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Original Article

Bifidobacterium alleviates guillain-barré syndrome by regulating the function of T17 cells

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Abstract: Objectives: Guillain-Barré syndrome (GBS) is a rare, autoimmune-mediated disease affecting 1.9/100,000 population worldwide. The use of Bifidobacterium as a probiotic is effective to alleviate GBS as they act by regulating immune system. In this study, we explore the protective effects of Bifidobacterium on GBS animal models and the concentrations of IL-17A, IFN-γ, IL-4, and Bifidobacterium in patients with GBS. Materials and Methods: The concentration of cytokines IL-17A, IFN-γ and IL-4 in plasma and CSF were determined by ELISA among 30 GBS patients and 20 healthy controls (HC). The concentration of Bifidobacterium was assayed by real-time PCR. The values of clinical parameters in the GBS patients were also measured. An Experimental Autoimmune Neuritis (EAN) animal model was established to support the protective role of Bifidobacterium in GBS patients. Results: The concentrations of plasma IL-17A, IFN-γ and CSF IL-17A in the acute phase of GBS patients were significantly higher than those in the HC. However, there was no significant difference in the levels of CSF IFN-γ and IL-4 between the GBS patients and the HC. The levels of plasma and CSF IL-17A were positively correlated with the GBS disability scale scores (GDSS) and the concentration of Bifidobacterium was negatively correlated with GDSS. Moreover, plasma and CSF IL-17A were negatively correlated with the concentration of Bifidobacterium. Treatment with Bifidobacterium significantly reduced the levels of plasma IL-17A in the EAN animal model. Conclusions: From this study it was concluded that Bifidobacterium reduces GBS by regulating the function of T17 cells.

Keywords: Guillain-barré syndrome, bifidobacterium, autoimmune neuritis, T17 cells

Introduction

Guillain-Barré syndrome (GBS) is a peripheral autoimmune neuropathy marked by progressive and potentially fetal ascending paralysis with loss of motor reflexes, peripheral nerve demyelination, and inflammatory cell infiltration [1]. The incidence of GBS is 1.9/100,000 worldwide. It occurs in both sexes but mostly men [2]. All parts of the world have similar rates of this disease. The initial symptoms are typically changes in sensation or pain along with muscle weakness, beginning in the feet and hands. Additionally, the symptoms of GBS are sudden severe radicular pain in the neck, shoulder, waist, symmetrical muscle weakness associated with depression, weakened or disappeared tendon reflexes [3]. During the acute phase, the disorder can be life-threatening with about 15% of patients developing weakness of the breathing muscles requiring mechanical ventilation. Most patients have upper respiratory or gastrointestinal tract infection prior to onset of the disease [4]. Patients with GBS show albuminocytological dissociation in the cerebrospinal fluid (CSF) with abnormally increased levels of proteins but without increased numbers of cells during the first week of the acute phase of GBS [5]. In GBS, there is ascending paralysis which often begins in the lower extremities and extends rapidly to the upper extremities trunk [6]. Serious cases may have paralysis of limbs, intercostal muscles, and diaphragm muscle weakness, respiratory weakness, or respiratory paralysis. The diagnosis of GBS is usually made on the basis of signs and symptoms which are supported by tests such as nerve conduction studies and examination of the cerebrospinal fluid. The exact cause of this disease is unknown. The

underlying mechanism of this disease is that the body's immune system mistakenly attacks the peripheral nerves and damages their myelin which was triggered by an infection, surgery, or vaccination. Molecular simulation theory states that some components of the pathogens mimic the structure of the peripheral nerves which leads to immune recognition errors and cause autoimmune T cells and autoantibodies attack on the peripheral nerve that ultimately results in peripheral nerve demyelination [7]. However, the association of components of pathogen with development of GBS has not been clarified.

