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Original Article

# Characterization of lymphocyte subsets in peripheral blood cells of children with EV71 infection

**Ming-Qi Zhao [a,1], Li-Hua Wang [a,b,1], Guang-Wan Lian [a,1], Zheng-Fang Lin [a], Ying-Hua Li [a], Min Guo [a], Yi Chen [a], Xiao-Min Liu [a], Bing Zhu [a,*]**

[a] *Virus Laboratory, Guangzhou Institute of Pediatrics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, 318 Renminzhong Road Yuexiu District, Guangzhou, 510120, China*

[b] *Department of Infectious Diseases, Xiaogan Central Hospital Affiliated to Wuhan University of Science and Technology, 6 Guangchang Road, Xiaogan, Hubei, 432000, China*

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**KEYWORDS**
Hand, foot, and mouth disease;
EV71;
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B10 cells;
Transcription factors

**Abstract** *Background:* Enterovirus 71 (EV71) is one of the major causative pathogens of hand, foot, and mouth disease (HFMD). Immune cells play a critical role in determining the outcomes of virus infection. We aimed to characterize the lymphocyte subsets and transcriptional levels of T lymphocytes-associated transcription factors in peripheral blood cells of children with EV71 infection.

*Methods:* Peripheral blood samples from 32 children with EV71 infection and 32 control subjects were included in this study. The frequencies of T-, B-lymphocytes, and their subsets were determined by flow cytometry. The expression of transcription factors, including T-bet, Gata3, ROR $\gamma$ t, Foxp3, TCF-1, and BCL-6 in the whole blood cells were evaluated by real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR).

*Results:* The frequencies of T cells, helper T cells (Th), cytotoxic T cells (Tc), IFN-$\gamma^+$ Th1, IFN-$\gamma^+$ Tc1, and regulatory T (Treg) cells were significantly decreased ($P < 0.01$) in children with EV71 infection. As for IL-$4^+$ Th2, IL-$4^+$ Tc2, IL-$17^+$ Th17, IL-$17^+$ Tc17, follicular helper T cells (Tfh), $CD3^+CD8^+$IL-$21^+$ T cells, $CD19^+$ B cells, and $CD19^+$IL-$10^+$ B10 cells, their frequencies were significantly increased in the EV71 group ($P < 0.01$). The EV71 group had lower mRNA expressions of T-bet, Gata3, and Foxp3 than the control group ($P < 0.05$), whereas the expressions of ROR $\gamma$ t, TCF-1, and BCL-6 showed no significant difference between two groups.

*Conclusions:* EV71 infection in children caused a decreased frequency of total Th, Tc and Treg cells, and increased percentages of B cell, Th2 and Th17 cells in blood.

* Corresponding author. Fax: +86 21 64085875.
*E-mail address:* zhubing2016@hotmail.com (B. Zhu).
[1] These authors contributed equally to this work.

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## Introduction

Enterovirus is a group of single positive-stranded ribonucleic acid viruses, which can be classified into five types, including polioviruses, coxsackie group A viruses (types 1–22, 24), coxsackie group B viruses (types 1–6), echoviruses (types 1–7, 9, 11–27, 29–34), and enteroviruses (types 68–71).[1] Characterized by lesions around hands, feet, anus, and oral mucosa,[2] hand, foot, and mouth disease (HFMD) is an emerging infectious disease caused by enterovirus. The main pathogenicity serotypes of enterovirus involved in HFMD include coxsackie group A (types 4–7, 9, 10, 16) and group B (types 1–3, 5), and the partial serotype of the echovirus and enterovirus 71 (EV71).[3] Epidemiology studies have shown that more than 40% cases of HFMD were due to EV71 infection, which can lead to death in certain conditions.[4] EV71 can cause severe complications of nervous system, including poliomyelitis-like paralysis, brain stem encephalitis, and aseptic meningitis.[5] In the past decade, EV71 infection has affected thousands of children suffering from neurological symptoms in the Asia-Pacific region, and caused hundreds of deaths.[4,6]

Previous studies have mainly focused on the inflammatory mediators and the viral proteins produced by EV71. For example, Chang et al. found that the production of Type 1 T helper (Th1) cells cytokine and proinflammatory cytokines were increased in peripheral mononuclear cells after EV71 stimulation *in vitro*, while EV71-related cases with pulmonary edema had significantly lower cellular IFN-$\gamma$.[7] Besides, the imbalances of Th1/Th2 and Th17/Treg ratios have been found to be existed in HFMD patients.[8] Moreover, the levels of IL-6 and IFN-$\gamma$ were increased in cerebrospinal fluid (CSF) from children with EV71 associated with meningoencephalitis.[9] Furthermore, the non-structural proteins 3C and 2A proteases of EV71 have been identified as the main antagonists of type I IFNs and relate to type I IFNs signaling.[10,11] These results suggest that EV71 infection can affect the differentiation and distribution of T subset.

IL-10-producing B cells, which are termed as B10 cells, server as a type of immune tolerance factor in immune system. Previously studies have demonstrated that B cells and IgG levels in EV71 infected cases were elevated and might be associated with neurological manifestations,[12] while the number of B cells was reduced in EV71-infected mice.[13] However, very limited studies were conducted to illustrate the changes of B cell subsets in EV71 infected children. In addition, the exact pathogenesis of EV71 on host immune responses, especially in terms of the impacts of EV71 infection on the percentages of T subpopulations and B10 population in children, has not been fully clarified. Therefore, in the present study, we attempted to delineate the outcomes of host immunological responses after EV71 infection, by analyses of the blood T and B lymphocytes subsets and mRNA expression levels of T lymphocyte-associated transcription factors. Our study can shed a light on utilizing the frequencies of lymphocytes in blood for the diagnosis and treatment monitoring of HFMD caused by EV71 infection.

