Immune Netw. 2024 Apr;24(2):e18
https://doi.org/10.4110/in.2024.24.e18
pISSN 1598-2629·eISSN 2092-6685

IMMUNE NETWORK

Brief Communication

Cytokine Storm Related to CD4⁺ T Cells in Influenza Virus-Associated Acute Necrotizing Encephalopathy

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Received: Sep 19, 2023
Revised: Apr 16, 2024
Accepted: Apr 18, 2024
Published online: Apr 29, 2024

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ABSTRACT

Acute necrotizing encephalopathy (ANE) is a rare but deadly complication with an unclear pathogenesis. We aimed to elucidate the immune characteristics of H1N1 influenza virus-associated ANE (IANE) and provide a potential therapeutic approach for IANE. Seven pediatric cases from a concentrated outbreak of H1N1 influenza were included in this study. The patients' CD4⁺ T cells from peripheral blood decreased sharply in number but highly expressed Eomesodermin (Eomes), CD69 and PD-1, companied with extremely high levels of IL-6, IL-8 in the cerebrospinal fluid and plasma. Patient 2, who showed high fever and seizures and was admitted to the hospital very early in the disease course, received intravenous tocilizumab and subsequently showed a reduction in temperature and a stable conscious state 24 h later. In conclusion, a proinflammatory cytokine storm associated with activated CD4⁺ T cells may cause severe brain pathology in IANE. Tocilizumab may be helpful in treating IANE.

Keywords: Influenza; Encephalopathy; Cytokine storm

INTRODUCTION

As the coronavirus disease 2019 (COVID-19) pandemic raised since Jan 2020, the levels of immunity against influenza in the population has waned, probably due to mask-wearing for prophylaxis against COVID-19. Influenza is an acute and highly infectious respiratory disease. The World Health Organization has estimated that an influenza pandemic could result in ≤4 million cases of severe infection and about 500,000 deaths annually (1). The prevalence of influenza is high in winter and spring, and children are more vulnerable to infection by influenza viruses than adults. The response to influenza A virus subtype H1N1 (influenza A (H1N1) virus) can be categorized into mild influenza and severe inflammatory

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
ANE, acute necrotizing encephalopathy; CNS, central nervous system; COVID-19, coronavirus disease 2019; CSF, cerebrospinal fluid; CT, computed tomography; Eomes, Eomesdermin; IANE, influenza virus-associated acute necrotizing encephalopathy; influenza A (H1N1) virus, influenza A virus subtype H1N1; MFI, mean fluorescence intensity; MRI, magnetic resonance imaging; RI, respiratory infection; USTC, University of Science and Technology of China.

Author Contributions
Data curation: Wang S, Chen M, Wang Y, Zhou H; Formal analysis: Wang S, Wang D, Wang X; Investigation: Chen M, Wang Y, Zhou H; Supervision: Lv Y, Wei H; Writing - original draft: Wang S, Wang D, Wang X, Zhou Y, Wei H; Writing - review & editing: Zhou Y, Lv Y, Wei H.

response syndrome (2). Proinflammatory cytokines and chemokines produced by Th cells have been implicated in the development of severe inflammatory response syndrome and in determining the outcome as well as the appearance of severe complications, of which acute necrotizing encephalopathy (ANE) is one of the deadliest.

ANE was first described by Mizuguchi et al. (3) in 1995 on the basis of its clinicopathological characteristics of rapid-onset seizures and severe neurological complications, which lead to a high prevalence of disability and death. Children constitute a vulnerable group for ANE, with a high mortality rate of nearly 30% (4). The main pathogens causing ANE are viruses, especially influenza A (H1N1) virus. However, the complex immune mechanism and pathogenesis orchestrating death from H1N1 influenza virus-associated ANE (IANE) are not known.

Herein, we reported the clinical characteristics and immune features determined using peripheral blood and cerebrospinal fluid (CSF) samples from seven confirmed pediatric cases of IANE, aiming to elucidate the immune mechanism of IANE and to provide a new therapeutic option.

MATERIALS AND METHODS

Collection of blood and CSF samples

This study is a prospective study utilizing residual samples. We collected data from pediatric department of First Affiliated Hospital of University of Science and Technology of China (USTC) between February 26 and December 28, 2023. All patients in this study were shown to be positive for influenza A (H1N1) virus infection by detection of nucleic acids in throat swabs.

The patients underwent blood routine test, liver and kidney function assessments and coagulation function test on the arrival at hospital. Residual blood samples were used to appraise peripheral immune cell subsets by flow cytometry and plasma samples were used for detection of inflammatory cytokines.

The diagnostic criteria for ANE as originally established by Mizuguchi in 1995 remain unamended to date (5). We used the definition of ANE in this study as defined by Mizuguchi et al. as follows: acute noninflammatory encephalopathy with an alteration in the level of consciousness; the demonstration by head computed tomography (CT) or magnetic resonance imaging (MRI) of bilateral symmetric thalamic lesions, other regions such as periventricular white matter, inner capsule, putamen, upper brainstem tegmentum, and cerebellar medulla can be involved; serum transaminase elevation but no hyperammonemia; CSF leucocyte count 8/mm^3 or less and the absence of any other reasonable explanation for the cerebral abnormalities (3,6). In this study, the IANE group consisted of pediatric patients who were diagnosed as showing ANE caused by virus H1N1 hospitalized in the pediatric intensive care unit. Pediatric patients who presented fever and cough caused by the influenza A (H1N1) virus but did not exhibit symptoms of severe H1N1 influenza were referred as respiratory infection (RI) group in the same hospital during this period. We selected the RI group as controls to elucidate the differential immunological profiles in peripheral blood between uncomplicated H1N1 RI and central nervous system (CNS) complications attributed to H1N1.

