

## Original Article

# Cellular immunity status and cytokine assay in enterovirus 71-infected children

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**Abstract:** Since 2008, Mainland China has experienced several epidemics of enterovirus 71 (EV71) infection. High hospitalization rates of pediatric patients during these epidemics have been a huge burden for public health care resources, with severe cases often leading to public panic. This study presents the results of comparing immunity status among patients with pediatric hand, foot, and mouth disease (HFMD), originating from EV71 at different stages of infection, to that of healthy control subjects. This study analyzed the cellular cytokine response of NK cells and CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, after EV71 antigen stimulation, as well as plasma cytokine levels. Results suggest that IFN- $\gamma$  and TNF- $\alpha$  play significant roles in cellular immunity and differences in plasma levels of IFN- $\gamma$ , IL-6, and TNF- $\alpha$  in different stages of infection may be used to predict progression of the disease.

**Keywords:** Children, enterovirus 71, cellular immunity, cytokine assay

## Introduction

Enterovirus 71 (EV71) is small, single-stranded, and positive-sense RNA virus from the Enterovirus genus in the family Picornaviridae [1]. Since first described in 1969 by Schmidt N et al. [2], outbreaks have been reported in Australia, Malaysia, Taiwan, and the mainland of China [3-7]. EV71-associated hand, foot, and mouth disease (HFMD), most frequently occurring in children ages 1 to 5, has become an important public health issue across the Asia-Pacific region. It is usually self-limited but may progress to cause aseptic meningitis, brainstem encephalitis (BE), and pulmonary edema (PE) [8, 9]. As most isolates of EV71 from fatal and nonfatal cases were found to be identical [10], host immunity status may play a critical role in progression of the disease. Several studies have analyzed the antiviral immunity of the disease. It has been confirmed by researchers that EV71 can productively infect human dendritic cells (DCs) and increase the antigen presentation capability of DCs [11]. It has also been found that cellular immunity is correlated with clinical outcome of EV71 infection [12,

13]. A study in Taiwan found significant elevation of plasma cytokines IL-10, IL-13, and IFN- $\gamma$  among patients with PE. Moreover, after administration of intravenous immunoglobulin, plasma cytokine IFN- $\gamma$ , IL-6, IL-8, IL-10, and IL-13 levels decreased among these patients [14, 15]. Immunity status of the patients was different at different stages of infection. This present study compared host antiviral immunity status of patients at different stages of infection to healthy control subjects, aiming to observe any discrepancies between them. Flow cytometry technology was used to analyze cellular cytokine response and plasma cytokine levels more precisely and directly.

## Subjects and methods

### Subjects

This study was approved by the West China Second University Hospital Research Ethics Committee. Informed parental consent was obtained for all subjects. A total of 50 EV71-infected patients and 20 healthy control subjects were enrolled. EV71 infection was con-

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**Table 1.** Plasma cytokine levels of patients infected with EV71 and healthy controls

Cytokine (pg/mL)	Healthy Control (n=20)	Acute Stage (n=50)	Recovery Stage (n=50)
IL-2	8.47 (3.90~15.26)	19.83 (10.25~29.3)	16.77 (6.36~39.19)
IL-4	4.94 (3.90~6.32)	13.36 (6.35~39.18)	6.51 (3.21~21.36)
IL-6	11.33 (5.67~18.96)	147.52 (48.19~515.48)	51.06 (10.26~241.24)
IL-10	8.19 (4.89~12.26)	77.21 (28.19~222.32)	44.34 (11.48~241.36)
TNF- $\alpha$	18.94 (10.25~29.92)	74.53 (15.78~266.33)	179.52 (18.14~609.32)
IFN- $\gamma$	42.45 (32.15~55.14)	200.92 (68.32~358.31)	92.85 (22.69~270.33)

Note: Data are mean followed with peak value and least value in the bracket. IFN, interferon; IL, interleukin; TNF, tumor-necrosis factor.

