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Increased effector γδ T cells with enhanced cytokine production are associated with inflammatory abnormalities in severe hand, foot, and mouth disease☆

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ABSTRACT

Background: Although γδ T cells have been reported to be closely related to the immunopathogenesis of some viral infectious diseases, the changes or roles of γδ T cells in the development of hand, foot, and mouth disease (HFMD) remain unclear.

Methods: Peripheral γδ T cells and their subsets were determined by surface (γδ TCR, Vδ1 TCR, Vδ2 TCR, CD45RA, and CD27) or intracellular (IFN-γ, TNF-α, CD107a, and Granzyme B) markers in healthy controls (HCs) and HFMD patients with FACS. The plasma levels of IFN-γ, TNF-α, IL-6, and MCP-1 were measured by ELISA. Differences in γδ T cells or their subsets and correlations between γδ T cells and inflammation indicators were statistically analyzed.

Results: Compared to HCs, HFMD patients showed increased effector γδ T and TNF-α^+γδ T cells and plasma TNF-α levels, especially in severe cases. In addition, significantly increased Vδ1 T and IFN-γ^+γδ T cells and other plasma inflammatory cytokines were further found in severe patients. Furthermore, EV71+ severe patients showed significantly increased effector and cytokine-producing γδ T cells, while the EV71− severe patients displayed significantly greater plasma cytokine levels. The percentage of IFN-γ^+γδ T or TNF-α^+γδ T cells was positively correlated with that of effector γδ T cells. There was a positive correlation between the proportion of Vδ1 T cells and white blood cell (WBC) count or the proportion of IFN-γ^+γδ T or TNF-α^+γδ T cells and neutrophil (N) count, while there was a negative correlation between Vδ2 T cells and WBC or N count. Moreover, the percentages of Vδ1 T and effector γδ T cells in the acute phase of disease declined significantly to normal levels during the recovery phase.

Conclusions: Increased effector γδ T cells with enhanced cytokine production were remarkably observed in severe HFMD patients, which was also associated with clinical inflammation parameters. These data indicated that γδ T cells might be involved in inflammatory abnormalities in severe HFMD.

Abbreviations: HFMD, hand, foot, and mouth disease; EV, enterovirus; HCs, healthy controls; WBC, white blood cell; LYM, lymphocyte; N, neutrophil; CRP, C reactive protein; Glu, glucose; ALT, alanine aminotransferase; PA, prealbumin; CK, creatine kinase; PCT, procalcitonin; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; MCP, monocyte chemotactic protein; MHC, major histocompatibility complex; NK, natural killer; iNKT, invariant natural killer T; TCR, T cell receptor; SEM, standard error of mean; Tfh, follicular T helper; Th17, T helper type 17; CHB, chronic hepatitis B; HBV-ACLF, HBV-associated acute-on-chronic liver failure; PFA, paraformaldehyde; EV71− HFMD, EV71 RNA (−) HFMD; EV71+ HFMD, EV71 RNA (+) HFMD; EV71− severe HFMD, EV71 RNA (−) severe HFMD; EV71+ severe HFMD, EV71 RNA (+) severe HFMD

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1. Introduction

Hand, foot, and mouth disease (HFMD) is a common contagious disease in children (always under 5 years of age), which is caused by multiple enteroviruses, most commonly enterovirus 71 (EV71) and coxsackievirus A16 (CV-A16) [1,2]. HFMD was first reported by New Zealand in 1957 and has become a global public health problem since then [3]. In recent decades, increased incidence rates and outbreaks of HFMD have been reported in Asia-Pacific regions, such as China, Singapore, Malaysia and Japan [3,4]. HFMD is characterized by a self-limiting illness with fever, rashes in the hands and feet or multiple oral ulcers [1,5]. However, some cases may have severe complications, such as aseptic meningitis, brain-stem encephalitis, neurogenic pulmonary edema, or even death [2,6]. Moreover, these severe cases were associated with EV71 infection. However, the exact pathogenesis of severe HFMD caused by EV71 has not been fully elucidated.

Recent studies have shown that several types of immune cells and inflammatory mediators are involved in the pathogenesis of HFMD with EV71 infection. Some studies have reported that the frequencies of circulating follicular T helper (Tfh) cells and T helper type 17 (Th17) cells increased in children with EV71 infection. In a mouse model of EV71 infection, significantly increased disease severity and a reduced survival rate were found in mice lacking B, $CD4^+$ T or $CD8^+$ T cells [7–9]. Meanwhile, innate immune cells also displayed an important role in EV71-induced HFMD. HFMD patients, especially those with encephalitis, showed decreased natural killer (NK) cell counts. Wang's report showed that $CD14^+$ cells isolated from normal monkeys could be infected by EV71 in vitro and produced higher levels of interleukin (IL)-8, IL-4 and IL-6. Moreover, invariant natural killer T (iNKT) cells had a protective effect during EV71 infection, which was dependent on the activation of TLR3 signaling in macrophages [10–12]. However, until now, there have been no reports regarding the roles of γδ T cells in the pathogenesis of HFMD.

γδ T cells, one type of innate immune cell, have a unique T cell receptor (TCR) γ and δ chains, and they represent approximately 2–10% of the overall human T cell population. Different from conventional αβ T cells, γδ T cells can be directly activated via the recognition of pathogen-associated or danger-associated molecular patterns, particularly natural phospho-antigens without major histocompatibility complex (MHC) restriction. After activation, γδ T cells exert cytotoxic activity against target cells or regulatory effects on other immune cells by cytokine production. γδ T cells often respond to stress quickly and play important roles in pathogen clearance, autoimmune diseases, inflammation or tissue homeostasis [13]. Human γδ T cells are composed of two major subsets, Vδ1 T and Vδ2 T cells, based upon different δ chain expression [14]. Vδ1 T cells are abundant in skin, gut and bronchial epithelial tissue, while the Vδ2 T subset comprises up to 50–95% of the total γδ T cells in the peripheral blood. Vδ1 T and Vδ2 T cells were found to expand and proliferate in some bacterial or viral infections, such as HIV, Mycobacterium tuberculosis, Influenza virus, Epstein–Barr virus. In our previous research, we examined changes in γδ T cells from patients with chronic hepatitis B (CHB) or HBV-associated acute-on-chronic liver failure (HBV-ACLF), and our results showed that these patients had lower Vδ2 T cell percentages than HCs. Moreover, another report indicated that the circulating Vγ9Vδ2 T cell frequency significantly decreased in chronic HCV patients compared to HCs [15–17].

Therefore, in this study, we intend to observe the changes in γδ T cells in patients with HFMD, especially those with EV71 infection, and to further explore the relationship between these changes and clinical parameters. This study will prompt new lines of investigation of the immunopathogenesis of HFMD.