Concurrently, during the same period, there were children presenting with fever and seizures post-H1N1 infection who did not progress to IANE (non-IANE group). Given the dangerous

progression of IANE, early detection, prompt diagnosis, and immediate treatment are crucial for improving the prognosis of affected children. Therefore, for pediatric patients with influenza A (H1N1) virus infections showing complex febrile convulsions and a poor mental state (non-IANE group), we conducted lumbar puncture to rule out other CNS infection after obtaining informed consent from their parents. The residual CSF samples from patients in IANE group and in non-IANE group were used for detection of inflammatory cytokines. The criteria for severe influenza in this study were based on the Expert Consensus on Childhood Influenza Diagnosis and Treatment of China (2020 edition) as there is no generally accepted definition for moderate-to-severe influenza to date (7). Notably, to rule out autoimmune encephalitis, residual plasma samples from the seven patients with IANE were used to test for Abs associated with autoimmune encephalitis.

This study was approved by the Institutional Review Board of the First Affiliated Hospital of USTC (2023-RE-118). Informed consent to participate in the study was obtained from the parents of the children. The research timeline was granted from February 2023 until the completion of the study.

Flow cytometry analysis

Blood samples were centrifuged (400×g, 10 min, 4°C) to obtain plasma. Cell pellets underwent lysis of RBCs using RBC Lysis Buffer (catalog number, 420302; Biolegend, San Diego, CA, USA). Suspensions of lymphocytes were stained with the human monoclonal Abs shown in **Supplementary Table 1**. The cells were then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set in accordance with the manufacturer's (eBioscience, San Diego, CA, USA) instructions. Intracellular staining was performed without re-stimulation. Homologous IgG molecules were used as negative-control Abs. Fluorescence-activated cell staining was performed using a flow cytometer (NovoCyte™ 3130) following the manufacturer's (ACEA Biosciences, San Diego, CA, USA) instructions.

Assessment of proinflammatory cytokines

The levels of proinflammatory cytokines in the plasma and CSF were measured using a cytometric bead array kit (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

For comparisons among the IANE, RI, and non-IANE groups, continuous variables showing a normal distribution (according to the Shapiro-Wilk test) were presented as mean ± SD and analyzed using conventional one-way ANOVA while those that did not show a normal distribution were presented as median (range) and analyzed using the Kruskal–Wallis test. Categorical variables were presented as number (%) and tested using the chi-square test or Fisher's exact test. For comparisons between two groups, the Student's *t*-test was utilized as data adhered to normal distribution. Mann-Whitney test was employed to analyze data that were not normally distributed. Statistical significance was indicated by two-tailed p-values < 0.05. Statistical analysis were performed using GraphPad prism v.9.3 software.

Results and Discussion

In early 2023, after three years of COVID-19 epidemic prevention and control, the first wave of an influenza outbreak occurred throughout China. During this period, we encountered seven confirmed pediatric cases of ANE caused by influenza A (H1N1) virus infection between February 26 and March 10 in the First Affiliated Hospital of USTC. Such a concentrated outbreak of ANE over a two-week period has not been reported before. Six of these

pediatric IANE cases (patient numbers 1 and 3–7) showed high fever and seizures with rapid progression to deep coma within several hours; intracranial CT scan showed low density in the thalamus and severe damage to the lateral ventricles (**Fig. 1A and B**, red arrow). The average interval from fever to coma was approximately 30 h; thus, brain dysfunction rapidly appeared in a short period of time, indicative of poor prognosis. Patients with IANE showed

