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Original Article

Expression and significance of $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood of patients with different phenotypes of Guillain-Barré syndrome

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Abstract: Objective: This study aims to investigate the changes of immune status and significance in patients with Guillain-Barré syndrome (GBS). Methods: The proportion of $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood before immunotherapy for 41 patients with GBS (including 29 classic type and 12 variant type) and 42 normal control patients (healthy volunteers) were evaluated by flow cytometry. And molybdenum three phenol red method was used to detect cerebrospinal fluid protein content of 28 patients with GBS (including 19 with classic type and 9 with variant type). Results: Compared with healthy control group, the $CD4^+CD25^+CD127^-$ of GBS group had obvious difference ($P<0.05$). Of which, the $CD4^+CD25^+CD127^-$ regulatory T cells of the classic GBS group had no significant changes compared with the variant group ($P>0.05$), as well as the cerebrospinal fluid protein content between classic and variant GBS groups. The decrease of the proportion of $CD4^+CD25^+CD127^-$ regulatory T cells suggested abnormal expression of immune function in GBS patients. Conclusion: The decrease of GBS regulatory T cell number or function indicated that the immune regulatory T cells mediated imbalance of immune regulation involved in the pathogenesis of GBS.

Keywords: Regulatory T cells, Guillain-Barré syndrome, flow cytometry

Introduction

Now that the Guillain-Barré syndrome (GBS) was considered as a group of autoimmune disease. The known pathogenesis was similar to certain components due to precursor of infected pathogen and peripheral nerve myelin, the immune system cannot correctly identify the allogeneic and autologous antigen after infection, then occurred error immune response antigen to component of autologous peripheral nerve, caused peripheral nerve demyelination, secondary to axonal degeneration for severe patients [1-3]. But its principles of immunology in the pathogenesis remained unclear.

Regulatory T cells ($CD4^+CD25^+$ Tregs) were a group of T cell subsets with immune regulation function different from helper T cell (Th1 and Th2) of the human body. Such cells had immunosuppressive properties, can maintain the immune tolerance and immune homeostasis, prevented the body's autoimmune diseases [4]. The previous study confirmed that $CD4^+CD25^{high}$ Treg cell number or function abnormality can cause a variety of autoimmune diseases, but the results were not entirely consistent [4-16]. In this study, three phase flow cytometry was used to detect $CD4^+CD25^+CD127^-$ proportion in peripheral blood of 41 GBS patients, and compared with the healthy control group, in order to explore the significance of $CD4^+CD25^+$ Tregs in the pathogenesis of GBS.

Materials and methods

Subjects

41 inpatients with GBS admitted in the Department of Neurology, the First Affiliated Hospital of Bengbu Medical College during January, 2012 to November, 2014 (excluding patients with other autoimmune diseases or receiving hormone therapy immune factors) were selected. The diagnosis and classification were in line with treatment guidelines of 2010 Chinese Guillain-Barré syndrome [17]. The blood samples were collected after the patients

Table 1. Comparison of proportion of $CD4^+CD25^+CD127^-$ Treg in $CD4^+$ T cells in peripheral blood of Guillain-Barré syndrome patients and healthy controls

Group	Typing	Cases	$CD4^+CD25^+CD127^-$ Treg detection values (%)
GBS	Classic	29	3.40±1.46*
	Variant	12	3.29±1.64*
	Total	41	3.37±1.49*
Control		42	6.26±1.72

Note: Total GBS patient group (including the classic and variant types) was significantly lower compared with the healthy control group (*P<0.05), the difference was statistically significant. Of which, compared GBS variant group with classic group, P>0.05, the difference was not statistically significant.

signing informed consent after admission, and performed lumbar puncture in 2-3 weeks after the incidence. 41 cases patients who enrolled in regulatory T cells detection, including 25 males and 16 females, aged 9-76 years with mean age of 45.8 years, 19 cases sub-type was the classic type, 12 cases of variant; 28 cases were enrolled in cerebrospinal fluid detection, including 15 males and 13 females, aged 9-70 years with mean age of 42.3 years old, 19 cases were classic type, 9 cases of variant type. The control group was the healthy subjects provided by the hospital medical center, a total of 42 people including 30 males and 12 females, aged 16-58 years with a mean age 40.1 years. The study was authorized by Bengbu Medical Ethics Committee, and all participants wrote informed consent. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Bengbu Medical College. Written informed consent was obtained from all participants.