2. Materials and methods

2.1. Subjects

In the cross-sectional study, a total of 66 HFMD patients and 26 age- and sex-matched healthy controls (HCs) were enrolled from the Children's Hospital of Chongqing Medical University, China, from August 2017 to March 2018. Mild HFMD was typically characterized by skin rashes on the hands and feet with a mild fever. Severe cases were identified when they showed severe central nervous system complications, such as encephalitis, meningitis or pulmonary angioneurotic edema. EV71 infection was confirmed as positive EV71 RNA identification from a throat swab or stool specimens by real-time PCR assay according to the manufacturer's instructions (Sansure Biotech, Hunan, China). Blood samples were collected at the time of admission and before any treatment intervention. Thus, among our 66 enrolled HFMD patients, 21 patients were grouped as mild cases and 45 patients as severe cases. Moreover, 53 patients underwent EV71 RNA detection, and 25 patients were identified as having EV71 infection. The subjects without any abnormal test results after thorough clinical and laboratory examinations were enrolled as the healthy controls.

For the longitudinal study, we recruited a total of 17 HFMD patients from November to December 2018. Blood samples were collected twice for each patient during the acute phase of illness and in the recovery phase.

HFMD patients were diagnosed based on their clinical manifestations and laboratory results according to the Chinese Ministry of Health diagnosis and treatment guidance for HFMD (version 2010). The study was approved by the ethics committee of the Children's Hospital of Chongqing Medical University. Written informed consent was obtained from all participants prior to collecting blood samples.

2.2. Sample collection

Approximately 2 mL of EDTA-K2 anticoagulated blood samples were collected from healthy children and HFMD patients (within 24 h of admission). Blood tests, including complete blood count, comprehensive metabolic panel, myocardial zymogram, procalcitonin (PCT) and C reactive protein (CRP), were performed at the Children's Hospital of Chongqing Medical University. Plasma samples were obtained by centrifuging at 2000 rpm for 10 min and stored at −80 °C for cytokine and chemokine tests later. Fresh isolated whole blood cells were stained for surface markers and intracellular cytokines and analyzed by FACS immediately.

2.3. Surface markers and intracellular cytokines assay by flow cytometry

Surface markers were analyzed by direct staining of fresh peripheral blood with an appropriate volume of fluorescent-labeled monoclonal antibodies. After 30 min of staining at 4 °C, erythrocytes were lysed using BD FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA) for 15 min at room temperature. Then, cells were washed in ice-cold FACS buffer (PBS plus 2% fetal calf serum) and analyzed by a BD FACSCanto™ II Flow Cytometer (BD Biosciences, San Jose, CA, USA) immediately. Peripheral blood samples were collected from HCs (n = 26) and HFMD patients (mild HFMD (n = 21), severe HFMD (n = 45), EV71− HFMD (n = 28), and EV71+ HFMD (n = 25), EV71− severe HFMD (n = 19) and EV71+ severe HFMD (n = 17)).

For intracellular cytokine staining, leukocytes were prepared first by NH_4Cl-induced RBC lysis. Then, cells were suspended in RPMI 1640 medium containing 10% FBS and stimulated by a Cell Activation Cocktail (with PMA, ionomycin, and Brefeldin A) (BioLegend, San Diego, CA, USA) for 6 h in a 37 °C incubator with 5% CO_2. Cells were collected and washed in FACS buffer, and then 10^6 cells were re-suspended in 100 μL FACS buffer and stained for surface markers. Next, cells were fixed with 4% paraformaldehyde (PFA) for 20 min at 4 °C and

permeabilized with 0.1% saponin (Sigma, St, Louis, MO, USA) for 10 min at room temperature. Finally, the cells were stained with cytokine antibodies for 1 h and analyzed by BD FACS Canto II cytometry immediately. Peripheral blood samples were collected from HCs (n = 26) and HFMD patients (mild HFMD (n = 14), severe HFMD (n = 29), EV71− HFMD (n = 18), and EV71+ HFMD (n = 16), EV71− severe HFMD (n = 13) and EV71+ severe HFMD (n = 10)).

FACS antibodies used in this study were as follows: PerCP/Cy5.5-conjugated anti-CD3 (clone OKT3, Cat: 317336), APC-conjugated anti-TCR γ/δ (clone B1, Cat: 331212), PE-conjugated anti-TCR Vδ2 (clone B6, Cat: 331408), PerCP/Cy5.5-conjugated anti-CD45RA (clone HI100, Cat: 304122), PE/Cy7-conjugated anti-CD107a (LAMP-1) (clone H4A3, Cat: 328618) and APC-conjugated anti-Granzyme B Recombinant (clone QA16A02, Cat: 372204) were purchased from BioLegend (San Diego, CA, USA). FITC-conjugated anti-TCR δTCS1 monoclonal antibody (clone TS-1, Cat: TCR2055) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). PE-Cyanine7-conjugated anti-CD27 (clone 0323, Cat: 25-0279-42), PerCP-Cyanine5.5-conjugated anti-interferon (IFN)γ (clone 4S.B3, Cat: 45-7319-42) and PE-Cyanine7-conjugated anti-tumor necrosis factor (TNF)α (clone MAb11, Cat: 25-7349-82) were purchased from eBioscience (San Diego, CA, USA).

Flow cytometry data were analyzed with BD FACSDiva Software or FlowJo software (version 7.6.1) (Tree Star, San Carlos, CA).

2.4. Detection of plasma cytokines or chemokines by enzyme-linked immunosorbent assay (ELISA)

The concentrations of plasma IFN-γ, TNF-α, IL-6 or monocyte chemotactic protein (MCP)-1 in HCs and HFMD patients were measured with a commercial ELISA Kit (Neobioscience, Shenzhen, China) according to the manufacturer's protocols. The minimum detectable levels values (pg/ml) were 0.98 (IFN-γ), 8 (TNF-α), 1.5 (IL-6), or 7.8 (MCP-1). Peripheral blood samples were collected from HCs (n = 26) and HFMD patients (mild HFMD (n = 14), severe HFMD (n = 29), EV71− HFMD (n = 18), and EV71+ HFMD (n = 16), EV71− severe HFMD (n = 13) and EV71+ severe HFMD (n = 10)).

2.5. Statistics

Statistical analysis was performed with SPSS (Version 23.0, IBM, Chicago, Illinois, USA) software. One-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks was applied for the comparisons among multiple independent variables. The Mann-Whitney *U* test was applied for comparisons between two groups. Dunn's Method or chi-squared test was used for multiple pairwise comparisons. A paired *t*-test was applied for comparison of two pairwise groups. Spearman's correlation coefficient was used to assess correlations between variables. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Clinical and laboratory characteristics of studied subjects