## Materials and methods

### Study population

The EV71 group consisted of thirty-two cases of children with EV71 infection, who were admitted to Guangzhou Women and Children's Medical Center (Guangzhou, China) between November, 2014 and July, 2015. Pharyngeal swabs took from the children with the onset of fever within three days, rash in one or more parts of mouth, buttocks, hands or feet were examined for the presence of common pathogens by real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) according to the protocols of the detection kits manufacturer (Daan Gene Co., Ltd of Sun Yat-Sen University, Shenzhen, China). All patients were conformed with the diagnostic criteria of guideline for the diagnosis and treatment of HFMD (2018 Edition).[3] Another thirty-two children were from the outpatient department of health care, and they had no fever, no use of medication, and no signs of infectious diseases for at least 14 days. Pharyngeal swabs from these children were negative for EV71, and they were enrolled in the control group over the same period. Other common pathogens, including coxsackievirus A16 (CoxA16), influenza virus (IFV), respiratory syncytial virus (RSV), adenovirus (ADV), human bocavirus (HBoV), human metapneumovirus (hMPV), parainfluenza virus (PIV), rhinovirus (RHV), mycoplasma pneumoniae (MP), chlamydia pneumoniae (CP), cytomegalovirus (CMV), Epstein–Barr virus (EBV), and herpes simplex virus (HSV), were found negative in both groups. Informed consent was obtained from the legal parents of the children, and all procedures involved in this study were approved by the Ethics Committee of Guangzhou Women and Children's Medical Center.

### Analysis of lymphocyte subsets by flow cytometry

After the enrollment of subjects, their peripheral venous blood samples were collected in the morning and anticoagulated with heparin. The BD Multitest IMK Kit (including CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC, CD3-FITC/CD16 + 56-PE/CD45-PerCP/CD19-APC) (catalog number 340503; BD Biosciences, CA, USA) was used to define total major lymphocyte subsets, including $CD19^+$ B cells, $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells. Regulatory T (Treg) cells were defined as $CD4^+CD25^+CD127^{-/lo}$ cells. The antibodies CD4-APC (allophycocyanin), CD25-FITC (fluorescein isothiocyanate), CD127-PE (phycoerythrin), CD3-PerCP

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**Table 1** Primer sequences of transcription factors for mRNA analysis by real-time PCR.

| Gene product | Primers Orientation | Sequence | Product size |
|---|---|---|---|
| T-bet | Forward | 5′-ATGTGACCCAGATGATTGTGC-3′ | 112bp |
| | Reverse | 5′-TGCGTGTTGGAAGCGTTG-3′ | |
| Gata3 | Forward | 5′-CAAAATGAACGGACAGAACC-3′ | 129bp |
| | Reverse | 5′-CCATTGGCATTCCTCCTC-3′ | |
| RORγt | Forward | 5′-GTGCTGGTTAGGATGTGCCG-3′ | 135bp |
| | Reverse | 5′-GTGGGAGAAGTCAAAGATGGA-3′ | |
| Foxp3 | Forward | 5′-GTTCCTCCACAACATGGACTAC-3′ | 171bp |
| | Reverse | 5′-GTGGCAGGATGGTTTCTGAAG-3′ | |
| TCF-1 | Forward | 5′-CCGCCTTCAATCTGCTCA-3′ | 178bp |
| | Reverse | 5′-TGCTTCTGGCTGATGTCCG -3′ | |
| BCL-6 | Forward | 5′-CTTTCCCCACGAGCCTACA-3′ | 161bp |
| | Reverse | 5′-CCCGTCATGGACCTGTTAA-3′ | |
| GAPDH | Forward | 5′-GAGTCAACGGATTTGGTCGT-3′ | 238bp |
| | Reverse | 5′-TTGATTTTGGAGGGATCTCG -3′ | |

(Peridinin Chlorophyll), CD8-APC, CD3-PE, CD8-PerCP, and CD19-APC were used for surface staining, while IL-4-PE, IFN-γ-FITC, IL-17-Alexa Flour 488, IL-21-Alexa Flour 647, and IL-10-PE were used for intracellular staining. All antibodies were purchased from BD Biosciences, CA, USA. Cell apoptosis was determined by flow staining with PI and Annexin V (a kit from Beyotime Biotechnology, China) according to the manufacturer's protocol.

For intracellular staining, the whole blood samples were incubated with phorbol myristate acetate (PMA; Sigma-Aldrich, MO, USA), ionomycin (Sigma-Aldrich) and brefeldin A (BFA; Sigma-Aldrich), and followed by staining with surface antibodies. After fixation and permeabilization with BD FACS Permeabilizing Solution (BD Biosciences), the cells were labeled with the indicated antibodies. In this study, $CD3^+CD8^-$ T cells were considered to represent $CD3^+CD4^+$ T cells because of the down regulating effect of CD4 surface antigen by PMA.[14] For intracellular staining of B10 cells, blood samples were incubated with 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) before being stimulated by PMA, ionomycin, and BFA. To avoid non-specific staining, samples were incubated with the Human TruStain FcX Fc Receptor Blocking Solution (BioLegend, CA, USA) before the specific surface staining. Data were acquired with the BD