firmed by positive EV71 specific IgM with a negative bacterial culture and clinical syndrome (containing fever and erythra on the hands, feet, and mouth) at the onset of the disease. All 50 patients were uncomplicated cases, without brainstem encephalitis (BE) and pulmonary edema (PE). Patients were aged 1 to 4 years old and first diagnosed in our hospital. BE was defined as myoclonus, nysagmus, ataxia, oculomotor palsies, and bulbar palsy, in various combinations, with or without neuroimaging. Autonomic nervous system (ANS) dysregulation was defined by the presence of mottled skin, cold sweating, tachycardia, tachypnea, and hypertension. PE was defined as respiratory distress symptoms and signs (tachypnea, tachycardia, rales, and frothy sputum) developing after ANS dysregulation, together with a chest radiograph showing bilateral pulmonary infiltrates without cardiomegaly. Venous blood samples were collected from patients within 2 days after admission (acute stage) and 2 days before being discharged from the hospital (recovery stage), respectively.

### Laboratory studies

EV71 specific IgM was measured at onset of disease by the Diagnostic Kit for IgM Antibody to EV71 (ELISA) (Beier Bioengineering, Beijing, China). Sensitivity and specificity were 85.1% and 97.0%, respectively. EV71 isolate (GenBank accession number) was purified and inactivated as an antigen for use in the stimulation of peripheral blood mononuclear cells (PBMCs), obtained from patients and healthy donors. To isolate PBMCs, heparinized blood samples were subjected to Ficoll-Hypaque gradient centrifugation (Pharmacia Diagnostics AB, Uppsala, Sweden). Cells at the interface were harvested, carefully, and washed twice with PBS. Isolated PBMCs were cultured in 24 holes ( $2 \times$

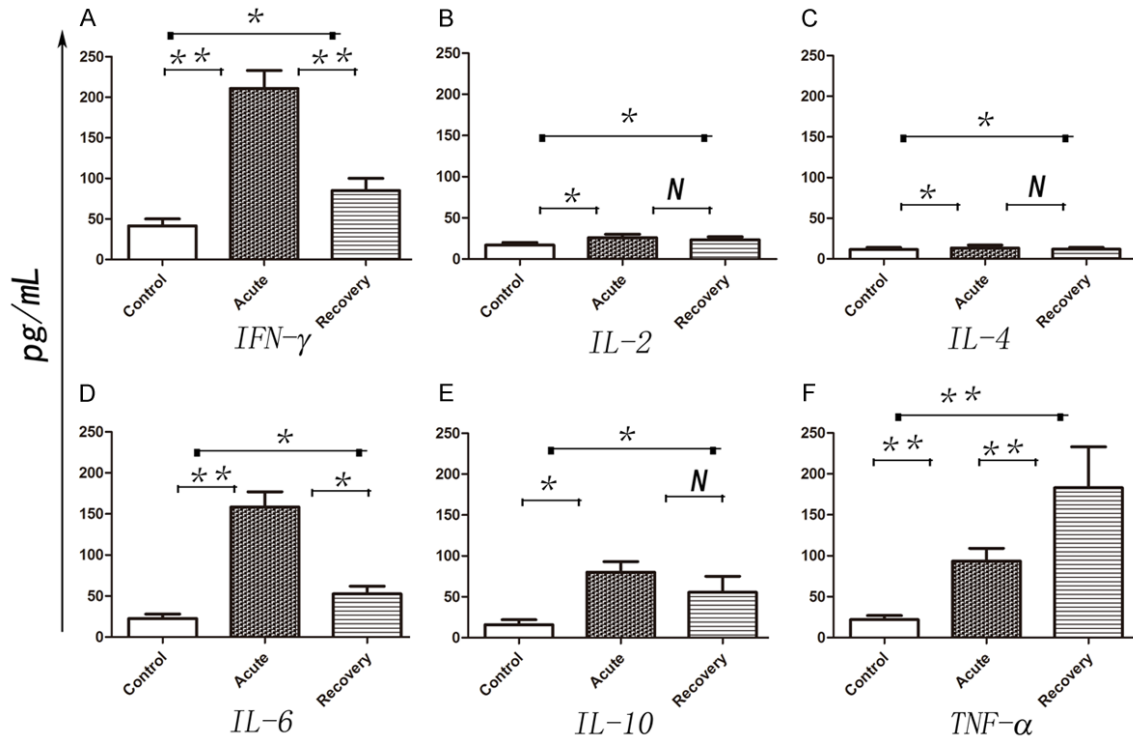
$10^6$  per hole in 1.5 mL of culture medium) in RPMI 1640 medium supplemented with 1 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Next, EV71 whole virus antigen or PBS was added in as a stimulate antigen (final concentration was 10  $\mu$ L/mL). The protein transport inhibitor Monensin (BD Biosciences) was added in (final concentration was 1  $\mu$ L/mL), as an impacting medium, 8 hours before staining for enrichment of cellular cytokines. PBMCs were cultured for 10 hours in an incubator at 37°C in 5% CO<sub>2</sub>.