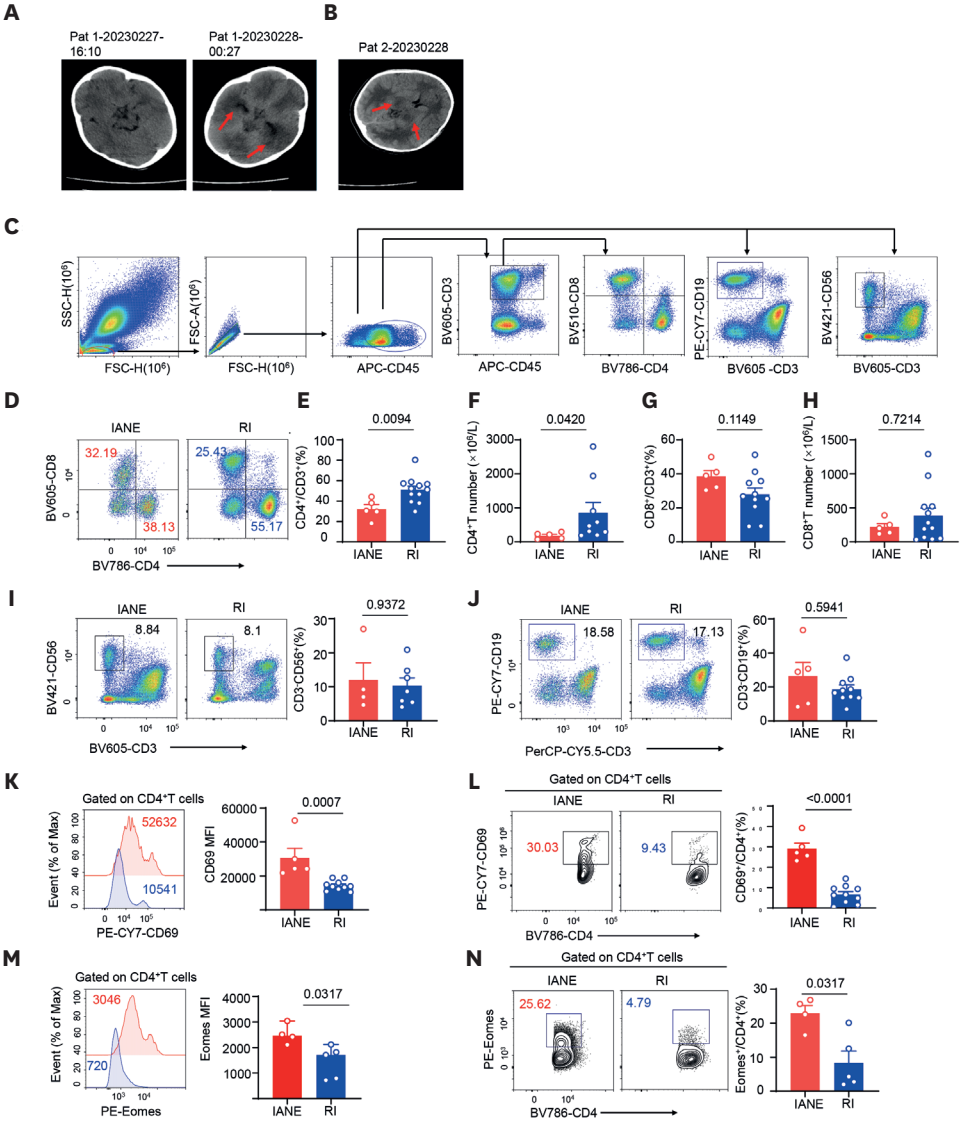

Figure 1. Head CT scan images and immune features in the peripheral blood of patients with IANE. (A, B) Intracranial CT scans of pediatric patients with IANE (the red arrow indicates that the changes in the brain parenchyma are mainly located in the thalamus). (C) Gating strategy for flow cytometry analysis of peripheral blood samples. (D-H) Flow cytometry showing the proportions of $CD4^+$ and $CD8^+$ in $CD3^+$ T cells and their numbers from the peripheral blood of pediatric patients with IANE (n=5) and pediatric patients with RI caused by H1N1 virus (RI; n=9–12). (I, J) Flow cytometry showing the proportions of $CD3^-CD56^+$ NK cells (IANE, n=4; RI, n=7) and $CD3^-CD19^+$ B cells (IANE, n=5; RI, n=10) isolated from the peripheral blood of patients with IANE and those with RI caused by H1N1 virus. (K, L) Comparison of MFI and proportion of CD69 expression on gated $CD45^+CD3^+CD4^+$ T cells between IANE group (n=5) and RI group (n=10). (M, N) Comparison of MFI and proportion of Eomes expression in gated $CD45^+CD3^+CD4^+$ T cells between IANE group (n=4) and RI group (n=5). (O, P) Comparison of MFI and proportion of CD44 expression on gated $CD45^+CD3^+CD4^+$ T cells between IANE group (n=5) and RI group (n=10). (Q) Comparison of the proportion of PD-1 on $CD45^+CD3^+CD4^+$ T cells from the peripheral blood of patients with IANE (n=5) and patients with RI caused by H1N1 virus (n=10). (R, S) Comparison of MFI and proportion of CD69 expression on gated $CD45^+CD3^+CD8^+$ T cells between IANE group (n=4) and RI group (n=10). (T, U) Comparisons of MFI and proportion of CD44 expression on gated $CD45^+CD3^+CD8^+$ T cells between IANE group (n=4) and RI group (n=9). (V) Comparison of the proportion of PD-1 on $CD45^+CD3^+CD8^+$ T cells from the peripheral blood of patients with IANE (n=5) and patients in RI group (n=9). The p-values are from the Mann–Whitney test or unpaired *t*-test. The $p<0.05$ indicated significant differences.

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https://doi.org/10.4110/in.2024.24.e18