A previous study highlighted that IFN-γ and IL-17 are produced by pro-inflammatory Th1 and Th17 cells and are important for the pathogenesis of GBS [8, 9]. T helper 17 cells (Th17) are a subset of pro-inflammatory T helper cells which produce cytokines such as IL-17 and IL-22. They play an important role in adaptive immunity and protect the body against pathogens through immune regulation, chronic inflammatory responses, and organism defenses. Dysregulation of Th17 cells has been associated with autoimmune disorders. IL-6 and IL-23 promote Th17 development [10]. The normal bacterial flora are found in or on our bodies on a semi-permanent basis without causing disease. They are widely distributed in the human body and play an important role in the development of Th differentiation through promoting release of a variety of cytokines such as IL-17 and IL-6. IFN-gamma can promote Th1 differentiation and IL-4 can promote differentiation of Th2 [12, 13] cells. Currently, plasmapheresis and intravenous immunoglobulins (IVIG) are the two main immunotherapy treatments for GBS. Apart from immunotherapy, management of pain and rehabilitation is also done for the management of GBS.

In this present study, we examined the levels of CSF and plasma cytokines IL-17A, IL-6, IFN-γ and IL-4 in 30 GBS patients as well as 20 healthy controls. Additionally, we explored the potential association of CSF and plasma cytokines level and Bifidobacterium with disease activity in GBS patients. The protective effects of Bifidobacterium on GBS were also confirmed in animal models. Hence, it is concluded that Bifidobacterium has protective effects which reduces Guillain-Barré syndrome by regulating the function of T17 cells.

Materials and methods

Participants

Thirty patients with GBS were recruited sequentially at the Neurology Department, and 20 age- and sex-matched healthy volunteers were recruited at the Physical Examination Center of First Affiliated Hospital of Bengbe Medical College from May 1st 2016 to Oct 1st 2017. Individual patients with GBS were diagnosed, according to the international diagnostic criteria [15]. Diagnosis of acute GBS was based on the following criteria: an acute progressive symmetrical weakness of the extremities with areflexia or hyporeflexia, albuminocytological dissociation in the CSF, and demyelinating/axonal neuropathy by electrophysiology. Albuminocytological dissociation was defined as abnormal levels of proteins but a total cell count of ≤10/mm^3 in CSF. Individual patients were excluded if she/he had a history of autoimmune diseases, such as multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and type 1 diabetes (T1D), or chronic inflammatory diseases, such as metabolic syndrome, type 2 diabetes (T2D), chronic cardiovascular disease, and malignancy, or a recent infection or if he/she was a heavy smoker.

The disease severity of individual patients with GBS was evaluated by experienced neurologists using the GBS disability scale scores (GDSS), a widely accepted scoring system to evaluate the functional status of GBS patients [16]. Briefly, the GBS at grade 0: normal neurological status; grade 1: minor symptoms, able to run; grade 2: limb weakness, able to walk 5 m unaided; grade 3: able to walk 5 m only with aid; grade 4: chair or bed bound; grade 5: requiring assisted ventilation; and grade 6: death. Written informed consent was obtained from individual participants, and the experimental protocol was approved by the Ethical Committee of the First Affiliated Hospital of Soochow University. Their demographic and clinical characteristics are summarized in **Table 1**. Individual patients were treated intravenously with 0.4 g/kg/d of immunoglobulin daily for 5 consecutive days.

Specimen collection and preparation

Blood samples and CSF samples of individual participants were collected within 48 h after

Table 1. Demographic and clinical characteristics of participants

	Healthy controls	GBS patients
Number	20	30
Age	35.4±6.5	36.7±7.8
Gender (M/F)	14/16	16/14
WBC in CSF (10^6/L)	0.53±0.25	0.61±0.21
WBC in plasma (10^9/L)	6.94±1.86	7.38±2.17
Albumin in CSF (g/L)	0.26±0.09	0.69±0.28**
Albumin in plasma (g/L)	41.37±4.19	42.11±3.08

**P < 0.01 vs. HC.

admission. The CSF samples were donated by those healthy controls, which was approved by the Ethical Committee of the First Affiliated Hospital of Soochow University. The blood and CSF samples were subjected to centrifugation, and the resulting plasma and CSF supernatants were stored at -80°C.

