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Effect of low-dose rituximab treatment on T- and B-cell lymphocyte imbalance in refractory myasthenia gravis

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ABSTRACT

We aimed to explore the effects of low-dose rituximab (RTX) on circulating T- and B-cell lymphocytes and the improvement of clinical symptoms in refractory myasthenia gravis (MG) patients. Fifteen patients with refractory MG were treated with a low dose of 600 mg RTX and were evaluated by serial-clinical scales, flow cytometry of peripheral blood T and B cells, and antibody titer before and after six months of RTX treatment. The quantitative MG score (QMGS), manual muscle testing (MMT), MG-related activities of daily living (MG-ADL) and MG-specific quality-of-life (QOL) were significantly improved and the average steroid-dosage reduction was 40% (p = .001) in refractory MG patients at six months after RTX infusion. Compared to eighteen non-refractory MG patients and eighteen Healthy controls, our study showed that the frequencies of circulating regulatory B cells (Bregs) and regulatory T cells (Tregs) were significantly lower and the expression of B-cell activating factor receptors (BAFF-Rs) was greater in refractory MG patients without RTX treatment. Importantly, 600-mg RTX was sufficient to deplete B cells and maintain low B-cell counts for up to six months after infusion. Additionally, a low dose of RTX further increased the frequencies of Tregs. Hence, there is an immune imbalance in circulating T- and B-cell lymphocytes in refractory MG patients compared to non-refractory MG patients. We conclude that remarkable T- and B-cell lymphocyte imbalance exists in refractory MG. Low-dose RTX can improve myasthenic symptom and deplete B cells and increase Tregs%.

1. Introduction

Myasthenia gravis (MG) is a rare autoimmune disorder of the neuromuscular junction that is characterized by fluctuating muscle weakness and fatigue (Lindstrom, 2000). Autoantibodies against the acetylcholine receptor (AChR), muscle-specific kinase (MUSK), and lipoprotein-related protein 4 (LRP4) are involved in the pathogenesis of MG (Verschuuren et al., 2013).

MG is usually treated effectively with glucocorticoids combined with traditional immunosuppressive (IS) agents, such as azathioprine, tacrolimus or mycophenolate mofetil. Nonetheless, 10%–15% of patients will develop to refractory MG, which fails to respond or accepts conventional IS therapies or induces adverse reactions (Silvestri and Wolfe, 2014). In the past decade, rituximab (RTX, a chimeric monoclonal antibody directed against cluster of differentiation [CD] 20^+B cells), has been reported to be successful in treating refractory MG (Díaz-Manera et al., 2012; Dustin et al., 2016; Zaja et al., 2000). Our previous observational study also indicated that 600 mg RTX may be sufficient in depleting B cells and improving the clinical symptoms of MG. RTX is assumed to act by depleting the precursors of plasma cells, although the exact mechanism remains unclear (Marino et al., 2018). It is currently believed that B-cell depletion could affect the activity of B cells, as well as the interaction of T and B lymphocytes.

B cells not only produce antibodies, but also participate in antigen presentation (conventional/effector B cells) and immune regulation (regulatory B cells, Bregs) (Krumbholz and Meinl, 2014). Human Bregs regulate macrophage and dendritic cell function, suppress proliferation of CD4+ T cells, and enhance expression of regulatory T cells (Tregs). In humans, the surface-marker phenotypes of Bregs include $CD19^+CD1d^+CD5^+$, $CD19^+CD24^+CD38^+$, and $CD19^+CD24^+CD27^+$ (Iwata et al., 2011; Kessel et al., 2012; Yoshizaki et al., 2012). The latter subset shares common cell-surface markers with memory B cells (Bmems), with approximately 60% expressing CD38. In our present study, we focused on $CD19^+CD1d^+CD5^+$ and $CD19^+CD24^+CD38^+$

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subsets in refractory MG patients accepting RTX treatment.

$CD4^+CD25^+$ Tregs regulate T-helper (Th) and B-cell immune responses and play a central role in maintaining immunological tolerance (Shin et al., 2015). The transcription factor Foxp3 has been found to be highly expressed in $CD4^+$ Tregs that play a critical role in maintaining self-tolerance and regulating immune responses (Sakaguchi et al., 2006). Several studies have demonstrated that Tregs are numerically and functionally impaired in MG patients, suggesting a key role for Treg abnormalities in MG pathogenesis (Balandina et al., 2005; Zhang et al., 2009). Furthermore, the Treg population is significantly lower in the active stage of MG (Nishimura et al., 2015).

