.00–2.20)	2.50 (1.65–3.00)	.003
Total fever duration, d	2.80 (2.50–4.50)	4.55 (4.00–5.60)	<.001
Blood routine examination			
WBC ( $\times 10^9/L$ )	12.06 (11.03–15.70)	11.07 (8.95–13.49)	.070
Neutrophil (%)	61.30 (43.70–75.60)	63.55 (49.35–71.38)	.687
Lymphocyte (%)	28.50 (18.58–44.63)	29.75 (23.05–42.88)	.525
Hemoglobin, g/L	119.53 $\pm$ 11.60	121.28 $\pm$ 9.37	.479
Platelet count, $\times 10^9/L$	284.11 $\pm$ 51.71	312.14 $\pm$ 70.72	.105
Biochemical test			
Blood glucose, mmol/L	5.23 (4.30–6.93)	5.75 (5.13–6.63)	.195
ALT, U/L	15.00 (12.00–22.00)	13.00 (10.25–17.00)	.183
CK-MB, U/L	22.00 (20.00–35.00)	28.00 (21.25–43.75)	.264
Creatinine, $\mu\text{mol/L}$	21.00 (19.00–24.00)	21.50 (18.00–25.00)	.822
Humoral immunity			
IgG, g/L	8.28 (5.99–10.16)	8.40 (6.96–10.17)	.381
IgA, g/L	0.42 (0.26–0.65)	0.57 (0.38–0.77)	.025
IgM, g/L	1.24 $\pm$ 0.55	1.68 $\pm$ 0.54	.002
C3, g/L	1.10 $\pm$ 0.28	1.21 $\pm$ 0.17	.036
C4, g/L	0.35 $\pm$ 0.13	0.39 $\pm$ 0.11	.274
CSF variables			
WBC, $\times 10^6/L$	4.00 (2.00–7.00)	95.00 (48.50–197.50)	<.001
Neutrophil%	–	50.00 (28.00–72.00)	–
Lymphocyte%	–	42.00 (21.00–66.00)	–
Total protein, g/L	156.00 (105.00–216.00)	263.00 (172.50–343.25)	<.001
Glucose, mmol/L	3.77 (3.48–4.09)	3.86 (3.40–4.38)	.582
Chloride, mmol/L	123.00 (122.00–127.00)	124.00 (121.00–126.00)	.951

ALT = alanine transaminase, CK-MB = creatine kinase isoenzyme MB, WBC = white blood cells.

study participants are summarized in Table 1. In short, no significant differences in age, gender, blood routine examination or biochemical test were found between encephalitis and FC patients. However, fever duration and length of hospital stay were longer in the encephalitis group than in the FC group, and

the differences were statistically significant. In addition, serum immunoglobulin (Ig)A, IgM, and C3 levels were higher in the encephalitis group compared to FC group. Furthermore, higher white blood cells (WBC) and protein concentrations in CSF were found in encephalitis patients than in FC cases.