ELISA for the measurement of IL-17A, IFN-γ and IL-4

The levels of IL-17A, IFN-γ and IL-4 in plasma and CSF in the HC and GBS patients were determined using commercially available human ELISA kits (BD Biosciences, San Jose, CA, USA), according to the manufacturers' instructions. Briefly, individual plasma and CSF samples were diluted 1:1 and tested in triplicate by ELISA. The concentrations of IL-17, IFN-γ and IL-4 of individual samples were determined using the standard curve established using standard sample provided.

Real time PCR to detect bifidobacterium

Fresh stool 2 g was collected and 18 ml of PBS was added, mixed thoroughly, and centrifuged at 400 × g for 5 min. The top phase was collected and centrifuged at 400 × g for 5 min for 3 times. The cells (1 ml of top phase) were collected at 9000 × g for 3 min, washed with PBS 4 times and re-dissolved in 0.1 ml distilled water. After 100°C 5 min heating, the cells were immediately put into the ice water. Pure culture as a positive template by a known amount of continuous dilution of Bifidobacterium longum (10^0-10^7 CFU/ml) fluorescence were used as a standard curve of Bifidobacterium. The primers used for Bifidobacterium were 5'-CTCCTGGAAACGGGTGG-3' and 5'-GGTGTTCTTCCCGATATCTACA-3'. Real time PCR was carried out at 95°C for 15 s, followed by 40 cycles of 94°C for 10 s, 55°C for 10 s, 74°C for 35 s, and then 74°C for 2 min.

Experimental autoimmune neuritis (EAN) animal model

Adult SD rats (40) weighing 180-200 g were purchased from experimental animal center of Soochow University. All procedures of animal handling were carried out in accordance with the protocols of the animal care guide lines of the Institutional Animal Care and Use Committee of Soochow University which approved the study. Establishment of the EAN rat model has been described previously [17]. In brief, EAN rats were induced by subcutaneous injection at hind feet with 100 μL of an emulsion containing 200 μg $P0_{180\text{-}199}$ peptide (KE Biochem Shanghai, China), 1 mg Bacillus Calmette-Guérin vaccine (Wanma pharmaceutical co., LTD, Zhejiang, China), and 1 mg Mycobacterium tuberculosis (Huayun, Guangzhou, China). Rats were scored at week 2 and week 4 after immunization for development of EAN as follows: 0 = normal, 1 = limp tail, 2 = mild paresis of the hind limbs, 3 = severe paraparesis or paraplegia of the hind limbs, and 4 = tetraparesis. Animals were randomly divided into four groups, including normal group, model group, intragastric administration of Bifidobacterium high dose (10^9 CFU/ml) and low dose (10^7 CFU/ml) groups. Each group contained five to six rats. Bifidobacterium prescription started at week 2 after immunization.

Electrophysiological examination of sciatic nerve

The rats were anesthetized by administering an intraperitoneal injection of pentobarbital sodium (30 g/L) at 45 mg/kg. After complete anesthesia, the skin was cut between the right biceps muscle of the thigh and semimembranosus muscle with blunt separation to expose the sciatic nerve. To guide the sensory nerve action potential, stimulating electrodes were inserted into the left plantar mucosa of the rats, and the recording electrodes were placed in the middle of the sciatic nerve. MS-302 multimedia biological signal recording system (Chengdu, China) was used to observe and record the latency of sensory nerve action

