

Clinical significance of inflammatory cytokine and chemokine expression in hand, foot and mouth disease

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Abstract. The present study examined the relationship between cytokine and chemokine expression and the clinical presentation of hand, foot and mouth disease (HFMD), which is currently unclear. The present study involved 28 patients with mild HFMD, 44 patients with severe HFMD and 26 healthy children. Venous blood was tested for cytokine [interleukin (IL)-4, IL-12, IL-18, tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ)] and chemokine expression [IL-8, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and IFN- γ -inducible protein-10 (IP-10)]. Stool samples from the patients were tested for enterovirus 71 (EV71) RNA using reverse transcription-polymerase chain reaction. The results indicated that all cytokine/chemokine levels were increased in patients with severe HFMD compared with in patients with mild HFMD or control subjects. In addition, RANTES, MCP-1, IL-4, IL-12 and IL-18 levels were higher in mild HFMD patients than in the controls. In patients with severe HFMD, all expression levels (with the exception of IL-8 and IL-4) were increased in patients with encephalitis plus pulmonary edema compared with those with encephalitis alone. Furthermore, all levels (with the exception of IL-8) were increased in EV71-positive patients compared with EV71-negative patients. In mild HFMD, all levels (with the exception of IL-8 and IL-4) were increased in EV71-positive patients compared with EV71-negative patients.

However, in severe HFMD, only RANTES, IP-10 and IFN- γ levels were increased in EV71-positive patients compared with EV71-negative patients. In the EV71-negative group, all levels were increased in severe HFMD compared with mild HFMD. In the EV71-positive group, all levels (with the exception of IL-8) were increased in severe HFMD compared with mild HFMD. These results indicated that cytokines and chemokines participate in HFMD pathogenesis, and may be useful to monitor disease progression and predict prognosis.

Introduction

Hand, foot and mouth disease (HFMD) is a common acute infectious disease caused by various human intestinal viruses. HFMD usually affects preschool children (age, <3 years) and has caused widespread epidemics in this population (1). It is commonly caused by enterovirus 71 (EV71) and coxsackievirus A16 (1) and EV71 leads to more HFMD compared with coxsackievirus A16. Meanwhile, EV71 is the most neurovirulent of enteroviruses and is responsible for fatal outcomes. EV71 is a member of an enterovirus group that belongs to the Picornaviridae family. Similar to other members of Picornaviridae, it is a non-enveloped, positive, non-segmented RNA virus, with a diameter of ~30 nm. Its genomic RNA is packaged within the capsid composed of 60 copies of 4 structural proteins, VP1, VP2, VP3 and VP4. It was first isolated in California, in 1969. Since the 1960s, EV71 has led to numerous epidemic outbreaks worldwide. Presently, it has become a major public health issue, especially in Asia-Pacific region (2).

The severity of HFMD varies; patients with mild HFMD present with mild fever and a self-limiting herpetic rash on the hands, feet and mouth. However, in patients with severe HFMD, the disease progresses rapidly and may involve meningitis, brainstem encephalitis, pulmonary angioneurotic edema, pulmonary hemorrhage, myocarditis, and even cardiopulmonary failure and mortality (3,4). Good oral and skin hygiene is crucial for patients with mild HFMD. Additionally, quarantine of infected individuals is also effective in order to limit the spread of the virus. Symptomatic supportive treatments should be conducted when required. No established antiviral treatments are available for severe HFMD. Intravenous

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immunoglobulin treatment has been reported to be effective and is used in various Asian countries; however, sufficient evidence for its effectiveness is lacking. Glucocorticoid may also be used to reduce brain and pulmonary edema. A comprehensive life support including fluid management, inotrope and respiratory support therapy is regularly conducted (5-7).

The pathogenesis of severe HFMD caused by EV71 infection is not fully understood. Numerous studies have demonstrated that cellular and humoral immune dysfunction in patients with HFMD, in particular the abnormal expression of inflammatory cytokines and chemokines and an imbalance in the expression of anti- and proinflammatory cytokines, is involved in the exacerbation of HFMD (8-11).

In the present study, the expression levels of inflammatory cytokines and chemokines were detected in peripheral blood samples obtained from children with EV71-induced HFMD. The relationship between abnormal cytokine/chemokine expression and various clinical presentations of HFMD was analyzed in order to clarify the mechanisms underlying the exacerbation of HFMD. The clinical significance of the results is discussed.