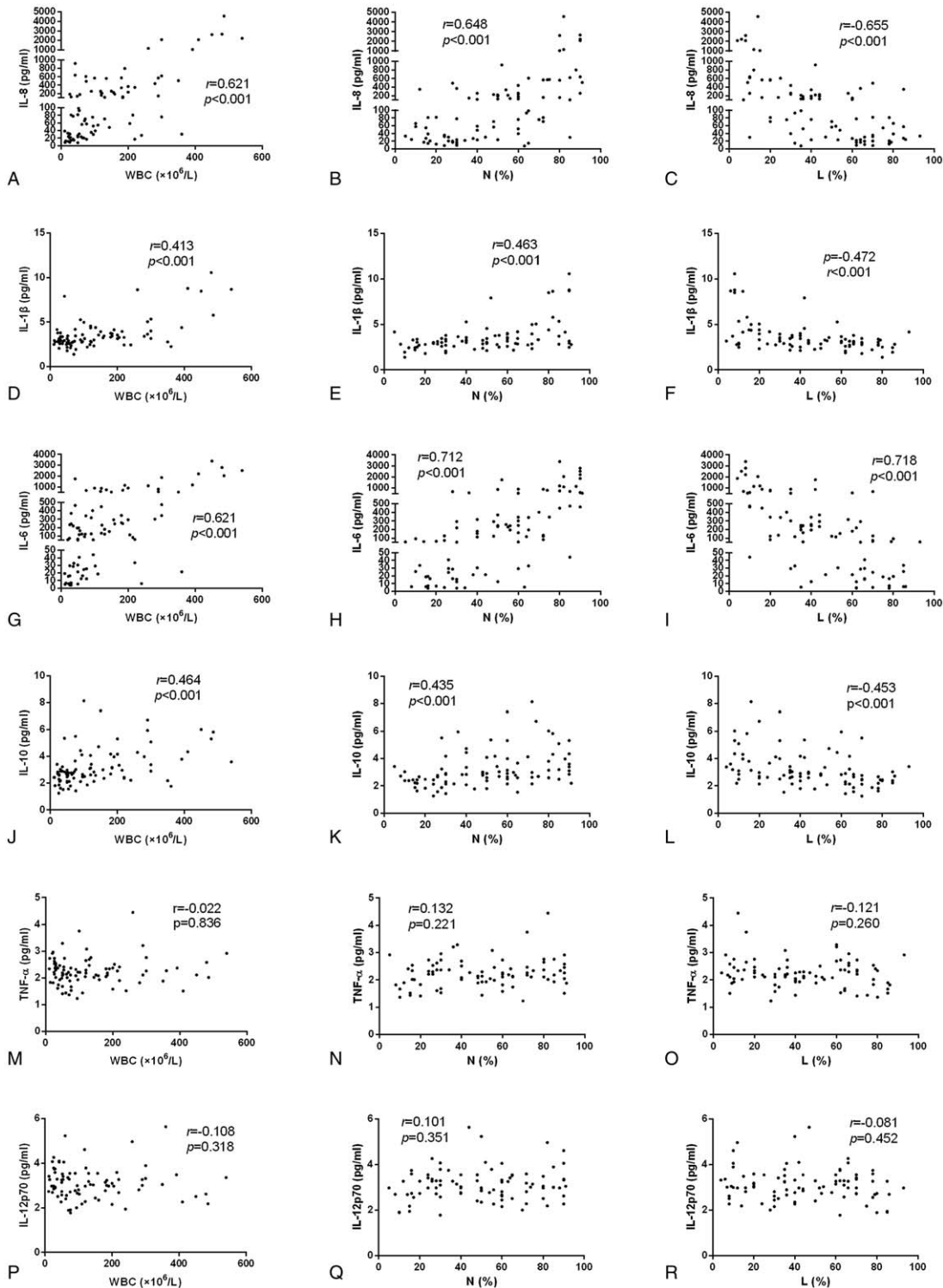


**Figure 1.** CSF inflammatory cytokine concentrations in EV71-related encephalitis patients and FC patients. (A) IL-8; (B) IL-1 $\beta$ ; (C) IL-6; (D) IL-10; (E) TNF- $\alpha$ ; (F) IL-12p70. EV71 = enterovirus 71, FC = febrile convulsion, IL = interleukin, TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .

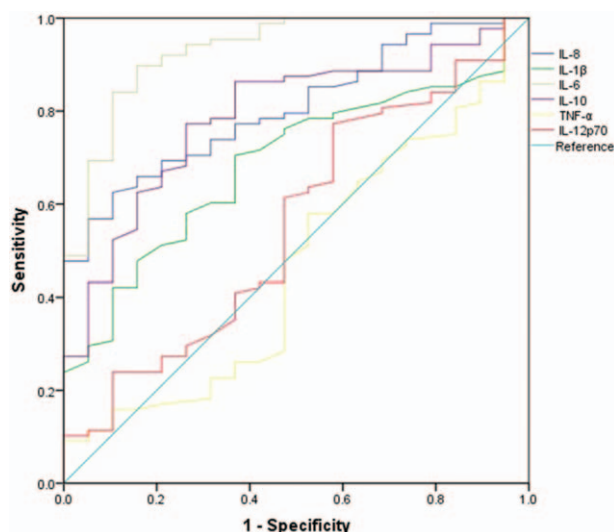
**3.2. Inflammatory cytokine expression in CSF**

The expression levels of IL-8, IL-1 $\beta$ , IL-6, and IL-10 were significantly higher in the encephalitis group than in the FC group

(87.31 [30.57–345.15] pg/mL vs 25.09 [9.87–37.24] pg/mL, 3.15 [2.75–3.80] pg/mL vs 2.70 [2.48–3.13] pg/mL), (158.14 [29.41–470.24] pg/mL vs 4.02 [3.24–6.65] pg/mL, 2.84 [2.38–



**Figure 2.** Correlation analysis of CSF inflammatory cytokine concentrations with CSF cytology variables in encephalitis patients depicted as scatter plots. (A–C) The correlation between IL-8 and CSF WBC, neutrophil percentage (N%) and lymphocytes percentage (L%), respectively; (D–F) the correlation between IL-1 $\beta$  and CSF WBC, N% and L%, respectively; (G–I) the correlation between IL-6 and CSF WBC, N% and L%, respectively; (J–L) the correlation between IL-10 and CSF WBC, N% and L%, respectively; (M–O) the correlation between TNF- $\alpha$  and CSF WBC, N% and L%, respectively; (P–R) the correlation between IL-12p70 and CSF WBC, N% and L%, respectively. CSF = cerebrospinal fluid, IL = interleukin, TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .



