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Short communication

Intravenous immunoglobulin promotes the proliferation of $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells and the cytokines secretion in patients with Guillain-Barré syndrome *in vitro*

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ABSTRACT

Intravenous immunoglobulin (IVIg) serves as the first line therapy in Guillain-Barré syndrome (GBS), however, its action mechanism remains unknown. We hereby stimulated peripheral blood mononuclear cells (PBMCs) from patients with GBS and healthy controls using IVIg and an IgG-derived natural Treg epitopes, namely Tregitopes. Our results showed that IVIg significantly promoted both the expansion of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) and secretion of IL-10 and TGF-β1 while Tregitopes promoted secretion of IL-10 and TGF-β1 only. Further study is necessary to elucidate the molecular mechanism of IVIg and Tregitopes on Tregs and the secretion of IL-10 and TGF-β1 in GBS.

1. Introduction

Guillain-Barré syndrome (GBS) is a post-infectious autoimmune disease affecting the peripheral nervous system (Shahrizaila and Yuki, 2011). $CD4^+CD25^+$ regulatory T cells (Tregs) is essential for maintaining the immune tolerance and was reported to decrease in the peripheral blood during early stage of both GBS and its animal model (Wang et al., 2014). The clinical recovery of the patients was accompanied by the restoration of the Tregs (Chi et al., 2007). Strategies to increase the amounts of Tregs have been explored for the treatment of GBS (Zhang et al., 2013).

Intravenous immunoglobulin (IVIg), a poly specific immunoglobulin G preparation from large pools of donor plasma, serves as the first line therapy in GBS. There was significant increase of Tregs frequency in patients with GBS after the IVIg treatment (Maddur et al., 2014), which suggests a role of IVIg in treatment of this disease. In 2008, an IgG-derived natural Treg epitopes, namely Tregitopes were reported, which bind to MHCII and promote the expansion of Tregs (De Groot et al., 2008). *In vivo* administration of Tregitopes with strong adjuvants, such as complete Freund's adjuvant does not induce Th1 or Th2 cytokine expression (Su et al., 2013). Tregitopes have been considered as a novel immunomodulatory approach for the suppression of immune responses as well as for treatment of autoimmune diseases (Su et al., 2013). We hereby investigated effects of IVIg and Tregitopes on Tregs from patients with GBS and the healthy donors *in vitro*.

2. Materials and methods

2.1. Patients and samples

A total of 18 patients fulfilled with the criteria of GBS (Asbury and Cornblath, 1990) and 18 of age, sex-matched healthy donors were recruited into this study. Our study received prior approval by the local Ethics Committee and written consent from each donor was obtained. For the patients, the blood was collected within 2 weeks after onset (acute phase) before treatment. IgG antibodies to gangliosides (GM1, GM1b, GD1a, GalNAc-GD1a, GD1b, GT1a and GQ1b) were detected as previously described (Wang et al., 2017).

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Table 1
Demographic and clinical features of patients with Guillain-Barré syndrome.

No.	Sex/Age	Antecedent infection	GBS-DS[a] at entry	Nerve conduction studies[b]	IgG antibodies to	Treatment
1	F/64	URTI	3	Demyelinating	–	ST
2	M/55	–	1	Equivocal	–	PE
3	M/43	URTI	4	Equivocal	GD1a, GD1b	IVIg
4	M/43	URTI	1	–	–	ST
5	F/74	–	4	Equivocal	GD1a, GD1b, GalNAc-GD1a	PE
6	M/62	–	3	Demyelinating	–	IVIg
7	F/81	–	4	Axonal	–	ST
8	F/60	–	4	–	GM1, GD1b	IVIg
9	M/70	–	3	Demyelinating	GM1, GD1b	IVIg
10	F/55	–	5	–	GM1, GD1b	PE
11	F/46	URTI	1	Equivocal	GD1b, GQ1b	PE
12	M/61	–	2	Axonal	–	ST
13	F/28	Diarrhea	4	Demyelinating	GM1, GD1a, GD1b	IVIg
14	F/60	–	1	Demyelinating	GM1	ST
15	M/48	Diarrhea	4	Axonal	GD1a, GD1b, GalNAc-GD1a	PE
16	M/44	–	2	Demyelinating	GM1	PE
17	F/76	–	3	Axonal	–	PE
18	F/50	–	4	Equivocal	–	ST

URTI, Upper respiratory tract infection.
ST, supportive treatment; IVIg, intravenous immunoglobulin; PE, plasma exchange.
[a] GBS-DS, Guillain-Barré syndrome disability score (Fokke et al., 2014).
[b] (Hadden et al., 1998).

