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Interleukin-7 promotes CD8$^+$ T cell activity in patients with enterovirus 71 associated encephalitis

Xiaojun Tian*, Zhou Su, Shuangxi Guo, Congcong Wang, Xueying Zhang, Jia Du

The First Ward in Department of Neurology, The First Affiliated Hospital of Xinxiang Medical University, Weihui, Xinxiang, Henan Province 453100, PR China

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ABSTRACT

Enterovirus 71 (EV71) always induces severe hand, foot, and mouth disease with neurological complications, such as encephalitis. Interleukin (IL)-7 augments CD8$^+$ T cells activity in chronic viral infection and cancers. However, few studies have focused on common γ-chain (γc) cytokine expression and regulatory function of IL-7 to CD8$^+$ T cells in EV71 associated encephalitis. In this study, twenty-one patients with EV71 associated encephalitis, twenty-seven patients with febrile convulsion (FC), and twenty healthy individuals were enrolled. γc cytokine (IL-2, IL-4, IL-7 and IL-15) concentration was measured by ELISA. IL-7 receptor α chain (membrane/soluble CD127) expression was also investigated. Purified CD8$^+$ T cells were stimulated with recombinant human IL-7 in vitro. The regulatory activity of IL-7 to CD8$^+$ T cells from peripheral blood and cerebrospinal fluids (CSF) was investigated in direct and indirect contact co-culture with U-87MG cells. IL-7 in the serum and CSF, but not IL-2, IL-4, or IL-15, was significant increased in EV71 associated encephalitis. Both total CD127 mRNA relative level and membrane/soluble CD127 expression was comparable among three groups. IL-7 stimulation promoted CD8$^+$ T cells proliferation, up-regulated perforin/granzyme B level, but reduced programmed death-1 expression in CD8$^+$ T cells from EV71 associated encephalitis patients. Cytotoxicity and interferon-γ production of CD8$^+$ T cells from peripheral blood and CSF was also augmented in response to IL-7 stimulation in both direct and indirect co-culture systems in EV71 associated encephalitis. The present data indicated that IL-7 induced cytolytic and non-cytolytic functions of CD8$^+$ T cells in EV71 associated encephalitis. IL-7 might be considered as one of the immunomodulatory therapeutic candidates for EV71 infection.

1. Introduction

Hand, foot, and mouth disease (HFMD), which is a common infectious disease in infants and young children, is primarily caused by enterovirus 71 (EV71) and coxsackievirus A16 [1–3]. HFMD is always self-limited, and is characterized by fever, vesicles on hands and feet, and/or ulcers in oral mucosa [4]. However, EV71 could also induce severe neurological complications, including encephalitis, brainstem encephalitis, and aseptic meningitis [5]. A small proportion of EV71-induced neurological complications may develop cardiopulmonary failure, which may be fatal [6]. There are no effective vaccines or specific therapeutic approaches for EV71 infection currently. Thus, it is pivotal to better understanding the neuropathogenesis of EV71 infection. It is well accepted that EV71 infection could not only induce direct cytopathic effect in various cell types such as neuronal cytoplasm [7], but also lead to dysregulation of cellular immunity and inflammatory responses [8–10]. Unfortunately, the mechanisms underlying EV71 associated neurological complications are not fully elucidated.

Common γ-chain (γc) cytokine family members include interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [11], which regulate development, proliferation, survival, and differentiation of immune cells [12]. γc cytokines collectively have broad actions and activate three major signaling pathways, including the MAP kinase, PI3-kinase, and (Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathways [13]. IL-7 and IL-15 mainly regulates T and NK cells, respectively, while defective signaling by IL-21 substantially induces the non-functional B cells [14]. Signaling through IL-7/CD127 (IL-7 receptor α chain) maintained homeostatic proliferation of naïve and memory CD4$^+$ T cells [15], and in vivo administration in humans also increased CD4$^+$ T cell counts in idiopathic CD4 lymphocytopenia [16]. IL-7 also mediated the homeostasis of naïve and memory CD8$^+$ T cells in vivo [17], and this process was probably dependent on induction of type I interferon (IFN) and suppression of programmed death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), especially

* Corresponding author at: The First Ward in Department of Neurology, The First Affiliated Hospital of Xinxiang Medical University, 88 Jiankang Rd, Weihui, Henan Province 453100, PR China.
E-mail address: xyyfytxj@sina.com (X. Tian).

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in chronic viral hepatitis infection [18,19]. IL-7 overcame chronic viral infection and limited organ pathology by engagement of multiple mechanisms, including down-regulation of critical repressor of cytokine signaling, amplification of cytokine production, enhancement of thymic output, and proliferation of viral specific and non-specific T cells [20].

Exhausted $CD8^+$ T cell responses contribute to viral persistence and tumor metastasis [21,22]. In contrast, excessive cytotoxic T cells in acute viral infection always lead to inflammatory cytokine storm, resulting in damage of organs and tissues [23]. However, few studies focused on the role of γc cytokines in EV71-associated diseases. Therefore, the purpose of this study was (i) to evaluate γc cytokines level in peripheral blood and CSF in EV71-induced encephalitis, and (ii) to investigate the potential regulatory role of IL-7 to $CD8^+$ T cells in EV71 associated encephalitis.

2. Materials and methods

2.1. Subjects

The study protocol was approved by the Ethics Committee of First Affiliated Hospital of Xinxiang Medical University, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients or guardians of each enrolled subjects before sampling. A total of 21 children with EV71 associated encephalitis were enrolled in this study. All children were hospitalized at The First Affiliated Hospital of Xinxiang Medical University from April 2016 to March 2018. EV71 infection was confirmed by EV71 VP1 gene detection in throat swabs, stools, or CSF using EV71 nucleic acid detection kit (Da'An Gene, Guangzhou, Guangdong Province, China). All enrolled patients were carefully examined and evaluated by senior physicians. The diagnosis of HFMD was made in accordance with the Guidelines for the Diagnosis and Treatment of HFMD (2010 Edited Version) by the Ministry of Health of the People's Republic of China [8]. Encephalitis was defined by the altered levels of consciousness (including lethargy, drowsiness or coma, seizures or myoclonus) with CSF pleocytosis [9]. Moreover, 27 patients with simple febrile convulsion (FC) and 20 healthy individuals were also enrolled. The diagnosis of FC was defined as generalized tonic-clonic seizures occurring in the first 48 h of a febrile illness and lasting for < 15 min, but without any focal signs or recurring within 24 h, or no associated evidence of intracranial infection or a metabolic disorder [9]. Lumbar puncture was performed in all EV71 associated encephalitis and FC patients, while peripheral blood samples were obtained from all enrolled subjects. The baseline characteristics of enrolled subjects were shown in Table 1.

2.2. $CD8^+$ T cells purification

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA). Cells from CSF were isolated by centrifugation at 1000 ×*g* for 10 min. $CD8^+$ T cells were purified using human $CD8^+$ T Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) following manufacturer's instruction, respectively. The purity of enriched $CD8^+$ T cells was > 95% according to flow cytometry determination.