**Table 2** Clinical characteristics of study population.

| Characteristic | EV71 group (N = 32) | Control group (N = 32) | P-value |
|---|---|---|---|
| Clinical symptoms | | | |
| Age (years) | 2.56 ± 1.15 | 2.62 ± 0.95 | 0.74 |
| Gender (male/female) | 17/15 | 16/16 | 0.80 |
| Peak Body Temperature (°C) | 38.90 ± 0.62 | 37.10 ± 0.56 | <0.001 |
| Rash | N = 32 | — | — |
| hand | 32, 100% | — | — |
| foot | 29, 90.63% | — | — |
| mouth | 27, 84.38% | — | — |
| crissum | 23, 71.88% | — | — |
| Cough | N = 10 | — | — |
| Flu-like symptoms | N = 3 | — | — |
| Blood routine measures | | | |
| White blood cell ($\times 10^9$/L) | 10.05 ± 2.63 | 6.92 ± 0.81 | <0.001 |
| Neutrophil ($\times 10^9$/L) | 5.34 ± 2.20 | 2.73 ± 0.43 | <0.001 |
| Monocyte ($\times 10^9$/L) | 0.60 ± 0.31 | 0.33 ± 0.09 | <0.001 |
| Lymphocyte ($\times 10^9$/L) | 4.02 ± 1.59 | 3.61 ± 0.72 | 0.24 |
| Eosinophil ($\times 10^9$/L) | 0.031 ± 0.028 | 0.209 ± 0.150 | <0.001 |
| Basophil ($\times 10^9$/L) | 0.025 ± 0.123 | 0.016 ± 0.014 | 0.044 |
| Red blood cell ($\times 10^{12}$/L) | 4.51 ± 0.26 | 4.58 ± 0.27 | 0.413 |
| Hemoglobin (g/L) | 119.39 ± 9.64 | 124.06 ± 7.52 | 0.098 |
| Platelet ($\times 10^9$/L) | 334.26 ± 71.97 | 328.50 ± 77.66 | 0.801 |
| Neutrophil (%) | 52.35 ± 15.67 | 40.06 ± 7.41 | 0.001 |
| Monocyte (%) | 5.65 ± 1.43 | 4.94 ± 1.39 | 0.111 |
| Lymphocyte (%) | 40.77 ± 14.70 | 51.88 ± 6.87 | 0.001 |

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FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

### Detection of the expression of transcription factors by RT-qPCR

Total RNA was extracted from the whole blood samples of the two groups using the HiPure Liquid RNA/miRNA Kit (Magen, Guangzhou, China), while cDNA samples were obtained by RNA reverse transcription with the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, China), according to the manufacturers' protocols. To quantify the expression levels of the transcription factors, including T-bet, Gata3, RORγt, Foxp3, TCF-1, and BCL-6, real-time quantitative PCR was conducted with SYBR Premix Ex Taq II (Takara Bio USA, Inc., Japan) using ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The sequences of primers are listed in Table 1. The thermal cycler conditions were as follows: single amplification cycle of denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. The housekeeping gene GAPDH was chosen as the internal control. Delta Ct (ΔCt) and delta-delta Ct (ΔΔCt) values were determined for relative quantification of gene expression. $\Delta Ct = Ct_{Gene} - Ct_{GAPDH}$. ΔΔCt = average of $\Delta Ct_{EV71\ group}$ - average of $\Delta Ct_{control\ group}$. The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used for determining the related fold changes of target genes.

### Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed with SPSS 18.0 software (SPSS Inc., IL, USA). Statistical analyses were performed with Student's *t*-test, and all tests were two-sided. A *P* value less than 0.05 was considered statistically significant.

## Results

### General characteristics of patients and healthy controls

A total of 32 children with confirmed EV71 infection were enrolled in this study, and this EV71 group included 17 males and 15 females (Table 2). The Age of patients ranged between 10 months and 6 years, and the average age was 2.56 ± 1.15 years. In the control group, there were 16 males and 16 females with an average age of 2.62 ± 0.95 years. The compositions of age and gender did not have a significant difference between the EV71 infected patients and the healthy controls ($P > 0.05$). Fever and rash were observed among all the EV71 infected subjects. While 31.25% of these subjects showed cough, 9.38% of them showed flu-like symptoms (Table 2).

### EV71 infected children had decreased percentages of total T, helper T and cytotoxic T cell populations in blood

As illustrated in Fig. 1, the percentages of $CD3^+$ total T cells, $CD3^+$ $CD4^+$ helper T cells (Th), and $CD3^+$ $CD8^+$ cytotoxic T cells (Tc) among the blood lymphocytes in the EV71 group were 55.95 ± 7.86%, 30.63 ± 7.22%, and 21.91 ± 4.56%, respectively. Whereas, the corresponding percentages of total T, Th, and Tc cells in the control group