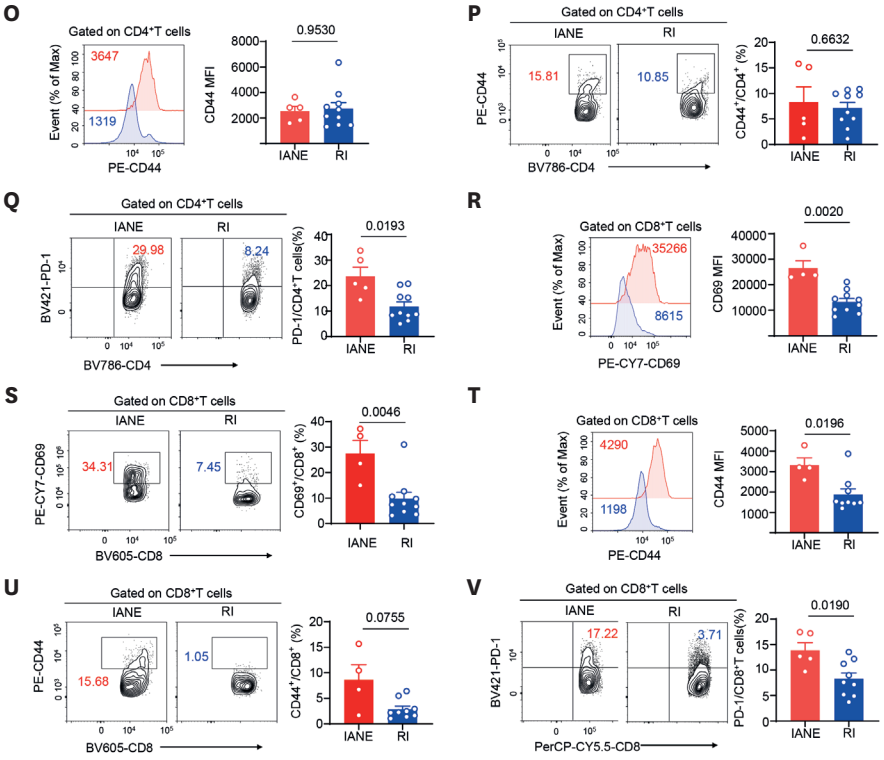

Figure 1. (Continued) Head CT scan images and immune features in the peripheral blood of patients with IANE. (A, B) Intracranial CT scans of pediatric patients with IANE (the red arrow indicates that the changes in the brain parenchyma are mainly located in the thalamus). (C) Gating strategy for flow cytometry analysis of peripheral blood samples. (D-H) Flow cytometry showing the proportions of $CD4^+$ and $CD8^+$ in $CD3^+$ T cells and their numbers from the peripheral blood of pediatric patients with IANE (n=5) and pediatric patients with RI caused by H1N1 virus (RI; n=9–12). (I, J) Flow cytometry showing the proportions of $CD3^-CD56^+$ NK cells (IANE, n=4; RI, n=7) and $CD3^-CD19^+$ B cells (IANE, n=5; RI, n=10) isolated from the peripheral blood of patients with IANE and those with RI caused by H1N1 virus. (K, L) Comparison of MFI and proportion of CD69 expression on gated $CD45^+CD3^+CD4^+$ T cells between IANE group (n=5) and RI group (n=10). (M, N) Comparison of MFI and proportion of Eomes expression in gated $CD45^+CD3^+CD4^+$ T cells between IANE group (n=4) and RI group (n=5). (O, P) Comparison of MFI and proportion of CD44 expression on gated $CD45^+CD3^+CD4^+$ T cells between IANE group (n=5) and RI group (n=10). (Q) Comparison of the proportion of PD-1 on $CD45^+CD3^+CD4^+$ T cells from the peripheral blood of patients with IANE (n=5) and patients with RI caused by H1N1 virus (n=10). (R, S) Comparison of MFI and proportion of CD69 expression on gated $CD45^+CD3^+CD8^+$ T cells between IANE group (n=4) and RI group (n=10). (T, U) Comparisons of MFI and proportion of CD44 expression on gated $CD45^+CD3^+CD8^+$ T cells between IANE group (n=4) and RI group (n=9). (V) Comparison of the proportion of PD-1 on $CD45^+CD3^+CD8^+$ T cells from the peripheral blood of patients with IANE (n=5) and patients in RI group (n=9). The p-values are from the Mann–Whitney test or unpaired *t*-test. The $p<0.05$ indicated significant differences.

decreased lymphocyte counts, severe coagulation disorders, and liver and kidney dysfunction (**Table 1**). The detailed characteristics and other laboratory findings of the IANE patients are listed in **Table 2**.

The inflammatory response in IANE has been suggested to be systemic, leading to multiple organ damage. We analyzed immune cells by flow cytometry to identify the proportion of reduced lymphocytes. We found that the proportion and number of $CD4^+$ T cells were significantly reduced while the proportion and number of $CD8^+$ T cells did not change significantly in the peripheral blood of pediatric patients with IANE (**Fig. 1C-H**). Moreover, the proportions of NK cells and B cells were nearly the same between the IANE group and RI group (**Fig. 1I and J**).

Although the number of $CD4^+$ T cells decreased significantly, these cells showed high expression levels of CD69 and Eomesodermin (Eomes) (**Fig. 1K-N**), indicating that these $CD4^+$ T cells were activated, while the mean fluorescence intensity and proportion of CD44 expression on gated $CD4^+$ T cells did not reach significant differences (**Fig. 1O and P**).

https://doi.org/10.4110/in.2024.24.e18

Table 1. Clinical characteristics and laboratory findings of patients in this study

Characteristics	IANE (n=7)	RI (n=20)	Non-IANE (n=6)	p-value*
Time from fever to seizure (h)	16.86±6.28	-	45.60±21.79	0.007
Time from fever to coma (h)	30.00 (19.50–36.25)	-	-	-
Time from fever to sample collection (h)	36.00 (24.00–40.00)	52.50 (31.25–75.00)†	23.00 (19.00–36.00)	0.022
Age, (mon)	88.00 (28.00–115.00)	48.50 (13.50–79.50)	68.50 (26.00–122.30)	0.508
Gender (male)	5 (71.40)	13 (65.00)	4 (66.67)	0.953
White blood cell count ($\times10^9$/L)	9.67 (7.41–14.61)	7.82 (5.03–10.46)	6.36 (4.75–7.65)	0.175
Neutrophil count ($\times10^9$/L)	5.85 (4.23–12.51)	3.41 (1.77–6.05)	3.00 (2.22–5.65)	0.184
Lymphocyte count ($\times10^9$/L)	0.70 (0.60–2.30)	2.75 (1.50–4.58)‡	1.65 (0.67–2.45)	0.033
Monocyte count ($\times10^9$/L)	0.14 (0.10–0.84)	0.56 (0.45–0.82)	0.71 (0.34–0.76)	0.260
Hemoglobin (g/L)	130.90±27.47	121.40±11.89	128.50±21.88	0.421
Platelet count ($\times10^9$/L)	170.40±88.63	304.80±101.10§	223.00±61.22	0.006
Activated partial thromboplastin time (s)	47.50±18.23	30.55±4.01§	31.60±7.78‡	0.002
Prothrombin time (s)	21.03±8.47	13.45±2.60§	12.45±2.11‡	0.003
D-dimer (mg/L)	33.50 (1.67–69.02)	0.32 (0.19–0.49)‖	0.40 (0.19–2.27)‡	0.001
Albumin (g/L)	42.19±2.50	42.44±3.05	41.68±5.22	0.113
Alanine aminotransferase (U/L)	50.00 (37.00–985.00)	19.50 (14.25–60.25)	21.50 (17.75–31.75)	0.096
Aspartate aminotransferase, U/L	105.60 (68.80–1,864.00)	37.25 (25.38–78.60)‡	43.50 (18.15–52.50)	0.027
Potassium (mmol/L)	4.12±0.97	4.37±0.52	4.39±0.51	0.651
Sodium (mmol/L)	136.50 (130.00–137.60)	137.50 (135.50–139.20)	134.00 (132.60–138.20)	0.143
BUN (mmol/L)	7.28±2.00	3.77±1.04¶	4.31±1.45§	<0.001
Creatinine (μmol/L)	61.27±34.99	28.97±8.96‖	38.47±13.23	0.013
CRP (mg/L)**	9.21 (5.00–28.80)	5.00 (5.00–8.55)	5.00 (5.00–14.39)	0.340
Procalcitonin (ng/mL)	13.37 (2.91–82.06)	NA	NA	
Antiviral medication	Intravenous infusion of Paramivir at the dosage of 10 mg/kg	Oseltamivir orally 2 mg/kg, divided into twice for 5 days	Oseltamivir orally 2 mg/kg, divided into twice for 5 days	-

Values are presented as median (interquartile range) or mean±SD or number (%).
NA, not available.
*p-values were from ordinary one-way ANOVA analysis or Kruskal-Wallis test. $p<0.05$ was considered statistically different; †Indicated $p<0.05$ when compared with non-IANE group; ‡Indicated $p<0.05$; §Indicated $p<0.01$; ‖Indicated $p<0.005$ and ¶Indicated $p<0.001$ when compared with IANE group; **Normal range of CRP is 0–5 mg/L.