In previous research, the presence and dynamics of B and T cells have not been clear in refractory MG patients. In this present study, we aim to explore the status of the immune balance between B and T cells in refractory and non-refractory MG patients compared with that of healthy controls, and the alteration of the immune balance after treatment with low-dose RTX, to better clarify the immunologic mechanism behind and clinical efficacy of low-dose RTX in the treatment of refractory MG.

2. Materials and methods

2.1. Patients and controls

In this present study, a total of thirty-three MG patients were recruited from 2014 to 2017 at Huashan Hospital, Shanghai Medical College, Fudan University, China. All of the patients met the following criterias: (1) age ≥ 18 years, and (2) definitive diagnosis of generalized MG. The clinical characteristics of the 33 MG patients are summarized in Table 1. All of the patients with a laboratory-confirmed MG autoantibody (31 anti-AChR, 1 anti-MUSK, and 1 dSN-MG) were divided into two groups: group A, refractory MG; group B, non-refractory MG. Eighteen healthy controls (HC) were recruited and were matched to the patients in terms of age, gender, and demographics , consisted of five males and thirteen females with a mean age of 31.6 ± 9.7 years.

The refractory MG group (group A) comprised 15 patients (Nos. 1–15) defined as being refractory based on exhibiting one of the followings: (1) having an unsatisfactory response to two immunomodulatory agents, at least one of which was prednisone/prednisolone, (2) unacceptable adverse reactions to conventional treatments, (3) requirement for repeated treatment with intravenous immunoglobulin(IVIG) or plasma exchange, or (4) frequent myasthenic crises (Silvestri and Wolfe, 2014).

The mean age of refractory MG patients in this present study was 34.4 ± 13.1 years. The average duration of the disease before RTX treatment was 57.3 ± 32.8 months (Table 2). Among them, the first eight patients (Table 1) were the same patients reported in our previous study (Jing et al., 2017).

The non-refractory MG group (group B) comprised 18 patients (Nos. 16–33) who responded well to conventional immunomodulatory therapies and for which their post-intervention status (PIS) was of a minimal manifestation status (MMS) or better. The mean age of non-refractory MG patients in this present study was 44.3 ± 13.9 years. The average duration of the disease was 43.1 ± 51.4 months (Table 2).

We analyzed peripheral blood samples from 33 MG patients and 18 healthy donors at Huashan Hospital, Shanghai Medical College, Fudan University. Clinical and laboratory assessments were performed at baseline and six months after RTX treatment. Evaluation included the follows: quantitative MG score (QMGS), MG-specific manual muscle testing (MMT), MG-related activities of daily living (MG-ADL) scale, and MG-specific quality-of-life (QOL)-15.

Thymic abnormalities were found in 11 patients who had undergone a thymectomy (one had hyperplastic thymus, while the other 10 had a thymoma). The patients did not have other autoimmune diseases, ongoing infection or malignancies. Blood samples from 18 HCs were obtained from those who were not receiving any treatment and absence of diseases.

The regimen of RTX infusion was a total dose of 600 mg, as used in our previous study (Jing et al., 2017), and was administered as follows: 100 mg on the first day (day 1) and 500 mg on the second day (day 2).

Written informed consent was obtained from each participant. The study was approved by the Medical Ethics Committee of Huashan Hospital, Shanghai Medical College, Fudan University.

2.2. Immunophenotyping by flow cytometry analysis

The whole-blood samples were collected in sterile heparin tubes during check-up visits. Peripheral blood mononuclear cells (PBMNCs) were separated by Ficoll density-gradient centrifugation and PBMNCs layers were resuspended in PBS at a concentration of 1×10^6 cells/mL.

Within 60 min of the blood draw, whole-blood samples were immunostained with monoclonal antibodies (mAbs) and the appropriate isotype controls for 30 min at 4 °C in the dark. T and B cells were analyzed for expression of surface markers using the following fluorescently labeled antibodies: anti-CD19-PE-Cy5, anti-CD27-PE, anti-BAFF-R-PE, anti-CD24-PE, anti-CD38-FITC, anti- CD5-PE and CD1d-FITC (eBioscience, San Diego, CA, USA); anti-CD3 PE-Cy5/CD4 PE/CD8 FITC Cocktail (BioLegend, San Diego, CA, USA). These surface markers were used to calculate the percentage of $CD19^+$B cells, $CD27^+$ Bmems, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD24^+CD38^+$, $CD5^+CD1d^+$ Bregs.

For Treg-staining assays, PBMNCs were washed three times with PBS and a Human Regulatory T Cell Staining Kit (eBioscience, San Diego, CA, USA), which included the use of FITC CD4, PE CD25, and PE-Cy5 FOXP3. Tregs were analyzed according to the manufacturer's instructions. The percentage of $FOXP3^+$Tregs ($CD4^+CD25^+FOXP3^+$) was counted in the $CD4^+$ population.

