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Peripheral T lymphocyte subset imbalances in children with enterovirus 71-induced hand, foot and mouth disease

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ABSTRACT

Inflammatory mediators (i.e. cytokines) play a pivotal role in the regulation of pathophysiological processes during EV71-induced hand, foot and mouth disease (HFMD). Different T cell subsets have distinct cytokine secretion profiles, and alteration in the T cell subsets frequency (imbalance) during infection leads to changed cytokine patterns. However, the effects of EV71 infection on T cell subsets were not clear. The objective of this study was to determine whether EV71-induced HFMD can be explained by the emergence of particular T-cell subsets (Th1, Th2, Tc1, Tc2, Th17, Tc17 and Treg cells) and the cytokine they produced (IFN-γ, IL-4, IL-17A and TGF-β1), as well as distinct responses to EV71 infection. We found that when compared to the control group, the percentage of Th1 and Tc1 cells was significantly higher in mild and severe HFMD group. Similar results were found in the Th1/Th2 ratio and IFN-γ levels. On the other hand, the percentage of Th17 cells and IL-17A levels were the highest in severe HFMD cases, and lowest in controls. Similar trend was also found for the Th17/Treg cell ratio. An optimal cutoff value of 2.15% for Th17 cell and 6.72 pg/ml for IL-17A provided a discriminatory value for differentiating the severity of HFMD cases by receiver operating characteristic curve analyses. These findings reveal that the Th1/Th2 and Th17/Treg imbalance exist in HFMD patients, suggesting their involvement in the pathogenesis of EV71 infection, which may have potential value as biomarkers.

1. Introduction

Hand, foot and mouth disease (HFMD) is a major infectious disease that is prevalent in infants and children <5 years old (Ho et al., 1999; Wang et al., 1999). Among the various enterovirus serotypes, enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are the most frequent strains that cause HFMD (Yang et al., 2011). Most HFMD cases are mild and self-limited, but some, especially EV71-induced cases may have serious complications, including aseptic meningitis, encephalitis, brainstem encephalitis (BE), poliomyelitis-like paralysis, pulmonary edema (PE), and even death (Ho et al., 1999; Wang et al., 1999). Since 1997, outbreaks of HFMD associated with EV71 have been witnessed worldwide, especially in Asia-Pacific region, leading to millions of cases and hundreds of deaths (Ahmad, 2000; Ho et al., 1999; Sanders et al., 2006; Yang et al., 2009). For instance, in mainland China, a total of 488,955 HFMD cases were reported nationwide including 126 fatal cases in 2008, with EV71 as the major pathogen (Yang et al., 2009). Thus, EV71 infection has emerged as an important public health concern.

Until now, the pathogenesis of EV71 infection is not completely clear. Recent studies have found that some inflammatory mediators were elevated after EV71 infection, including IFN-γ (a Th1 cytokine), IL-13 (a Th2 cytokine), IL-6 (a pleiotropic cytokine), IL-1β (a pro-inflammatory cytokine), IL-10 (an immunoregulatory cytokine), and the chemokines IL-8 and IP-10, which are correlated with disease severity and outcome (Chang et al., 2006; Lin et al., 2002; Wang et al., 2003, 2008). A mouse model study showed that mice lacking CD4+ or CD8+ T cells developed more severe disease, compared to cellular-immune competent mice upon EV71 infection (Lin et al., 2009). Observations in an EV71-infected neonate mouse model also showed that sustained high levels of IL-6 might cause tissue damage (Khong et al., 2011). Altogether, these studies

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underscore the importance of cellular immunity in the pathogenesis of EV71-mediated disease.

T lymphocytes, as important players in immune regulation, are classified into CD4+ T cells (including Th1, Th2, Th17, and Treg subsets) and CD8+ T cells (including Tc1, Tc2, Tc17) based on their surface phenotype, cytokine production patterns, as well as the functional characteristics (Saxena et al., 2011; Zhou et al., 2009). In brief, Th1 and Tc1 cells produce type 1 cytokines (IL-2, TNF-α, and IFN-γ), whereas Th2 and Tc2 cells secrete type 2 cytokines (IL-4, IL-5, and IL-13). Similarly, Treg cells are characterized by their IL-10 and transforming growth factor (TGF-β) secretion capacities, while Th17 and Tc17 cells are characterized by a strong IL-17-producing capacity. The imbalance between various T-cell subsets accounts for different immune pathologies, including infectious disease. For example, as opposed to pro-inflammatory Th1/Th17 responses favoring the development of chronic *Staphylococcus aureus* implant infection, Th2/Treg responses provide a protective mechanism against chronic infection (Prabhakara et al., 2011). Ye et al. (2010) found that the frequencies of intrahepatic Th17 and Th1 cells in patients with hepatitis B virus (HBV)-associated liver dysfunction were much higher than that of Th2 and Treg cells, indicating inappropriate and excessive Th17 and Th1 effector responses may be involved in the pathogenesis of HBV-associated liver inflammation and hepatocellular damage. Patients with severe A/H1N1 pneumonia exhibit cellular Th2/Tc2 responses that likely contribute to the poor prognosis observed in these patients (Zuniga et al., 2011). Likewise, a prominent shift to a Th2/Tc2 phenotype in patients with condyloma acuminatum (CA) supports the notion that the dominantly repressed activation of the Th1 and Tc1 phenotype is associated with the pathogenesis of CA (Xu et al., 2009). These evidences together highlight the crucial role of T-cell subsets imbalance in immune-mediated infections.