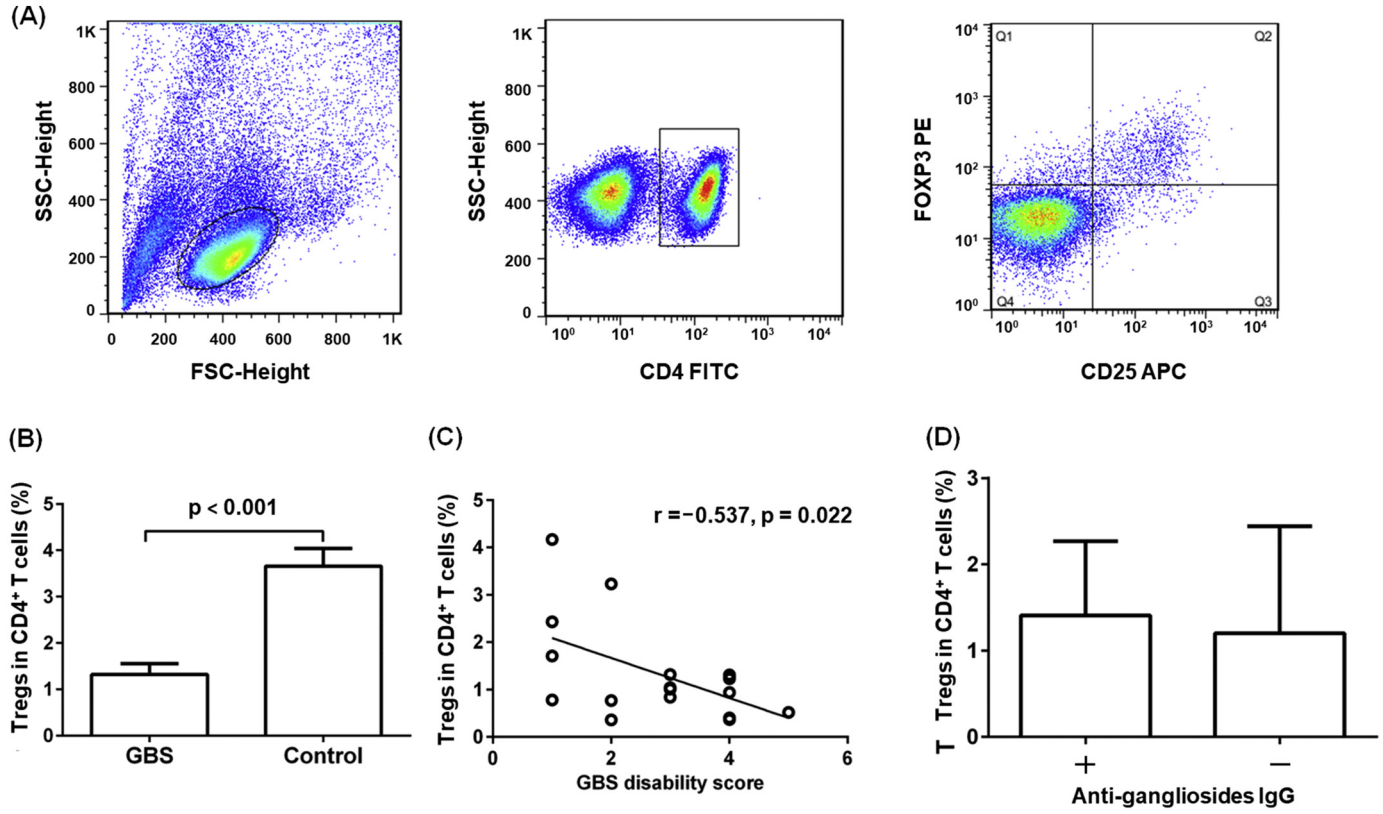

Fig. 1. Reduced Tregs in peripheral circulation of patients with GBS during the acute phase.
(A) R1 = lymphocyte; R2 = $CD4^+$ T cells; Q2 = Treg cells ($CD25^+Foxp3^+$). (B) A significant reduced of Tregs in peripheral circulation of patients with GBS at the acute phase than healthy controls. (C) Spearman's correlation analysis showed that percentage of Tregs was negatively correlated with the disability scores of patients with GBS at entry. (D) There was no difference in percentage of Tregs between anti-gangliosides IgG positive and negative group.