The samples were immediately analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA).

2.3. MG autoantibody detection

Serum samples were obtained from 18 non-refractory MG and 15 refractory MG patients before and after six months of administration of RTX. For measurement of serum MG autoantibodies, sera were immediately separated from whole blood by centrifugation (3000 rpm, 10 min) and stored at −20 °C. Serum AChR-autoantibody titers were evaluated with an enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (RSR Limited, Cardiff, UK) and quantified by reference to standard curves. The results are expressed as nmol/L of AChR protein bound, and positivity was defined as ≥0.45 nmol/L.

Serum MuSK antibody was tested by ELISA (IBL International GMBH, Hamburg,Germany), using a high sensitive protocol (range 0.4–12 U/mL).

2.4. Statistical analysis

Statistical analyses were conducted with SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA), and figures were generated using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Paired Student's *t*-test or non-parametric Wilcoxon test was used to compare the experimental data from patients pre- and post-RTX treatment. Unpaired Student's t-test or Mann–Whitney *U* tests was used for all of the other comparisons among two independent groups. Results are expressed as mean ± SD. A statistical probability of $p < .05$ was considered significant. Significance was defined as $P < .05$ and is expressed in the individual figures as $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ and $^{****}P < .0001$.

Table 1
Characteristics of enrolled MG patients.

Patient No	Sex	Age (years)	Disease duration (months)	All previous therapies	Maximum disease severity (MGFA)	Postintervention status (MGFA)*	AChR-ab(nmol/L)	
							Pre RTX	Post RTX
1	F	25	96	Pyrid, Pred, AZA, Tacro, IVIg	IIIb	I	11.1	4.7
2	F	37	68	Pyrid, Pred, Cyclo, Tacro, IVIg, Tx (thymoma)	VIb	I	15.9	15.7
3	F	19	62	Pyrid, Pred, Cyclo, Tacro, IVIg, Tx(Thymic hyperplasia)	IIIb	I	11.5	12.5
4	F	61	6	Pyrid,Pred,Tacro, IVIg,PE	IIb	MM-3	3.0	3.3
5	M	30	48	Pyrid,Pred,AZA , Tx(thymoma)	IIIb	I	11.6	9.4
6	F	36	103	Pyrid,Pred,IVIg,Tacro, AZA,Cyclo,PE, Tx (thymoma)	V	I	7.9	3.7
7	F	32	17	Pyrid,Pred,AZA,Tacro, IVIg,Tx(thymoma)	VIb	I	5.89	4.38
8	F	40	76	Pyrid,Pred,AZA,Tacro, Tx(thymoma)	IIIb	I	2.61	1.95
9	F	32	98	Pyrid,Pred,Cyclo,Tacro, IVIg	IIIb	U	8.01	8.01
10	F	54	98	Pyrid,Pred,Cyclo, AZA, IVIg, Tx(thymoma)	IIb	U	7.12	5.13
11	F	50	50	Pyrid, Pred, Cyclo, AZA, Tacro, IVIg, Tx (thymoma)	IIIb	I	8.02	7.23
12	F	35	22	Pyrid, Pred, AZA, Tacro	IIb	MM-3	——[a]	——[a]
13	F	24	33	Pyrid, Pred, AZA, Tacro, Tx(thymoma)	VIb	I	7.90	6.71
14	F	11	20	Pyrid, Pred, Tacro, MMF, IVIg	IIIb	I	7.90	7.34
15	F	30	62	Pyrid, Pred, AZA, Tacro	IIIb	U	8.0	6.6
16	M	57	19	Pyrid, Pred	IIa	MM-3	——[b]	
17	F	40	48	Pyrid, Tacro	IIb	MM-1	6.32	
18	F	59	24	Pyrid, Pred, AZA	IIIb	MM-3	1.30	
19	M	46	15	Pyrid, Pred	IIb	CSR	2.79	
20	F	26	21	Pyrid, Pred	IIb	MM-1	3.28	
21	F	27	191	Pyrid, Pred, Tacro, IVIg	IIb	MM-3	7.67	
22	M	65	25	Pyrid, Pred, AZA	IIb	MM-3	8.02	
23	F	53	15	Pyrid, Pred	IIb	MM-3	3.70	
24	M	55	39	Pyrid, Pred, AZA	IIIb	MM-3	7.12	
25	F	54	28	Pyrid, Pred, Tacro	IIa	MM-3	6.42	
26	F	50	23	Pyrid, Pred, Tacro	IIb	MM-3	5.21	
27	F	34	25	Pyrid, Pred, AZA	IIb	MM-1	6.81	
28	F	51	174	Pyrid, Pred, Tx(thymoma)	IIa	MM-3	6.91	
29	M	43	23	Pyrid, Pred	IIIa	MM-3	5.06	
30	M	61	31	Pyrid, Pred	IIb	MM-3	4.23	
31	M	26	19	Pyrid, Pred	IIb	MM-3	7.79	
32	F	23	23	Pyrid, Pred	IIIb	MM-3	4.84	
33	F	27	33	Pyrid, Pred, AZA , Tx(thymoma)	IIb	MM-3	7.02	