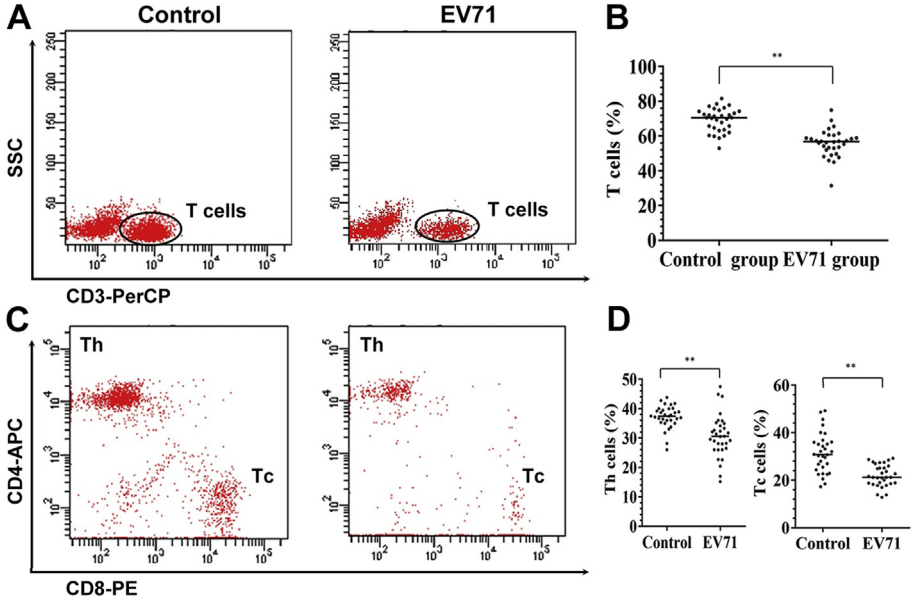


**Figure 1.** The percentages of total T, Th, and Tc cells in blood lymphocytes were decreased in EV71 infected patients. **(A—D)** Patients in the EV71 group had significantly reduced percentages of $CD3^+$ total T cells (A—B) and $CD3^+CD4^+$ Th cells and $CD3^+CD8^+$ Tc cells (C—D) in blood lymphocytes. Representative flow profiles of blood cell staining using BD MultiTEST kit are shown in A and C, while summarized percentages of indicated T cell populations in blood lymphocytes are summarized in B and D. Blood cells were pre-gated on total lymphocytes in A and C. n = 32 for the control group, and n = 32 for the EV71 group. $^{**}P < 0.01$.

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were 69.39 ± 6.71%, 37.21 ± 3.84%, and 31.49 ± 8.32%, respectively. Statistically, the frequencies of total T cells (Fig. 1A and B), Th cells, and Tc cells (Fig. 1C and D) among the blood lymphocytes were significantly decreased in the EV71 group (all $P < 0.01$).

## Blood Th and Tc cells from the EV71 group had lower frequency of IFN-γ production and higher frequency of IL-4 production

As shown in Fig. 2A and Fig. 2B, both the percentages of $CD3^+CD8^-$IFN-$\gamma^+$ Th1 cells and $CD3^+CD8^+$ IFN-$\gamma^+$ Tc1 cells among the corresponding Th or Tc subsets in the EV71 group were significantly lower than those in the control group (4.56 ± 1.78% vs. 11.58 ± 5.18%, 13.82 ± 6.78% vs. 21.73 ± 10.70%). In contrast, the percentages of $CD3^+CD8^-$IL-$4^+$ Th2 cell and $CD3^+CD8^+$IL-$4^+$ Tc2 cells in the EV71 group were significantly higher than those in the control group (3.06 ± 1.53% vs. 1.58 ± 0.75%, 1.24 ± 0.89% vs. 0.57 ± 0.35%). As a result, the Th1/Th2 (Fig. 2C) and Tc1/Tc2 (Fig. 2D) ratios were significantly decreased in the EV71 group compared with control group.

## Blood samples from the EV71 group exhibited higher frequencies of IL-17- and IL-21-expressing cells in Th and Tc cells, and lower frequency of Tregs in Th cells

The percentages of IL-17-expressing Th17 (IL-$17^+CD3^+CD8^-$) cells and IL-17-expressing Tc17 (IL-$17^+CD3^+CD8^+$) cells among the corresponding Th cells or Tc cells were significantly increased in the EV71 group, compared with the control group (1.38 ± 0.98% vs. 0.61 ± 0.37%, 2.83 ± 1.09% vs. 1.67 ± 0.97%) (Fig. 3A). Similarly, blood Th cells and Tc cells from the EV71 group also had higher ratios of IL-21-expressing Tfh (IL-$21^+CD3^+CD8^-$) cells and IL-21-expressing IL-$21^+CD3^+CD8^+$ cells, respectively, compared with the control group (2.40 ± 1.50% vs. 0.96 ± 0.69%, 0.83 ± 0.52% vs. 0.19 ± 0.22%) (Fig. 3B). In contrast, the percentages of $CD4^+CD25^+CD127^{-/lo}$ Treg cells among the $CD4^+$ Th cells were significantly decreased in the EV71 group