2.2. PBMCs preparation and intervention

About 10 ml of venous blood from each subject was collected into heparin-anticoagulated vacuum tubes. 100 µl of the whole blood was directly used for Tregs analysis by flow cytometry and the other blood were used for the PBMCs preparation as previously described (Wang et al., 2013). The PBMCs were seeded into a 6-well culture plate (2×10^6 per well) and cultured with RPMI 1640 medium containing 10% of fetal bovine serum, 50 U/ml-50 µg/ml of penicillin-streptomycin. The cells were cultured with anit-CD3 (5 µg/ml), anti-CD28 (2 µg/ml) and recombinant IL-2 (10 µg/mL) (Abcam, Cambridge, USA) for 72 h and further divided into different groups with different treatment. For Lipopolysaccharide (LPS) and IVIg group, the PBMCs were stimulated by LPS (1 mg/ml) (Sigma, MO, USA) and IVIg (Hualan

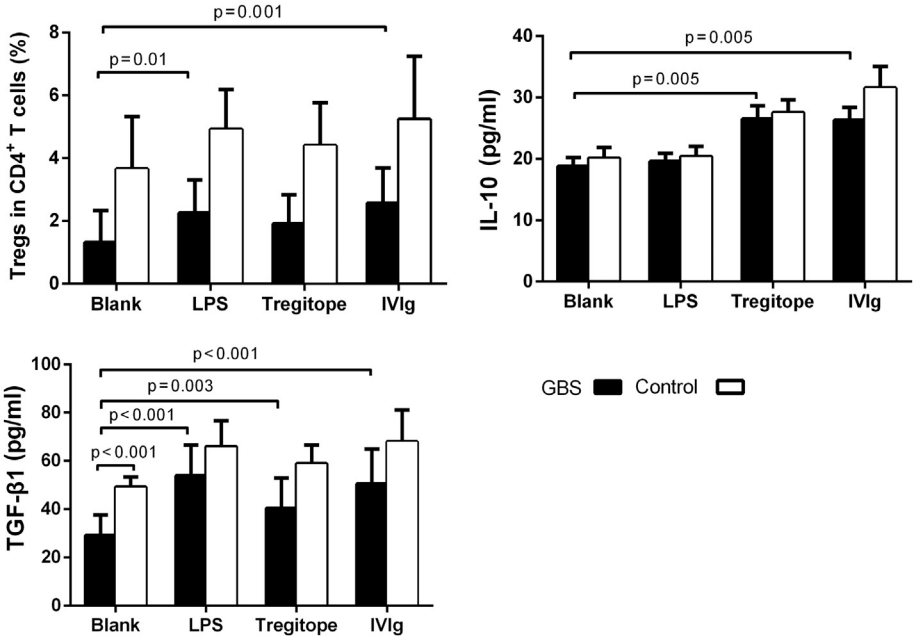

Fig. 2. Effects of IVIg and Tregitopes on expansion of Tregs and secretion of TGF-β1 and IL-10 in cultured PBMCs.
The PBMCs from both patients with GBS and the controls were divided into blank group, LPS group, IVIg group and Tregitope group. The cells and supernatants were harvested for the analysis of Tregs, IL-10 and TGF-β1. (A) The frequency of Tregs in LPS group and IVIg group was significantly higher than that in the blank group from both GBS patients and the controls. There was no difference in frequency of Tregs between Tregitope group and the blank group. (B)The levels of IL-10 in IVIg group and Tregitope group were significantly higher than those in the blank group. There was no difference in levels of IL-10 between LPS group and the blank group. (C) The levels of TGF-β1 in LPS group, Tregitope group and IVIg group were significantly higher than those in the blank group. In blank group, there were higher levels of TGF-β1 in culture of PBMCs from healthy controls than GBS patients. Representative results from experiments were shown as mean ± standard deviation.

Biological, Henan, China) (10 mg/ml), respectively. For Tregitope group, Tregitope 167 and 289 described as previously described (De Groot et al., 2008) were synthesized by 21st Century Biochemicals (Marlborough, MA, USA) (purity > 80%) and equally mixed as the stimulus (50 μg/ml). The medium was changed every 3 days and on day 7, the cells and supernatants were respectively harvested for the analysis of Tregs, IL-10 and TGF-β1.

2.3. Flow cytometry

The antibodies or their isotype controls used below were purchased from BD Biosciences (San Jose, CA). In brief, 100 μl of the whole blood or 1×10^6 PBMCs (50 μl–100 μl) were incubated with a cocktail containing anti-human CD4 (FITC, clone OKT4) (FITC mouse IgG2b K isotype control), CD25 (APC, clone BC96) (APC mouse IgG1 K isotype control), for 30 min at room temperature (RT) in the darkness. After wash, the cells were incubated with anti-human Foxp3 (PE, clone 236A/E7) (PE mouse IgG1 K isotype control) at RT for 30 min in the darkness. About 3–5 $\times 10^4$ $CD4^+$ cells/sample were acquired to analyse the percentage of $CD4^+CD25^+Foxp3^+$ cells using a FACS Calibur (BD Biosciences).

2.4. ELISA

The levels of secreted IL-10 and TGF-β1 were determined using ELISA kits (Abcam) as per instruction. The culture supernatants and standards were respectively submitted to appropriate well for incubation overnight at 4 °C with gentle shaking. After wash, biotinylated detection antibodies were added and incubated for one hour at RT with gentle shaking. Then, HRP-Streptavidin solution was added and incubated for 45 min at RT with gentle shaking. Finally, TMB substrate reagent was added to visualize the binding of IL-10 and TGF-β1. The absorbance at 450 nm was measured using a ChroMate® Microplate Reader (Awareness, Palm City, USA). The final results were determined according to the standard curve.