A total of 26 HCs and 66 HFMD patients were recruited in our cross-sectional study. The detailed clinical and laboratory data of HFMD patients and HCs are summarized in Table 1. In the 66 patients, 21 patients (31.8%) were diagnosed with mild HFMD, and 45 (68.2%) were diagnosed with severe HFMD. Of the 53 patients who underwent EV71 RNA detection, 25 (47.2%) patients were positive for EV71 RNA and were classified as a group of EV71-infected patients (EV71+ HFMD). There were no significant differences in age and gender distribution between the HFMD subgroup and HCs, except for patients in the EV71− HFMD group who were significantly younger than HCs (P = 0.027) or EV71+ HFMD patients (P = 0.002). Encephalitis was the most common CNS complication in most (97.8%) of the severe patients or 88% of EV71+ patients displayed in our study. Although HFMD patients showed no significant changes in peripheral lymphocyte (LYM) number, all HFMD subgroups showed significantly higher white blood cell (WBC) (except EV71− HFMD group) and neutrophil (N) counts than the HC group, moreover, EV71+ patients displayed the highest WBC and neutrophil counts. In addition to the blood cell count, prealbumin (PA) levels in all HFMD subgroups were significantly lower than HCs ($P < 0.001$). Other laboratory measurements were also investigated, such as CRP, alanine aminotransferase (ALT), glucose (Glu), creatine kinase (CK) or PCT. Differences in PCT levels were statistically significant between the mild HFMD (0.92 ng/mL) and severe HFMD groups (0.95 ng/mL) (P = 0.041) or between the EV71− HFMD (0.9 ng/mL) and EV71+ HFMD groups (1.3 ng/mL) (P = 0.045). Glu levels in the severe HFMD group (5.8 mmol/L) were significantly higher than in the mild HFMD group (4.9 mmol/L) (P = 0.006).

Moreover, the mild or severe HFMD patients were further subdivided into EV71− or EV71+ subgroups. However, statistics analysis cannot be performed to compare the differences between HCs and EV71− or EV71+ mild HFMD patients because few patients in mild HFMD subgroup (data not shown).

3.2. The percentage of Vδ2 T cells decreased significantly in HFMD patients when they were grouped by disease severity

Peripheral blood was collected to detect the percentage of γδ T cells and their subsets using the FACS method. Representative FACS figures are shown in Fig. 1. To comprehensively analyze the differences in these proportions between HC and HFMD patients, HFMD patients were subdivided into mild and severe groups or into EV71− and EV71+ groups according to the disease severity or EV71-RNA detection results, respectively. Differences were statistically analyzed between these HFMD subgroups and the HC group.

As shown in Fig. 2, our results first displayed that the percentages of total peripheral γδ T cells did not differ between any of the HFMD subgroups and HCs (Fig. 2A–C), although a slightly increased percentage was found in EV71− patients. There was a tendency of an increased percentage of Vδ1 T cells in patients compared to that in healthy controls (15.8%), but a significant difference was found only in severe HFMD patients (27.2%) (P = 0.03). Furthermore, HFMD patients also showed a tendency of a lower proportion of Vδ2 T cells than HCs (59.7%), and a significant difference was displayed in either the mild (44.5%) (P = 0.033) or the severe (46.8%) (P = 0.041) HFMD group. Furthermore, there were no significant differences in the percentage of Vδ1 T or Vδ2 T cells between HCs and EV71− or EV71+ HFMD patients or between HCs and EV71− or EV71+ severe HFMD patients.

3.3. Higher percentages of effector γδ T cells were displayed in EV71-infected severe HFMD patients

Then, effector or memory γδ T cells were detected with the expression of surface molecules CD45RA and CD27 by flow cytometry as shown in Fig. 1. γδ T cells can be distinguished into naïve cells ($CD45RA^+CD27^+$), central memory cells ($CD45RA^-CD27^+$), effector memory cells ($CD45RA^-CD27^-$) or effector cells ($CD45RA^+CD27^-$), in which effector cells exert cytotoxicity and cytokine secretion quickly and effectively.

Our results showed that in healthy controls, the average percentage of naïve, central memory, effector memory or effector γδ T cells was 30.0%, 41.3%, 12.9% or 15.7%, respectively. The proportion of naïve γδ T cells increased significantly in both EV71+ HFMD (38.3% on average) and EV71+ severe HFMD patients (39.1% on average) when compared to HC and EV71− HFMD or EV71− severe HFMD patients ($P < 0.05$). HFMD patients exhibited a significantly lower percentage of central memory γδ T cells than healthy controls, and the lowest percentage found in the EV71+ severe HFMD group (21.1% on average) ($P < 0.001$). No significant differences in the proportion of

Table 1
Clinical and laboratory characteristics of enrolled HC and HFMD patients (grouped by disease severity or detection results of EV71 RNA).

	HC	HFMD (by disease severity)		HFMD (by EV71 RNA)	
		Mild	Severe	EV71 −	EV71 +
Cases, n	26	21	45	28	25
Male, n (%)	16 (61.5)	13 (61.9)	28 (62.2)	17 (60.7)	15 (60.0)
Age, months	37.2 ± 33.6	24.0 ± 13.2	27.6 ± 14.4	21.6 ± 12.0*,&&	32.4 ± 15.6
Encephalitis, n (%)	0 (0)	0 (0)	44 (97.8)	18 (64.3)	22 (88.0)
WBC, $\times 10^9$ cells/L	7.9 ± 2.4	10.6 ± 4.8*	10.6 ± 5.1**	9.8 ± 4.5	11.2 ± 4.5**
LYM, $\times 10^9$ cells/L	3.9 ± 1.6	4.1 ± 2.3	3.8 ± 2.0	3.9 ± 2.0	4.2 ± 2.5
N, $\times 10^9$ cells/L	3.5 ± 1.5	5.9 ± 3.9*	6.4 ± 4.3***	5.4 ± 3.5*	6.6 ± 3.4***
PA, mg/L	192.9 ± 39.0	132.6 ± 29.6***	145.7 ± 36.0***	136.2 ± 34.6***	152.8 ± 36.6***

Note: Data are shown as mean ± SD. In total 66 HFMD patients, 53 have been tested for EV71 RNA. HFMD: hand, foot, and mouth disease; HC: healthy control; EV: enterovirus; WBC: white blood cell; LYM: lymphocyte; N: neutrophil; PA: prealbumin.
* $P < 0.05$ vs. HC group.
** $P < 0.01$ vs. HC group.
*** $P < 0.001$ vs. HC group.
&& $P < 0.01$ vs. EV71 + HFMD group.

effector memory γδ T cells were noted among the HCs and HFMD subgroups, except for a lower percentage in the mild group (9.8% on average) than in the severe group (14.0% on average) ($P = 0.041$). For effector γδ T cells, HFMD patients were found to have greater percentages than the HC group, which was especially noticeable in the severe HFMD group (23.7% on average) ($P = 0.021$) (Fig. 3A–C).

3.4. Enhanced IFN-γ and TNF-α production of γδ T cells in EV71 + severe HFMD patients

Cytokine production and cytotoxicity are the most important functions of γδ T cells. To further elucidate the functional state of increased effector γδ T cells, IFN-γ or TNF-α production and cytotoxicity of γδ T cells were analyzed via flow cytometry upon in vitro stimulation with ionomycin and PMA. FACS figures are shown in Fig. 1. A tendency toward an increased percentage of IFN-γ^+γδ T or TNF-α^+γδ T cells was found in HFMD patients compared to healthy controls. The highest percentage of IFN-γ^+γδ T or TNF-α^+γδ T cells was shown in the EV71 + severe HFMD group (15.4% or 24.5% on average, respectively). The production of both cytokines increased significantly in both severe HFMD and EV71 + severe HFMD patients when compared to HCs. In addition, significant differences in the percentage of TNF-α^+γδ T cells were also observed between HCs and EV71 − or EV71 + HFMD groups (Fig. 4A–C).