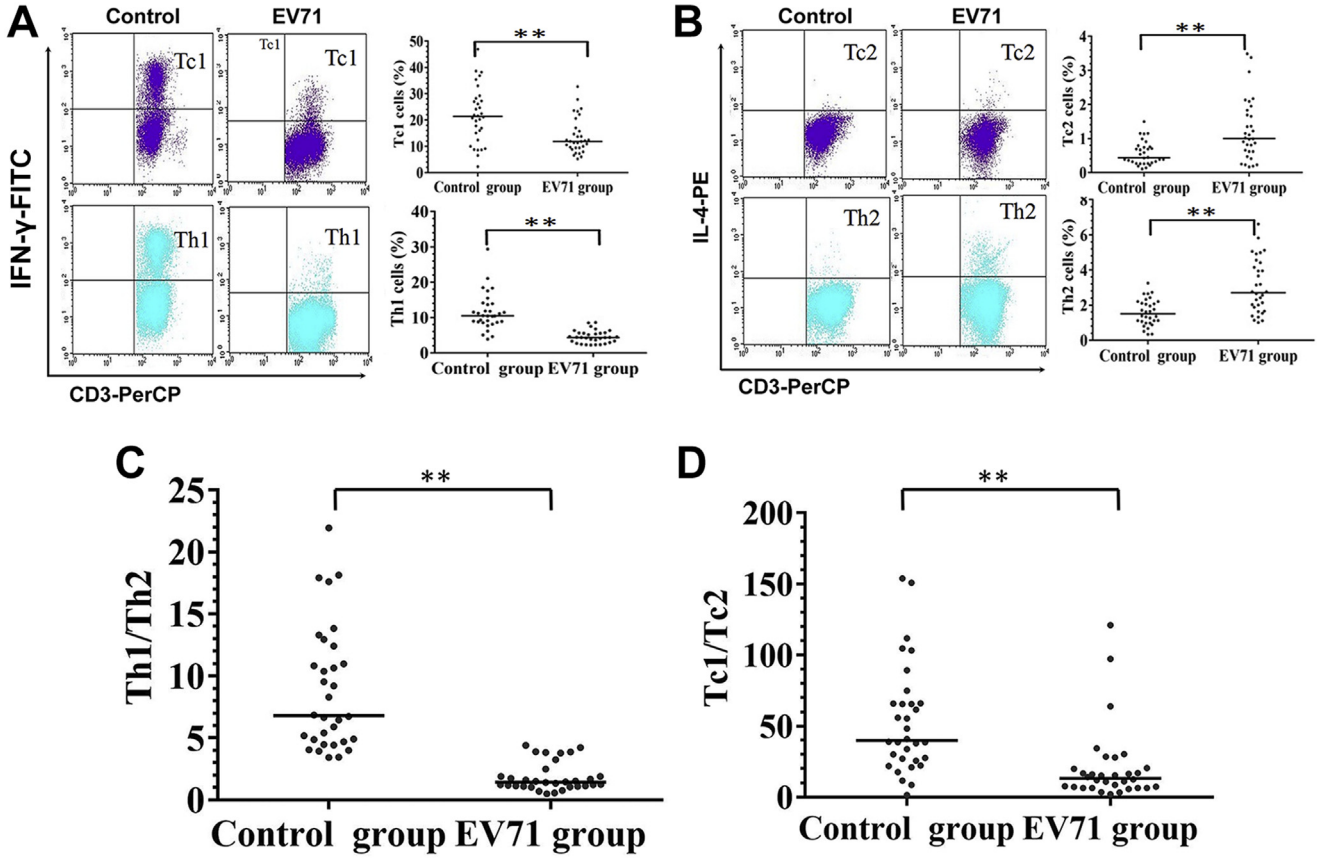


**Figure 2.** Blood Th and Tc cells from the EV71 group had lower frequency of IFN-γ production and higher frequency of IL-4 production. **(A—B)** The expression frequencies of IFN-γ and IL-4 in blood $CD3^+CD8^+$ (Tc) cells and $CD3^+CD8^-$ (Th) cells from the control group and the EV71 group. After stimulation with PMA, ionomycin and BFA, the whole blood cells were subjected to intracellular staining for IFN-γ (A) and IL-4 (B). Representative flow profiles are shown on left and the summarized frequencies of indicated cells are shown on right. Tc1, $CD3^+CD8^+$ IFN-$\gamma^+$ cells; Th1, $CD3^+CD8^-$ IFN-$\gamma^+$ cells; Tc2, $CD3^+CD8^+$ IL-$4^+$ cells; Th2, $CD3^+CD8^-$ IL-$4^+$ cells. **(C—D)** The ratios of Thl/Th2 and Tc1/Tc2 in the control group and the EV71 group. n = 32 for the control group, and n = 32 for the EV71 group. Each dot in the column scatter plot represents an individual sample. $^{**}P < 0.01$.

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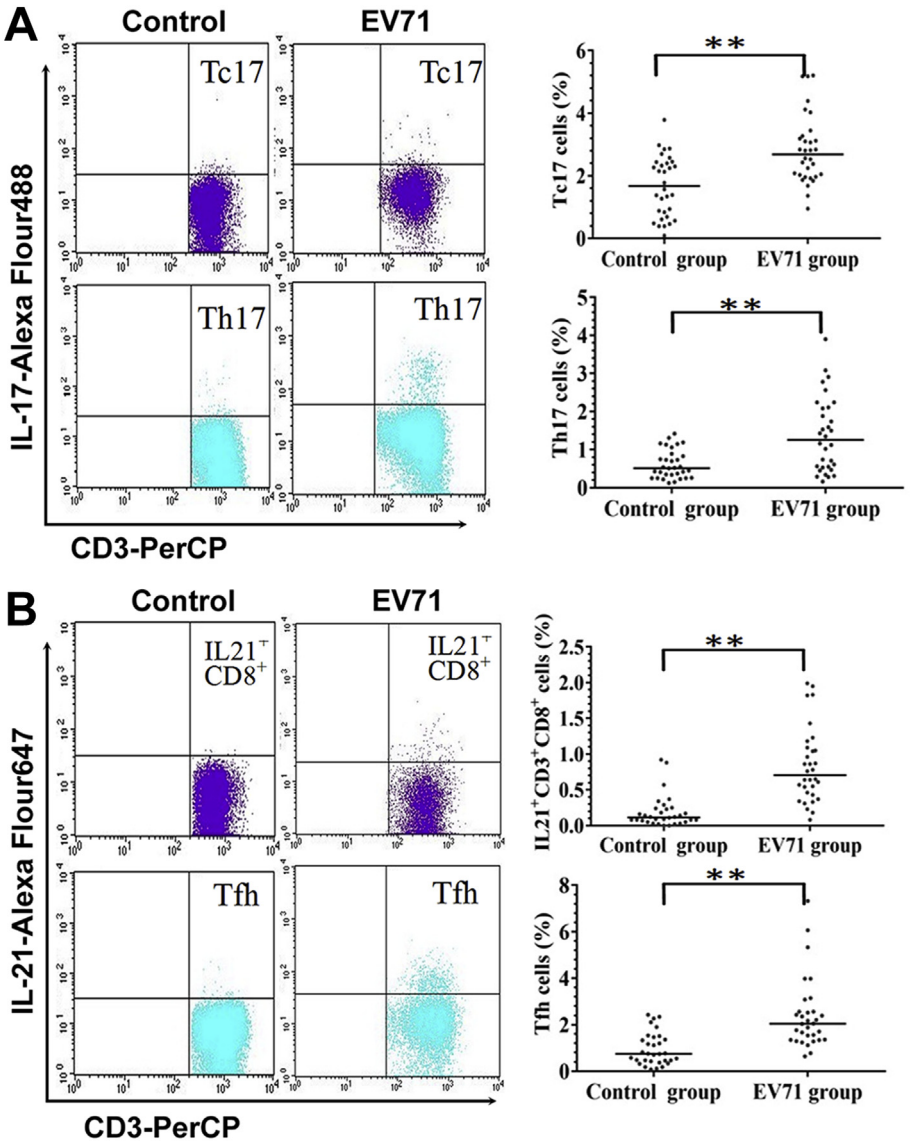