The involvement of different T cell subsets in EV71-induced HFMD had also been under investigation. For example, clinical studies have shown that Th17 were involved in the pathological process of EV71 infection (Chen et al., 2012), and CD4+ and CD8+ T lymphocytes are associated with disease activity and adverse outcomes (Wang et al., 2003). However, to date no systematic analysis of the effect of EV71 infection on T cell subsets in HFMD patients had been conducted. Furthermore, as there are still no approved vaccines or drugs available for the prevention of EV71 infection or effective clinical treatment, a better understanding of its pathogenesis might lead to new therapeutic strategies. Therefore, in the present study, to examine whether peripheral blood T cell subset (including Th1, Th2, Th17, Treg, Tc1, Tc2 and Tc17) homeostasis was disrupted in HFMD patients, we examined the distribution of these T cell subsets and the corresponding inflammatory mediators (including IL-4, IFN-γ, IL-17A and TGF-β1). We found that imbalances of T cell subsets, specifically, upregulated Th1/Th2 and Th17/Treg ratio occurred during EV71 infection, which might contribute to the pathogenesis of HFMD.

2. Materials and methods

2.1. Case selection

Patients were identified by senior physicians with special training according to the Chinese Ministry of Health diagnostic criteria of HFMD (2008) (http://www.moh.gov.cn/mohbgt/s9503/200812/38494.shtml). In accordance with the guideline, HFMD was characterized by oral ulcers and skin lesions on the hands and feet, with fever or not. EV71 infection was defined as the successful detection of EV71 nucleic acid in the throat swabs or stool specimens, which were collected from each patient on the day of admission. Patients treated with intravenous immunoglobulin (IVIG) or steroids prior to blood sampling were excluded from this study. Thus, a total of 95 EV71-infected HFMD patients (aged 0.3–5.7 years), who were admitted to Children's Hospital, Zhejiang University School of Medicine and Hangzhou Children's Hospital during May–August 2011, were finally enrolled in this study. They were then grouped according to their clinical characteristics. In detail, patients with any evidence of central nervous system (CNS) complications, such as aseptic meningitis, encephalitis, and poliomyelitis-like paralysis, were classified into the severe group, while the remaining patients were classified into the mild group due to the absence of CNS complication. Simultaneously, 20 age-matched children who were hospitalized for minor surgery (such as hydrocele, inguinal hernia and redundant prepuce) without any symptoms or signs of HFMD were included as controls at the same time. This study was approved by and carried out under the guidelines of the Ethic Committee of the Children's Hospital, Zhejiang University School of Medicine and Hangzhou Children's Hospital. Written informed consent was obtained from all children participants' guardians involved in this study.

2.2. Sample collection

Clinical samples including throat swabs or stool specimens were collected only from HFMD patients. The specimens were immediately placed in virus transport media tubes (Yocon, Beijing, China) and stored at −80 °C until use. Simultaneously, blood samples from all participants were collected in Vacutainer-heparinized tubes (Becton Dickinson Medical Devices Co. Ltd., CA, USA). Plasma were isolated by centrifugation at 2000 rpm for 10 min at 4 °C and stored at −80 °C until analysis. Peripheral blood mononuclear cells (PBMCs) were separated with lymphocytes separation reagents (Axis-shield, Oslo, Norway) according to the manufacturer's instructions. PBMCs were suspended at a density of 2×10^6 cells/ml in RPIM 1640 (Gibco, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco). All the clinical samples from HFMD patients mentioned above were taken at the same time and within 3 days from the HFMD symptom onset.

2.3. RNA extraction and identification of EV71

Viral RNA was extracted from throat swabs or stool specimens using the viral RNA extraction kit (Haofeng Biotechnology Co., Ltd., Hangzhou, China) in accordance with the protocol provided. Detection of EV71 was based on the TaqMan technology using the enterovirus nucleic acid detection kit (Da An Gene Co. Ltd., Guangzhou, China). Then EV71 were analyzed using one-step primescript RT-PCR kit (Takara, Kyoto, Japan) according to the manufacturer's recommendations. In brief, the RT-PCR thermal profile consisted of 25 min at 40 °C, 3 min at 94 °C, and then followed by 40 cycles of 15 s at 93 °C and 45 s at 55 °C. All the RT-PCR processes, including reverse transcription, amplification, detection, and data analysis were carried out using the 7500 real-time RT-PCR system (Applied Biosystems, CA, USA).

2.4. Flow cytometry analysis

For the analysis of Th1, Th2, Th17, Tc1, Tc2 and Tc17 cells, the cell suspension was stimulated with 25 ng/ml phorbol myristate acetate (PMA, Sigma–Aldrich, USA) plus 1 μg/ml ionomycin (Sigma–Aldrich) in the presence of 2 μM monensin (Biolegend, San Diego, CA, USA) for 4 h at 37 °C with 5% CO_2. Because PMA may down-regulate CD4+ surface antigen to a varying degree, thus making identification of CD4+ cells difficult, CD4+ lymphocytes were indirectly detected by identifying CD3+CD8− cells (Sewell et al., 1997). The cells were incubated with