2.5. Statistical analysis

Continuous and normally distributed data were analysed using independent samples *t*-test or one-way analysis of variance. The relationship between the percentage of Tregs and the GBS disability score (GBS-DS) at entry (Fokke et al., 2014) was analysed by Spearman's correlation. Analysis was performed with the SPSS 20.0 analysis software by IBM (Armonk, NY), and a p value of < 0.05 was considered significant.

3. Results

The details of patients with GBS were shown in Table 1. The percentage of Tregs in patients with GBS at the acute phase (1.32 ± 0.99) was significantly less than those in the healthy controls (3.65 ± 1.63) ($p < .001$). The percentage of Tregs was negatively correlated with the GBS-DS at entry ($r = -0.537$, $p = .02$) (Fig. 1).

As shown in Fig. 2, for the PBMCs from patients with GBS, there was higher frequency of Tregs in both LPS group and IVIg group than the blank group ($p = .01$ and $p = .001$, respectively). For PBMCs from healthy controls, there was higher frequency of Tregs in both LPS group and IVIg group than the blank group ($p = .013$ and $p = .012$, respectively). There was no difference in frequency of Tregs between Tregitope group and the blank group in PBMCs from both GBS patients and the controls. In the supernatants of cultured PBMCs from both GBS patients and the controls, there were higher levels of TGF-β1 in LPS group, Tregitope group and IVIg group than the blank group. In blank group, there were higher levels of TGF-β1 in culture of PBMCs from healthy controls than GBS patients. There were higher levels of IL-10 in both Tregitope group and IVIg group than blank group. There was no difference in levels of IL-10 between LPS group and blank group.

4. Discussion

We hereby demonstrated the direct effects of IVIg on the proliferation of Tregs and the cytokine secretion in cultured PBMCs from the patients with GBS. Tregs play a critical role in the maintenance of host immune tolerance and preventing the autoimmunity conditions (Sakaguchi et al., 2008). Dysfunction of Tregs or their insufficient number contributes to the autoimmune and inflammatory conditions, whereas expansion of Tregs or recovery of their functions ameliorates the autoimmune diseases (Sakaguchi et al., 2008). The frequency of Tregs in peripheral circulation of patients with GBS was negatively correlated with the clinical severity of the patients. The reduced Tregs may be insufficient to maintain the immune homeostasis and result in the autoimmune response in patients with GBS. The IgG antibodies

against the gangliosides have been frequently presented in the patients with GBS, especially the axonal subtypes (Koga et al., 2003). In this study, there was no difference in the percentage of Tregs in patients with and without anti-gangliosides IgG antibodies, which may be caused by our limited samples and deserve further confirm by larger cohort studies. Notably, there was low frequency of patients with antecedent infection, which may be due to our small sample size.

IVIg may expand the Tregs by modulating the functions of antigen presenting cells, especially the dendritic cells (Trinath et al., 2013). IVIg internalized into the dendritic cells may be further processed to the tolerogenic fragment, such as Tregitopes, or the pro-inflammatory fragment, which promote the expansion of Tregs or other effector T cells (De Groot et al., 2008).In this study, we tried different concentration of Tregitopes and gradient-time culture of PBMCs; however, no effects of Tregitopes on expansion of Tregs *in vitro* were observed. High expression of TGF-β1 and IL-10 are important to maintain the suppressive effects of Tregs (Li et al., 2018). IVIg could increase the cytokines secretion and pathways in order to create tolerance (Maddur et al., 2014). Our results demonstrated that IVIg could enhance the secretion of TGF-β1 and IL-10 in cultured PBMCs from GBS and the controls. Notably, although no effects of Tregitopes on expansion of Tregs, the Tregitopes significantly promoted the levels of TGF-β1 and IL-10 in culture of PBMCs. Being different from previous study, our study demonstrated that Tregitopes displayed no effects on expansion of Tregs but enhanced the tolerogenic functions of this T cells subpopulation, which deserves further study to clarify the mechanism.

To sum up, our study confirmed that insufficient Tregs was related with the clinical severity of the patients with GBS. IVIg exerted its therapeutic effects in GBS *via* promoting the expansion of Tregs and the secretion of TGF-β1 and IL-10. It is necessary to investigate whether IVIg acts in other autoimmune diseases *via* similar mechanism. Tregitopes also promoted secretion of TGF-β1 and IL-10 in cultured PBMCs; however, its potentials in GBS deserve more investigation using experiments *in vivo* and *vitro*.

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Declaration of competing interest

The authors declare no conflicts of interests.

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