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Original Article

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Th17 cells were recruited and accumulated in the cerebrospinal fluid and correlated with the poor prognosis of anti-NMDAR encephalitis

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Abstract

Anti-*N*-methyl-*D*-aspartate-receptor (NMDAR) encephalitis is an autoimmune disorder characterized by memory deficits, psychiatric symptoms, and autonomic instability. The lack of suitable biomarkers targeting anti-NMDAR encephalitis makes the immunotherapy and prognosis challenging. In this study, we found that the Th17 cells were significantly accumulated in the cerebrospinal fluid (CSF) of anti-NMDAR encephalitis patients than that of control individuals. The concentration of the cytokines and chemokines including interleukin (IL)-1β, IL-17, IL-6, and CXCL-13 were significantly increased in the CSF of anti-NMDAR encephalitis patients. IL-6 and IL-17 were found to promote the differentiation of CD4+ T cells into Th17 lineage. The chemotaxis assay showed that CCL20 and CCL22 play essential roles in the migration of Th17 cells. Notably, the correlation between the expression of IL-17 and the outcome of anti-NMDAR encephalitis patients was analyzed. The data showed that high level of IL-17 was significantly correlated with the limited response to the treatment and relapse of anti-NMDAR encephalitis patients. Our results suggested the potential important involvement of IL-17 in anti-NMDAR encephalitis.

Key words: NMDAR, Th17, cerebrospinal fluid, IL-17

Introduction

Anti-*N*-methyl-*D*-aspartate-receptor (NMDAR) encephalitis is an autoimmune disorder characterized by the focal or diffuse neural inflammation mediated by autoantibodies against the NR1 subunit of the anti-NMDAR encephalitis [1–6]. Since the first description in 2007, over 1000 cases of the autoimmune anti-NMDAR encephalitis have been reported worldwide [7,8]. Currently, anti-NMDAR encephalitis has been considered as the most common type of autoimmune encephalitis and is associated with a set of psychiatric and neurological symptoms with rapid onset [9,10]. The typical manifestations of anti-NMDAR include memory loss, consciousness deficits, and autonomic instability [11]. A recent study showed that the cells in the germinal center reactions that contribute to the production of NR1-IgG might be the promising target for the anti-NMDAR encephalitis treatment [12]. The treatment of anti-NMDAR encephalitis requires early recognition and the reduction of anti-NMDAR antibodies, however, ~20%–25% of patients suffer disease relapse after the first-line treatment.

Histopathologic examinations of post-mortem brain tissues of the anti-NMDAR encephalitis patients have revealed the perivascular and intraparenchymal T cells [13]. It has been well documented that T cells play important roles in mediating host defense against bacteria, viruses, and fungi as well as commensal [14–16]. Naive T cells can be differentiated into specific T cell subsets including T helper (Th1), Th2, and Th17 in response to specific cytokine stimulation [17]. Among them, Th17 cells are characterized by its

stability to secret proinflammatory cytokine IL-17, which are differentiated from native T cells in the presence of TGF-β and IL-6 [18]. Th17 cells have been reported to enhance immunity against extracellular bacterial infections by recruiting neutrophils [19,20]. Excessive activation of Th17 is tightly associated with the immunopathology of autoimmune diseases [21,22]. Although T cells occur in the anti-NMDAR encephalitis, the cell amount and differentiation as well as the migration of T cells, especially Th17 cells, in the initiation and prognosis of anti-NMDAR encephalitis still remains largely unknown.

In this study, we collected the cerebrospinal fluid (CSF) and peripheral blood samples from the anti-NMDAR encephalitis patients and control participants. Significant accumulation of Th17 cells was observed in the CSF originated from the anti-NMDAR patients in comparison with that of the control group. To further understand the function of Th17 cells in anti-NMDAR encephalitis, the underlying mechanisms that are responsible for the differentiation and chemotaxis of Th17 cells were investigated. Notably, higher number of Th17 cells was found to be associated with the poor prognosis of anti-NMDAR encephalitis. These findings suggested the potential application of Th17 cells in the diagnosis and treatment of anti-NMDAR encephalitis.