Materials and methods

Patient selection. The present study involved 72 children (39 male, 33 female) with mild or severe HFMD who were treated at Hangzhou Sixth People's Hospital (Hangzhou, China) between March 2011 and June 2013. All diagnoses were made using the Foot and Mouth Disease Treatment Guidelines (2010) issued by the Ministry of Health and the Expert Consensus on the Clinical Treatment of Severe EV71 Infection (6,7). Patients who received immunomodulators, or who had other viral or bacterial infections or other immune disorders, were excluded.

In addition, 26 healthy children (control group), who had normal findings on physical examination from Hangzhou Sixth People's Hospital, were recruited. Verbal consent was obtained from all participants. Ethical approval (no. 2017-11) for the present study was obtained from the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (Hangzhou, China).

Sample collection. Venous blood samples (3 ml) were collected from all children in the HFMD and control groups within 24 h of admission. The blood samples were collected in EDTA tubes and centrifuged at $3,000 \times g$ for 15 min at room temperature to obtain plasma samples, which were subsequently stored at -80°C until further analysis. In addition, stool samples were collected from the children with HFMD, and these were subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for the detection of EV71 RNA.

EV71 detection. A diagnostic PCR-fluorescence probing kit (cat. no. DA-BT109) for enterovirus RNA (Da An Gene Co., Ltd., Guangzhou, China) was used for the detection of EV71. The RNA extraction reagent was conducted in the PCR-fluorescence probing kit. All forward and reverse primer sequences used were contained within the kit. The procedure was conducted according to the manufacturer's protocol. The

thermal cycling parameters were as follows: cDNA synthesis at 42°C for 25 min; denaturation at 94°C for 3 min; and 40 cycles at 93°C for 15 sec and 55°C for 45 sec. RT-PCR was performed on the CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). If test sample had a typical S-shaped amplification curve and the Cq value (12) was lower than 34.9, it was deemed to be positive, otherwise it was negative.

Cytokine and chemokine detection. Plasma expression levels of the cytokines interleukin (IL)-4, IL-12, IL-18, tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), and the chemokines IL-8, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and IFN- γ -inducible protein-10 (IP-10) were determined using double-antibody sandwich enzyme-linked immunosorbent assays (ELISA; R&D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocols. The catalogue numbers for all ELISA kits used were as follows: IL-4, D4050; IL-12, D1200; IL-18, 7620; TNF- α , DTA00C; IFN- γ , DIF50; IL-8, D8000C; RANTES, DRN00B; MCP-1, DCP00; IP-10, DIP100.

Statistical analysis. Data were analyzed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA) and were representative of three independent experiments expressed as the mean \pm standard deviation. Multi-sample means were detected using one-way ANOVA analysis of variance and the Student's t-test. The multiple comparisons test was adjusted by Bonferroni test. Correlations were analyzed using Pearson's correlation analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Demographic data. Of the 72 study participants (39 male, 33 female), 28 (17 male, 11 female; average age, 2.3 ± 0.8 years) had mild HFMD and 44 (22 male, 22 female; average age, 2.1 ± 0.7 years) had severe HFMD. The control group included 16 males and 10 females, with an average age of 2 ± 0.6 years. No significant differences in age or gender were present between the mild HFMD, severe HFMD and control groups.

Cytokine and chemokine expression. The plasma expression levels of all chemokines (IL-8, RANTES, MCP-1, IP-10; Fig. 1A) and cytokines (IL-4, IL-12, IL-18, TNF- α , IFN- γ ; Fig. 1B) were significantly increased in the severe HFMD group compared with the mild HFMD and normal control groups ($P < 0.01$). In addition, the expression levels of the chemokines RANTES and MCP-1 (Fig. 1A) and the cytokines IL-4, IL-12 and IL-18 (Fig. 1B) were significantly increased in the mild HFMD group compared with the normal control group ($P < 0.05$).

The children in the severe HFMD group were further divided into those with encephalitis (E group; $n=26$; 14 male, 12 female) and those with both encephalitis and pulmonary edema (E+P group; $n=18$; 8 male, 10 female). No significant differences in age or gender were present between the E and E+P groups. The plasma expression levels of all chemokines, with the exception of IL-8 (Fig. 2A), and all cytokines, with