Cell staining for flow cytometry cells were stained for surface markers with the following antibodies: anti-CD3-PB, anti-CD4-APC/Cy7, anti-CD8 $\alpha$ -perCP/cy5.5, anti-CD56-APC, and anti-CD107 $\alpha$ -FITC; Interface markers were: anti-IFN $\gamma$ -FITC, anti-TNF $\alpha$ -PE/cy7, and anti-IL2-PE. These antibodies and their isotype-matched control Abs of relevant specificity were purchased from BD (BD Biosciences). Intracellular staining was performed after cell fixation and permeabilization using a BD Fix and Perm Kit (BD Biosciences, USA). All samples were acquired using a Bectan Coulter Gallios instrument (Bectan Coulter Company, USA) and analyzed by means of Flowjo software (version 7.5).

### Plasma cytokine detection

Plasma concentrations of IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$  and IFN- $\gamma$  were quantitatively determined by cytometric bead array (CBA) kit - BD™ CBA Human Th1/Th2 Cytokine Kit II (BD Biosciences, USA), as previously described in the literature [16], according to manufacturer instructions. Samples were acquired using a Bectan Coulter Gallios instrument (Bectan Coulter Company, USA) and analyzed by means of Flowjo software (version 7.5).

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**Figure 1.** Comparison of plasma cytokine levels among patients infected with EV71, during acute and recovery stages, to those of healthy control subjects. Longitudinal axis shows the concentration of cytokines (pg/mL). \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ ; N: not significant. EV71, enterovirus 71; IFN, interferon; IL, interleukin; TNF, tumor-necrosis factor; Control, healthy control; Acute, acute stage; Recovery, recovery stage.

### Statistical analysis

Student's t-test was used for continuous data. Nonparametric data without normal distribution were tested by Mann-Whitney U-test. All analyses were performed by means of SPSS software (version 17.0; SPSS).  $P < 0.05$  was considered statistically significant.

### Results

#### Clinical features and outcomes

A total of 50 patients with EV71 infection and 20 healthy control subjects were enrolled in the study. Median age at onset of disease was 1.50 (1~4) years. Male to female ratio was 3:2 for EV71 cases and 1:1 for healthy controls. Median length of stay for patients was 7 (5~11) days. All 50 patients were completely recovered when discharged from the hospital.