Materials and Methods

Patients

Patients who were admitted to the Haikou People's Hospital between August 2013 and October 2016 with a definitive diagnosis of anti-NMDAR encephalitis were recruited in this study. The inclusion criteria included: (i) positive CSF and serum anti-NMDAR antibody; (ii) evidence of CNS inflammation through magnetic resonance imaging (MRI); (iii) adequate amounts of CSF and serum samples; (iv) clinical follow-up of at least 8 months. Patients randomly selected with other non-inflammatory neurologic disorders and negative autoantibody test were recruited as controls. Patients enrolled in this study have no other diseases and have not received any immunosuppressive therapies before this investigation. The CSF and serum samples collected from patients were kept at −20°C after the antibody test and thawed once for the analysis of cytokine/chemokine. This study was approved by the Haikou people's Hospital. Written informed consent was obtained from all the participants. The basic clinical features of all the participants are summarized in **Table 1**.

CD4+ T cell culture and *in vitro* differentiation

Peripheral blood mononuclear cells were isolated from heparinized peripheral blood samples of anti-NMDAR encephalitis patients and healthy controls by Ficoll gradient centrifugation (Lymphoperp; Nycomed Pharma, Zürich, Switzerland). For positive selection of CD4+ T cells, MACS CD4 microbeads (Miltenyi Biotec, Auburn, USA) were used. The purification of CD4+ T cells was confirmed by the flow cytometery (95%). The purified CD4+ T cells were cultured in RPMI-1640 medium containing 10% FBS (Gibco, Carlsbad, USA), 100 μg/ml streptomycin and 100 U/ml penicillin.

For the cell differentiation, CD4+ T cells were activated with the 5 μg/ml plate bound anti-CD3 (Biolegend, Shanghai, China) and 2 μg/ml anti-CD28 (eBioscience, San Diego, USA), 10 ng/ml IL-6, 10 ng/ml IL-17, and 10 μg/ml anti-INFγ for 5 days in an atmosphere at 37°C with 5% CO_2. To trigger the production of IL-17, cells were treated with 10 ng/ml phytohemagglutinin (PHA; Beyotime, Shanghai, China) and 500 ng/ml iomomycin (Beyotime, Shanghai, China) for 5 h. Cells that secret IL-17 from the above differentiated CD4+ T cells were isolated with the IL-17 Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from the cells using Trizol Reagent (Life technologies, Carlsbad, USA). The first-strand cDNA was synthesized by reverse transcription with the 5× All-In-One RT Master Mix (Applied Biological Materials Inc, Richmond, Canada). The quantitative real-time PCR (qRT-PCR) assay was performed with the Bio-Rad Fast EvaGreen Super mix (Bio-Rad, Hercules, USA) using the IQ5 real-time PCR system (Bio-Rad). The PCR reaction conditions were set as follows: 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The relative mRNA expression was normalized to that of *GAPDH* and calculated with the $2^{-\Delta\Delta CT}$ method. The primer sequences are summarized in **Table 2**. All the experiments were performed in triplicate.

Flow cytometry

Cells (1×10^6) harvested from the CSF or peripheral blood were resuspended in 100 μl PBS. The surface and intracellular staining of T cell markers was performed with anti-CD4 conjugated with PE-Cy5 and anti-human IL-17-APC antibody (BD Biosciences, San Diego, USA), respectively. Cells were pretreated with 25 ng/ml PMA

Table 1. The clinical characteristics of anti-NMDAR patients

	Anti-NMDAR $N = 60$	Control $N = 60$	*P*-value
Age	33.5 ± 8.3	37.3 ± 10.4	>0.05
Sex ratio (female:male)	1.4	1.5	>0.05
Clinical symptom, *n* (%)			
Cognitive impairment	28 (46.7)	N/A	<0.001
Psychiatric symptom	60 (100.0)	N/A	<0.001
Seizures	38 (63.3)	N/A	<0.001

Table 2. The sequences of primers used in this study

Gene name	Primer sequence (5′→3′)
IL-17	Forward: TGTCCACCATGTGGCCTAA
	Reverse: GTCCGAAATGAGGCTGTCTTTGA
RORC	Forward: ACCTCACCGAGGCCATTCAG
	Reverse: TAGGCCCGGCACATCCTAAC
CD45RO	Forward: AGAATACTGGCCATCGATGG
	Reverse: GCTGAACGCATTCACTCTCCT
CCR7	Forward: GATCCTATGACCAGCGACTGTC
	Reverse: AGTAGCTTCCAATGCCCACCAAA
CD62L	Forward: CTCCTTGCCAGCCAAATGATAA
	Reverse: CCTCTTCATTCCAGTGGCAGTC
CCR2	Forward: CTGTCCACATCTCGTTCTCGGTTTA
	Reverse: CCCAAAGACCCACTCATTTGCAGC
CCR4	Forward: CAACCTGCTCAACCTTGCCATCT
	Reverse: CCACCGCCTTGTTCTTCTTCTCAT
CCR6	Forward: ATCCTGCCAGAGCGAAAAGC
	Reverse: CATTGTCGTTATCTGCGGTCTCAC
GAPDH	Forward: GCACCGTCAAGGCTGAGACC
	Reverse: ATGGTGGTGAAGACGCCAGT