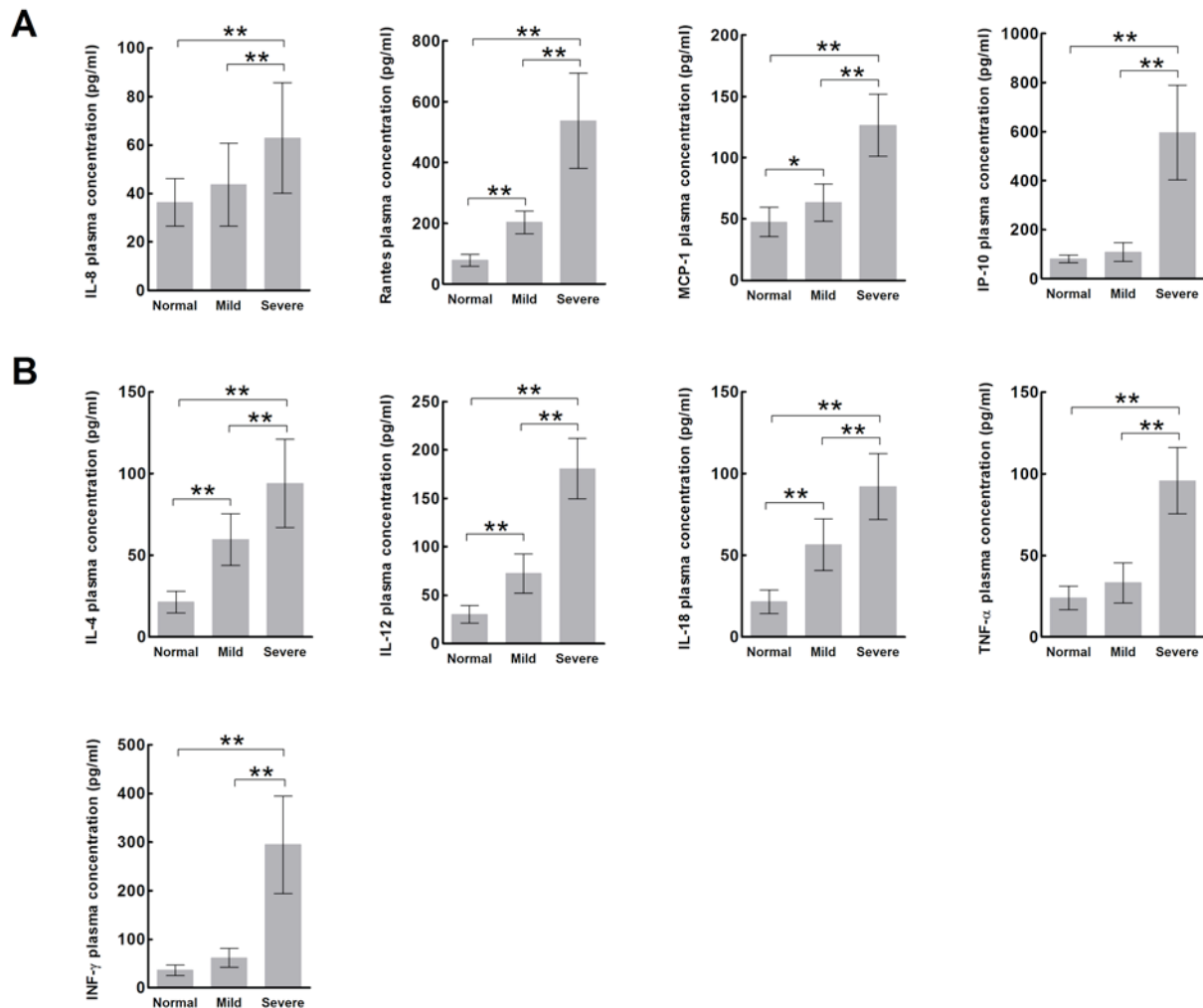


Figure 1. Expression of inflammatory (A) chemokines and (B) cytokines in children with severe and mild hand, foot and mouth disease. * $P < 0.05$, ** $P < 0.01$. IL, interleukin; RANTES, regulated on activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1; IP-10, IFN- γ -inducible protein-10; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ .

the exception of IL-4 (Fig. 2B) were significantly increased in the E+P group compared with the E group ($P < 0.05$).

EV71 RNA detection in stool samples and its correlation with plasma cytokine and chemokine expression. Stool samples from the children with HFMD were analyzed for the presence of EV71 RNA using RT-PCR (Table I). The plasma expression levels of all chemokines, with the exception of IL-8 (Fig. 3A), and all cytokines (Fig. 3B) were significantly increased in children whose stool samples tested positive for EV71 RNA compared with children whose stool samples were negative for EV71 RNA ($P < 0.05$). In the mild HFMD group, the plasma expression levels of all chemokines, with the exception of IL-8 (Fig. 4A), and all cytokines, with the exception of IL-4 (Fig. 4B) were significantly increased in the EV71-positive patients compared with the EV71-negative patients ($P < 0.05$). However, in the severe HFMD group, only the expression levels of RANTES (Fig. 4A), IP-10 and IFN- γ (Fig. 4B) were significantly increased in the EV71-positive patients compared with the EV71-negative patients ($P < 0.05$).