Activated Eomes+ CD4+ T cells have been considered to be biomarkers and therapeutic targets for CNS inflammation (8,9); therefore, we speculated that these Eomes+ CD4+ T cells may play an inflammatory role in IANE progression.

To investigate whether the significant decrease in the number of CD4+ T cells was related to their functionality, we evaluated PD-1 expression. We found that the percentages of PD-1+ CD4+ T cells from the peripheral blood of pediatric patients with IANE were elevated in comparison with those in patients with RI (**Fig. 1Q**). It has been recognized that a subset of CD4+Foxp3- T cells with high PD-1 expression inhibits T cell functions in the context of tumor (10). Neonatal CD4+ T cells with PD-1 expression can perform activation-induced events by producing Th1 cytokines in acute bacterial infection (11). Our study showed that the decreased CD4+ T cells in the peripheral blood of pediatric patients with IANE were activated and exhibited high levels of PD-1 expression, indicating that this population of CD4+ T cells may possess the ability to secrete inflammatory cytokines in IANE, rather than being exhausted CD4+ T cells.

Similarly, during acute infection, high PD-1 expression is not a marker of T cell exhaustion (12) and PD-1-expressing severe acute respiratory syndrome coronavirus-2-specific CD8+ T cells are functional in patients with COVID-19 (13). In the present study, CD8+ T cells of patients with IANE were also activated due to high expression of CD69, CD44, and especially PD-1 when compared to patients with RI (**Fig. 1R-V**), indicating that activated CD8+ T cells may play a role in combating influenza A (H1N1) virus infection rather than being in an exhausted state.

https://doi.org/10.4110/in.2024.24.e18

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https://doi.org/10.4110/in.2024.24.e18

Table 2. The details of seven pediatric patients with IANE

Patients	Sex	Age	Clinical presentation	Time interval (fever to coma/ fever to sample collection)	Antiviral treatment for influenza	IVIG	Glucocorti-coid	Plasma exchange	Tocili-zumab	Ritux-imab	Outcome at discharge	CSF cell count (×10⁶/L)	CSF protein level (g/L)	IL-6 level in CSF (pg/ml)	IL-8 level in CSF (pg/ml)	IL-10 level in CSF (pg/ml)	IFN-α level in CSF (pg/ml)	IL-17A level in CSF (pg/ml)	IL-1β level in CSF (pg/ml)	IFN-γ level in CSF (pg/ml)	H1N1 nucleic acid testing (throat swabs)	Respiratory virus nucleic acid testing*	Virus detected in CSF†	Antibodies associated autoimmune encephalitis in plasma‡	Blood ammonia§ (μmol/L)
1	M	7 yr	High fever, frequent seizures	34 h/36 h	Peramivir 10 mg/kg, daily for consecu-tive 5 days	1 g/kg, daily, for 2 days	Methylpred-nisolone 20 mg/kg daily for 3 days	Yes	No	No	Survive, right limb muscle strength level 3, left muscle strength is normal	1	0.4	25.95	85.94	3.15	1.46	18.01	1.7	2.94	Positive	Negative	Negative	Negative	<8.7
2	F	2 yr	High fever, frequent seizures	-/20 h	Peramivir 10 mg/kg, daily for consecu-tive 3 days	1 g/kg, daily, for 2 days	Methylpred-nisolone 20 mg/kg daily for 3 days	Yes	8 mg/kg, once	No	Seizures alleviated, consciousness stable 24 h af-ter Tocilizumab administration and brought home by her parents	0	1.15	4,677.92	3,562.07	21.78	6.97	2.14	1.38	1.75	Positive	Negative	Negative	Negative	18.8
3	M	1 yr 9 mon	Higher fever, cough, frequent seizures	6 h/26 h	Peramivir 10 mg/kg, daily for consecu-tive 2 days	400 mg/kg, consecutive 2 days	Methylpred-nisolone 20 mg/kg, daily, for 2 days	Yes	No	No	Deep coma, pu-pil light reflex disappeared, transferred to local hospital	0	9.3	4,437.26	15,474.56	72.88	48.6	19.45	4.39	3.48	Positive	Negative	Negative	Negative	17.7
4	F	9 yr	Fever, frequent seizures, ventricular tachycardia	24 h/28 h	Peramivir 10 mg/kg, daily for consecu-tive 2 days	400 mg/kg, consecutive 2 days	Methylpred-nisolone 20 mg/kg, daily, for 2 days	Yes	No	No	Died after 2-day hospital-ization	0	4.5	291.76	7,055.79	9.36	4.12	6.15	1.87	1.89	Positive	Negative	Negative	Negative	<8.7
5	M	4 yr 10 mon	Fever, seizures, cardiac arrest	35 h/52 h	Peramivir 10 mg/kg, daily	2 g/kg, once	Methylpred-nisolone 20 mg/kg once	No	No	No	Died 11 h after hospital admission	-	-	-	-	-	-	-	-	-	Positive	Negative	-	Negative	31.9
6	M	9 yr	High fever and headache, disturbance of consciousness	26 h/38 h	Peramivir 10 mg/kg, daily	1 g/kg daily, consecutive 2 days	Methylpred-nisolone 20 mg/kg daily for 2 days	Yes	No	No	Died after 2-day hospital-ization	2	>18	11,949.47	12,667.18	167.62	930.66	18.73	61.56	6.19	Positive	Negative	Negative	Negative	9
7	M	8 yr	Chest tightness, chest pain, dyspnea, seizures	40 h/40 h	Peramivir 10 mg/kg, daily, for consecu-tive 5 days	1 g/kg, daily, for 2 days	Methylpred-nisolone 20 mg/kg daily for 3 days	No	No	No	Hospitalized for 31 days and died	12	2.04	4,930.95	15,474.56	36.34	3.59	20.93	3.9	3.88	Positive	Negative	Negative	Negative	13.7

M, male; F, female; IVIG, intravenous immunoglobulin.