Moreover, the cytotoxicity of γδ T cells was estimated by the proportion of CD107a and Granzyme B in our study (FACS figures shown in Fig. 1). All the differences were rarely meaningful except for the proportion of Granzyme B$^+$γδ T cells between mild HFMD (37.4% on average), severe HFMD (38.7%) or EV71 − severe HFMD (38.6%) patients and HCs (50.5%) ($P < 0.05$) (Fig. 4D–F).

Then, Spearman's correlation coefficient was used to evaluate the correlation between the surface molecules and intracellular cytokines of γδ T cells in HFMD patients. As shown in Fig. 5, the proportion of IFN-γ^+γδ T or TNF-α^+γδ T cells was positively correlated with the proportion of effector memory or effector γδ T cells but negatively correlated with the proportion of central memory cells. Other insignificant correlation analyses were not shown.

3.5. Changes in plasma inflammatory cytokines and chemokines

The systemic inflammatory status of HFMD patients was assessed via the quantification of plasma inflammatory cytokines (IFN-γ, TNF-α,

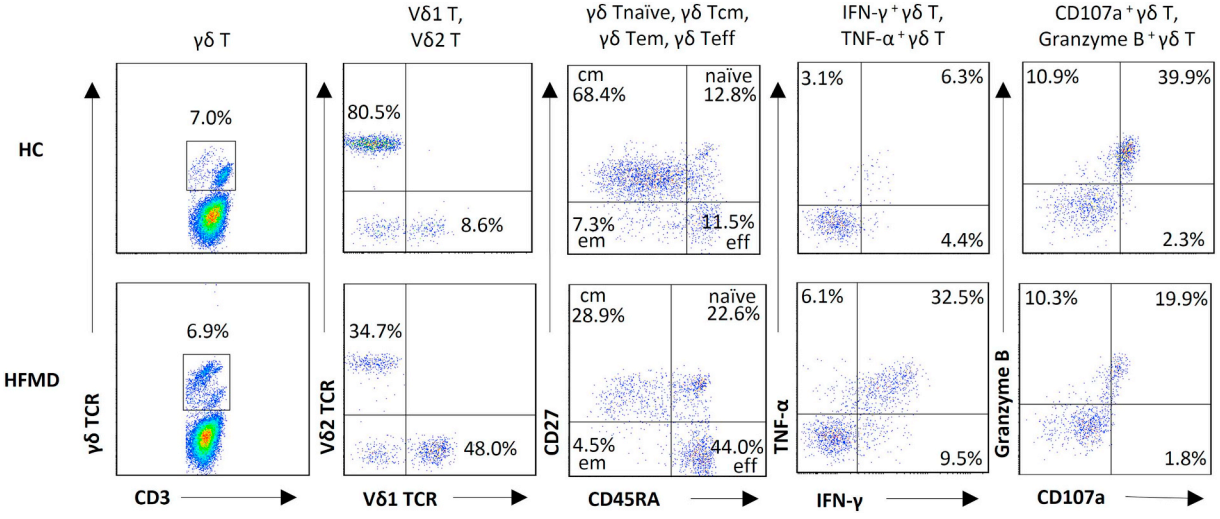

Fig. 1. Representative FACS figures of γδ T cells and their subsets, including γδ T, Vδ1 T, Vδ2 T, CD45RA$^+$CD27$^+$γδ T (γδ Tnaïve), CD45RA$^-$CD27$^+$γδ T (γδ Tcm), CD45RA$^-$CD27$^-$γδ T (γδ Tem), CD45RA$^+$CD27$^-$γδ T (γδ Teff), IFN-γ^+γδ T, TNF-α^+γδ T, CD107a$^+$γδ T and Granzyme B$^+$γδ T cells, from HCs and HFMD patients. Direct cell surface staining of peripheral blood was used to detect γδ T, Vδ1 T, Vδ2 T, γδ Tnaïve, γδ Tcm, γδ Tem, and γδ Teff cells. An intracellular cytokine staining assay was applied to evaluate the other intracellular markers in γδ T cells (IFN-γ, TNF-α, CD107a and Granzyme B) after stimulating WBC with PMA and ionomycin. The percentage of each type of cell is shown in this figure, along with the percentage of γδ T in CD3$^+$ T cells and the percentage of the subset in total γδ T cells.

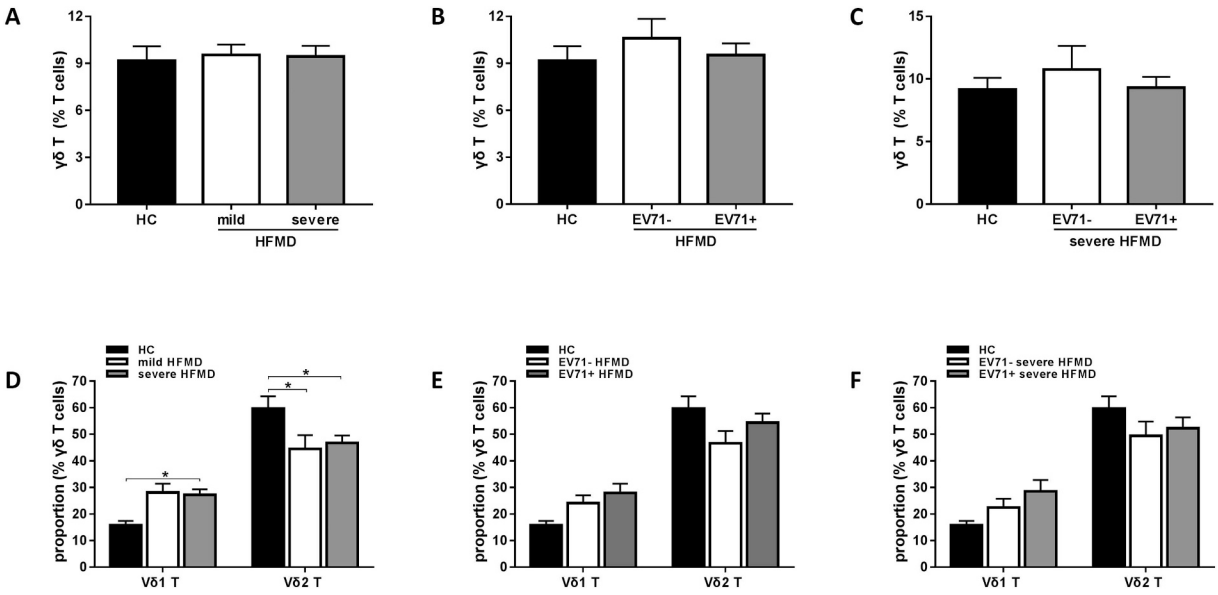

Fig. 2. The proportions of peripheral γδ T cells (in $CD3^+$ T cells), subset Vδ1 T cells and Vδ2 T cells (in total γδ T cells) in the HC group and HFMD patients grouped by disease severity or by EV71 RNA detection results. Peripheral blood samples were collected from HCs and HFMD patients. γδ T cells and their subsets were analyzed by flow cytometry. Differences in percentage of γδ T, Vδ1 T or Vδ2 T cells were statistically analyzed among HCs (n = 26), mild HFMD (n = 21) and severe HFMD (n = 45) groups (A and D, respectively), among HCs, EV71− HFMD (n = 28), and EV71+ HFMD (n = 25) groups (B and E, respectively), and among HCs, EV71− severe HFMD (n = 19) and EV71+ severe HFMD (n = 17) groups (C and F, respectively). Data are shown as the mean ± SEM. (*P < 0.05).