The children with HFMD were regrouped according to their EV71 status. Of the 72 study patients, 33 (18 male, 15 female) were EV71 negative, and 39 (21 male, 18 female)

were EV71 positive. Of the 33 EV71-negative patients, 17 (10 male, 7 female) had mild HFMD and 16 (8 male, 8 female) had severe HFMD. Of the 39 EV71-positive patients, 11 (7 male, 4 female) had mild HFMD and 28 (14 male, 14 female) had severe HFMD (Table I).

In the EV71-negative group, the plasma expression levels of all cytokines (Fig. 5A) and chemokines (Fig. 5B) were significantly increased in children with severe HFMD compared with children with mild HFMD ($P < 0.01$). In the EV71-positive group, the plasma expression levels of all cytokines, with the exception of IL-8 (Fig. 5A), and all chemokines (Fig. 5B) were significantly increased in children with severe HFMD compared with children with mild HFMD ($P < 0.01$).

Discussion

The immune system is a complex, sophisticated and coordinated network, and its dynamic equilibrium sustains health. Upon invasion by microorganisms, inflammatory mediators are generated in 'cascades' through a series of pathways, which results in systemic inflammatory response syndrome. In addition, the excessive generation of inflammatory mediators may lead to tissue and organ damage; therefore, various

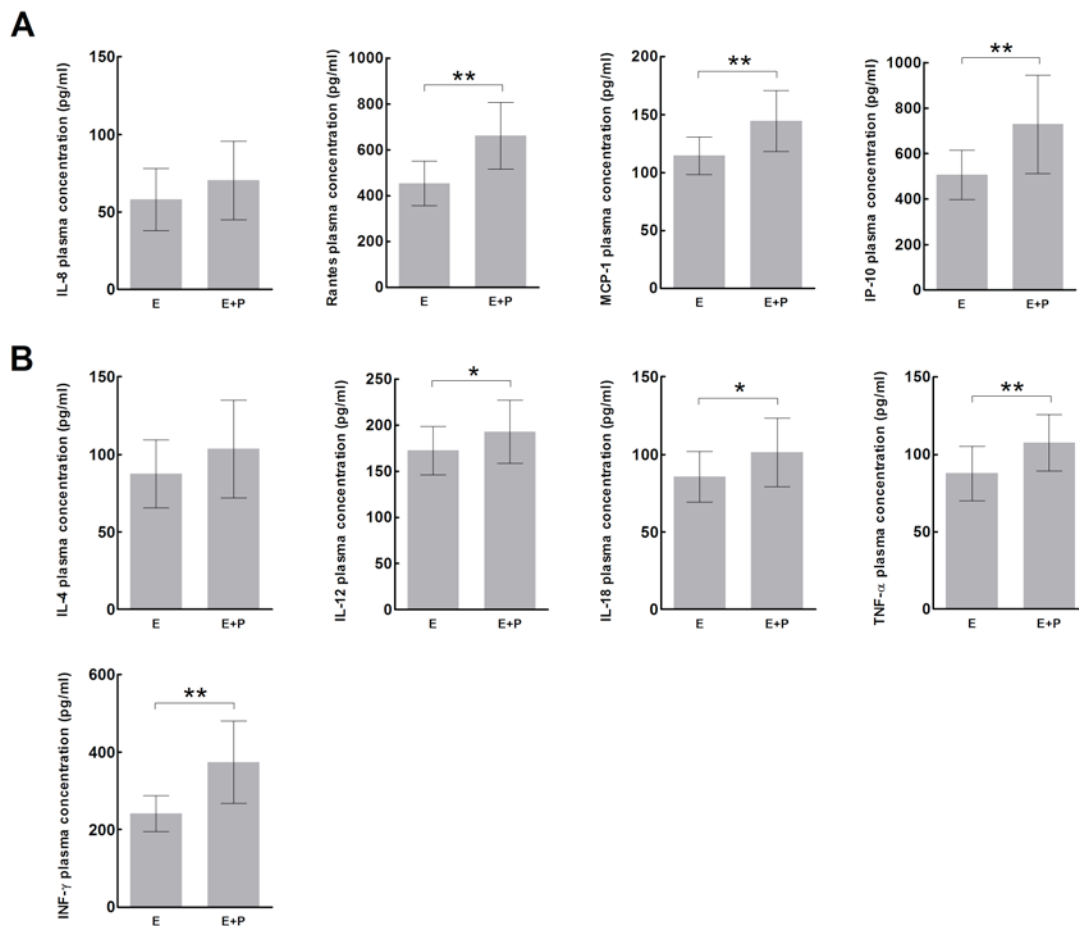


Figure 2. Expression of inflammatory (A) chemokines and (B) cytokines in children with severe hand, foot and mouth disease, in the E and E+P groups. * $P<0.05$, ** $P<0.01$. E, encephalitis group; E+P, encephalitis with pulmonary edema group; IL, interleukin; RANTES, regulated on activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1; IP-10, IFN- γ -inducible protein-10; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ .

anti-inflammatory mediators are produced to inhibit excessive inflammatory responses and protect the body from immune damage (13). Pro- and anti-inflammatory mediators coexist in the infection-induced immunological stress response, and the growth, decline and equilibrium of the two determine the evolution, development and prognosis of infectious disease (14).

In children, the immune system is not fully developed, which facilitates the invasion of pathogenic microorganisms. As a result, children, particularly preschoolers, exist in a physiological immunocompromised state, and their specific and non-specific immune responses are poor compared with those of adults. This may partially explain why preschoolers are susceptible to HFMD.

Following EV71 infection, susceptible cells and non-specific immune cells are stimulated to produce cytokines including IFNs, TNF- α and IL-12. These cytokines are involved in the early control of viral replication and infection. The virus further induces the production of chemokines, including IL-8, MCP-1 and IP-10, which contribute to the recruitment of non-specific immune cells, including natural killer cells, monocytes and macrophages, to the infection site. Furthermore, the activation of these cells by cytokines results in the secretion of inflammatory mediators and cytokines, interference with viral replication and the death of virus-infected host cells. These responses expand and enhance anti-infection

immunity and accelerate recovery from HFMD, leading to a self-limiting disease (10,11,15-17). However, the pathogenic mechanisms that lead to the exacerbation of EV71 infection and to abnormal cytokine expression remain unknown.

In the present study, the relationship between plasma cytokines (IL-4, IL-12, IL-18, TNF- α , IFN- γ) and chemokines (IL-8, RANTES, MCP-1, IP-10), and disease severity (mild vs. severe groups), complications (E vs. E+P groups) and fecal EV71 status (EV71-positive vs. EV71-negative groups) was examined in children with HFMD. The results demonstrated that cytokine and chemokine expression levels were i) significantly increased in the severe HFMD group compared with the mild HFMD and normal control groups; ii) significantly increased in the E+P group compared with the E group; and iii) significantly increased in the EV71-positive group compared with the EV71-negative group, regardless of disease severity. These results suggested that EV71 infection-induced abnormal expression of cytokines and chemokines was involved in the exacerbation of HFMD.

It has previously been demonstrated that cytokines, including IL-1 β , IL-6, IL-18, TNF- α and IFN- γ , have inhibitory effects on the replication and infective ability of numerous viruses, and that these compounds are major components of the early non-specific immune response to viral infections (18). Conversely, chemokines, including IL-8, RANTES, MCP-1 and IP-10, are involved in the recruitment and activation

Table I. Hand, foot and mouth disease groups and EV71 status.

Group	EV71-PCR (+)		EV71-PCR (-)	
	No. (M/F)	Average age (y)	No. (M/F)	Average age (y)
Mild	11 (7/4)	2.3±0.8	17 (10/7)	2.2±0.9
Severe (E)	17 (8/9)	1.9±0.7	9 (6/3)	2.1±0.6
Severe (E+P)	11 (6/5)	2.1±0.8	7 (2/5)	2.2±0.6

EV71, enterovirus 71; PCR, polymerase chain reaction; M, male; F, female; E, encephalitis; P, pulmonary edema.