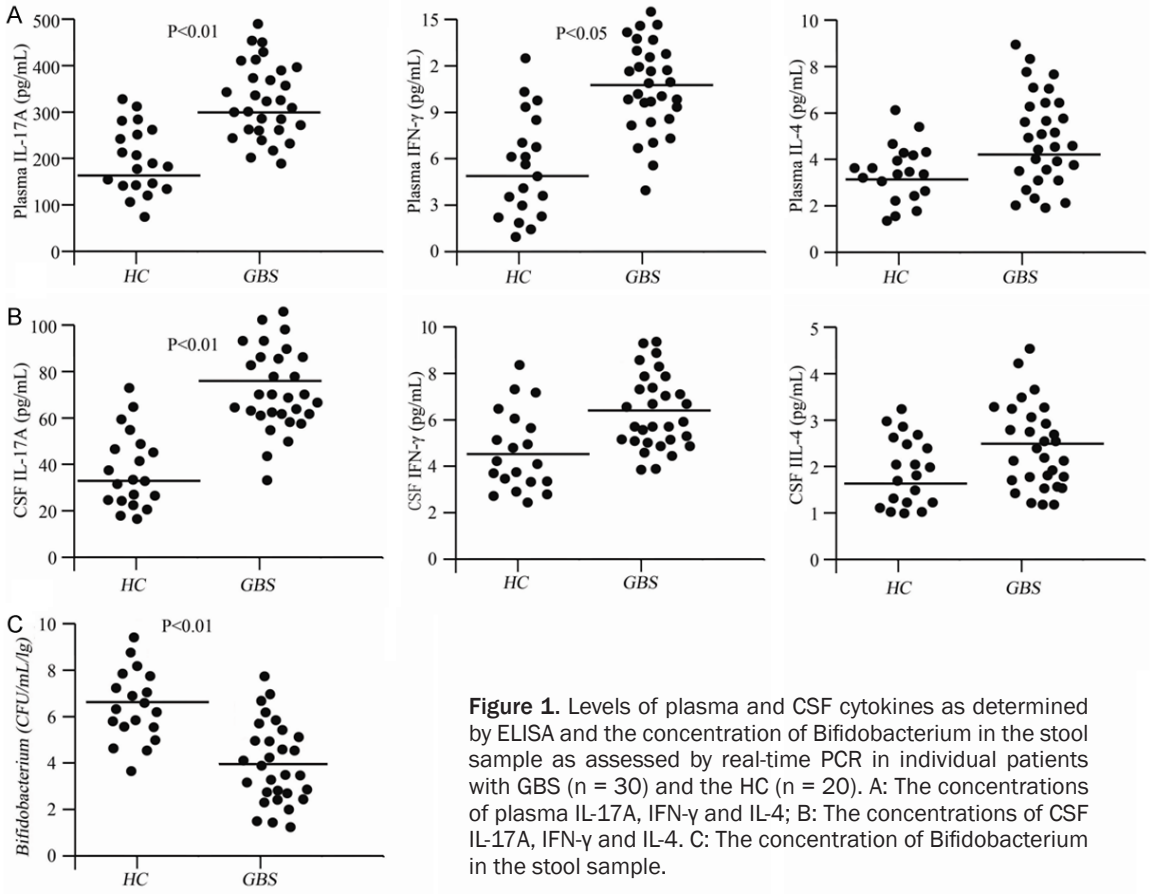

Figure 1. Levels of plasma and CSF cytokines as determined by ELISA and the concentration of Bifidobacterium in the stool sample as assessed by real-time PCR in individual patients with GBS (n = 30) and the HC (n = 20). A: The concentrations of plasma IL-17A, IFN-γ and IL-4; B: The concentrations of CSF IL-17A, IFN-γ and IL-4. C: The concentration of Bifidobacterium in the stool sample.

potential, the sciatic nerve conduction velocity and amplitude.

Statistical analysis

Statistical comparisons among different groups were done using the student *t* test and one-way analysis of variance (ANOVA) using SPSS 18.0 software. The relationship between the variables was evaluated using the Spearman rank correlation test. A two-side *P* value of < 0.05 was considered statistically significant.