2.3. Cell culture

Purified $CD8^+$ T cells were stimulated with recombinant human IL-7 (Peprotech, Rocky Hill, NJ, USA; final concentration: 5 ng/mL) for 12 h. Cells and supernatants were harvested for further experiments. Human glioblastoma cell line U-87MG cells, which were HLA-A2 restricted, were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2 condition.

2.4. Direct and indirect contact co-culture system

Purified $CD8^+$ T cells from HLA-A2 restricted patients with EV71 associated encephalitis and healthy individuals were stimulated with recombinant IL-7 (Peprotech; final concentration: 5 ng/mL) for 12 h. After washed twice, 10^5 of stimulated $CD8^+$ T cells were co-cultured in direct or indirect contact with 5×10^5 of U-87MG cells, which have been demonstrated as HLA-A2 restricted [24]. In direct contact co-culture system, $CD8^+$ T cells and U-87MG cells were directly mixed together in the presence of anti-CD3/CD28 (eBioscience, San Diego, CA, USA; final concentration: 1 ng/mL). In indirect contact coculture system, $CD8^+$ T cells were seeded into the upper chamber of Transwell plate (Corning, Corning, NY, USA) with anti-CD3/CD28 stimulation, while U-87MG cells were seed into lower chamber of plates. $CD8^+$ T cells and U-87MG cells were separated by a 0.4 μm membrane, which allowed passage of soluble factors only. The supernatants were harvested 48 hour post-coculture for further experiments.

2.5. Enzyme linked immunosorbent assay (ELISA)

The expression of cytokines, including IL-2, IL-4, IL-7, IL-15, soluble CD127 (sCD127), IFN-γ, and tumor necrosis factor-α (TNF-α), was measured using commercial ELISA kits (R&D systems, Minneapolis, MN, USA; or Boster, Wuhan, Hubei Province, China) following manufacturer's instruction.

2.6. Flow cytometry

PBMCs were stained with anti-CD3 FITC (BD Bioscience, San Jose, CA, USA), anti-CD8 APC (BD Bioscience), and anti-CD127 PE (BD Bioscience). In certain experiments, purified $CD8^+$ T cells were stained with anti-CD8 APC (BD Bioscience), anti-PD-1 (CD279) FITC (BD Bioscience), and anti-CTLA-4 (CD152) PE (BD Bioscience). Stained cells were acquired using FACS Calibur flow cytometer (BD Bioscience), and were analyzed using FlowJo for Windows Version 8.4.2 (Tree Star, Ashland, OR, USA).

2.7. Cellular proliferation assay

Cellular proliferation was assessed using Cell Counting Kit-8 (CCK-8, Beyotime, Wuhan, Hubei Province, China) following manufacturer's

Table 1
Baseline clinical characteristics of enrolled subjects.

	EV71 encephalitis	Febrile convulsion	Healthy individuals
Cases (n)	21	27	20
Gender (M/F)	14/7	19/8	12/8
Age (months)[a]	36.7[16.8, 81.6]	32.0[9.6, 60.4]	37.1[17.1, 62.3]
Blood WBC ($\times 10^9$/L)	13.88 ± 4.12	15.67 ± 6.01	8.98 ± 2.12
CSF WBC ($\times 10^6$/L)[a]	167.1[22.3, 546.8]	5.33[2.19, 17.16]	Not available
CSF total protein (g/L)[a]	338.7[102.4, 781.7]	107.7[51.11, 266.8]	Not available
CSF glucose (mmol/L)	4.17 ± 1.14	4.08 ± 0.77	Not available

[a] Data were shown as median[Q1, Q3].

Table 2
Primer sequences for real-time PCR.

Primer	Sequence
CD127 sense	5′-AAA GTT TTA ATG CAC GAT GTA GCT T-3′
CD127 anti-sense	5′-TGT GCT GGA TAA ATT CAC ATG C-3′
Perforin sense	5′-CGC CTA CCT CAG GCT TAT CTC-3′
Perforin anti-sense	5′-CCT CGA CAG TCA GGC AGT C-3′
Granzyme B sense	5′-TGG GGG ACC CAG AGA TTA AAA-3′
Granzyme B anti-sense	5′-TTT CGT CCA TAG GAG ACA ATG C-3′
PD-1 sense	5′-GCG TGA CTT CCA CAT GAG C-3′
PD-1 anti-sense	5′-GCA GGC TCT CTT TGA TCT GC-3′
CTLA-4 sense	5′-CCT GGA GAT GCA TAC TCA CAC ACA-3′
CTLA-4 anti-sense	5′-GGA TTT CAG CGG CAC AAG G-3′
β-Actin sense	5′-TGG CAC CAG CAC AAT GAA-3′
β-Actin anti-sense	5′-CTA AGT CAT AGT CCG CCT AGA AGC A-3′

instruction.

2.8. Real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized from total RNA using PrimeScript™ RT Reagent Kit (TaKaRa, Beijing, China) with random hexamers. Real-time PCR was performed using TB Green *Premix Ex Taq* II (Tli RNaseH Plus) (TaKaRa). The relative gene expression was quantified using $2^{-\Delta\Delta CT}$ method using ABI7500 System Sequence Detection Software (Applied Biosystems, Foster, CA, USA). The primers sequences were shown in Table 2.

2.9. Western blot

Western blot was performed as previously described [8]. Cells were lysed in 2 × SDS loading buffer for 15 min on ice, and supernatants were harvested by 13,000 × *g* for 1 min. Total proteins were separated on SDS-PAGE using Mini-protean 3 electrophoresis cell systems (Bio-Rad, Hercules, CA, USA), and were electroblotted into a PVDF membrane. The membrane was soaked in PBS containing 5% non-fat milk and 0.05% Tween 20. PVDF membrane was then incubated in the presence of anti-STAT5 (Abcam, Cambridge, MA, USA; 1: 1000 dilution), anti-phosphorylated STAT5 (Abcam; 1: 1000 dilution), or anti-GAPDH (Abcam; 1: 2000 dilution). Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody IgG (Abcam; 1: 2000 dilution) was then added for additional 2 h incubation. Antibody-antigen complexes were visualized by enhanced chemiluminescence (Western Blotting Luminol Reagent, Santa Cruz, CA, USA).

2.10. Enzyme-linked immunospot assay (ELISPOT)

The secretion of perforin and granzyme B by $CD8^+$ T cells was measured by Human Perforin ELISPOT Kit (Abcam) and Human Granzyme B ELISPOT Kit (Abcam) following manufacturer's instruction, respectively.

2.11. Cytotoxicity of U-87MG cells

The cytotoxicity of U-87MG cells were assessed by measurement of lactate dehydrogenase (LDH) level in cultured supernatants using LDH Cytotoxicity Assay Kit (Beyotime) following manufacturer's instruction. Low level LDH control was presented by U-87MG cells, while high level LDH control was presented by U-87MG cells treated with Triton X-100. The percentage of cytotoxicity was calculated in the following equation: (experimental value-low level control)/(high level control-low level control) × 100% [25].

2.12. Statistical analyses

All data were analyzed using SPSS version 21.0 for Windows (SPSS, Chicago, IL, USA), and data were presented as mean ± standard deviation or median[Q1, Q3]. Student's *t*-test, One way ANOVA, or SNK-*q* test was used for comparison among groups. Paired *t*-test was used for comparison prior to and post-stimulation. All tests were two-tailed, and P values < 0.05 were determined to be statistical differences.