Regulatory T cell detection

Specimen collection: EDTA-K2 anticoagulant vacuum blood was used to collect 2 ml venous blood. Two streaming dedicated pipes were numbered to be Tr1 and Tr2. Added 20 μl Anti-CD25-FITC (No. 3,295,612, US BD Biosciences), Anti-CD4-Percp (No. 3,311,518, US BD Biosciences) and the Anti-CD127-PE (No. 3,276,859, US BD Biosciences) to Tr2 pipe, respectively. 20 μL of isotype antibody were added to Tr1 tube join as negative control at the same time. Added 100 μL of whole blood to Tr1 and Tr2 tubes, respectively, mixed evenly, incubated in 35°C water bath in dark for 15 min. 2.0 mL of Lysing hemolytic solution (NH_4Cl) were added to the two tubes, and bathed in 35°C water in dark for 10 min. Centrifuged at 500 rev/min for 5 min, the supernatant was discarded. Saline (PBS) cells were washed twice with 1500 rpm/min centrifugal 5 min, the supernatant was discarded. 0.5 ml of 1% paraformaldehyde (PFA) was added to each group to fix. Flow cytometry (FACS Calibur, purchased from BD Biosciences) was used to test.

According to the lateral light scattering (FSC) and longitudinal optical (SSC) scattering scatterplot, set the lymphocyte gate. 10000 lymphocytes of each specimen was collected, the results were analyzed by Cell Quest software.

Determination of protein content

Specimen collection: a dry tube (Z) vacuum, 2 ml of cerebrospinal fluid specimens (not hemolysis) was centrifuged at 500 rev/min for 5 min, and 5 μL of the supernatant were collected and numbered to be T1. 5 μL of standard product (excluding serum albumin solution) (Code: 8050-717, China Ruiyuan biotechnology) was collected and number to be T2, then added 300 μL of the reagent (the main component was pyrogallol red buffer), mixed and incubated at 37°C for 600 s, measured the absorbance A after water blank to be zero, according to CSF protein (mg/dl) = standard sample concentration ×A/standard A, the data were statistically analyzed.

Statistical analysis

All data were expressed as mean ± standard deviation ($\bar{x}$ ± s). SPSS17.0 software package was used for statistical analysis. The difference between the t test was compared. $P<0.05$ was considered to be statistically significant.

Results

Comparisons between GBS and healthy control groups: GBS total patient group (including the classic type and variant) had significantly reduced compared with the healthy control group, the difference was statistically significant ($P<0.05$; **Table 1**; **Figure 1A**, **1B**).

Comparisons between classic GBS and variant groups: compared with the GBS variant group, the ratio of $CD4^+CD25^+CD127^-$ regulatory T cells in the classic group had no significant differ-