(Sigma, St Louis, USA) for 5 h at 37°C. At the same time, 10 μg/ml brefeldin (Maokang Biotechnology, Shanghai, China) was added into the cells for the last 4 h of the incubation. The FACS analysis was performed by fixing the cells with FACS Lysing solution (BD Bioscience) and detected using the BD FACSCaliber flow cytometer (BD Bioscience).

Cytokines and chemokine measurements

The expression levels of IL-2, IL-6, IL-10, and IFN-γ were detected using the Bio-Plex Cytokine Assay System (Bio-Rad) according to the manufacturer's instructions. Additionally, the concentrations of IL-1β, IL-17, and CXCL-13 were measured with the enzyme-linked immunosorbent assay (ELISA) using the human ELISA kit (Invitrogen, Carlsbad, USA). All the experiments were performed in triplicate.

Chemotaxis assay

The migration of Th17 cells was detected using the 24-well transwell plates (Costar, Cambridge, USA). CCL20 or CCL22 was added into the lower chambers to induce the chemotaxis with the indicated concentration. The Th17 cells from the *in vitro*-differentiated CD4+ T cells were seeded into the upper wells of the transwell membranes and incubated at 37°C for 8 h. For the blocking experiments, the anti-CCL20 antibody (Invitrogen) or anti-CCL22 antibody (Invitrogen) was added at the concentration of 10 μg/ml to the Th17 cells and pre-incubated for 2 h at 37°C. After the incubation for 2 h, the cells on the upper surface of the membrane were removed by washing with PBS. The migrated cells in the lower compartment were counted. The chemotaxis index was calculated by dividing the number of cells counted by the number of the cells in the media wells. The experiment was performed in triplicate.

Statistical analysis

Data were presented as the mean ± standard deviation. The statistical analysis was performed by Student's *t*-test or one-way ANOVA with the SPSS statistical software version 16 (SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant.

Results

Th17 cells were significantly accumulated in the CSF of anti-NMDAR encephalitis patients

To detect the involvement of Th17 in the development of anti-NMDAR encephalitis, the CSF and peripheral blood samples from 60 randomly selected anti-NMDAR encephalitis patients and patients with non-inflammatory disorders were collected as the control. The CD4+ T cells in each sample were isolated and the percentage of Th17 in both CSF and peripheral from different individuals were examined by the FACS analysis. The data showed that compared with that of the control group, Th17 cells were significantly accumulated in the CSF but not in the peripheral blood of the anti-NMDAR encephalitis patients (**Fig. 1A**). Additionally, the mRNA and protein expression levels of CD45RO, CD62L, and CCR7 in Th17 cells were detected by qRT-PCR and western blot analysis, respectively. As shown in **Fig. 1B,C**, both the mRNA and protein expression of all these three cell markers in anti-NMDAR encephalitis were increased in comparison with that of the controls. These results demonstrated that Th17 cells were significantly enriched in the CSF of anti-NMDAR encephalitis patients, which indicated the possible involvement of Th17 cells in the progression of anti-NMDAR encephalitis.

IL-6 and IL-17 promoted the differentiation of Th17 cells *in vitro*

To understand the differentiation mechanism of Th17 cells during anti-NMDAR encephalitis, the expression levels of these cytokines including IL-1β, IL-2, IL-6, IL-10, IL-17, CXCL-13, and IFN-γ in the CSF from the NMDAR patients and control individuals were measured by the ELISA analysis. As indicated in **Fig. 2A**, patients with anti-NMDAR encephalitis showed increased CSF IL-1β, IL-6, IL-17, and CXCL-13 levels compared with those of the control (**Fig. 2A**). The expression levels of IL-2, IL-10, and IFN-γ remain unchanged between different groups (**Fig. 2A**).