#### Plasma cytokine levels

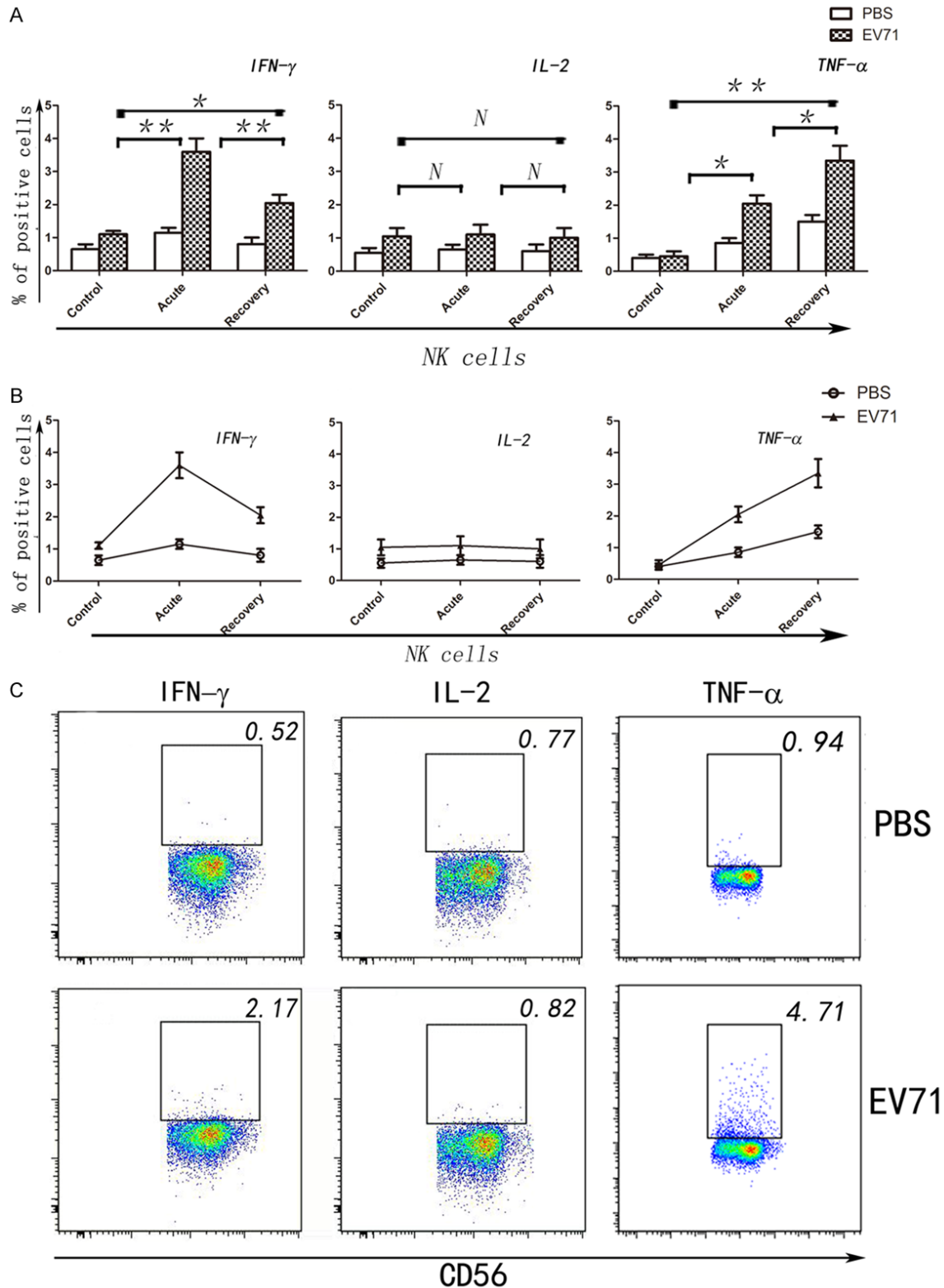
**Table 1** and **Figure 1** compare plasma cytokine levels of patients, during the acute and recovery

stages, to those of healthy control subjects. Compared with healthy control subjects, there was significant elevation of plasma cytokine levels of IFN- $\gamma$ , IL-6, and TNF- $\alpha$  ( $p < 0.01$ , **Figure 1A, 1D, 1F**) among patients at the acute stage. During the recovery stage, there was a decline in cytokine levels of IL-6 ( $P < 0.05$ , **Figure 1D**) and IFN- $\gamma$  ( $p < 0.01$ , **Figure 1A**), but an increase of cytokine levels of TNF- $\alpha$  ( $p < 0.01$ , **Figure 1F**), compared to the acute stage. There was very little increase of cytokine levels among the 3 cytokines, IL-2, IL-4, and IL-10 ( $p < 0.05$ , **Figure 1B, 1C, 1E**), during infection, compared to healthy control subjects.