*Respiratory virus nucleic acid testing including influenza B virus/adenovirus/respiratory syncytial virus/parainfluenza virus type I and type III/COVID-19, the term “negative” means all the above virus nuclear acid of throat swabs were tested negative. Patient No.5 underwent cardiopulmonary resuscitation upon admission and was unable to undergo a lumbar puncture for cerebrospinal fluid collection; †Virus detected in CSF was used next-generation sequencing of pathogenic microorganisms conducted in infectious disease department of our hospital. ‡Abs associated autoimmune encephalitis in plasma include those targeting AMPA1/2 glutamate receptors, MOG Ab IgG, CASPR2, GABA B receptor, LGI1, and NMDA glutamate receptors. The term “negative” means all these Abs were detected negative; §The normal range of blood ammonia was 9–33 μmol/L in the lab of our hospital.

To further explore the CNS inflammatory response, we analyzed the protein level and cellular composition in the CSF from pediatric patients with and without IANE. The CSF protein concentrations increased sharply in patients with IANE. The number of cells in the CSF from IANE patients were either slightly elevated or within normal ranges and cellular composition in CSF did not differ significantly between two groups (**Fig. 2A-F**), indicating that the neurological damage in IANE may stem from pathogenic necrotic alterations associated with systemically activated CD4⁺ T cells.

Next, we aimed to determine the key proinflammatory cytokines in patients with IANE. We measured the concentrations of the Th cell-related cytokines IL-6, IL-8, IL-10, IFN-α, IL-17A, IL-1β, and IFN-γ in the CSF and plasma. The levels of IL-6, IL-8, IL-10, and INF-α in the CSF of patients with IANE were markedly higher than those in the controls (**Fig. 2G**). These differences in cytokine levels were also observed in plasma, except for IFN-α (**Fig. 2H**). Then, we conducted additional analysis on whether the CD4⁺ T cells of peripheral blood expressed IL-6 and IL-8. We found that the IL-6 and IL-8 expressions from CD45⁺CD3⁺CD4⁺ T cells were significantly higher in pediatric patients with severe influenza than those in the RI group, while the differences in IL-6 and IL-8 expressions from CD45⁺CD3⁺CD8⁺ T cells were almost negligible (**Fig. 2I-L**). These data suggest that activated CD4⁺ T cells could be linked to an intense inflammatory response in IANE progression because these inflammatory mediators have been shown to be the primary cause of cytokine storms in patients with severe acute

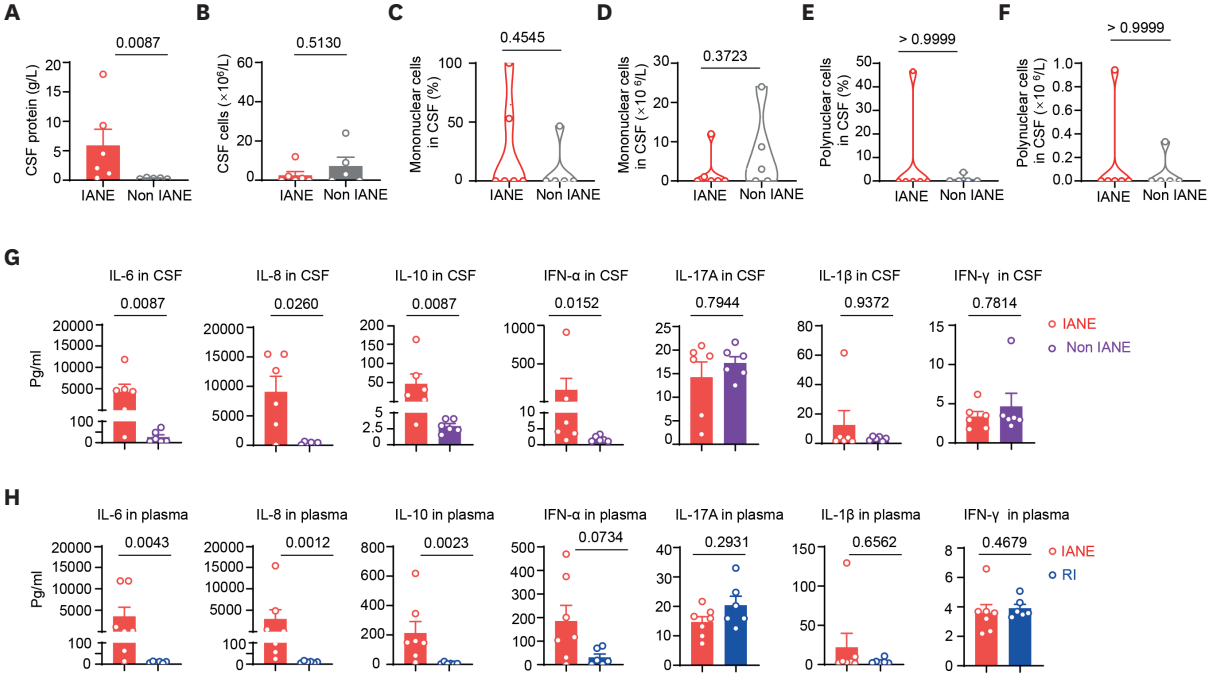

Figure 2. Detection of inflammatory cytokines. (A, B) Comparisons of protein concentrations and cell numbers in the CSF of pediatric patients with IANE (n=6) and without IANE (n=5). (C, D) Comparisons of the proportion and number of mononuclear cells in the CSF between pediatric patients with IANE (n=6) and without IANE (n=5). (E, F) Comparisons of the proportion and number of polynuclear cells in the CSF between pediatric patients with IANE (n=6) and without IANE (n=5). (G) Comparisons of the levels of inflammatory cytokines in the CSF between pediatric patients with IANE (n=6) and without IANE (n=6). (H) Comparisons of the levels of inflammatory cytokines from the plasma of patients with IANE (n=7) and without IANE (n=6). (I, J) Comparisons of the proportions of IL-6 and IL-8 expression from CD45⁺CD3⁺CD4⁺ T cells in the peripheral blood between pediatric patients with severe influenza caused by H1N1 (n=5) and those in RI group (n=7). (K, L) Comparisons of the proportions of IL-6 and IL-8 expression on CD45⁺CD3⁺CD8⁺ T cells in the peripheral blood between pediatric patients with severe influenza caused by H1N1 (n=5) and those in RI group (n=7). Patient 5 in IANE group happened cardiac arrest, so lumbar puncture was unavailable to perform. The p-values are from the Mann–Whitney test or unpaired *t*-test. The $p<0.05$ indicated significant differences. (continued to the next page)