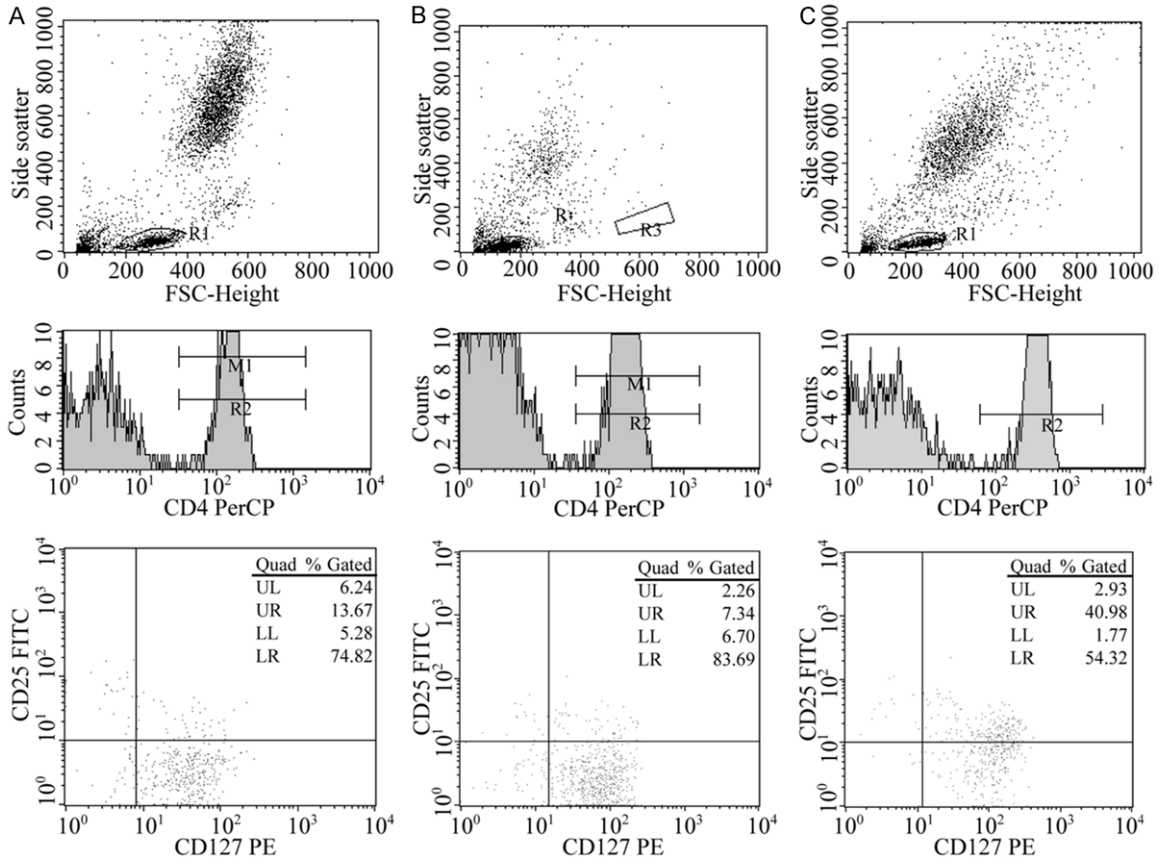

Figure 1. Comparisons of flow cytometry charts for $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood of healthy, classic and variant GBS type patients. A: Flow cytometry chart for $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood of healthy patients, and gated the $CD4^+$ T lymphocyte. B: Flow cytometry charts for $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood of classic GBS type patients. C: Flow cytometry charts for $CD4^+CD25^+CD127^-$ regulatory T cells in peripheral blood of variant GBS type patients.

Table 2. Comparison of cerebrospinal fluid protein content between the patients with classic and variant Guillain-Barré syndrome (mg/dl)

Typing	Cases	Cerebrospinal fluid protein content
Classic	19	1.07±0.41
Variant	9	0.99±0.28

Note: Cerebrospinal fluid protein content changes of patients with classic and variant GBS had no significant differences (P>0.05), the differences were not statistically significant.

ence (★*P*>0.05), the difference was not statistically significant, as well as the cerebrospinal fluid protein content (★★*P*>0.05; **Table 1**; **Figure 1B**, **1C**).

Discussion

Sakaguchi et al. [18] formally proposed by animal experiments in 1995 that $CD4^+CD25^+$ T cells had function of active immune suppression, then such cells were widely paid attention to by immunologists. As different from the immune suppressor T cells in the traditional theory, these cells were named for life "$CD4^+$-$CD25^+$ regulatory T cells" ($CD4^+CD25^+$ Tregs). Previous studies for classical Scurfy mouse model [19, 20] suggested that FoxP3 and its coding products were sensitive and specific markers of regulatory T cells, which played an important role in the regulation of T cell development and function. But Shevach research team [21, 22] found that different from rodents, Foxp3 expression in $CD4^+CD25^+$ Treg in human had differences; and it was thought that Foxp3 was not regulatory T cell-specific markers. Previous literature confirmed that 5%-10% of $CD4^+CD25^+$ Treg cells existed in peripheral blood of human, but CD25 expression in peripheral blood of human and mouse was significantly different. The number of CD25 on the $CD4^+$ T cell surface was uniform, there was a

clear demarcation between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, which was a single cell population independent of each other. While the CD25 expression on human $CD4^+$ T cell surface can be divided into weak, medium, strong three levels according to the number or fluorescence intensity. The precious studies suggested that CD25 strongly positive $CD4^+$ T cells ($CD4^+CD25^{high}$) can specifically expressed $CD4^+CD25^+$ Treg cells [23]. But function molecules (Foxp3) of regulatory T-cell located in the nucleus, the detection of which needed rupture markers, test procedures were cumbersome. Liu et al. [24] found in 2006 that the membrane surface molecules IL-7 receptor CD127 expression was negatively correlated with FoxP3, this molecule inhibited $CD4^+$ Treg cell function. Through detecting human Treg cell subpopulations CD127 molecules in peripheral blood of patients with type 1 diabetes, the molecule was proved to be used as Tregs cell surface markers. Study of Yu et al. [25] confirmed that low expression of CD127 molecule was an inherent characteristic of Tregs cells, $CD4^+CD25^+CD127^-$ markers can accurately assess the number of human Treg cells and subsequent function of regulatory T cells in vitro. The studies of Klein et al. [26] believed that as $CD4^+CD25^+$ Treg cell surface markers in human peripheral blood, two indicators ($CD4^+CD25^+CD127^-$ T cells and $CD4^+CD25^+$FoxP3) had good correlation, and $CD4^+CD25^+CD127^-$ mark method was thought to be more ideal indicators of human CD4 than $CD4^+CD25^+$FoxP3 mark method. Saison et al. [5] studied the human autoimmune disease systemic lupus erythematosus and demonstrated that $CD4^+CD25^+CD127^-$ T cells was positive correlated with $CD4^+CD25^+$FoxP3. Therefore, this study used $CD4^+CD25^+CD127^-$ as a detection indicator for $CD4^+CD25^+$ Treg. The proportional changes of $CD4^+CD25^+CD127^-$ in patients with GBS were detected, and $CD4^+CD25^+CD127^-$ in GBS pathogenesis were analyzed to explore the clinical application significance.