#### Antiviral cytokine expression of NK cells after stimulation of EV71 antigen

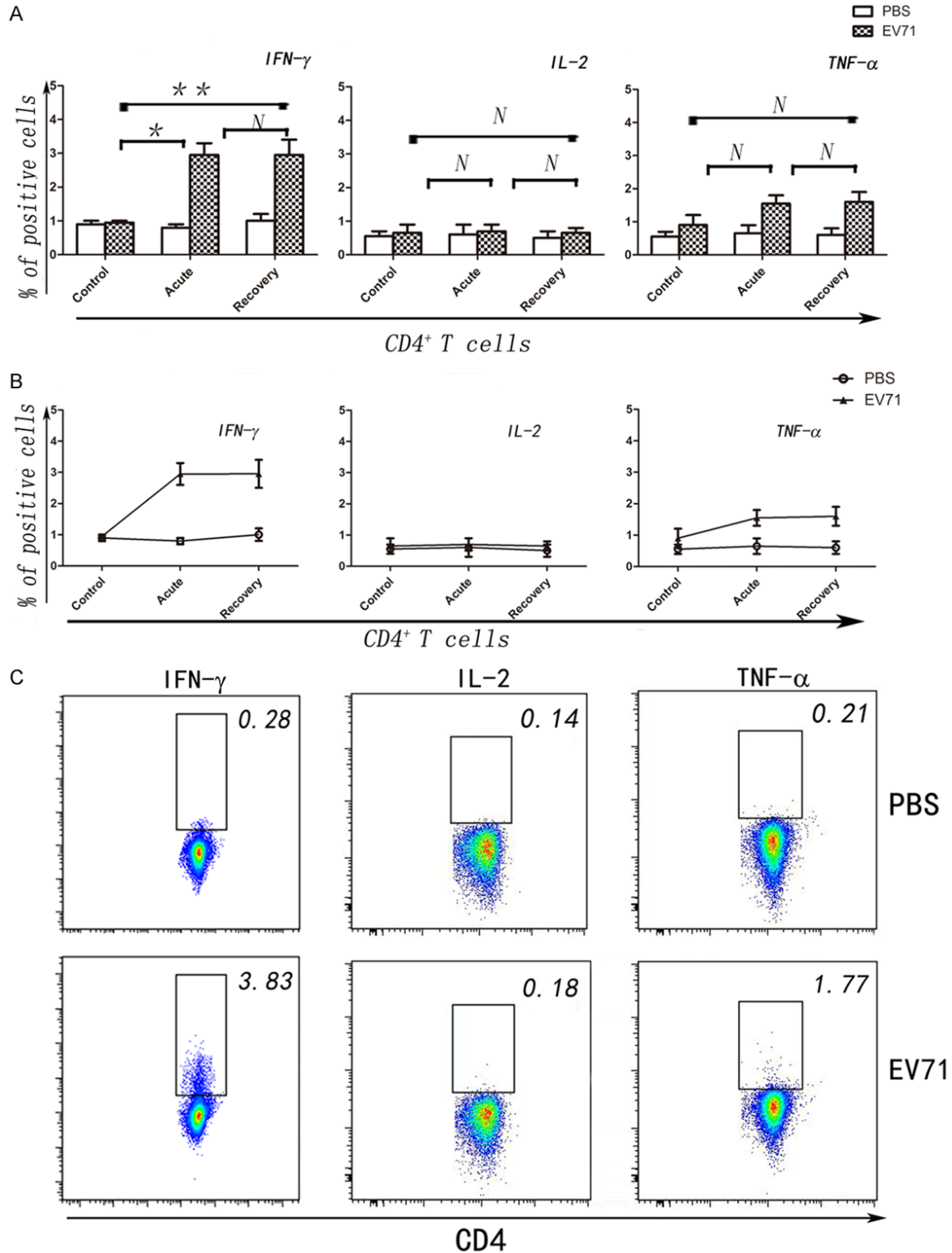
To determine the antiviral cytokine (IFN- $\gamma$ , IL-2 & TNF- $\alpha$ ) response of NK and CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, PBMCs were stimulated with EV71 antigen. As shown in **Figure 2A**, EV71 antigen increased IFN- $\gamma$  and TNF- $\alpha$  expression in NK cells among the acute stage and recovery stage. IFN- $\gamma$  expression at the acute stage was higher