**Figure 3.** Blood samples from the EV71 group exhibited higher frequencies of IL-17- and IL-21-expressing cells in Th and Tc cells. **(A—B)** The expression frequencies of IL-17 and IL-21 in blood $CD3^+CD8^+$ (Tc) cells and $CD3^+CD8^-$ (Th) cells from the control group and the EV71 group. After stimulation with PMA, ionomycin and BFA, the whole blood cells were subjected to intracellular staining for IL-17 (A) and IL-21 (B). Representative flow profiles are shown on left and the summarized frequencies of indicated cells are shown on right. Tc17, $CD3^+CD8^+$ IL-$17^+$ cells; Th17, $CD3^+CD8^-$ IL-$17^+$ cells; Tfh, $CD3^+CD8^-$ IL-$21^+$ cells. n = 32 for the control group, and n = 32 for the EV71 group. Each dot in the column scatter plot represents an individual sample. $^{**}P < 0.01$.

(6.22 ± 1.22% vs. 7.92 ± 1.92%) (Fig. 4A and B). Consequently, compared with the control group, the Th17/Treg ratio in the EV71 group was markedly higher (Fig. 4C).

## Blood samples from the EV71-infected patients had higher frequencies of B cells and IL-10-expressing B10 cells

As shown in Fig. 5A, the percentage of $CD19^+$ B cells among the total lymphocytes showed a statistically significant increase in the blood of patients with EV71 infection than that of the subjects in the control group (30.00 ± 7.62% vs. 18.48 ± 6.10%, $P < 0.01$). In addition, after stimulation with LPS, PMA and ionomycin, the $CD19^+$ B cells in the EV71 group had significantly higher percentage of IL-10-expressing B10 (IL-$10^+CD19^+$) cells than that in the control group (3.03 ± 1.37% vs. 1.82 ± 0.85%, $P < 0.01$) (Fig. 5B).

## The expression of transcription factors T-bet, Foxp3, and Gata3 were significant down-regulated in the blood from patients with EV71 infection

Compared with the control group, the mRNA expression levels of the examined transcription factors T-bet, Foxp3, and Gata3 were significantly decreased in the EV71 group, while other transcription factors RORγt, TCF-1, and BCL-6 did not show significant differences in mRNA expression. If

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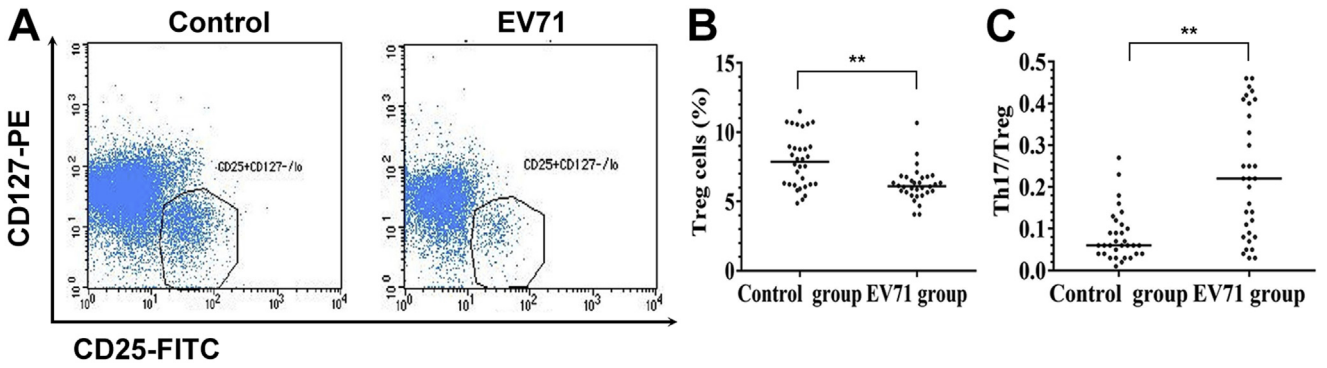


**Figure 4.** Blood samples from the EV71 group exhibited lower frequency of Tregs in Th cells. **(A—B)** Blood $CD4^+$ Th cells from the EV71 group had lower frequency of Treg cells than that from the control group. Representative flow profile of blood Treg staining after pre-gating on $CD4^+$ lymphocytes are shown in A, while summarized percentages of Tregs in Th cells are shown in B. The $CD4^+CD25^+CD127^{-/lo}$ cells were used to represent Treg cells. **(C)** Compared with control group, the EV71 group had a significantly increased ratio of Th17/Treg in blood. n = 32 for the control group, and n = 32 for the EV71 group. Each dot in the column scatter plot represents an individual sample. $^{**}P < 0.01$.

the normalized expression level of each gene in the control group was set as 1, the mRNA expression levels of T-bet, Gata3, RORγt, Foxp3, TCF-1, and BCL-6 in the peripheral blood of children with EV71 infection were 0.41, 0.27, 2.10, 0.33, 0.60, and 2.02, respectively (Fig. 6).