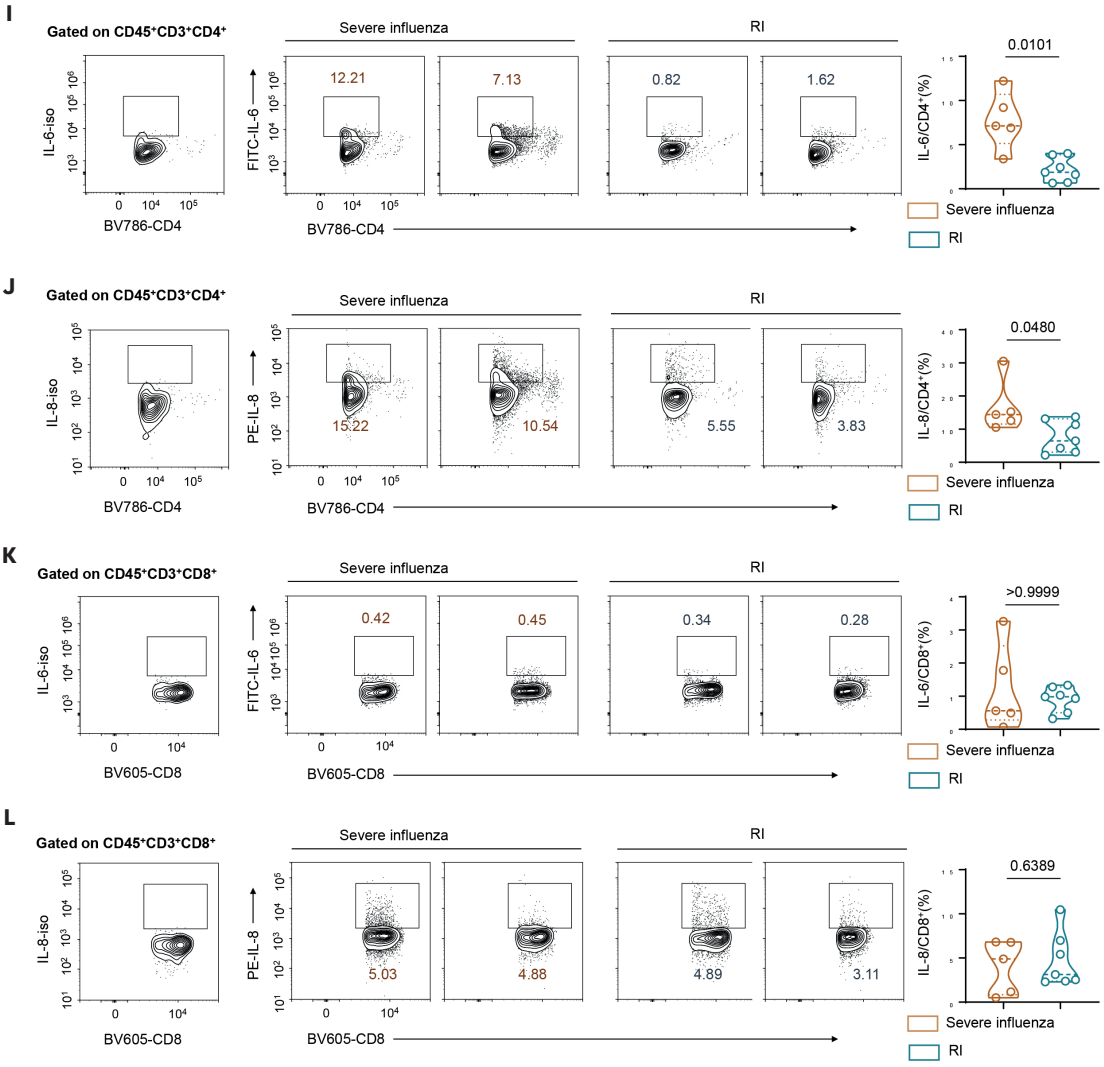

Figure 2. (Continued) Detection of inflammatory cytokines. (A, B) Comparisons of protein concentrations and cell numbers in the CSF of pediatric patients with IANE (n=6) and without IANE (n=5). (C, D) Comparisons of the proportion and number of mononuclear cells in the CSF between pediatric patients with IANE (n=6) and without IANE (n=5). (E, F) Comparisons of the proportion and number of polynuclear cells in the CSF between pediatric patients with IANE (n=6) and without IANE (n=5). (G) Comparisons of the levels of inflammatory cytokines in the CSF between pediatric patients with IANE (n=6) and without IANE (n=6). (H) Comparisons of the levels of inflammatory cytokines from the plasma of patients with IANE (n=7) and without IANE (n=6). (I, J) Comparisons of the proportions of IL-6 and IL-8 expression from $CD45^+CD3^+CD4^+$ T cells in the peripheral blood between pediatric patients with severe influenza caused by H1N1 (n=5) and those in RI group (n=7). (K, L) Comparisons of the proportions of IL-6 and IL-8 expression on $CD45^+CD3^+CD8^+$ T cells in the peripheral blood between pediatric patients with severe influenza caused by H1N1 (n=5) and those in RI group (n=7). Patient 5 in IANE group happened cardiac arrest, so lumbar puncture was unavailable to perform. The p-values are from the Mann–Whitney test or unpaired *t*-test. The $p<0.05$ indicated significant differences.

respiratory syndrome coronavirus-2 infections (14-16). Clinical details of pediatric patients with severe influenza were listed in **Table 3**.