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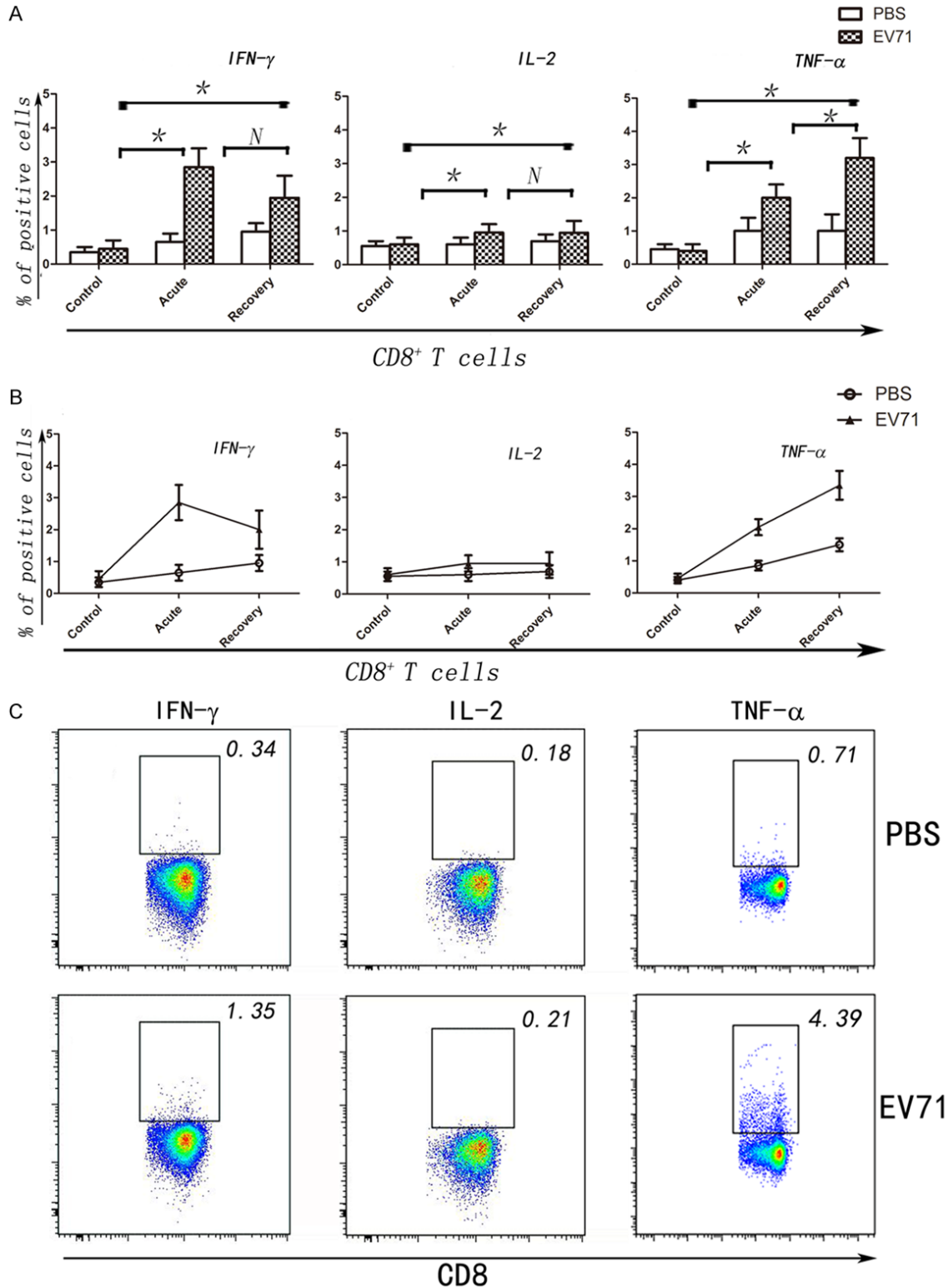
**Figure 2.** Antiviral cytokine expression of NK cells after stimulation of EV71 antigen. A, B: The antiviral cytokine response of NK cells during acute stage (n=50) and recovery stage (n=50) of infection, as well as healthy control (n=20). Longitudinal axis shows the percentage of cytokine positive cells. C: A representative flow plot for cellular cytokines within NK cells is shown (numbers in pictures show the percentage of cytokine positive cells with and without the EV71 antigen). Data shown are mean  $\pm$  s.e.m. \*\*: p<0.01; \*: p<0.05; N: not significant. NK, natural killer; EV71, enterovirus 71; IFN, interferon; IL, interleukin; TNF, tumor-necrosis factor; Control, healthy control; Acute, acute stage; Recovery, recovery stage.