Because of the significantly increased levels of IL-6 and IL-17 in the CSF of anti-NMDAR encephalitis, we tried to figure out the regulation of these cytokines on the differentiation of Th17 cells. To this end, naïve CD4+ T cells were isolated from the control subjects and the cytokines were added into the cells, respectively. As shown in **Fig. 2B**, both the mRNA and protein level of IL-17 were significantly enhanced with the addition of IL-6 and IL-17. To further confirm this observation, both the mRNA and protein expression of nuclear receptor ROR-gamma (RORC), another specific marker of Th17 cells, were measured. It was also increased in the presence of IL-6 and IL-17 (**Fig. 2C**). These results suggested that IL-6 and IL-17 promoted the differentiation of CD4+ T cells into Th17 lineage.

Previous studies have demonstrated that the JAK/STAT-signaling pathway, especially STAT3, regulates the differentiation and function of Th17 cells, however, the involvement of STAT3 in the differentiation of Th17 cells in anti-NMDAR encephalitis remains largely unknown. To answer this question, naïve CD4+ T cells were treated with cryptotanshinone, the specific inhibitor of STAT3, and the expression levels of IL-17 and RORC were detected. As shown in **Fig. 2D,E**, treatment with the cryptotanshinone significantly decreased the level of IL-17 and RORC, indicating critical involvement of STAT3 in the differentiation of Th17 cells.

CCL20 and CCL22 promoted the migration of Th17 cells

To understand the molecular mechanism by which Th17 cells are recruited into the encephalitis tissues, the expressions of chemotaxis receptors including CCR2, CCR4, and CCR6 of Th17 cells were detected in the CSF from anti-NMDAR encephalitis patients and control individuals. As shown in **Fig. 3A**, the levels of these chemotaxis receptors were significantly increased in anti-NMDAR encephalitis patients compared with those of control group. To further support this observation, the concentration of chemokine CCL20 and CCL22 was also detected by the ELISA assay. The data showed that in the CSF from anti-NMDAR encephalitis patients, the concentration of CCL20 and CCL22 was significantly increased in comparison with that from the control individuals (**Fig. 3B,C**).

To detect the chemotactic ability of Th17, Th17 cells isolated from the healthy controls were seeded into the upper insert, CCL20 and CCL22 were used to induce the chemotaxis of Th17 cells, respectively. The results showed that the addition of CCL20 and CCL22 significantly promoted the chemotactic ability of Th17 cells (**Fig. 3D**). To address the involvement of CCL20 and CCL22 in the chemotaxis of Th17 cells, Th17 cells were incubated with the monoclonal antibodies against CCL20 and CCL22, respectively, and then the chemotactic ability of Th17 was detected. As shown in **Fig. 3E**, the addition of anti-CCL20 and anti-CCL22 antibodies markedly