IL-6 has been recently reported to play a key part in CNS inflammation (16,17), which can exacerbate the cytokine storm. The IL-6 level in the CSF of pediatric patients with IANE was much higher than that in plasma. These data suggest that IL-6 plays a central role in the cytokine storm of the nervous system, which resulted in severe neurologic deficits and even multiple organ failure in patients with IANE. The inconsistencies in the findings for different cellular immunological indicators in IANE patients can be attributed to the varying amounts of residual samples after essential clinical testing, and the limitations in obtaining

https://doi.org/10.4110/in.2024.24.e18

Table 3. The details of five pediatric patients with severe influenza

Patients	Sex	Age	Clinical presentation	WBC ($\times 10^9$/L)	Neutrophil ($\times 10^9$/L)	Lymphocytes ($\times 10^9$/L)	Chest X-ray	Clinical treatment	Outcome
1	M	9 yr	High fever and cough severely, tachypnea	12.13	8.83	2.7	Patchy areas of increased density in the lower lobes of both lungs	Oseltamivir taken orally for consecutive 5 days; atomized inhalation of Budesonide suspension for 3 days	Temperature normal and cough less
2	F	8 yr	High fever and cough with wheeze; tachypnea; sluggish response; wheezing sounds in both lungs	9.77	6.55	2.3	Patchy opacities in the right upper lobe and both lower lobes of the lungs	Oseltamivir taken orally for consecutive 5 days; Azithromycin at a dosage of 10 mg/kg orally for consecutive 3 days, and Methylprednisolone at a dosage of 2 mg/kg once daily via intravenous infusion for 3 days	Wheezing resolved, coughing and wheezing symptoms reduced, remaining afebrile
3	M	6 yr	Fever and cough with difficulty in breathing; lip cyanosis	6.52	4.15	1.5	Patchy shadows in the upper lobes of both lungs	Oral oseltamivir for 5 days, along with oxygen therapy	Breath difficulty relieved, cough lessened, discharged
4	M	10 yr	Fever, cough, symptoms of lethargy and fatigue accompanied by intermittent tremors of the limbs, diminished breath sounds in the right lung	7.65	3.31	3.6	Atelectasis in the lower lobe of the right lung	Oseltamivir taken orally for consecutive 5 days, bronchial lavage was performed via bronchoscopy	Temperature normal, cough reduced, tremors of the limbs disappeared
5	M	2 mon	Six days of persistent fever, coughing, and wheezing, Respiratory rate 70 breaths per minute, rales and wheezing in both lungs	13.55	6.25	5.7	Streaky opacities	Budesonide suspension nebulization; oral prednisone at a dosage of 1 mg/kg daily divided into twice	Symptoms alleviated 5 days later and discharge

Severe influenza is classified as if one or more of the following conditions are present according to Expert Consensus on Childhood Influenza Diagnosis and Treatment of China (2020 edition): 1. Respiratory distress and/or rapid breathing: children over 5 years old with a rate >30 breaths/min; children aged 1–5 yrs >40 breaths/min; infants aged 2–12 mon >50 breaths/min; newborns to 2 mon old >60 breaths/min; 2. Altered mental status: sluggish response, drowsiness, restlessness, seizures, etc.; 3. Severe vomiting, diarrhea, and signs of dehydration; 4. Oliguria: urine output in children <0.8 mL/(kg·h), or daily urine volume in infants <200 mL/m^2, preschool children <300 mL/m^2, school-aged children <400 mL/m^2, children over 14 years old <17 mL/h, or the presence of acute renal failure; 5. Complications with pneumonia; 6. Significant worsening of pre-existing underlying diseases; 7. Other clinical conditions requiring hospitalization for treatment.

larger sample sizes or collecting additional samples. Therefore, we could only prioritize the testing of some key indicators in accordance with the experimental design, which provided an important foundation for understanding the immunopathogenesis of IANE.

Intravenous immunoglobulin-G, glucocorticoids, and related support therapy have been used to treat IANE (18), but have not shown satisfactory therapeutic effects. In the present study, six patients admitted to the hospital showed IANE onset in ≤2 or 3 days, whereas patient 2 showed IANE onset within 20 h with high fever and frequent seizures. After detecting a significant increase in the IL-6 level in the plasma of patient 2, she was administered intravenous tocilizumab (8 mg/kg bodyweight) promptly. She did not experience convulsions subsequently. Moreover, her temperature decreased and consciousness was clear and stable 24 h after tocilizumab administration. Subsequently, she was discharged from the hospital and brought home by her parents after a 3-day hospitalization.

Notably, extremely high levels of IL-6 were detected in both the CSF and plasma samples of IANE patients. Therefore, we suggest that tocilizumab may be appropriate to counteract very early-onset IANE to block downstream IL-6 signaling and reduce the risk of organ dysfunction. However, this suggestion needs to be verified in clinical trials. Moreover, the incidence of IANE is very low, increasing the difficulty of etiological research and limiting the feasibility of conducting effective clinical trials. Nevertheless, our work provides a potentially

valuable treatment option for IANE patients, since no effective treatments specifically for IANE are available at present.

In conclusion, our results demonstrate that systemically activated CD4+ T cells may link to the cytokine storm causing IANE. We speculate that tocilizumab may help block the very early stage of the cytokine storm in pediatric patients with IANE, but this finding needs to be verified by clinical trial confirmation.

ACKNOWLEDGEMENTS

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences, Grant No. XDB0490000. This work was also supported by the National Natural Science Foundation of China (Nos. U19A2024, 81930037 to H.W.), the Anhui Natural Science Foundation (No. 2208085J38 to Y.Z.) and the Fundamental Research Funds for the Central Universities (No. YD9110002019 to D.W.). This research has been approved by human research review boards in the in the first affiliated hospital of USTC.

SUPPLEMENTARY MATERIAL

Supplementary Table 1

The antibodies used for surface and intracellular staining

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