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**Figure 3.** Antiviral cytokine expression of CD4<sup>+</sup> T-cells after stimulation of EV71 antigen. A, B: The antiviral cytokine response of CD4<sup>+</sup> T-cells during acute stage (n=50) and recovery stage (n=50) of infection, as well as healthy controls (n=20). Longitudinal axis shows the percentage of cytokine positive cells. C: A representative flow plot for cellular cytokines within CD4<sup>+</sup> T cells is shown (numbers in pictures show the percentage of cytokine positive cells with and without the stimulation of EV71 antigen). Data shown are mean ± s.e.m. \*\*: p<0.01; \*: p<0.05; N: not significant. EV71, enterovirus 71; IFN, interferon; IL, interleukin; TNF, tumor-necrosis factor; Control, healthy control; Acute, acute stage; Recovery, recovery stage.

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**Figure 4.** Antiviral cytokine expression of CD8<sup>+</sup> T-cells after stimulation of EV71 antigen. A, B: The antiviral cytokine response of CD8<sup>+</sup> T cells during acute stage (n=50) and recovery stage (n=50) of infection, as well as healthy controls (n=20). Longitudinal axis shows the percentage of cytokine positive cells. C: A representative flow plot for cellular cytokines within CD8<sup>+</sup> T-cells is shown (numbers in pictures show the percentage of cytokine positive cells with and without the stimulation of EV71 antigen). Data shown are mean  $\pm$  s.e.m. \*\*: p<0.01; \*: p<0.05; N: not significant. EV71, enterovirus 71; IFN, interferon; IL, interleukin; TNF, tumor-necrosis factor; Control, healthy control; Acute, acute stage; Recovery, recovery stage.

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**Table 2.** T-cell and NK cell counts in patients with EV71, at different stages of infection, to healthy control subjects

Cell Category	Healthy Control (n=20)	Acute Stage (n=50)	Recovery Stage (n=50)
CD3 <sup>+</sup> T cells	1720 (1022~2324)	1570 (1125~1994)	1683 (1184~2207)
CD4 <sup>+</sup> T cells	1152 (482~1585)	1205 (414~1459)	1088 (572~1506)
CD8 <sup>+</sup> T cells	514 (246~729)	583 (223~751)	601 (214~813)
NK cells	362 (160~572)	241 (98~389) <sup>a</sup>	226 (104~411) <sup>b,c</sup>

Note: Measurement unit is cells/mm<sup>3</sup>. There were no significant differences in cell counts among the 3 groups in any of the T-cell subpopulations. <sup>a</sup>P<0.05 vs. Healthy control group; <sup>b</sup>P<0.05 vs. Healthy control group; <sup>c</sup>P>0.05 vs. Acute stage group.

than that at recovery stage ( $p < 0.01$ ). Regarding TNF- $\alpha$ , it was lower at acute stage than the recovery stage ( $p < 0.05$ ). However, EV71 antigen did not induce significant cytokine response in NK cells among the healthy controls.

### *Antiviral cytokine expression of CD4<sup>+</sup> T-cells after stimulation of EV71 antigen*

This study further determined the antiviral cytokine response of CD4<sup>+</sup> T-cells. As shown in **Figure 3A**, EV71 antigen only induced IFN- $\gamma$  expression of CD4<sup>+</sup> T-cells and IFN- $\gamma$  expression, at the recovery stage, was not significantly different from that at acute stage. Moreover, EV71 antigen did not induce significant IL-2 and TNF- $\alpha$  response in CD4<sup>+</sup> T-cells.

### *Antiviral cytokine expression of CD8<sup>+</sup> T-cells after stimulation of EV71 antigen*

When determining the antiviral cytokine response of CD8<sup>+</sup> T-cells, it was found that EV71 antigen induced IFN- $\gamma$ , IL-2, and TNF- $\alpha$  expression of CD8<sup>+</sup> T-cells. However, only for TNF- $\alpha$ , the cytokine expression was higher at the recovery stage than acute stage ( $p < 0.05$ , **Figure 4A**). EV71 antigen did not induce a significant cytokine response in CD8<sup>+</sup> T-cells among healthy controls.