3. Results

3.1. IL-7 expression was down-regulated in both peripheral blood and CSF in EV71 associated encephalitis

We firstly screened γc cytokines (IL-2, IL-4, IL-7, and IL-15) expression in peripheral blood and CSF by ELISA. There were no statistical differences of IL-2 (Fig. 1A), IL-4 (Fig. 1C), or IL-15 (Fig. 1G) level among EV71 associated encephalitis, FC patients, and healthy individuals (SNK-*q* tests, all $P > 0.05$). CSF IL-2 (Fig. 1B), IL-4 (Fig. 1D), and IL-15 (Fig. 1H) expression was comparable between EV71 associated encephalitis and FC patients (Student's *t*-tests; all $P > 0.05$). Importantly, serum IL-7 level was notably reduced in patients with EV71 associated encephalitis (31.95 ± 11.37 pg/mL) in comparison with in FC patients and healthy individuals (SNK-*q* tests, all $P < 0.0001$, Fig. 1E). However, there was no remarkable difference of serum IL-7 between FC and healthy controls (51.89 ± 16.76 pg/mL vs. 51.15 ± 15.54 pg/mL; SNK-*q* tests, $P = 0.878$, Fig. 1E). Furthermore, IL-7 expression was also significantly down-regulated in CSF from EV71 associated encephalitis (10.59 ± 3.14 pg/mL) than from FC patients (13.62 ± 3.49 pg/mL; Student's *t*-test, $P = 0.0028$, Fig. 1F). However, there were no correlation between IL-7 expression (either in peripheral blood or in CSF) and clinical index (Pearson correlation tests, all $P > 0.05$).

3.2. CD127 level was comparable among EV71 associated encephalitis, FC, and healthy individuals

CD127 mRNA relative level in PBMCs or cells from CSF was semi-quantified by real-time PCR. CD127 mRNA in PBMCs did not reveal significant difference among EV71 associated encephalitis, FC, and healthy individuals (One way ANOVA, $P = 0.837$, Fig. 2A). There was also no remarkable difference of CD127 mRNA in CSF between EV71 associated encephalitis and FC (Student's *t*-test, $P = 0.759$, Fig. 2B). Soluble CD127 in the serum and CSF was measured by ELISA. Similar to the trend of CD127 mRNA, there was comparable serum sCD127 level EV71 associated encephalitis (100.2 ± 19.00 pg/mL), FC (103.5 ± 12.65 pg/mL), and healthy individuals (103.6 ± 13.93 pg/mL) (One way ANOVA, $P = 0.707$, Fig. 2C). sCD127 expression in CSF did not reveal notable difference between EV71 associated encephalitis and FC (39.05 ± 17.88 pg/mL vs. 33.81 ± 13.93 pg/mL; Student's *t*-test, $P = 0.260$, Fig. 2D). Membrane CD127 (mCD127) expression on $CD8^+$ T cells were analyzed by flow cytometry. The representative flow dots for $CD3^+CD8^+$ analysis and histogram for CD127 were shown in Fig. 2E. Mean fluorescence intensity (MFI) value corresponding to mCD127 on peripheral $CD8^+$ T cells was comparable among EV71 associated encephalitis (28.05 ± 6.68), FC (25.47 ± 12.65), and healthy controls (25.87 ± 8.21) (One way ANOVA, $P = 0.468$, Fig. 2F). There was no remarkable difference of mCD127 expression on $CD8^+$ T cells from CSF between EV71 associated encephalitis and FC (40.38 ± 15.23 vs. 38.96 ± 13.76; Student's *t*-test, $P = 0.737$, Fig. 2G).