Results

Demographic and clinical characteristics of study subjects

Thirty GBS patients and 20 healthy controls (HC) were recruited. Their demographic and clinical characteristics are summarized in **Table 1**. There was not a significant difference in the distribution of age and gender between the GBS patients and the HC. Moreover, there was not a significant difference in the values of albumin in plasma and WBC in the CFS and plasma between the GBS patients and the HC. However, the concentrations of CSF albumin in the patients were significantly higher than that in the HC ($P < 0.01$).

Higher levels of IL-17A and lower levels of bifidobacterium were detected in patients with GBS

Pro-inflammatory responses have been associated with the pathogenesis of GBS, and IL-17A, released by Th17 cells, is commonly detected in inflammatory tissues to induce immune pathological damage [17]. To investigate the potential role of these cytokines, we examined the concentrations of plasma IL-17A, IFN-γ and IL-4 in 30 patients with GBS and 20 HC by ELISA. We found that the concentrations of plasma IL-17A and IFN-γ in the patients were significantly higher than those in the HC (**Figure 1A**, $P < 0.01$ and $P < 0.05$). Characterization of CSF cytokines revealed that the levels of CSF IL-17A in the patients were significantly higher than those in the HC (**Figure 2B**, $P < 0.01$). In con-

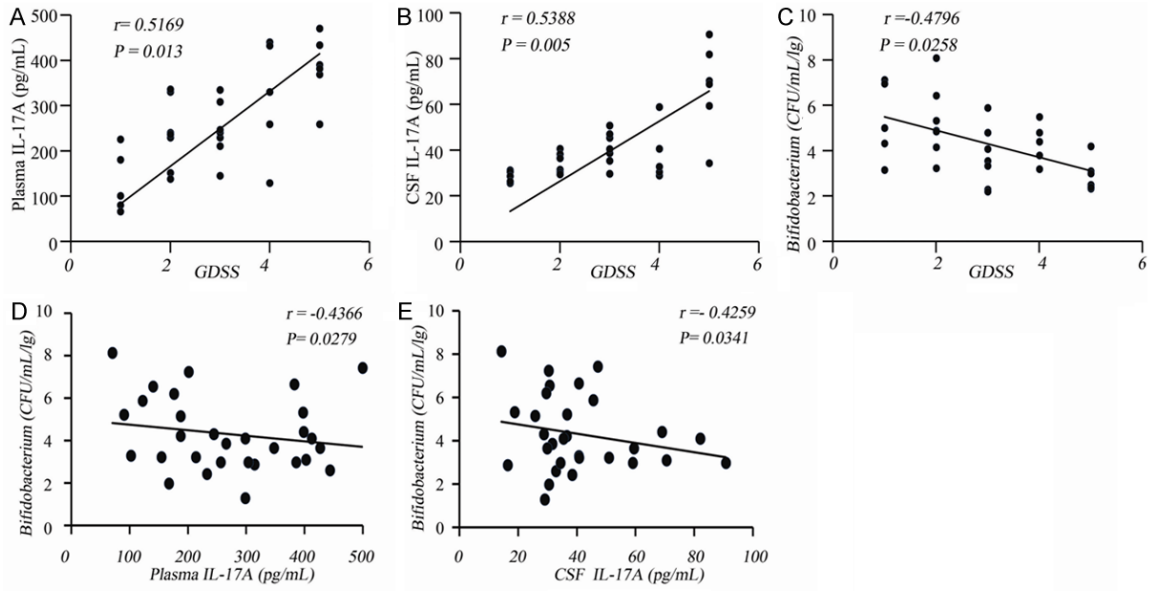

Figure 2. Correlation analyses between the levels of IL-17A and Bifidobacterium and the values of GDSS in GBS patients. A: Plasma IL-17A was correlated positively with GDSS; B: CSF IL-17A was correlated positively with GDSS; C: The concentration of Bifidobacterium was correlated negatively with GDSS; D: Plasma IL-17A was negatively correlated with the concentrations of Bifidobacterium; E: CSF IL-17A was negatively correlated with the concentrations of Bifidobacterium.