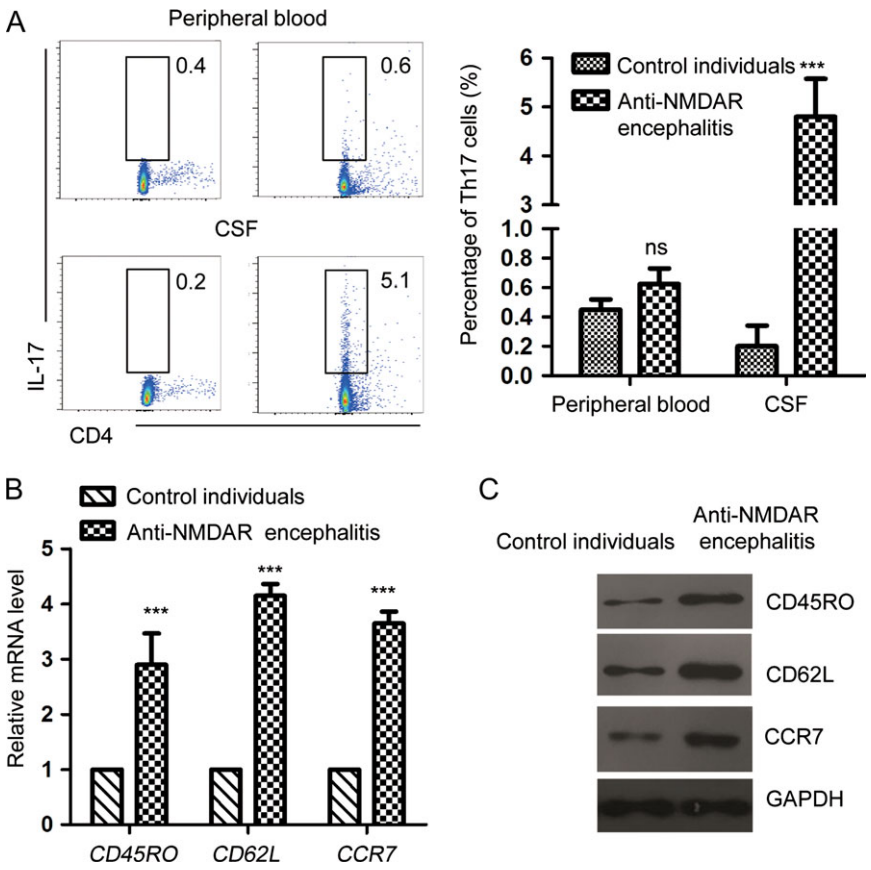

Figure 1. Th17 cells were accumulated in the CSF of anti-NMDAR encephalitis patients (A) The percentage of Th17 cells in the peripheral blood and CSF from control individuals or anti-NMDAR encephalitis was determined by FACS analysis. (B,C) The expression levels of CD45RO, CD62L, and CCR7 of the Th17 cells from the CSF of anti-NMDAR encephalitis patients and control individuals were detected by the qRT-PCR and western blot analysis. The expression of GAPDH was used as the normalization control. ***$P < 0.001$. ns, no significance.

decreased the chemotaxis of Th17 cells. These results demonstrated that both CCL20 and CCL22 were associated with the migration and accumulation of Th17 cells in the CSF of NMDAR patients.

The accumulation of Th17 cells was positively correlated with the poor prognosis of the anti-NMDAR encephalitis patients

The significant accumulation of Th17 cells in the CSF of anti-NMDAR encephalitis patients suggested the potential application of Th17 cells in the treatment of anti-NMDAR encephalitis in the clinical practice. To test this hypothesis, the correlation between the expression of IL-17 with the outcome of the anti-NMDAR encephalitis patients who received early treatment (within 60 days of symptom onset) and first-line immunotherapies were analyzed. As shown in **Fig. 4A**, among the 60 patients, those with higher level of IL-17 ($n = 43$) at the initial evaluation presented relative poor prognosis (mRS score ≥3) during the 8-month follow-up. After the determination of IL-17, 5 of the 17 (29.4%) patients with favorable outcomes and 28 of the 43 (65.1%) patients with poor improvement received second-line therapy.

After the second-line treatment, 10 patients showed clinical relapses. Among them, seven (70.0%) patients who had normal IL-17 concentration at remission developed relapse with elevated level of IL-17 during relapse (**Fig. 4B**). And two samples showed unchanged level of IL-7 from the remission to the relapse phase. Additionally, one patient showed high level of IL-17 during the remission but low level of IL-17 in the relapse. The statistical analysis indicated that high expression of CSF IL-17 was correlated with the clinical relapse of anti-NMDAR encephalitis.

Discussion

Anti-NMDAR encephalitis is a severe autoimmune disorder characterized by prominent psychiatric and neurologic symptoms [6,23,24]. Extensive studies have identified that the increased amount of multiple immune cells and cytokines contributed greatly to the pathogenesis of anti-NMDAR encephalitis [11,13]. Interestingly, Kothur *et al.* [25] showed that the combination of several cytokine/chemokines including CSF TNF-α, IL-10, IL-6, CXCL-13, and CXCL10 might act as biomarkers for diagnosing and monitoring active intrathecal inflammation. The function of Th17 in anti-NMDAR encephalitis has attracted wide attention, however, the accumulation, differentiation, and the correlation between Th17 cells with the outcome of anti-NMDAR encephalitis patients still remains largely unknown. In this study, we found that Th17 cells were significantly increased in the CSF from the NMDAR patients. Addition of IL-6 and IL-17 promoted the differentiation of CD4+ T cells into Th17 cells. A recent study showed that blockade of IL-6 with tocilizumab led to the remission of anti-NMDAR encephalitis patient [26]. Further studies demonstrated that CCL20 and CCL22 play critical roles in the chemotaxis of Th17 cells. Notably, our results also revealed that high