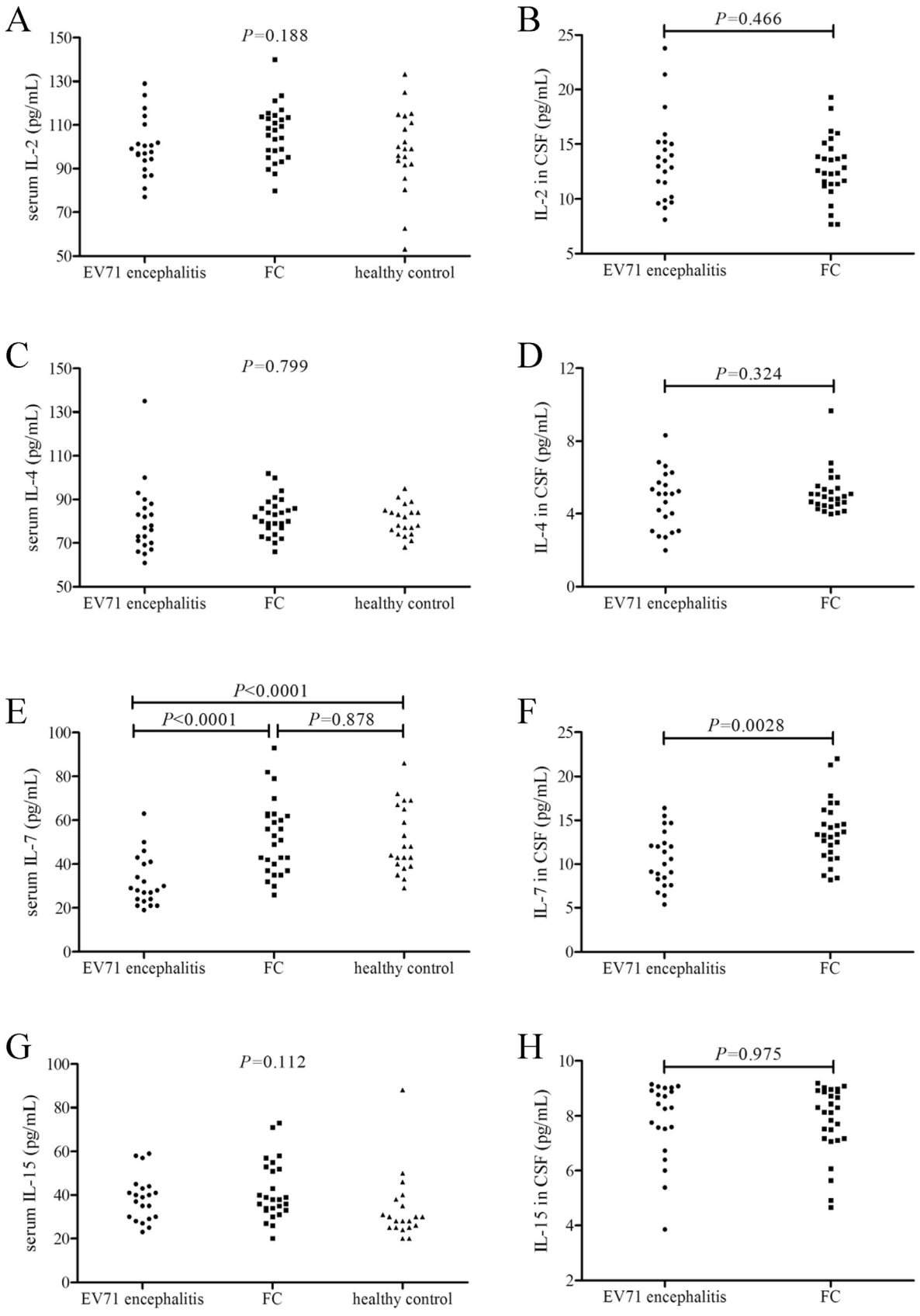

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Fig. 1. γc cytokines expression in the serum and CSF of EV71 associated encephalitis (n = 21), febrile convulsion (FC, n = 27), and healthy individuals (n = 20). γc cytokine (IL-2, IL-4, IL-7, and IL-15) levels were measured by ELISA. (A) Serum IL-2 was comparable among EV71 associated encephalitis, FC, and healthy individuals. (B) IL-2 expression in CSF was comparable among EV71 associated encephalitis and FC. (C) Serum IL-4 was comparable among EV71 associated encephalitis, FC, and healthy individuals. (D) IL-4 expression in CSF was comparable among EV71 associated encephalitis and FC. (E) Serum IL-7 was significantly reduced in EV71 associated encephalitis in comparison with FC and healthy individuals. (F) IL-7 expression in CSF was also down-regulated in EV71 associated encephalitis than in FC. (G) Serum IL-15 was comparable among EV71 associated encephalitis, FC, and healthy individuals. (H) IL-15 expression in CSF was comparable among EV71 associated encephalitis and FC. Individual level of each subject was shown. SNK-*q* test or Student's *t*-test was used for comparison.

3.3. IL-7 promoted proliferation and perforin/granzyme B expression, while reduced PD-1 expression in CD8$^+$ T cells from EV71 associated encephalitis patients

We selected peripheral CD8$^+$ T cells from thirteen patients with EV71 associated encephalitis. CD8$^+$ T cells were also purified from CSF of seven patients with EV71 associated encephalitis, whose WBC counts in CSF were higher than 100×10^6/L. 5×10^4 of CD8$^+$ T cells were stimulated with recombinant human IL-7 for 12 h. Recombinant human IL-7 stimulation induced the phosphorylation of STAT5 (Fig. 3A). CCK-8 results showed that in vitro IL-7 stimulation promoted proliferation of CD8$^+$ T cells from both peripheral blood ($[0.81 \pm 0.13] \times 10^5$ vs. $[0.81 \pm 0.13] \times 10^5$; paired *t*-test, $P = 0.0009$, Fig. 3B) and CSF ($[0.87 \pm 0.10] \times 10^5$ vs. $[0.79 \pm 0.11] \times 10^5$; paired *t*-test, $P = 0.033$, Fig. 3B). mRNA relative levels corresponding to perforin and granzyme B were semi-quantified by real-time PCR. IL-7 stimulation significantly elevated the perforin and granzyme B mRNA level in CD8$^+$ T cells from both peripheral blood and CSF (paired *t*-tests, all $P < 0.01$, Fig. 3C and D). 2×10^4 of CD8$^+$ T cells from peripheral blood of nine EV71 associated encephalitis, and from CSF of five EV71 associated encephalitis were stimulated with recombinant human IL-7. The secretion of perforin and granzyme B by CD8$^+$ T cells was analyzed by ELISPOT. The spot-forming cells (SFC)/10^5 CD8$^+$ T cells of both perforin and granzyme B were elevated in response to IL-7 stimulation (paired *t*-test, all $P < 0.05$, Fig. 3E and F).

Furthermore, mRNA relative levels corresponding to PD-1 and CTLA-4 were semi-quantified by real-time PCR. IL-7 stimulation down-regulated PD-1 mRNA relative level in CD8$^+$ T cells from both peripheral blood and CSF (paired *t*-tests, all $P < 0.0001$, Fig. 4A). However, CTLA-4 mRNA relative level was not significantly affected in response to IL-7 stimulation (paired *t*-tests, all $P > 0.05$, Fig. 4B). PD-1 and CTLA-4 positive cells in peripheral CD8$^+$ T cells were also analyzed by flow cytometry. The representative plots prior to and post IL-7 stimulation were shown in Fig. 4C and D, respectively. Similar with the trend of mRNA changes, percentage of PD-1$^+$ cells was reduced in response to IL-7 stimulation ($3.13 \pm 0.69\%$ vs. $3.51 \pm 0.74\%$; paired *t*-test, $P = 0.0078$, Fig. 4C), while CTLA-4$^+$ cells percentage was comparable prior to and post IL-7 stimulation ($1.92 \pm 0.21\%$ vs. $1.90 \pm 0.11\%$; paired *t*-test, $P = 0.712$, Fig. 4D).

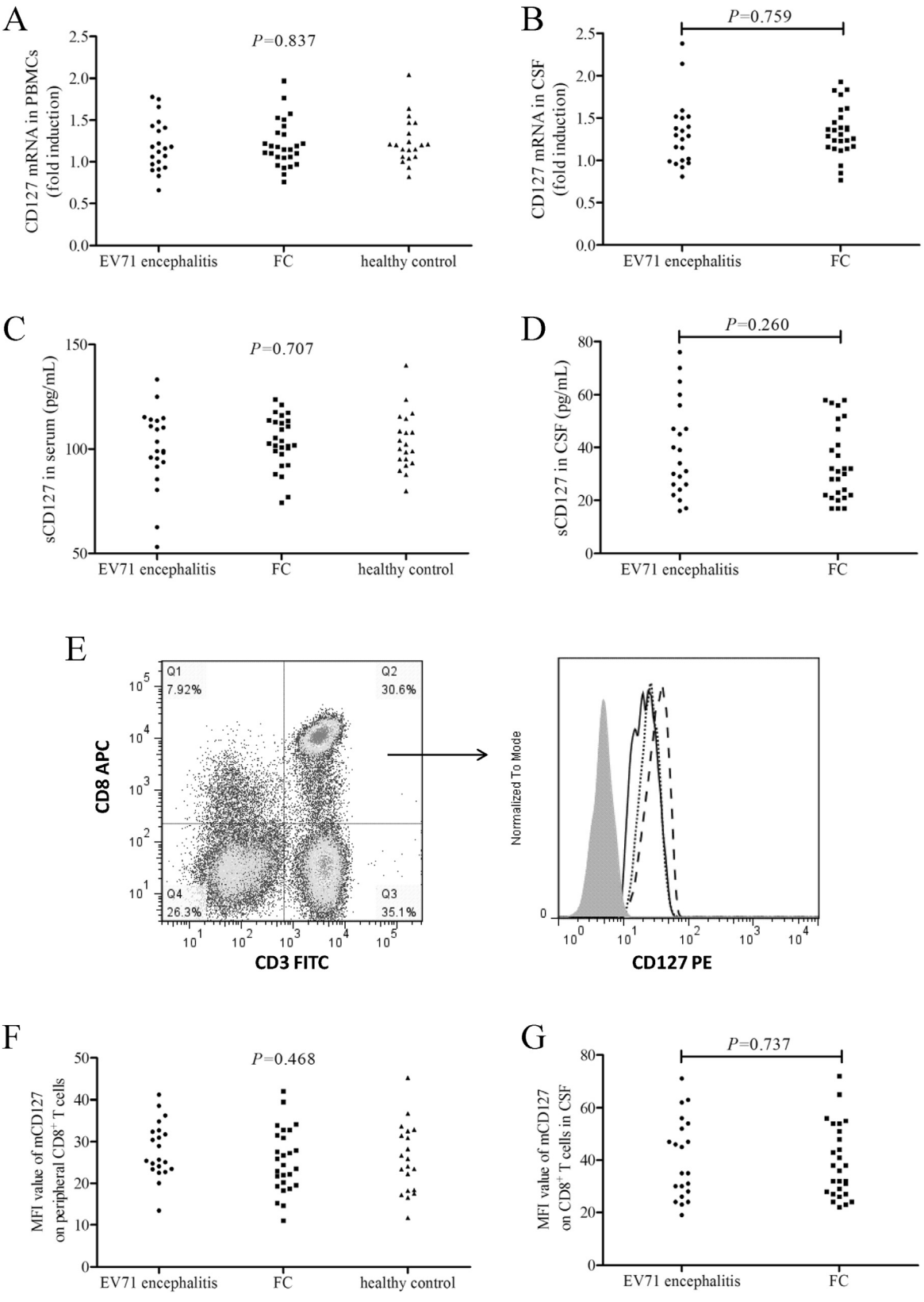
3.4. IL-7 elevated cytolytic and non-cytolytic activity of CD8$^+$ T cells from EV71 associated encephalitis patients