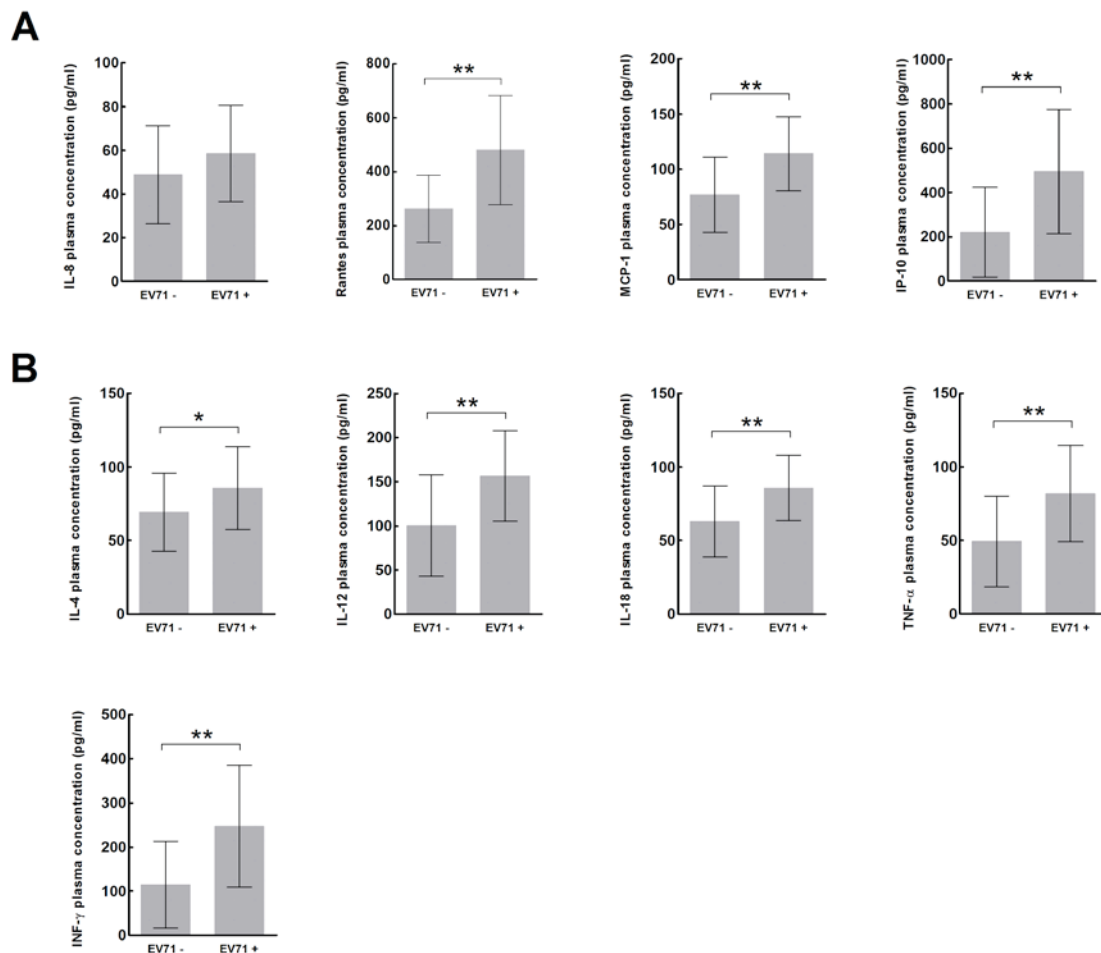


Figure 3. Expression of inflammatory (A) chemokines and (B) cytokines in patients with hand, foot and mouth disease whose stool samples tested positive or negative for EV71 RNA. *P<0.05, **P<0.01es. EV71, enterovirus 71; IL, interleukin; RANTES, regulated on activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1; IP-10, IFN-γ-inducible protein-10; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

of immune cells (19), thereby further enhancing antiviral immunity. Overexpression or imbalances in the expression of these immune factors aggravates the inflammatory response to infection, causing damage to infected tissues and leading to loss of or disordered organ function. Under these circumstances, the vulnerable immune system of children is unable to effectively eliminate the virus and control inflammation, which results in further spread of the virus and eventually causes severe HFMD.

Lin *et al* (10) confirmed the aforementioned mechanism by demonstrating that the peripheral expression levels of IL-1β

and several other inflammatory cytokines were significantly increased in patients with EV71-induced HFMD with both encephalitis and pulmonary edema, as compared with in those with only encephalitis, those with mild symptoms or normal controls. A previous study reported similar results; demonstrating that the expression levels of inflammatory cytokines, including IFN-γ, MCP-1 and IP-10 in the peripheral blood and cerebrospinal fluid were significantly increased in patients with pulmonary edema compared with patients with encephalitis or patients with mild disease (11). Similar results have been reported for the inflammatory cytokine TNF-α,