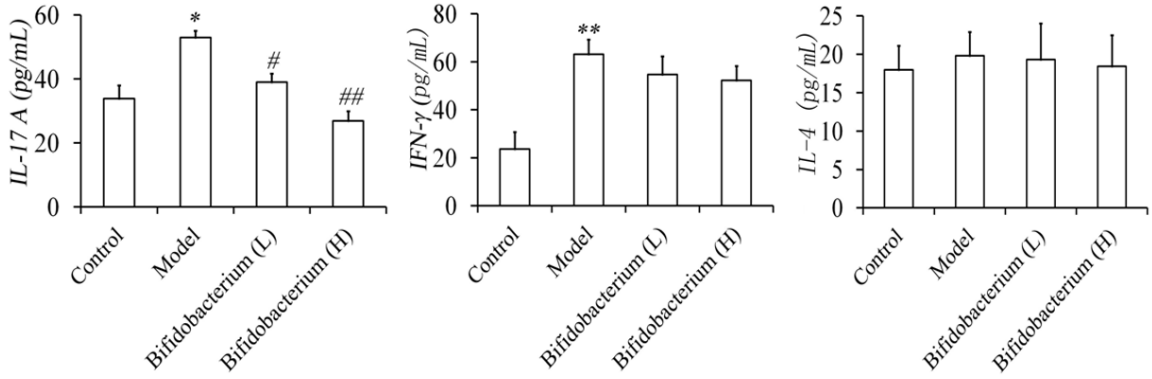

Figure 3. Effects of Bifidobacterium on plasma IL-17A, IFN-γ and IL-4 were assayed by ELISA (n = 6). Treatment with Bifidobacterium significantly reduces the levels of plasma IL-17A in EAN animal models. $^{**}P < 0.01$ and $^{*}P < 0.05$ vs. Control; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ vs. Model.

trast, there was no significant difference in the levels of CSF IFN-γ and IL-4 between the GBS patients and the HC. In contrast, the levels of Bifidobacterium in the GBS patients were significantly lower than those in the HC (**Figure 1C**, $P < 0.01$)

Serum and CSF IL-17A are correlated with the concentration of bifidobacterium and values of GDSS in GBS Patients

We next analyzed potential relationships between the levels of IL-17A and the values of GDSS in those patients. We found that the concentrations of plasma and CSF IL-17A were correlated positively with GDSS in GBS patients (**Figure 2A**, **2B**, R = 0.5169 and P = 0.013; R = 0.5388 and P = 0.005, respectively). Similarly, the concentrations of Bifidobacterium were correlated negatively with GDSS in GBS patients (**Figure 2C**, R = 0.4796 and P = 0.0258). Further analysis revealed that the concentrations of plasma and CSF IL-17A were negatively correlated with the concentrations of Bifidobacterium (**Figure 2D**, **2E**, R = 0.4366 and P = 0.0279; R = 0.4259 and P = 0.0341, respectively). These

Table 2. Electrophysiological examination of sciatic nerve

Group	n	Velocity (m/s)	Amplitude (ms)	Latency (mV)
Control	6	40.33±7.54	1.03±0.16	40.66±4.09
Model	6	20.67±6.22**	1.86±0.15**	22.08±4.46**
Bifidobacterium (low)	6	31.12±6.82#	1.39±0.08#	29.43±5.07#
Bifidobacterium (high)	6	36.37±5.08##	1.57±0.14#	35.28±5.23##

**P < 0.01 vs. Control; ##P < 0.01 and #P < 0.05 vs. Model.

data suggest that increased levels of IL-7A and decreased concentration of Bifidobacterium may be associated with the development of GBS.

Bifidobacterium improves symptoms and reduces the levels of IL-17A

Two weeks after the Bifidobacterium treatment, we found that the concentration of plasma IL-17A in Bifidobacterium groups was significantly lower than those in model group, while there was no significant difference in the levels of plasma IL-4 (**Figure 3**). When compared with the model group, Bifidobacterium treatment promoted conduction velocity and amplitude of sciatic nerve and reduced the latency of sensory nerve action potential (**Table 2**, $P < 0.05$). Therefore, Bifidobacterium treatment reduced symptoms and reduced the levels of plasma cytokines in EAN animal models.