We then selected peripheral CD8$^+$ T cells from ten HLA-A2 restricted patients with EV71 associated encephalitis c while CD8$^+$ T cells were only selected from six HLA-A2 restricted patients due to limited cell numbers. CD8$^+$ T cells were cultured with or without IL-7 for 12 h. Cells were washed twice, and 10^5 of stimulated CD8$^+$ T cells were co-cultured in direct or indirect contact with 5×10^5 of U-87MG cells for another 48 h. Supernatants were harvested for further experiments. CD8$^+$ T cells from healthy controls induced higher target cell death in both direct and indirect contact co-culture system in comparison with CD8+ T cells from EV71 associated encephalitis (paired *t*-tests, all $P < 0.0001$, Fig. 5A). IFN-γ, but not TNF-α, was increasing secreted by CD8$^+$ T cells from healthy controls (Student *t*-test, $P < 0.05$, Fig. 5B). In direct contact co-culture system, IL-7 stimulation increased target U-87MG cell death when co-cultured with CD8$^+$ T cells from both peripheral blood ($28.40 \pm 6.85\%$ vs. $12.87 \pm 2.71\%$; paired *t*-test, $P < 0.0001$, Fig. 5A) and CSF ($29.50 \pm 4.76\%$ vs. $11.92 \pm 4.20\%$; paired *t*-test, $P = 0.0021$, Fig. 5A). IFN-γ production was also elevated in direct contact co-culture system of U-87MG cells with CD8$^+$ T cells in response to IL-7 stimulation (peripheral: 79.70 ± 8.59 pg/mL vs. 42.50 ± 8.28 pg/mL, CSF: 82.83 ± 8.80 pg/mL vs. 48.83 ± 10.57 pg/mL; paired *t*-tests, $P < 0.0001$ and $P = 0.0030$, Fig. 5B). In indirect contact co-culture system, target U-87MG cell deaths were also elevated when co-cultured with IL-7 induced CD8$^+$ T cells (peripheral: $4.92 \pm 1.02\%$ vs. 3.65 ± 0.57 pg/mL, CSF: 5.13 ± 0.67 pg/mL vs. 4.32 ± 0.51 pg/mL; paired *t*-tests, $P = 0.0030$ and $P = 0.019$, Fig. 5A). IL-7 stimulation also induced higher IFN-γ secretion by CD8$^+$ T cells in indirect contact co-culture system (peripheral: 16.43 ± 4.01 pg/mL vs. 11.84 ± 2.86 pg/mL, CSF: 15.38 ± 3.16 pg/mL vs. 11.03 ± 1.15 pg/mL; paired *t*-tests, $P = 0.020$ and $P = 0.0088$, Fig. 5B). However, TNF-α production by CD8$^+$ T cells did not reveal significant changes in response to IL-7 when cultured with U-87MG cells in either direct or indirect contact co-culture system (paired *t*-tests, all $P > 0.05$, Fig. 5C).

4. Discussion

In the current study, we screened γc cytokines expression in patients with EV71 associated encephalitis. Only IL-7, but not other γc cytokines (IL-2, IL-4, or IL-15) was reduced in either in peripheral blood and CSF was observed in patients with EV71 associated encephalitis, which was consistent with the previous reports that IL-2 and IL-4 expression in CSF was comparable in EV71-associated meningoencephalitis [26]. Moreover, membrane and soluble CD127 was comparable between EV71 associated encephalitis and controls. Recombinant human IL-7 in vitro promoted both cytolytic and non-cytolytic activities of peripheral and CSF-derived CD8$^+$ T cells from EV71 associated encephalitis patients, which presented as elevated cytotoxicity and enhanced IFN-γ secretion. This process was accompanied by augmenting CD8$^+$ T cell proliferation, increasing perforin/granzyme B, and decreasing PD-1 expression. The present results suggested a critical immunoregulatory property of IL-7 to CD8$^+$ T cells and a potential therapeutic target of IL-7 in EV71 associated encephalitis.

IL-7 is well accepted to maintain T cells homeostasis. Intermittent IL-7 signaling, which were interrupted by T cell antigen receptor, promoted CD8$^+$ T cell survival and preserving CD8$^+$ T cell homeostasis. In contrast, continuous IL-7 signaling induced proliferation and IFN-γ of naïve CD8$^+$ T cells, leading to IFN-γ-triggered cell death [27]. Chronic viral infection and cancer induced down-regulation of IL-7 in both peripheral blood and tissue-residency. Decreased IL-7 could not maintain the cytotoxicity of CD8$^+$ T cells [19,28], which partly contributed to immunotolerance and resulted in viral persistence and tumor metastasis. Thus, recombinant human IL-7 provided a perspective on the opportunities for clinical application and has been used in clinical trials for some kinds of chronic diseases [29]. More importantly, Plumb et al. revealed that IL-7 signaling was necessary for generation strong widespread and viral specific CD4$^+$ and CD8$^+$ T cell response in acute influenza A infection [30]. In vivo IL-7 administration also effectively protected CD4$^+$ T-cell pool during acute phase of simian immunodeficiency virus infection in macaques [31]. Although several previous studies screened cytokine and chemokine profile in serum and CSF in EV71-induced diseases [9,32], there was no evidence on IL-7



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expression in EV71 associated HFMD and encephalitis. To the best of our knowledge, we firstly reported that IL-7 level was robustly down-regulated in both serum and CSF from EV71 associated encephalitis, which was consistent with the findings in chronic hepatitis C virus infection [19]. This indicated that no matter in acute or chronic infection status, virus itself might be a critical factor for suppressing IL-7 production. Furthermore, controversy remained as to IL-7 receptor α chain CD127 expression in diseases. McLaughlin et al. showed that