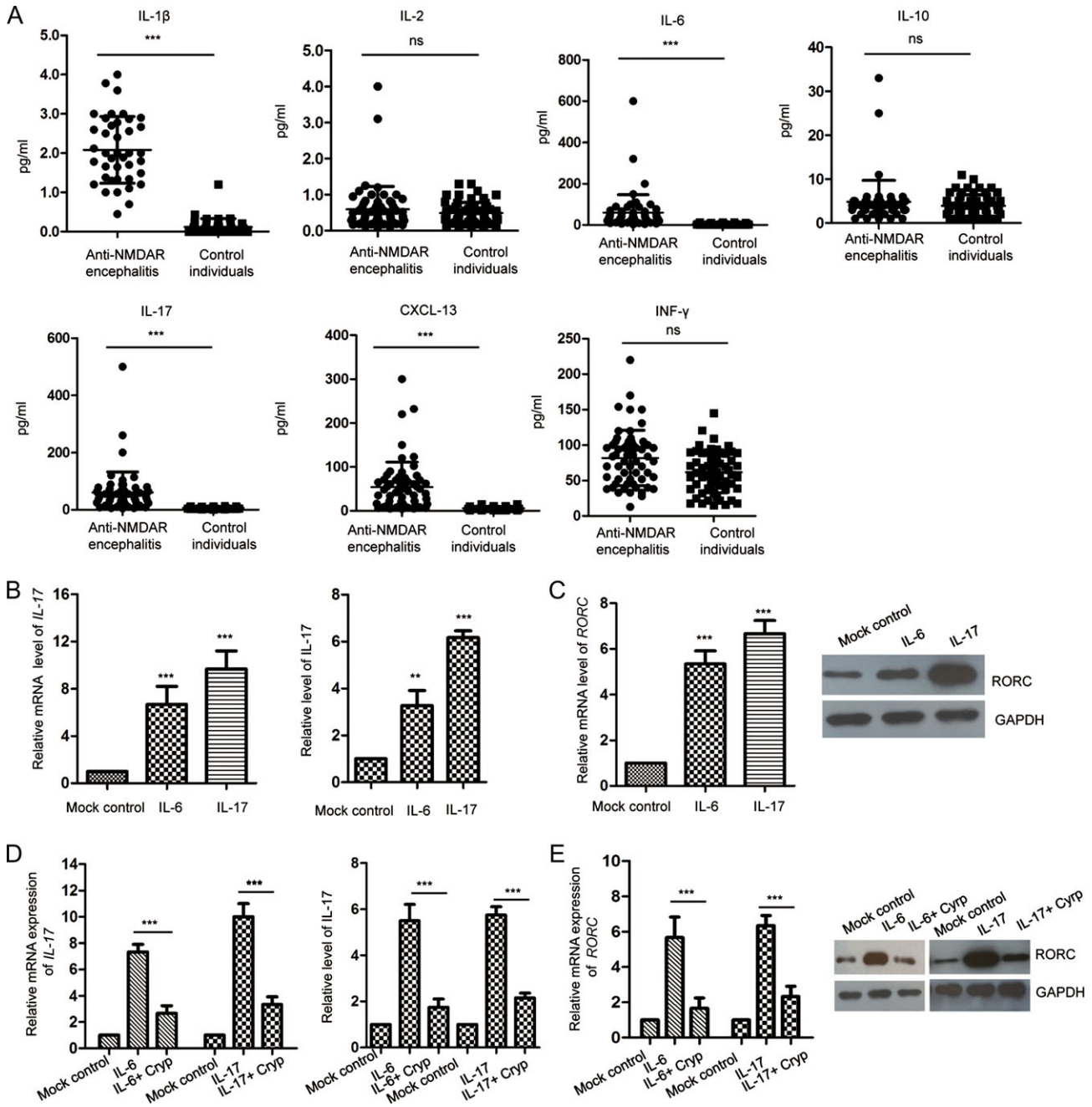

Figure 2. IL-6 and IL-17 promoted the differentiation of Th17 cells *in vitro* (A) The concentration of the CSF cytokine and chemokine in anti-NMDAR encephalitis patients and control individuals. (B,C) Naïve CD4+ T cells were treated with 10 ng/ml IL-6 or 10 ng/ml IL-17 for 5 days. The relative expressions of IL-17 and RORC in the cells were detected by the qRT-PCR, ELISA, or western blot analysis, respectively. Medium was used as the mock control. (D,E) Naïve CD4+ T cells were treated with cryptotanshinone in combination with IL-6 or IL-17, and the expressions of IL-17 and RORC were examined. $^{**}P < 0.01$, $^{***}P < 0.001$. ns, no significance.

expression of CSF IL-17 was significantly correlated with the clinical relapse of anti-NMDAR encephalitis patients.