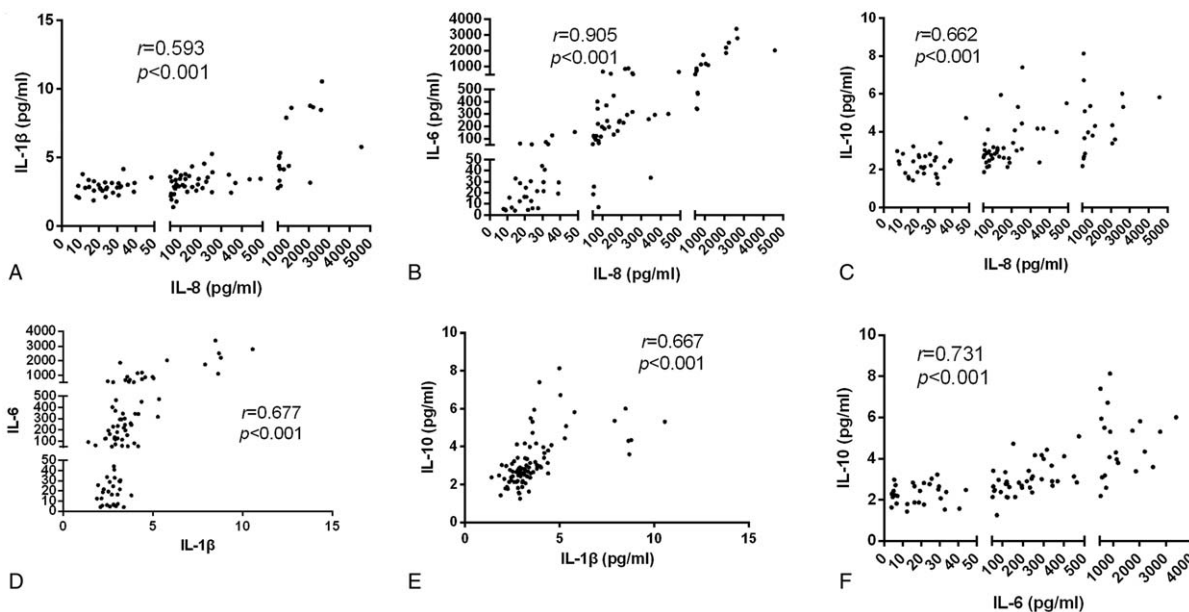
**Figure 3.** ROC analysis of CSF inflammatory cytokines (including IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and IL-12p70) in the encephalitis and FC groups. FC = febrile convulsion, IL = interleukin, ROC = receiver operator characteristic, TNF- $\alpha$  = tumor necrosis factor- $\alpha$ .

3.77 pg/mL vs 2.08 [1.91–2.50] pg/mL, respectively) (Fig. 1A–D). However, CSF TNF- $\alpha$  and IL-12p70 levels did not differ significantly between the 2 groups (2.21 [1.95–2.46] pg/mL vs 2.21 [1.99–2.62] pg/mL, 3.11  $\pm$  0.71 pg/mL vs 2.85  $\pm$  0.86 pg/mL, respectively) (Fig. 1E and F).

In encephalitis group, correlation analysis showed IL-8 and IL-6 levels strongly correlated with CSF cytology variables (including WBC, neutrophil percentages, and lymphocyte percentages) (Fig. 2 A–C, G and H), while IL-1 $\beta$  and IL-10

levels moderately correlated with CSF variables (Fig. 2 D–F, J–L). Inversely, no correlation was noted between CSF TNF- $\alpha$  or IL-12p70 levels, and cytology variables in encephalitis patients (Fig. 2 M–R). Similarly, there was no correlation between CSF inflammatory cytokines (IL-8, IL-1 $\beta$ , IL-10, TNF- $\alpha$ , and IL-12p70) levels, and CSF WBC in FC patients, except IL-6 (see Figure, Supplemental Content 1, <http://links.lww.com/MD/D525>, which shows correlation between CSF inflammatory cytokine concentrations, and CSF WBC in FC patients).