Fig. 2. CD127 level in the serum and CSF of EV71 associated encephalitis (n = 21), febrile convulsion (n = 27), and healthy individuals (n = 20). CD127 mRNA relative level was semi-quantified by real-time PCR, while sCD127 expression was measured by ELISA. (A) CD127 mRNA in PBMCs was comparable among EV71 associated encephalitis, FC, and healthy individuals. (B) There was also no remarkable difference of CD127 mRNA in CSF between EV71 associated encephalitis and FC. (C) Serum sCD127 level was comparable among EV71 associated encephalitis, FC, and healthy individuals. (D) There was also no remarkable difference of sCD127 expression in CSF between EV71 associated encephalitis and FC. (E) The representative flow dots for $CD3^+CD8^+$ analysis and histogram for CD127 were shown. The dark grey shadow presented isotype control, the black line the EV71 associated encephalitis, the dot line the FC, and the dash line the healthy individuals. (F) MFI corresponding to mCD127 on peripheral $CD8^+$ T cells was comparable among EV71 associated encephalitis, FC, and healthy controls. (G) There was no remarkable difference of mCD127 expression on $CD8^+$ T cells from CSF between EV71 associated encephalitis and FC. Individual level of each subject was shown. One way ANOVA or Student's *t*-test was used for comparison.

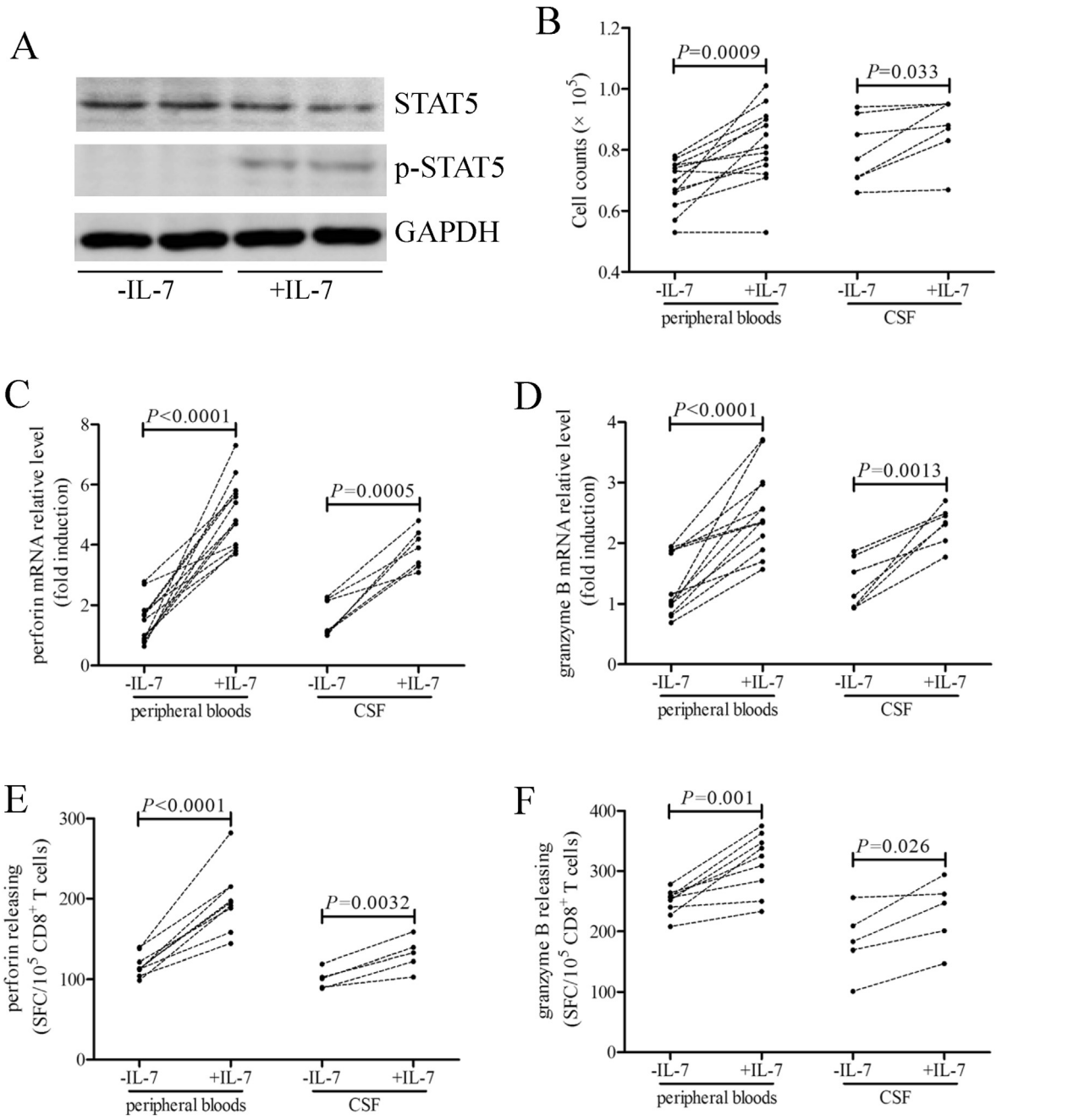

Fig. 3. IL-7 promoted proliferation and perforin/granzyme B expression in $CD8^+$ T cells in patients with EV71 associated encephalitis. $CD8^+$ T cells were purified from peripheral blood of thirteen EV71 associated encephalitis patients, as well as from CSF of seven EV71 associated encephalitis patients. Purified $CD8^+$ T cells were stimulated with recombinant human IL-7 for 12 h. (A) Total STAT5 and phosphorylated STAT5 was measured by Western blot. IL-7 stimulation induced STAT5 phosphorylation in $CD8^+$ T cells. (B) Cellular proliferation was assessed by CCK-8 methods. IL-7 stimulation promoted $CD8^+$ T cells proliferation. mRNA relative levels corresponding to (C) perforin and (D) granzyme B were semi-quantified by real-time PCR. IL-7 stimulation significantly elevated (C) perforin and (D) granzyme B mRNA level in response to IL-7 stimulation. The secretion of (E) perforin and (F) granzyme B by $CD8^+$ T cells, which were purified from peripheral blood of nine EV71 associated encephalitis and CSF of five EV71 associated encephalitis, was analyzed by ELISPOT. The spot-forming cells (SFC)/10^5 $CD8^+$ T cells of both (E) perforin and (F) granzyme B were elevated in response to IL-7 stimulation. Individual level of each subject was shown. Paired *t*-test was used for comparison.