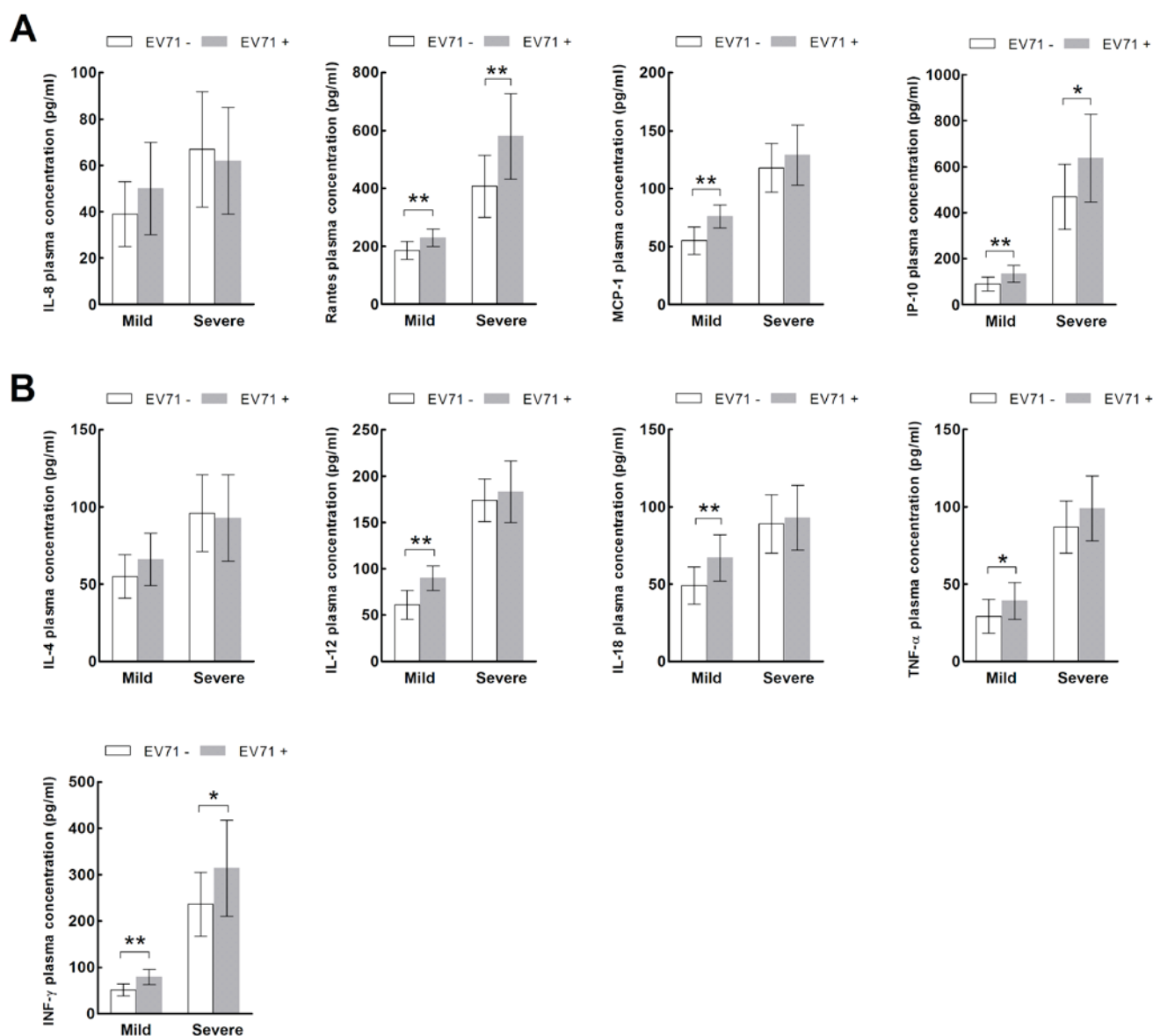


Figure 4. Expression of inflammatory (A) chemokines and (B) cytokines in the mild and severe hand, foot and mouth disease groups according to EV71 status. * $P < 0.05$, ** $P < 0.01$. EV71, enterovirus 71; IL, interleukin; RANTES, regulated on activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1; IP-10, IFN- γ -inducible protein-10; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ .

which was reported to have an abnormally high expression in patients with HFMD displaying pulmonary edema and encephalitis (20).

These results indicated that in the early stages of infection the increase in the levels of various cytokines and chemokines may contribute to the early activation of specific and non-specific immunity. This maximizes the mobilization of various types of immune cells to participate in anti-EV71 infection immunity for the alleviation of or recovery from the disease. When the immune system fails to effectively clear the virus and control inflammation, the disease is exacerbated. Overexpression of inflammatory cytokines and chemokines in infected tissues induces excessive immune responses and results in cytokine cascade reaction and cytokine storm through autocrine and paracrine mechanisms, resulting in severe local and systemic inflammation, and eventually tissue damage and organ dysfunction.