The area under the ROC curve (AUC) indicates the clinical usefulness of the tested cytokines in distinguishing EV71-related encephalitis from FC (Fig. 3) (see Table, Supplemental Content 2, <http://links.lww.com/MD/D526>, which illustrates ROC analysis of CSF inflammatory cytokines in HFMD patients with encephalitis). In detail, we noticed that the AUC of IL-6 (0.931) was larger than the area of IL-8 (0.805), IL-1 $\beta$ , (0.688) and IL-10 (0.789). In contrast, AUC of TNF- $\alpha$  (0.466) and IL-12p70 (0.556) were not statistically significantly larger in comparison to AUC = 0.5 (borderline of the diagnostic usefulness of the test) ( $P = .645$ , and  $P = .444$ , respectively). ROC curves also illustrated that the best cut off value 56.97 pg/mL of IL-8, 2.81 pg/mL of IL-1 $\beta$ , 10.62 pg/mL of IL-6, and 2.35 pg/mL of IL-10 had sensitivity, and specificity for EV71-related encephalitis as 62.5% and 89.5%, 70.5% and 63.2%, 89.8% and 84.2%, 77.3% and 73.7%, respectively. Furthermore, IL-8, IL-1 $\beta$ , IL-6, and IL-10 were positively correlated with each other; especially IL-8 was strongly correlated with IL-6 ( $r = 0.905$ ,  $P < .001$ ) (Fig. 4). Additionally, logistic regression analysis revealed that IL-6 rather than IL-8, IL-1 $\beta$  or IL-10 was independently related to EV71-related encephalitis (odds ratio [OR] = 23.241; 95% confidence interval 4.740–113.952;  $P < .001$ ) (see Table, Supplemental content 3, <http://links.lww.com/MD/D527>, which demonstrates the logistic regression analysis for inflammatory cytokines contributing to EV71-related encephalitis).



**Figure 4.** Interrelation analysis between the inflammatory cytokines (including IL-8, IL-1 $\beta$ , IL-6, and IL-10) in the encephalitis depicted as scatter plots. (A–C) the relationship between IL-8 and IL-1 $\beta$ , IL-6, and IL-10, respectively; (D and E) the relationship between IL-1 $\beta$  and IL-6 and IL-10, respectively; (F) the relationship between IL-6 and IL-10. IL = interleukin.

#### 4. Discussion

Immune system is a complex, sophisticated, and coordinated network for sustaining health, while altered cellular response was thought to contribute to the exacerbation of EV71 infection.<sup>[13,14]</sup> Still, how EV71 disturbs the equilibrium of host immunity, is not well understood. In the present study, we found overproduction of pro-inflammatory cytokines (IL-8, IL-1 $\beta$ , and IL-6) as well as for IL-10 (a rather anti-inflammatory cytokine) in CSF of EV71-related encephalitis patients, indicating the appearance of a broader range of these inflammatory cytokines were responsible for severer clinical signs in EV71-infected children compared to FC patients. Similarly, there are several papers confirming that the enhanced expression of IL-1 $\beta$ , IL-6, IL-8, and IL-10 is associated with severe encephalitis caused by EV71 infection.<sup>[9,13,15–17]</sup> All these reports indicate that EV71 may play a critical role in HFMD by altering cytokine production. Furthermore, we found strong relationship among IL-1 $\beta$ , IL-6, IL-8, and IL-10, showing a complex pattern of cytokine co-expression. It was demonstrated that EV71 directly influences antiviral inflammatory responses by targeting various inflammatory cellular signaling pathways (eg, interferon, retinoic-acid inducible gene I, interferon regulatory factor, toll-like receptor, and nucleotide-binding site leucine-rich repeat-dependent signaling pathways). Likewise, severe cases of HFMD are associated with extreme inflammatory cytokine storm.<sup>[13]</sup> These literature reports suggest that a coordinated immune response related to a rise of inflammatory cytokine exists. Taken together, these evidence imply that EV71 infection strongly stimulate the secretion of cytokine, leading to excessive intensification of inflammation and even resulting in fatal complications (eg, cardiopulmonary collapse).