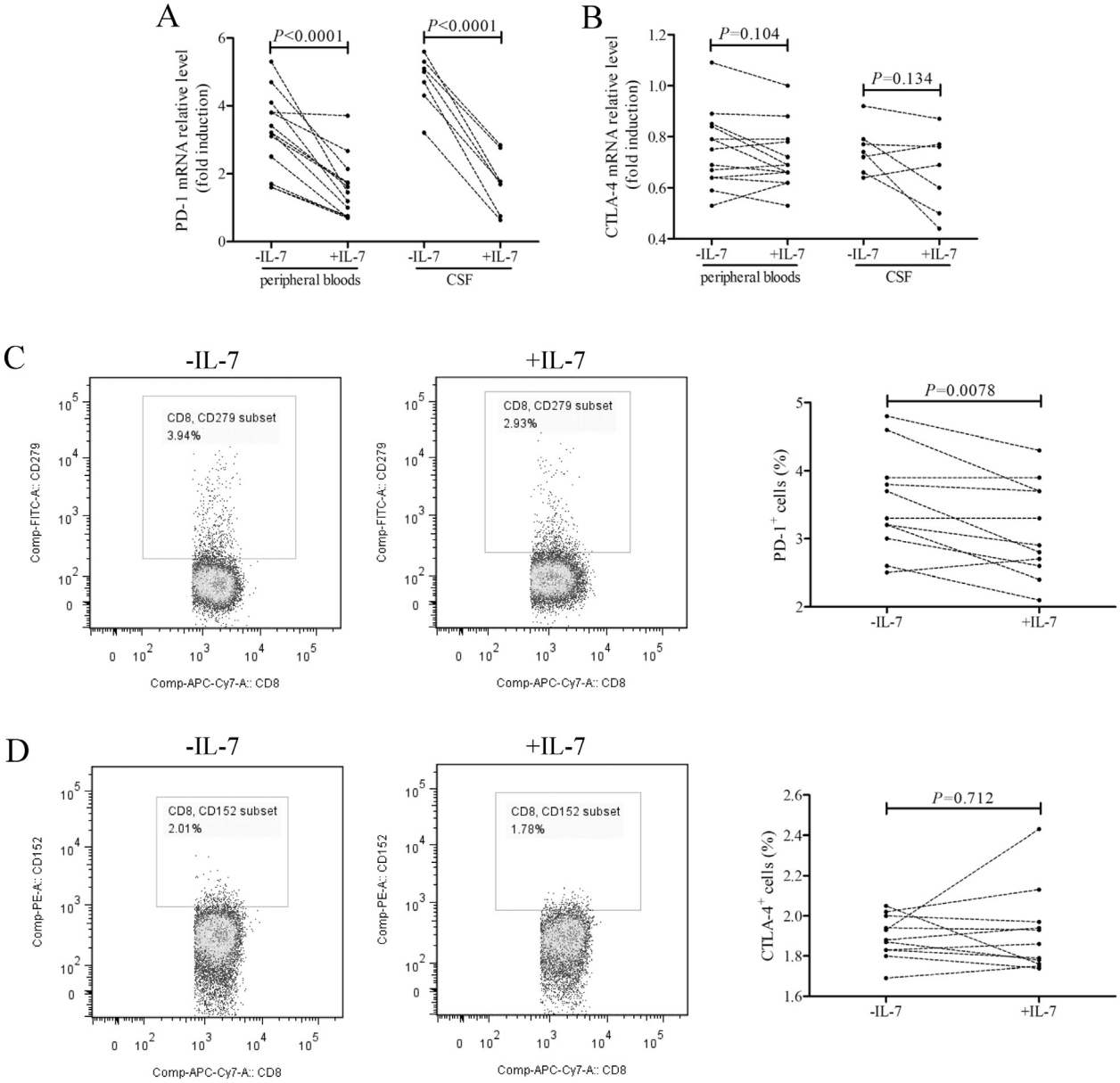

Fig. 4. IL-7 suppressed co-inhibitory molecules expression in CD8$^+$ T cells in patients with EV71 associated encephalitis. CD8$^+$ T cells were purified from peripheral blood of thirteen EV71 associated encephalitis patients, as well as from CSF of seven EV71 associated encephalitis patients. mRNA relative levels corresponding to (A) PD-1 and (B) CTLA-4 were semi-quantified by real-time PCR. IL-7 stimulation down-regulated (A) PD-1 mRNA relative level in CD8$^+$ T cells. (B) CTLA-4 mRNA relative level was not significantly affected in response to IL-7 stimulation. (C) PD-1 and (D) CTLA-4 positive cells in peripheral CD8$^+$ T cells were also analyzed by flow cytometry. The representative plots prior to and post IL-7 stimulation were shown. (C) Percentage of PD-1$^+$ cells was reduced in response to IL-7 stimulation. (D) CTLA-4$^+$ cells percentage was comparable prior to and post IL-7 stimulation. Individual level of each subject was shown. Paired *t*-test was used for comparison.

reduced levels of CD127 on both CD4$^+$ and CD8$^+$ T cells in viremic HIV-positive patients, and this process might be specifically regulated by HIV Tat protein [33]. However, there was no significant difference of CD127 expression on both peripheral and liver-resident CD8$^+$ T cells between healthy individuals and viral hepatitis-related hepatocellular carcinoma [19,28]. We also found that soluble CD127 in peripheral blood and CSF as well as membrane CD127 on peripheral and CSF-derived CD8$^+$ T cells was comparable between normal controls and EV71 associated encephalitis, suggesting that EV71 infection might not affect IL-7 receptor. Thus, the mechanism underlying IL-7 signaling in the pathogenesis of EV71 related diseases still needs further elucidation.

The binding of IL-7 to IL-7 receptor activates JNK-STAT signaling pathway, leading to the biological effects exertion of IL-7 signaling. In vitro recombinant human IL-7 stimulation to CD8$^+$ T cells from EV71 associated encephalitis induced STAT5 phosphorylation, indicating the activation of IL-7 signaling pathway. EV71 infection could result in cytopathic effects to infected cells. Thus, the cytolytic activity of CD8$^+$ T cells is important for viral clearance by destroying EV71-infected cells. Moreover, antiviral immunity to EV71 also involves cytokines (including IFN-γ and TNF-α) [32]. The cytokine-mediated non-cytolytic function could directly inactivate viral replication without killing infected cells [34,35]. Phillips et al. set up in vitro co-culture models, which allowed to assess the cytolytic and non-cytolytic activities of CD8$^+$ T cells independently and to dissect these two different functions to target cells [25]. The direct and indirect contact co-culture system has been widely used in for CD8$^+$ T cell function in viral infections [19,36] and cancers [28,37–39]. In this study, using human effector CD8$^+$ T cells from EV71 associated encephalitis and target human glioblastoma cells, we demonstrated that peripheral and CSF-derived