Regulatory T cells (Tregs) mainly had two characteristics including immune incompetence and immunosuppressive, the mechanism of action was negative role in immune regulation by dependent direct contact inhibition and independent secretion of cytokine inhibition. Its molecular basis of the immune inhibitory mainly ① through suppressing effector T cells by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) dependent direct cell contact manner; ② inhibiting $CD4^+$ T cell activation and proliferation by secreting inhibitory cytokines IL-10 or TGF-β, or directly inhibiting $CD8^+$ effector T cells and their precursor cells; ③ inhibiting $CD4^+$ T cell activation and proliferation by increasing the metabolism of tryptophan (Trp) via acting on antigen-presenting cells (APC) mode; ④ $CD4^+CD25^+$ Treg inducing local formation of target cells into micro-cells immunosuppressive environment, so that part of $CD4^+CD25^+$ T cells can be induced by $CD4^+CD25^+$ Treg and secreted IL-10 or TGF-β to exert immunosuppressive effects [6]; ⑤ $CD4^+CD25^+$ Treg also interfering with the proliferation of $CD8^+$ T effector T cells, inhibiting the secretion of cytokines and the ability of cytotoxicity through this local microenvironment [4, 15, 16].

GBS was a $CD4^+$ T cell-dependent autoimmune disease, $CD4^+$ T cell activation was the key to the disease [7]. The study found that the decrease of $CD4^+CD25^+$ Treg before immunotherapy in GBS group was significantly different from the normal control group, but there was no significant difference (**Table 1**). The level changes of cerebrospinal fluid protein content of GBS classic group had no significant difference compared with the variant group (**Table 2**), suggesting that $CD4^+CD25^+$ Treg played an important role in the pathogenesis, development process of GBS patients. The cerebrospinal fluid protein content levels before immunotherapy in patients with GBS had no significant correlation with the types of disease, which was consistent with the existing literature. The effective human serum immunoglobulin therapy prompted the process of GBS immune involved in both humoral and cellular immunity [8, 10]. The number decrease or function decline of $CD4^+CD25^+$ Treg in peripheral blood of GBS may be due to: 1) decreased production: decreased production or dysfunction of Treg cells caused by thymic dysfunction in patients with GBS; 2) the abnormal distribution: Treg cells migrated to the nerve root with lesions and locally gathered around small blood vessels, then resulted in a reduction in the number of peripheral blood; 3) abnormal differentiation: a significant increase in inflammatory cytokines such as interleukin-6 (IL-6) as a result of inhibition of the differentiation of Treg cells. $CD4^+CD25^+$ Treg played a negative role in immune regulation through regulating effector T cells, that is, $CD4^+CD25^+$ Treg played roles of direct contact

inhibition and secreting of inhibition cytokines IL-10 and TGF-β by CTLA-4 and glucocorticoid-induced tumor necrosis by cytotoxic factor receptor (GITR) to suppress autoreactive T cell proliferation and activation and played a negative role in immunoregulatory. Thus, the decline number of or function for Treg cells with immune suppression perhaps resulted in immune regulation dysfunction in GBS patients, inhibited effector T cell function decline and triggered an autoimmune response, caused immune damage, finally lead to the occurrence of the disease [4, 27].

$CD4^+CD25^+$ Treg cell was an important factor in maintaining immune homeostasis itself. The treatment of autoimmune diseases by inducing autologous exogenous $CD4^+$ T cells for the $CD4^+CD25^+$ Treg cells in vitro and transferred into the patients has been confirmed in animal experiments and some autoimmune diseases [11-14, 28]. Clinical widely use of $CD4^+CD25^+$ Treg had an important role in autoimmunity, transplantation immunology, infectious immunity, tumor immunology and reproductive immunology. However, the mechanisms and activation pathways of $CD4^+CD25^+$ Treg in disease was not entirely clear, which required the joint efforts of colleagues, to find a new theory for the clinical application of $CD4^+CD25^+$ Treg in autoimmune diseases.

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Disclosure of conflict of interest

None.

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