### *Cell counts*

**Table 2** compares the counts of T-cells and NK cells of patients, during acute and recovery stages, to those of healthy control subjects. There were no significant differences in cell counts among the 3 groups in any of the T-cell subpopulations. NK cell counts, however, in patients during acute and recovery stages, were lower than the healthy control group ( $p < 0.05$ ).

## Discussion

To the best of our knowledge, this is the first study to quantitatively investigate cellular immunity, during different stages of infection, among EV71-infected children using flow cytometry. This study analyzed the IFN- $\gamma$ , TNF- $\alpha$  and IL-2 response of NK and CD4<sup>+</sup> T and CD8<sup>+</sup> T-cells, during different

stages of EV71 infection, among pediatric patients. It was found that IFN- $\gamma$  from NK and CD4<sup>+</sup> T and CD8<sup>+</sup> T-cells increased during the inflammatory response in the acute stage. TNF- $\alpha$  response of NK and CD8<sup>+</sup> T-cells was critical in the recovery stage, but IL-2 did not play a significant role in the antiviral reaction. Results of plasma cytokine assay found an increase in IFN- $\gamma$ , IL-6, and IL-10 plasma cytokine levels in the acute stage, with a decrease in levels of the 3 cytokines in the recovery stage. TNF- $\alpha$  was dramatically higher in the recovery stage than acute stage. It was also found that NK cell counts of EV71-infected children were significantly lower than in healthy children, however, their T-cell counts were not significantly different. Since the patients enrolled in this study were uncomplicated cases, without brainstem encephalitis (BE) and pulmonary edema (PE), their immunity statuses reflected the general situation of anti-EV71 immunity of common children. Results of this present study may be used to predict progression of the disease. This study also suggests that there may be a therapeutic use for IFN- $\gamma$  in the treatment of EV71 infection.

In 2000, two studies investigated relevant nucleotide sequences and the whole genome to compare EV71 isolates from fatal and non-fatal cases. Most EV71 isolates, however, have been found to be identical or nearly identical [17, 18]. It seems that host factors may be critical to the outcome of EV71 infection. In 2003, researchers in Taiwan found that DCs presented high levels of MHC molecules rather than MHC II molecules after being infected by EV71 [13]. In 2003, a study in Taiwan found significant elevation of plasma cytokines IL-10, IL-13, and IFN- $\gamma$  among patients with PE [15, 23]. A study in 2006 showed that cases with pulmonary

edema (PE) had dramatically weaker cellular immunity compared with other uncomplicated cases [14]. There are differences between different stages of infection due to the host's immunity status. It is regretful that most of these studies, above, did not consider infection stages as a factor in their research.

A belief that acute inflammatory reactions are the main causes of deaths has promoted the use of anti-inflammatory drugs, such as corticosteroids and immunoglobulin, to treat severe cases. Their effectiveness, however, has remained uncertain [19, 20]. Since the present results demonstrate that IFN- $\gamma$  plays a critical role in the anti-EV71 inflammatory reaction, use of IFN- $\gamma$  to enhance cellular immunity in patients with insufficient immunity may be a novel strategy in for treatment of EV71 infections. In the case of poliomyelitis, IFN- $\gamma$  treatment of age-dependent poliomyelitis-susceptible mice protected them from paralytic disease [21]. It has also been reported that the combined use of IFN- $\gamma$  and IFN- $\alpha$  has a strong synergistic effect on severe acute respiratory syndrome-associated coronavirus (SARS-CoV) replication [22].

The present research shows that plasma levels of IFN- $\gamma$ , IL-6, and TNF- $\alpha$  in different stages, may be used to predict progression of the disease. Use of IFN- $\gamma$  may be a novel way to treat these immunocompromised children with virus infections, but further clinical studies regarding safety and efficacy are necessary. Since an EV71 vaccine is unavailable, induction of the appropriate Th1 cellular response may be critical to vaccine development, aiming to prevent severe EV71 disease. The present study also provides information concerning Th1 cellular response of EV71 infections that may prove useful in the development of a high-efficiency EV71 vaccine.

### Disclosure of conflict of interest

None.

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