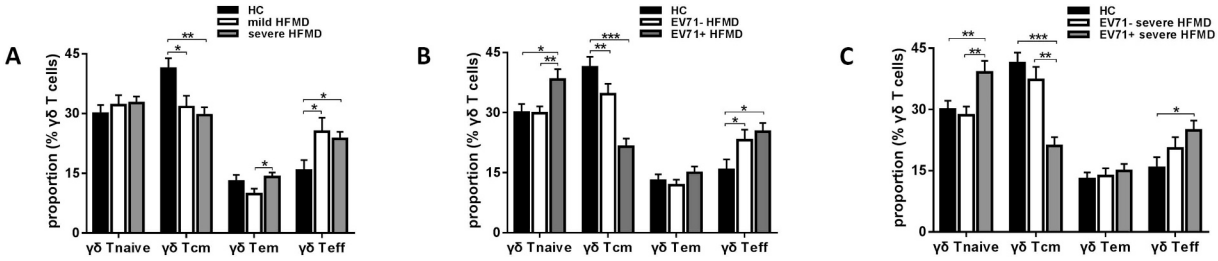

Fig. 3. The percentages of naïve γδ T (γδ Tnaive, $CD45RA^+CD27^+$), central memory γδ T (γδ Tcm, $CD45RA^-CD27^+$), effector memory γδ T (γδ Tem, $CD45RA^-CD27^-$) or effector γδ T (γδ Teff, $CD45RA^+CD27^-$) cell subpopulations in HFMD patient subgroups and HCs. Peripheral blood samples were collected from HCs and HFMD patients. γδ T subsets were analyzed by flow cytometry. Statistical analysis was performed to compare the differences between HCs (n = 26) and mild (n = 21) or severe HFMD (n = 45) subgroups (A), between HCs and EV71− (n = 28) or EV71+ HFMD (n = 25) subgroups (B), or between HCs and EV71− (n = 19) or EV71+ severe HFMD (n = 17) subgroups (C). Data are shown as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

and IL-6) and chemokine (MCP-1) levels by ELISA. In short, HFMD patients showed a trend of increased cytokines or chemokine levels compared to HCs. It was notable that severe HFMD patients differed significantly from HC in all of these cytokines. In these severe patients, the EV71− severe subgroup showed significantly higher levels of all four cytokines, while the EV71+ severe subgroup did not, except with TNF-α. EV71− HFMD also displayed significant differences in the levels of TNF-α, IL-6 or MCP-1, while EV71+ HFMD patients had significant differences only in the level of TNF-α (Fig. 6A–C).

3.6. Correlation analysis of γδ T subsets with inflammation indicators in HFMD patients

In addition, the correlation between γδ T subsets and inflammation indicators, including plasma IFN-γ, TNF-α, IL-6, MCP-1 or PCT levels and WBC or N count, were analyzed in this study. Our analysis showed no significant correlation between plasma cytokines or chemokine and γδ T cell subsets (data not shown). The WBC count was positively correlated with the percent of Vδ1 T cells (r = 0.286, P = 0.02) and negatively correlated with the proportion of Vδ2 T cells (r = −0.351, P = 0.004). Moreover, the N count was positively correlated with the proportion of IFN-γ^+γδ T (r = 0.338, P = 0.027) or TNF-α^+γδ T cells (r = 0.336, P = 0.028) and negatively correlated with the proportion of Vδ2 T cells (r = −0.248, P = 0.039). Additionally, the PCT level was positively correlated with the percentages of naïve γδ T cells (r = 0.321, P = 0.009) or TNF-α^+γδ T cells (r = 0.520, P < 0.001) (Fig. 7A–G). No other meaningless correlation analysis was shown.

3.7. Decreased proportion of Vδ1 T and effector γδ T cells in the recovery phase of HFMD

To observe dynamic changes of γδ T cells in the course of HFMD, kinetic analysis of γδ T cells and subtypes in the same patient was performed during the acute and recovery phases of illness. A total of 17 patients were observed in this longitudinal study. Twelve of the 17 patients were male, and the average age was 22.8 months. At the time of admission, 7 patients had mild HFMD, and 10 patients with severe HFMD had encephalitis. All these patients recovered or improved by treatment when they were discharged.

As shown in Fig. 8, the peripheral WBC and N counts of patients were significantly reduced in the recovery phase. At the same time, the proportion of Vδ1 T and effector γδ T cells in the acute phase (mean 15.1% and 11.7%, respectively) declined markedly to 7.7% and 7.8% (mean), respectively, in the recovery phase, and the differences were statistically significant (P = 0.025 and P = 0.009, respectively). However, the percentages of γδ T, Vδ2 T and central memory γδ T cells did