In conclusion, during the development and progression of HFMD, various cytokines and chemokines are involved in the occurrence of inflammation. Some of these compounds clear the virus, whereas others exacerbate the disease. However, at present, the immune pathogenesis of HFMD, its cytokine expression patterns and the changes in these patterns are not clear. Further investigation into the involvement of HFMD-associated cytokines and chemokines in the pathogenesis of the disease is required to search for cytokine indices to be used to monitor disease progression, determine prognosis, and guide clinical diagnosis and treatment.

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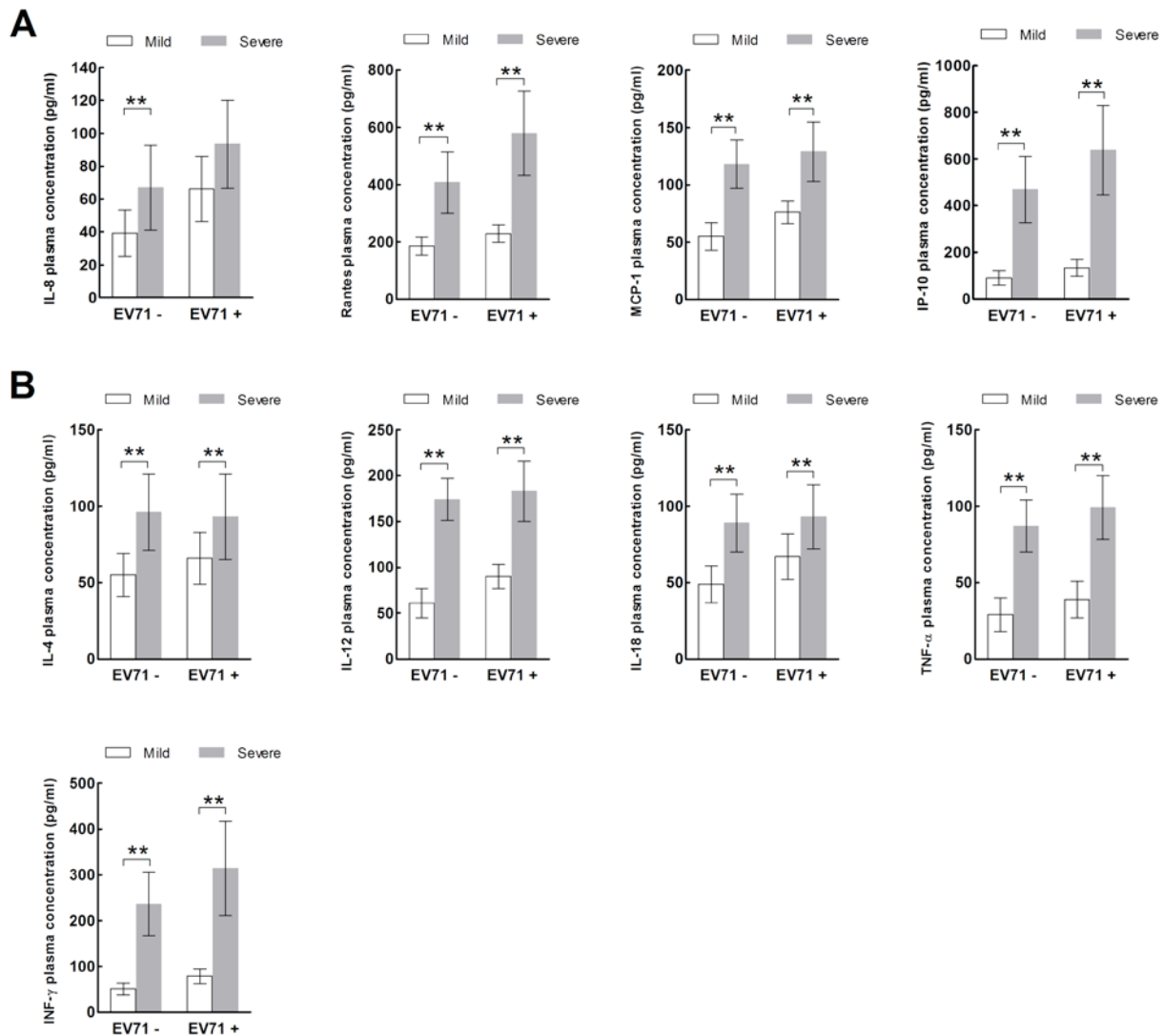


Figure 5. Expression of inflammatory (A) chemokines and (B) cytokines in the EV71-positive and EV71-negative groups according to severity of hand, foot and mouth disease. ** $P < 0.01$. EV71, enterovirus 71; IL, interleukin; RANTES, regulated on activation, normal T cell expressed and secreted; MCP-1, monocyte chemoattractant protein-1; IP-10, IFN- γ -inducible protein-10; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ .

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