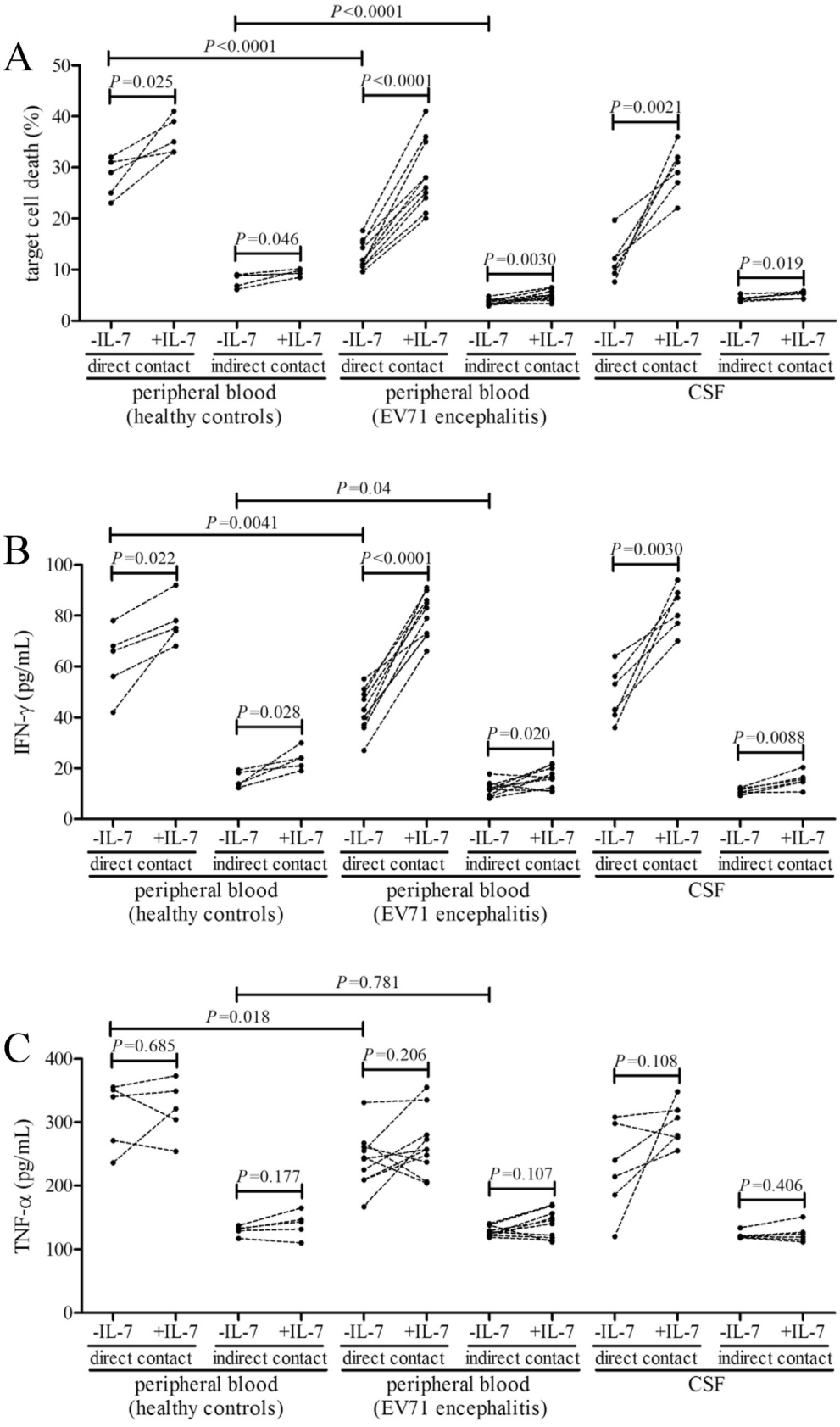

Fig. 5. IL-7 induced cytolytic and non-cytolytic activity of $CD8^+$ T cells from patients with EV71 associated encephalitis. $CD8^+$ T cells were purified from peripheral blood of ten HLA-A2 restricted EV71 associated encephalitis patients and five healthy controls, as well as from CSF of six HLA-A2 restricted EV71 associated encephalitis patients. $CD8^+$ T cells were cultured with or without IL-7 for 12 h. Cells were washed twice, and 10^5 of stimulated $CD8^+$ T cells were co-cultured in direct or indirect contact with 5×10^5 of U-87MG cells for another 48 h. (A) Target U-87MG cell death was calculated by measuring LDH expression in the supernatants. Increased target cell deaths were found in both direct and indirect co-culture system when $CD8^+$ T cells were stimulated with IL-7. (B) IFN-γ and (C) TNF-α production in the supernatants was measured by ELISA. (B) IFN-γ expression was elevated in both direct and indirect co-culture system when $CD8^+$ T cells were stimulated with IL-7. (C) TNF-α secretion by $CD8^+$ T cells did not reveal significant changes in response to IL-7 in either direct or indirect contact co-culture system. Individual level of each subject was shown. Paired *t*-test was used for comparison.

$CD8^+$ T cells could induce target cell death in both direct and indirect contact co-culture systems. IL-7-stimulated $CD8^+$ T cells revealed elevated cytotoxicity, which presented as increased target cell death. In indirect contact co-culture system, target cell death was also increased when co-cultured with IL-7-treated $CD8^+$ T cells, suggesting non-cytolytic function were also involved in induction of target cell death. IFN-γ, but not TNF-α, was more responsible for the non-cytolytic activity of $CD8^+$ T cells in EV71 associated encephalitis.

The potential mechanisms of IL-7-mediated elevation of $CD8^+$ T cells activity were also investigated. Previous study in chronic viral infection suggested that multiple mechanisms contributed to IL-7-mediated antiviral effects [20]. IL-7 mediated selective expansion of tumor-redirected cytotoxic T cell response without enhanced activity of regulatory T cells [40]. We also found that IL-7 promoted the proliferation of purified $CD8^+$ T cells from EV71 associated encephalitis patients in vitro. $CD8^+$ T cells exerted cytotoxic activity mainly through perforin-granzyme molecules pathway [41]. Perforin and granzyme was pore-forming molecules, and presented granule exocytosis [39]. Li et al. found that inhibition of Notch signaling promoted $CD8^+$ T cells activity by up-regulation of perforin in lung adenocarcinoma [39]. We found that IL-7 stimulation also enhanced perforin and granzyme B mRNA relative levels in $CD8^+$ T cells from EV71 associated encephalitis. Moreover, co-inhibitory molecules, especially PD-1 and CTL-4, could induce $CD8^+$ T cell exhaustion. IL-7 augmented polyfunctional viral specific CD8+ T cells responses without altering PD-1 expression in chronic lymphocytic choriomeningitis mouse model [42]. In contrast, IL-7 maintained liver-resident $CD8^+$ T cells response via regressing PD-1 expression in acute viral hepatitis [18] and hepatocellular carcinoma [28]. Herein, we revealed that IL-7 stimulation down-regulated PD-1, but not CTLA-4, expression in $CD8^+$ T cells from EV71 associated encephalitis, indicating IL-7 suppressed PD-1 signaling pathway in EV71 associated encephalitis. Further in vivo experiments are needed for confirming the role of IL-7 in CD8+ T cells regulation in EV71 related diseases.

In conclusion, decreased IL-7 in peripheral blood and CSF might be insufficient for maintaining $CD8^+$ T cells function, leading to exacerbation of EV71 associated encephalitis patients. Recombinant human IL-7 induced cytolytic and non-cytolytic functions of $CD8^+$ T cells in EV71 associated encephalitis. Multiple mechanisms, including promotion of cellular proliferation, enhancement of perforin/granzyme B, and inhibition of PD-1, were involved in IL-7-mediated $CD8^+$ T cells activation. IL-7 might be considered as one of the immunomodulatory therapeutic candidates for EV71 infection.

Declaration of Competing Interest

The authors declare that there are no competing interests associated with the manuscript.

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