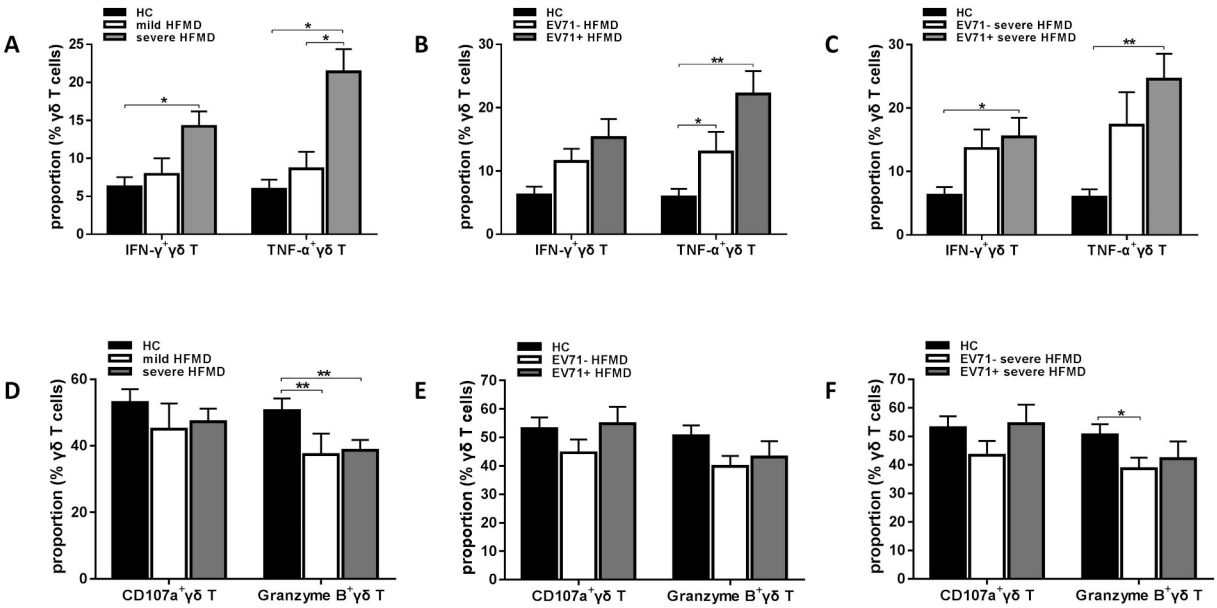

Fig. 4. The percentages of peripheral IFN-γ^+, TNF-α^+, CD107a$^+$ or Granzyme B$^+$ γδ T cells in HFMD patients and HCs. Peripheral blood samples were collected from HC and HFMD patients. γδ T cell subsets were analyzed with intracellular cytokine staining and flow cytometry. Statistical analysis was performed to compare the differences between HCs (n = 26) and mild (n = 14) or severe HFMD (n = 29) subgroups (A and D, respectively), between HCs and EV71− (n = 18) or EV71+ HFMD (n = 16) subgroups (B and E, respectively), or between HCs and EV71− (n = 13) or EV71+ severe HFMD (n = 10) subgroups (C and F, respectively). Data are shown as the mean ± SEM. (*P < 0.05; **P < 0.01).

not differ significantly between the acute and recovery phases of the disease.

4. Discussion

HFMD, mainly caused by EV71 and CA16, is a common infectious disease for preschool-aged children, which was verified to be related to some kinds of immune cells, such as NK and Th17 cell [7–12]. However, little information is currently available about the association between γδ T cells and HFMD. Therefore, it is worth investigating the phenotypic and functional changes of γδ T cells to understand their roles in the development of HFMD. In our study, a total of 26 HCs and 66 HFMD patients were enrolled for γδ T cell detection. Similar to the previously report [1], there were approximately 1.7 times more male HFMD patients than female patients in our study, indicating that HFMD has a higher prevalence in male children. The reasons for more severe HFMD patients than mild patients in our study were that the majority of HFMD cases are generally self-limiting and most of the mild patients would not be admitted to the hospital.

First, differences in the percentages of γδ T, Vδ1 T or Vδ2 T cells were statistically analyzed among HC, mild HFMD and severe HFMD groups. Our data showed no significant changes of γδ T cells, while

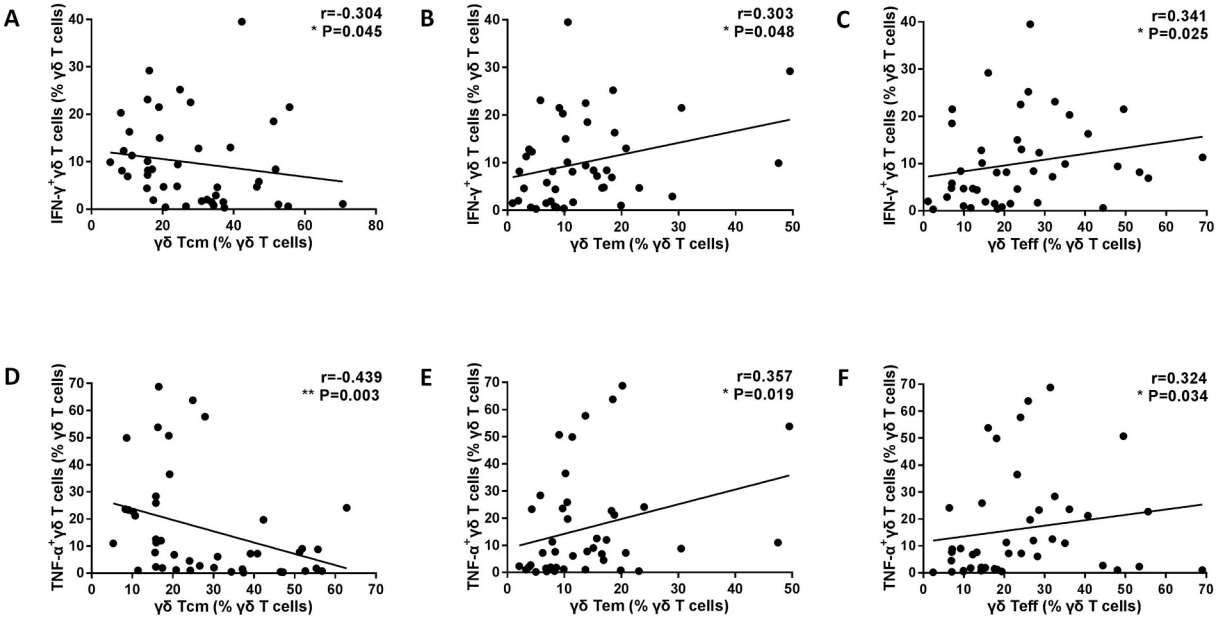

Fig. 5. Spearman's correlation of the percentage between surface molecules and intracellular cytokines of γδ T cells. A significant correlation was determined between the proportion of CD45RA$^-$CD27$^+$γδ T (γδ Tcm), CD45RA$^-$CD27$^-$γδ T (γδ Tem) or CD45RA$^+$CD27$^-$γδ T (γδ Teff) cells and IFN-γ^+γδ T (A, B and C, respectively) or TNF-α^+γδ T cells (D, E and F, respectively). (*P < 0.05; **P < 0.01).

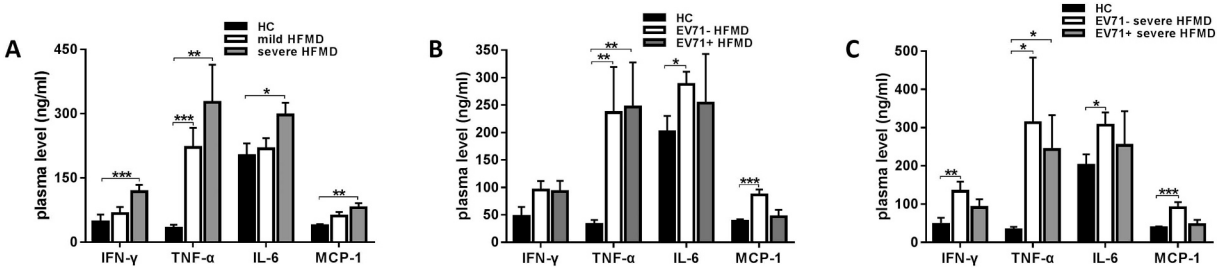

Fig. 6. The plasma levels of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) or monocyte chemotactic protein-1 (MCP-1) in HFMD patients and HCs. Peripheral blood samples were collected from HCs and HFMD patients. Cytokine levels were assayed by ELISA. Statistical analysis was performed to compare the differences between HCs (n = 26) and mild (n = 14) or severe HFMD (n = 29) subgroups (A), between HCs and EV71− (n = 18) or EV71+ (n = 16) HFMD subgroups (B), or between HCs and EV71− (n = 13) or EV71+ severe HFMD (n = 10) subgroups (C). Data are shown as the mean ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