## Discussion

In this study, we demonstrated that the percentages of T lymphocyte subpopulations and B lymphocytes populations in the peripheral blood of children with EV71 infection

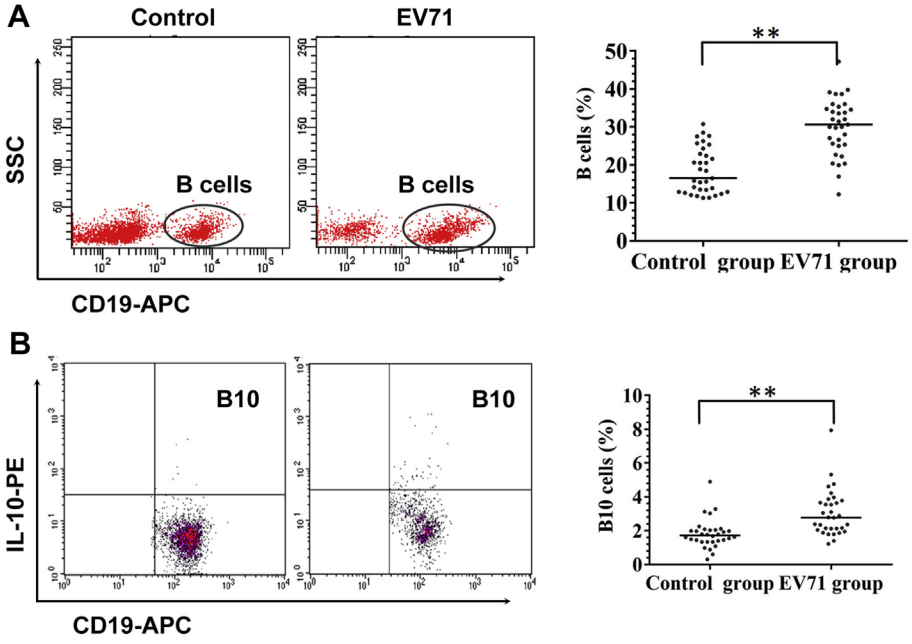


**Figure 5.** Blood samples from the EV71-infected patients had higher frequencies of B cells and IL-10-expressing B10 cells in lymphocytes. **(A)** The EV71 group had significantly higher frequency of $CD19^+$ B cells in blood lymphocytes than the control group. Representative flow profile of CD19 staining after pre-gating on whole lymphocytes are shown on left, while the summerized frequencies of $CD19^+$ B cells in lymphocytes are shown on right. **(B)** B cells from the EV71 group had higher frequency of IL-10 expression after stimulation with LPS than that from the control group. LPS was added into the whole blood cells, which were subsequently treated with PMA, ionomycin and BFA. After incubation for 5 h, blood cells were subjected to intracellular staining for IL-10. Representative flow profile of CD19 and IL-10 staining on pre-gated lymphocytes are shown on left, while the summerized data on percentages of IL-10-expressing $CD19^+$ B cells (B10) in total B cells are shown on right. n = 32 for the control group, and n = 32 for the EV71 group. Each dot in the column scatter plot represents an individual sample. $^{**}P < 0.01$.

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significantly changed, which suggested that the cellular immune response was potentially involved in the pathogenesis of HFMD in children.

The percentages of total T cells in blood lymphocytes, the frequencies of Th, Tc in total blood T cells, and the ratios of Th1 and Tc1 cells in corresponding Th and Tc cells were decreased, which indicated imbalanced distribution of T lymphocytes during EV71 infection, when compared with that from the control subject. Damsker et al. found that the lack of IFN-γ not only could play a protective role, but also increase the susceptibility to disease in a mouse model.[15] We found low-level of IFN-γ in Th1 cells during EV71 infection. Considering the critical roles of IFN-γ in Tc differentiation and cytotoxicity,[16,17] this might be associated with the inefficient differentiation of Tc precursor cells into Tc1 and compromised cytotoxic effect of Tc1 cells to protect the patient from EV71 infection. Moreover, a reduction in the level of eosinophil in EV71 group may be associated with ineffective production of Th1 type cytokines.[18,19] Cytokines produced by activated Th2 cells have strong inflammatory and chemotactic effects[20,21] Wei et al. have showed that elevated antigen-specific Th2 type response tended to cause a more prolonged period of high fever and a longer duration of illness in HFMD.[22] Consistently, in our study, the levels of IL-4 cytokine released by Th2 cells were increased, and the Th1/Th2 ratio was significantly decreased in the EV71 group, which might contribute to a series of inflammation associated clinical manifestations in the patients.

Treg cells are a group of cells with immunomodulatory functions for maintaining immune homeostasis *in vivo*. Here, we found decreased percentages of Treg cells and increased percentages of Th17, Tfh and $CD8^{+}IL\text{-}21^{+}$ T cells in blood $CD4^{+}$ T cells during EV71 infection. Treg cells have been demonstrated to inhibit the activity of Th cell through the mechanisms of direct cell contact and inhibition of cytokine secretion.[23] It can be speculated that the T cells activation might be down-regulated in the EV71 infected patients, since the percentages of Treg cells decreased in the blood of patients.

IL-17A, as a pro-inflammatory cytokine, stimulates the production of CXCL1, CXCL5, CXCL6 and other cytokines, and promotes neutrophil differentiation, maturation, and migration.[23,24] In this study, the percentages of Th17 cells in $CD4^{+}$ T cells and blood neutrophils concentration were increased in children with EV71 infection, which suggested that the activation of Th17 response was involved in the pathogenesis of EV71 infection and might partially explain the significant increase of neutrophils in HFMD patients. In addition, we here found that the percentages of Th and Tc cells in peripheral blood of children with HFMD were significantly decreased. This may contribute to the attenuation on the differentiation and proliferation of Th17 cells, which is consistent with a previous report from Chen et al.[25]

Tfh cells are a group of Th cell subsets in lymphoid follicles, and their role in cellular and humoral immune response is mainly through the production of cytokine IL-