Discussion

GBS is a rare disease in which the immune system attacks the peripheral nervous system and damages myelin insulation. GBS can affect the nerves that control muscle movement as well as those that transmit pain, temperature, and touch sensations. This can result in muscle weakness and loss of sensation in the legs and/or arms. The specific etiology of GBS is not clear, however it is known that it is post-infectious disease as many patients report an infectious illness in the weeks prior to the onset of GBS. It is believed that GBS is associated with infections of Campylobacter jejuni, cytomegalovirus, Epstein-Barr virus, influenza A, Mycoplasma pneumoniae, Haemophilus influenzae, hepatitis, and more recently, Zika virus. Much research also supports the "molecular simulation" theory that the genetic predisposition of exposure to certain environmental factors (such as infection, cold, etc.) leads to exposure of self-antigen, which activates the immune cells to secrete pro-inflammatory cytokines such as IL-17A, IFN-γ and IL-4 inducing an abnormal autoimmune response to components of the myelin sheath. Activated immune cells induce production of antibodies in the response of antigens that cross-react with specific gangliosides and glycolipids, such as GM1 and GD1b that are distributed throughout the myelin sheath of peripheral nerves. Previous studies have suggested that systemically and locally released pro-inflammatory Th1 and Th17 are important players in the pathogenesis of GBS [18, 19]. However, it is unclear how these pro-inflammatory T-cell responses are regulated during the pathogenic process. Th1 cell cytokines such as IFN-γ, IL-2, IL-12 and TNF activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. Th17 cells are a fairly new discovery in immunity in that they mainly secrete IL-17. Th17 may play an essential role in protection against certain extracellular pathogens such as Klebsiella, pneumonia, Bacteroides fragilis, Borrelia burgdoferi, mycobacterium tuberculosis, and fungal species. Th17 cells with specificity for self-antigens are highly pathogenic and have been thought to lead to the development of inflammation and severe autoimmunity. In this study, we examined the concentrations of IL-17A, IFN-γ and IL-4 in plasma and CSF of 30 patients with GBS and 20 healthy controls. We found that the concentrations of plasma pro-inflammatory cytokines such as IL-17A and IFN-γ in the patients were significantly higher than those in the control. Similarly, the levels of IL-17A in CSF of the patients were significantly higher than those in the control. More importantly, the levels of IL-17A in CSF and plasma were correlated positively with the patients with GBS, indicating Th17-cell immunity is crucial for the pathogenesis of GBS, which is consistent with previous findings. Accordingly, expression of Bifidobacterium was significantly decreased in patients with GBS, which was negatively correlated with the CSF and plasma IL-17A.

In the animal model of EAN, we also found that concentrations of IL-17A in plasma and CSF were significantly higher than in the control. Furthermore, the levels of plasma IL-17A were

correlated positively with the development of EAN. In addition, we found that Bifidobacterium administration significantly reduced the levels of IL-17A in plasma. Therefore, our data clearly demonstrate that treatment with Bifidobacterium not only alleviates the disease activity but also inhibits Th17 differentiation in GBS patients. Conceivably, the levels of IL-17A in plasma and CSF may be important for evaluating the therapeutic efficacy in GBS patients.

In summary, our data show higher levels of IL-17 in plasma and CSF of patients with GBS. The concentrations of IL-17A in plasma and CSF were correlated positively with the GDSS in GBS patients and negatively correlated with Bifidobacterium. Treatment with Bifidobacterium not only significantly alleviated the disease activity, but also reduced the levels of IL-17A. Our findings may provide new insights into the regulation of inflammatory responses during the pathogenic process of GBS and suggest that the levels of plasma and CSF IL-17A may be important for evaluating the disease severity and therapeutic efficacy in GBS patients.

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Disclosure of conflict of interest

None.

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