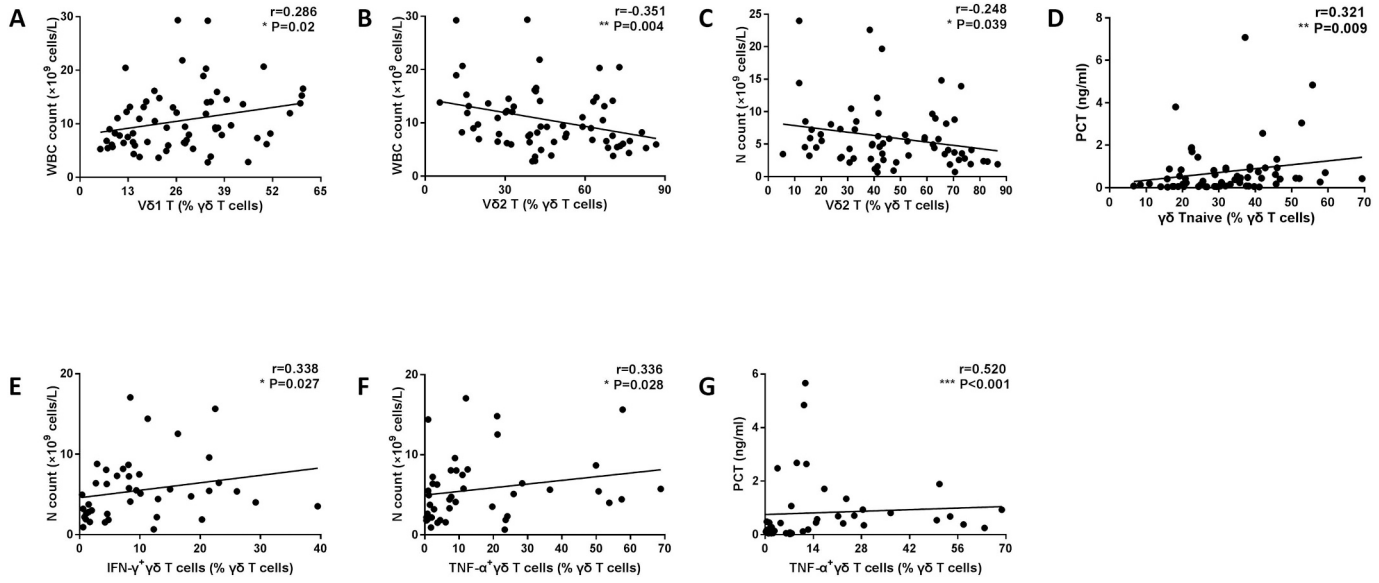

Fig. 7. Spearman correlation analysis between γδ T subsets and inflammation indicators. The significant correlation between the proportion of γδ T subsets and the white blood cell (WBC) count, neutrophil (N) count or procalcitonin (PCT) level (A–G). (*P < 0.05; **P < 0.01; ***P < 0.001).

increased percentages of Vδ1 T cells and decreased Vδ2 T cells in HFMD patients compared to HCs, and this result was similar to the trend of γδ T cells and subsets in the CHB study from our group [15,18]. Circulating γδ T cells are primarily composed of the Vδ1 T and Vδ2 T cell subsets, in which the Vδ2 T cell subset is the majority (approximately 50% to 95%). A significant increase in the ratio of Vδ1 T cells has been reported in some bacterial or viral infections [19–21]. In HIV infection, Vδ2 T cells decreased and Vδ1 T cells increased at the same time [22], and amplification of Vδ1 T cells could protect against similarly timed infections by filling the loss of $CD4^+$ T cells [23]. Additionally, it was reported that enhanced Vδ1 T cells and decreased Vδ2 T cells would promote the development of ACLF [15]. For CHB patients, severe liver damage and immunopathogenesis were related to reduced Vδ2 T cells, which could inhibit the pathogenic Th17 cell response [23]. Furthermore, both Vδ1 and Vδ2 T cells could release inflammatory cytokines [18,22]. Therefore, in our study, the increase of Vδ1 T cells might be involved in antiviral or inflammatory immune responses in severe HFMD.

γδ T cells could be divided into memory or effector cells based on the expression of CD45RA and CD27: naïve (Tnaïve: $CD45RA^+CD27^+$), central memory (Tcm: $CD45RA^-CD27^+$), effector memory (Tem: $CD45RA^-CD27^-$), and effector cells (Teff: $CD45RA^+CD27^-$). Tnaïve and Tcm cells are located in secondary lymphoid tissues with no direct effector function. Both Tem and Teff cells express chemokine receptors and can migrate into the inflamed area to exert their effector functions. Tnaïve cells have the longest antigenic response time and then decreased from Tem to Tcm cells, while the proliferation ability increased progressively from Tem and Tcm to Tnaïve cells. Teff showed the highest activity in cytokine production [24,25]. In our study, a lower percentage of γδ Tcm cells and a higher percentage of γδ Teff cells in HFMD patients than HCs indicated that the γδ T cells in the HFMD patients might have weaker responsiveness but greater cytokine reactivity.

Subsequently, severe HFMD patients showed a significant increase in both IFN-γ^+γδ T and TNF-α^+γδ T cells. Many cytokines will be produced by activated γδ T cells, in which IFN-γ and TNF-α are the most important [24,26]. Other studies have shown that γδ T cells play an important role in controlling viral infection by releasing cytokines. Concretely, HIV-1-infected patients had expanded IFN-γ-producing Vδ1 T cells [27]. Another study reported that recurrent wheezing after bronchiolitis might be partially caused by the suppressed IFN-γ production of γδ T cells in RSV-infected children [28]. IFN-γ and TNF-α were secreted by Tem and Teff if they are triggered through phosphoantigen or CD16 [29]. Our results also showed that the percentages of IFN-γ^+γδ T and TNF-α^+γδ T cells were positively correlated with γδ Teff or γδ Tem cells and negatively correlated with central memory γδ T cells. Taken together, these findings suggest that γδ T cells produce inflammatory factors when encountering proper stimuli to maintain the balance of inflammation and tolerance. These results were consistent with the characteristics of Tcm, Tem or Teff in other reports [24].