Th17 cells promote tissue inflammation by inducing the production of proinflammatory cytokine including IL-22, IL-21, and IL-17, which are critical for tissue homeostasis and regeneration [27–32]. Since first described in experimental autoimmune encephalomyelitis, aberrant expression of IL-17 has been observed in many types of human diseases [32–34]. Among them, increased number of Th17 cells and serum IL-17 level has been reported in systemic sclerosis, a complex inflammatory, and fibrotic autoimmune disease [35]. IL-17 was reported to downregulate the tight junction molecules and facilitates the movement of leukocyte across the blood–brain barrier [36]. The enhanced accumulation of Th17 cells is related to disease progression, and immunosuppressive treatment significantly decreases the number of Th17 cells [37,38]. Interestingly, the IL-17 secreted by Th17 cells was reported to enhance cancer-elicited inflammation and prevent cancer cells from immune surveillance, which gives impetus to tumor development [39]. In this study, the recruitment of Th17

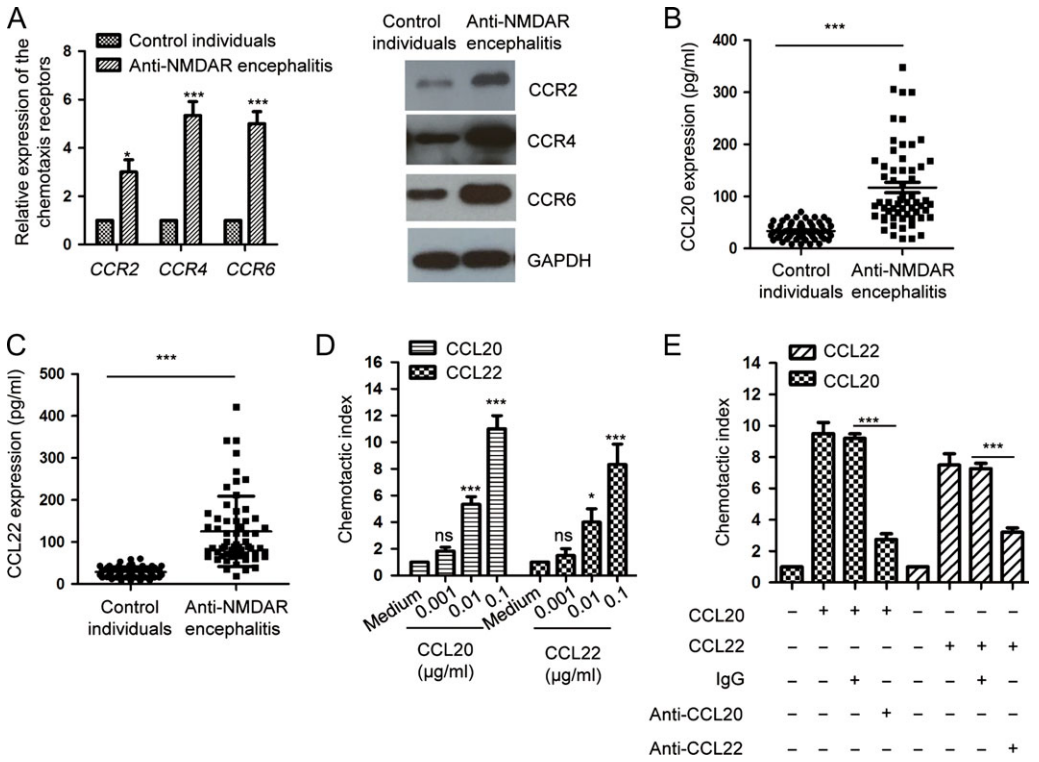

Figure 3. CCL20 and CCL22 promoted the migration of Th17 cells (A) The relative mRNA and protein levels of the chemotaxis receptors of Th17 cells were determined by the qRT-PCR and western blot analysis, respectively. (B,C) The expression of CCL20 and CCL22 in the CSF of anti-NMDAR encephalitis patients and control individuals was determined. (D) CCL20 or CCL22 was added into the cells with the indicated concentration and the migration of Th17 cells was compared with that of the control group. (E) Th17 cells were incubated with anti-CCL20 or anti-CCL22 antibody, and the cell migration was evaluated by the chemotactic index. $*P < 0.05$, $***P < 0.001$. ns, no significance.