The main function of IL-1 $\beta$  is to upregulate inflammation, and stimulate the secretion of IL-6, and IL-8.<sup>[18]</sup> IL-6 is a strong inducer of acute phase responses, and promotes inflammation, while IL-8 recruits, and activates circulating leukocytes.<sup>[19,20]</sup> In our study, we observed an increase in these 3 cytokines and correlation among them in EV71-infected patients with encephalitis. These evidences suggest that IL-1 $\beta$ , IL-6, and IL-8 synergize to induce CNS immune damage in EV71-related encephalitis and the locally pronounced “water-fall” inflammation seems responsible. In contrast, IL-10 as an anti-inflammatory cytokine, overturns the activation of leukocytes and the production of proinflammatory cytokines.<sup>[21]</sup> Although IL-10 increased in patients with EV71-associated encephalitis, the early production of IL-10 is relatively insufficient in controlling IL-6 and IL-8 at high levels. Therefore, there may be a strategic approach to use anti-inflammatory agents (eg, solasodine) to ameliorate the EV71-induced CNS damage.<sup>[22–24]</sup>

The AUC indicates the clinical usefulness of an inflammatory biomarker of the presence of encephalitis in children with EV71-induced HFMD. In this study, we observed statistically significantly larger AUCs for the tested inflammatory cytokines (with the exception of TNF- $\alpha$  and IL-12p70) compared to AUC = 0.5, indicating IL-8, IL-1 $\beta$ , IL-6, and IL-10 have potential diagnostic power for HFMD patients with encephalitis. Especially, the AUC of IL-6 (0.931) was the largest out of all the tested parameters in the encephalitis group, implying the strongest discriminatory power. Furthermore, the combination marker of the 4 inflammatory cytokines (IL-8, IL-1 $\beta$ , IL-6, and IL-10) did not show an elevation AUC (0.912), and its sensitivity in detecting encephalitis was 80.7% (data not shown), demonstrating the

diagnostic efficiency of the combination marker did not show superior to that of IL-6. Additionally, CSF IL-6 level >10.62 pg/mL provided maximum sensitivity (89.8%) and specificity (84.2%), showing IL-6 could represent good predictor for the distinguishing EV71-related encephalitis from FCs. Similarly, Lee et al also showed serum IL-6 could be used as an indicator of the presence of aseptic meningitis in children with EV71-induced HFMD.<sup>[25]</sup> Likewise, serum IL-6 >70 pg/mL was found to be the best predictor for EV71 encephalitis with PE.<sup>[9]</sup> Thus, to some extent, our results are complementary to previous studies.<sup>[9,25]</sup> In relation to these results, we believe that the value of IL-6 could be a significant marker for the HFMD patient with neurological complications. Moreover, we found IL-6 (OR = 23.241) had the strongest association with encephalitis of all investigated markers. A mouse model study showed that anti-IL-6-treated mice showed reduced tissue damage, absence of splenic atrophy, increased immune cell, and improved survival rates when compared to untreated EV71-infected mice.<sup>[26]</sup> An in-vitro experiment demonstrated that EV71-induced autophagy regulates the production of IL-6 through the p38 mitogen-activated protein kinase and extracellular regulated protein kinase signaling pathways.<sup>[27]</sup> Collectively, these observations indicate IL-6 may represent an interesting therapeutic target to provide protection from disease progression and improve disease outcome in children with EV71 infection.

Several studies have shown that high levels of cytokines (eg, TNF- $\alpha$  and IL-1 $\beta$ ) released during the fever are considered to be a factor implicated in the onset of FC.<sup>[28,29]</sup> Thus, the CSF from healthy children may be more specific for reflecting the normal condition of brain; however, we do not routinely collect CSF in children without suspected CSN infection for ethical issues. Still, despite this limitation, the present study clearly demonstrates differences in the inflammatory cytokine profiles in CSF from EV71-related encephalitis and FC.

#### 5. Conclusion

We detected that the CSF levels of 4 inflammatory cytokines, IL-8, IL-1 $\beta$ , IL-6, and IL-10, were significantly elevated in HFMD patients with EV71-related encephalitis. In particular, ROC analysis and logistic analysis indicated that IL-6 appears to be the best candidate for EV71-related encephalitis diagnosis and potential therapy target. Thus, our findings provide further insight into the pathogenesis of EV71 infection, and may facilitate design of novel interventions for EV71 infection. Further investigation is required to search for EV71-related cytokine indices that can be used to monitor disease progression, determine prognosis, and guide clinical diagnosis and treatment.

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#### Author contributions

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**Writing – review and editing:** Yingchun Xu, Shuxian Li, Chunyan Cai.

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