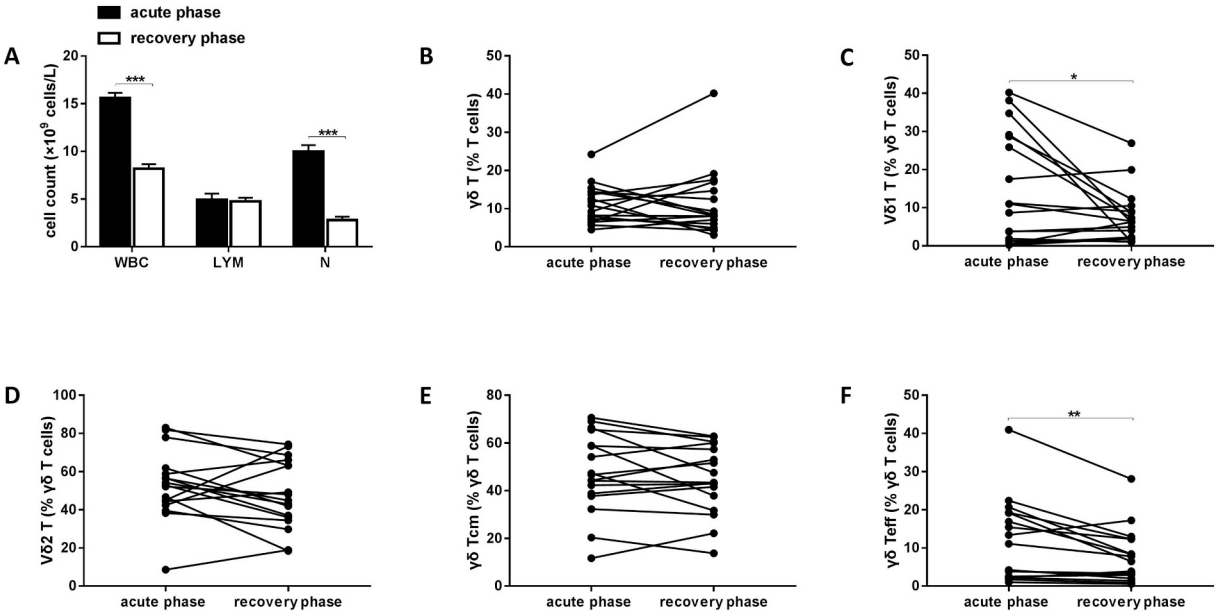

Fig. 8. Dynamic analysis of peripheral γδ T cells and subsets in the acute and recovery phases of disease from 17 HFMD patients with flow cytometry. A, cell count of white blood cells (WBC), lymphocytes (LYM) and neutrophils (N); B, proportion of peripheral γδ T cells (in $CD3^+$ T cells); C and D, proportion of Vδ1 T and Vδ2 T cells (in total γδ T cells), respectively; E and F, proportion of central memory γδ T (γδ Tcm, $CD45RA^-CD27^+$) and effector γδ T (γδ Teff, $CD45RA^+CD27^-$) cells (in total γδ T cells), respectively. A paired *t*-test was performed to compare the differences between the acute (n = 17) and recovery (n = 17) phases. Data are shown as the mean ± SEM. (*P < 0.05; **P < 0.01).

As reported, not only immune cells but also inflammatory cytokines are involved in the progression of HFMD [30]. Lee, J.Y., et al. found that EV71-induced HFMD patients with aseptic meningitis had high levels of TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, and IL-13 compared to the control group, and IL-6 was indicated as an indicator in EV71-induced HFMD children with aseptic meningitis [31]. Previous studies have shown that the concentrations of IFN-γ, IL-17A, IL-6, IL-21 or MCP-1 in severe HFMD with or without EV71 infection were significantly higher than those in HCs [8,30,32]. Here, we assessed plasma levels of IFN-γ, TNF-α, IL-6, and MCP-1 in HCs and HFMD patients by ELISA. Similar to previous research reports [32], levels of IFN-γ, TNF-α, and MCP-1 in severe HFMD cases were markedly higher than HCs. Furthermore, severe HFMD also showed a sharply increased percentage of IFN-γ^+γδ T and TNF-α^+γδ T cells. Taken together, our results suggest that effector γδ T cells might contribute to inflammatory abnormalities in severe HFMD by enhancing IFN-γ and TNF-α production [30].

In EV71+ severe HFMD patients, WBC and N count significantly increased compared to HCs in our study, indicating that EV71+ severe HFMD might cause inappropriate inflammation in the progression of the disease. These data were consistent with previously reported studies [5,9,30]. PA is a negative acute-phase protein that is downregulated during inflammation [33]. In our study participants, patients with HFMD had significantly lower PA than HCs, which might be associated with inflammation. Hence, the relationship between the clinical inflammation parameters, such as WBC, N or PCT, and γδ T cell subsets in HFMD patients was also analyzed. Notably, the percentage of Vδ1 T cells was positively correlated with the WBC count, while the WBC or N count was negatively correlated with the proportion of Vδ2 T cells. Moreover, the correlation between the percentage of TNF-α^+γδ T cells and the PCT level was positive. In addition, the percentage of IFN-γ^+γδ T or TNF-α^+γδ T cells was positively correlated with the N count. According to a study using *Mycobacterium avium*-infected mouse models, γδ T cells could cause an influx of neutrophils and tissue damage in the lungs and revealed pathogenic functions of γδ T cells [34]. These results implied that γδ T cells participate in the regulation of the inflammatory reaction in the progression of HFMD.

As shown in some reports, non-EV71-infected HFMD patients have increased over the years, and another enterovirus, such as CV-A6, has gradually replaced EV71 to become the dominant pathogen. Therefore, we observed differences in γδ T cells between EV71− and EV71+ HFMD patients. HFMD patients were divided into EV71− and EV71+ HFMD subgroups, and severe HFMD patients were further divided into EV71− and EV71+ severe HFMD subgroups. Furthermore, most of the changes in γδ T cells in EV71+ severe patients were similar to the severe group, such as decreased Tcm, increased Teff, IFN-γ^+γδ T and TNF-α^+γδ T cells. Thus, our present results support the speculation that EV71+ severe, but not EV71− severe, patients were the most important part of γδ T cell changes among all severe HFMD patients. For the plasma inflammatory cytokines, both EV71− and EV71+ HFMD patients showed an increasing trend compared to HCs; however, only EV71− patients displayed significantly increased levels. Nevertheless, the differences between patients with EV71− and EV71+ HFMD or EV71− and EV71+ severe HFMD were inconsistent with relevant research results [32]. The possible reason was that other enteroviruses could cause a more severe systemic inflammatory response in patients with HFMD or severe HFMD.

Similar to some of the above results, our longitudinal study showed that the percentages of Vδ1 T cells and effector γδ T cells in the acute phase of illness declined significantly to a normal level during disease recovery and were accompanied by decreased white blood cell and neutrophil counts. No significant difference was found in the proportion of γδ T cells. Therefore, Vδ1 T cells or effector γδ T cells might be subsets of γδ T cells involved in the progression of HFMD.

In conclusion, this study discovered that the increased effector function of γδ T cells in severe patients and γδ T cell subsets was related to clinical inflammation parameters. Therefore, we inferred that γδ T cells might contribute to inflammatory abnormalities in severe HFMD by enhanced IFN-γ and TNF-α production. This is the first study to explore the relationship between γδ T cells and HFMD. However, it is difficult to determine the mechanism of γδ T cells in HFMD in vivo.

Author contributions

Conceived and designed the research: MC and HMX. Sample and data collection: YXS, ZJP, and HMX. Performed the experiments: YXS, ZJP and WX. Analyzed the results: YXS, LZ, MLP, PH and, MC. Wrote the paper: YXS, HMX and MC. All authors commented on the manuscripts of the article.

Competing interests

All authors have no potential competing interests.

Acknowledgements

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