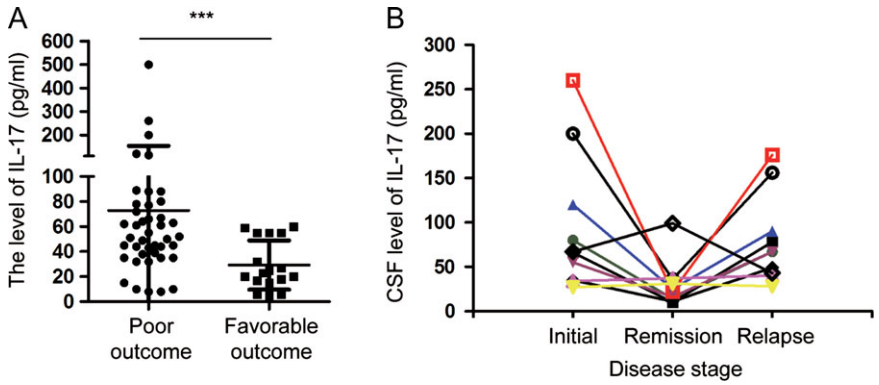

Figure 4. Increased level of IL-17 in the CSF was associated with the poor outcome of the anti-NMDAR encephalitis patients (A) The concentration of IL-17 in the CSF of anti-NMDAR encephalitis patients with poor or favorable outcome was compared. (B) The concentration of IL-17 in the patients with relapse. $***P < 0.001$.

cells in the CSF of anti-NMDAR encephalitis patients suggested the involvement of Th17 cells in the pathologies of anti-NMDAR encephalitis. The underlying molecular mechanism by which Th17 cells regulate the initiation and progression of NMDAR deserves further investigation.

Previous studies have demonstrated that multiple factors are required to trigger the differentiation of Th17 cells [40–43]. It has been reported that IL-21 secreted by the Th17 cells, together with TGF-β stimulated the differentiation of CD4+ T cells in the Th17 lineage, which induced the mRNA expression levels of both IL-17 and RORC. Notably, RORC is also required for the Th17 differentiation [44–46]. Consistent with these data, our results showed that the addition of IL-6 and IL-17 promoted the differentiation of CD4+ T cells. Inhibition of STAT3 significantly attenuated the production of IL-17, indicating the decreased differentiation of CD4+ T cells. These results suggested the essential role of IL-6, IL-17, and STAT3 activity in inducing the differentiation of Th17 cells in anti-NMDAR encephalitis patients. Beyond this, the chemotaxis of Th17 cells is another important element to explore the function of Th17 cells in NMDAR. It has been reported that the release of CCL20, the

cytokine for CCR6-expressing cells, plays critical roles in trafficking CCR6-positive lymphocytes into the inflammatory sites, which may promote the progression of chronic diseases [47]. In this study, we found that both CCL20 and CCL22 were highly expressed in the CSF from anti-NMDAR patients. Addition of CCL20 and CCL22 antibodies markedly decreased the chemotaxis of Th17 cells, which indicated the essential role of CCL20 and CCL22 in the migration of Th17 cells. This result indicated that blocking the interaction of CCL20/CCR6 might be a novel strategy in the treatment of anti-NMDAR encephalitis. Further studies are encouraged to explore other factors that may contribute to the chemotaxis of Th17 cells in anti-NMDAR encephalitis.

To better characterize the potential clinical application of Th17 cells, we evaluate the correlation between the concentrations of IL-17 with the outcome of NMDAR patients. Patients with limited response to therapy had higher level of IL-17 in comparison with that of patients with favorable outcome of therapy. The increased level of IL-17 might contribute to the resistance of immunotherapy of anti-NDMAR encephalitis. A recent study showed that modulating the level of IL-17 with secukinumab, a human monoclonal antibody that selectively binds to IL-17, was an effective and safe treatment therapy for adult psoriasis patients [48]. Combining the previous studies and our results, we proposed that blocking the IL-17 might be a promising strategy for anti-NMDAR encephalitis in the future.

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