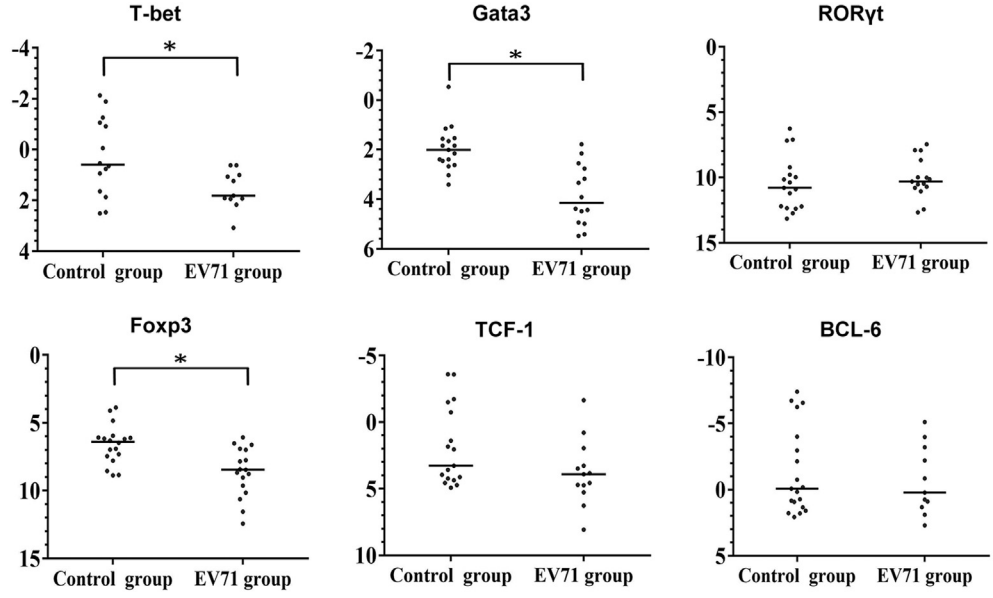


**Figure 6.** The mRNA expression levels of 6 T lymphocytes-related transcription factors in blood samples from the control group and the EV71 group. The mRNA expression levels of 6 transcription factors, including T-bet, Gata3, RORγt, Foxp3, TCF-1, and BCL-6 in whole blood samples from the control subjects and EV71 infected patients were measured using real-time quantitative PCR. The expression levels of these transcription factor were expressed as ΔCt, where $\Delta Ct = Ct_{Gene} - Ct_{GAPDH}$ for each sample. The lower ΔCt value means the greater amount of mRNA expression. The Y-axis has been reversed to better illustrate the effect. Fold changes of $2^{-\Delta\Delta Ct}$ in T-bet, Gata3, RORγt, Foxp3, TCF-1, and BCL-6 were 0.41, 0.27, 2.10, 0.33, 0.60, and 2.02, respectively. Although RORγt, TCF-1, and BCL-6 had slightly different expressions in blood samples from the EV71 group, there was no statistically significant difference between two groups. n = 20 for the control group, and n = 20 for the EV71 group. Each dot in the column scatter plot represents an individual sample. *$P < 0.05$.

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21.[26] Tfh cells secreted IL-21 may promote the proliferation and differentiation of Th17 cells, thereby destroying the balance of Treg/Th17. In addition, we observed that the percentages of Tfh cells in Th cells, $CD8^{+}IL\text{-}21^{+}$T cells in Tc cells, and B cells in total lymphocytes were increased, suggesting that IL-21 may indirectly promote the proliferation of B cells and antibody production.[27]

B10 cells are a class of B cell subsets that play a negative regulatory role in the immune response, mainly through the secretion of IL-10, which can directly interact with T cells and participate in the body's immune response. Previous studies showed that infection can induce the production of B10 cells,[28,29] and the B10 cells in peripheral blood of patients with virus infection (human immunodeficiency, HIV; chronic hepatitis B virus, CHB) were significantly higher than those of healthy counterparts.[30,31] Here, we found that both the percentages of total B cells and B10 cells in the EV71 group were significantly higher than those in the control group. Similarly, by analyzing the lymphocytes of children with severe and critical HFMD in our hospital, our previous work also revealed that the marked increase of B cell frequency in blood may reflect the critical stage of HFMD.[32] IL-10 is an anti-inflammatory factor which not only inhibits Th1, but also inhibits Th2 reactions.[33] IL-10 can be produced by both T cells and B cells, while this study focused only on IL-10 secreted by B cells, which is one of the limits in this study. However, more investigation on the IL-10 regulatory pathways in blood lymphocytes from EV71 infected children will be performed in further experiments. Taken together, the data suggest that heightened B10 cells may be involved in EV71 pathogenesis, and the excessive immune response may increase the risk of EV71 infection. It would be of interest to elucidate the precise function of B10 cells in EV71 infection in the further study.

T-bet, Gata3, RORγt, Foxp3, TCF-1, and BCL-6 are major transcription factors of T lymphocyte subsets. To gain a general impact of EV71 infection on the regulation of master regulators of T cells, we evaluated the mRNA expression of these transcription factors in peripheral blood cells. In our study, only the mRNA levels of T-bet, Gata3, and Foxp3 significantly decreased in the EV71 group. In addition, the levels of IL-4 cytokine released by Th2 cells in the peripheral blood of children with HFMD were increased, while the mRNA expression of Th2 transcription factor Gata-3 was decreased. Since we measured the transcriptional levels of whole blood cells but not purified T cells, this might be due to the generally negative regulatory effect of EV71 infection on ubiquitous Gata-3 expression, in which EV71 infection might regulate the stability of Gata-3.[34]

## Conclusion

The impairment of immune function in HFMD children with EV71 infection is mediated through a complex network of lymphocytes and their interaction with cytokines and transcription factors. In particular, the cumulative changes in percentages of T cell subsets and B cell that lead to imbalance of immune system would be crucial for the development of the disease, and may become the key indicators in diagnosis and treatment of HFMD. However, how EV71 infection can affect the differentiation and proliferation of these lymphocytes is still not clear, which needs further investigation in future.

## Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2019.03.001.