Abbreviations: AZA = Azathioprine; Cyclo = Cyclophoshamide; IVIg = Intravenous Immunoglobulin ; Pred = Prednisone; PE = Plasma Exchange; Pyrid = Pyridostigmine ; Tacro = Tacrolimus ; Tx = Thymectomy; CSR = Complete Stable Remission; I = Improved (MGFA-PIS); MM = Minimal Manifestations; U = Unchanged.

[a] The antibody of MG patient Nos. 9 was MUSK antibody, the titer before RTX treatment was 12 U/mL, and 0.34 U/mL after treatment.

[b] Non-refractory MG patient Nos. 16 with double seronegative MG (dSN-MG), who was negative for anti-AChR and anti-MuSK antibodies.

* :pt 1–15 was defined as the status at six months after 600 mg RTX.

3. Results

3.1. The proportion of Bregs and Bmems

Before RTX treatment, we firstly quantified the proportions of the total $CD19^+$B cells of $CD27^+$ Bmems and Bregs in the refractory MG, non-refractory MG, and HC groups.

No significant difference in total $CD19^+$ B cell percentages was found between groups (refractory MG, 10.9 ± 6.1%; non-refractory MG, 9.3 ± 3.7%; HC, 11.9 ± 3.5%; Fig. 1A). The proportions of $CD27^+$ Bmems out of the total $CD19^+$ B cells were significantly increased in refractory MG patients (38.8 ± 14.6%) compared with those in the HC group (26.6 ± 10.1%, $P < .05$). Similarly, the increase in the proportions of $CD27^+$ Bmems in non-refractory MG patients (36.8 ± 14.4%) was still significant compared to that in the HCs ($P < .05$). The percentages of Bmem levels were not significantly different between refractory and non-refractory MG patients (Fig. 1B).

There was an obviously reduced expression of $CD19^+CD5^+CD1d^+$ Bregs out of the total $CD19^+$ B cells in the refractory MG (20.3 ± 2.5%; $P < .0001$) and non-refractory MG patients (24.1 ± 3.4%; $P = .001$) compared with the HCs (28.0 ± 3.3). Meanwhile, the percentages of $CD19^+CD5^+CD1d^+$ Bregs were significantly lower in refractory MG patients compared with those in non-refractory MG patients (Fig. 1C). A comparison of the percentages of $CD19^+CD24^+CD38^+$ Bregs out of the total $CD19^+$ B cells between groups produced essentially the same results (Fig. 1D). The percentages of $CD19^+CD24^+CD38^+$ Bregs were significantly lower in the refractory MG patients (0.2 ± 0.6%; $P < .0001$) and non-refractory MG patients (1.9 ± 4.2%, $P < .0001$) compared with those in the HC group (6.1 ± 2.7%).

3.2. The expression of BAFF-R on peripheral blood B cells

The percentages of BAFF-R $^+$B cells out of the total $CD19^+$ B cells were significantly lower in non-refractory MG patients (85.2 ± 20.9%) than in refractory MG patients (97.5 ± 2.5%; $P = .003$) or HCs (94.6 ± 9.8%; $P = .011$). The percentages of BAFF-R^+B cells in refractory MG patients did not differ significantly from those in the HC group. (Fig. 1E).

3.3. The proportions of Tregs

The percentages of Tregs out of the $CD4^+$ T cells were significantly lower in the refractory MG patients (1.9 ± 1.0%) than in the non-

Table 2
Comparison of selected attributes of patients with refractory and non-refractory myasthenia gravis.

	Group A refractory MG	Group B non-refractory MG	P
Gender (male/female)	1/14	7/11	< 0.05
Age(years):mean ± SD (range)	34.4 ± 13.1 (11–61)	44.3 ± 13.9 (23–65)	< 0.05
Disease duration(months)	57.3 ± 32.8	43.1 ± 51.4	> 0.05
MGFA: number			
I	0	0	
IIa	0	3	
IIb	3	11	
IIIa	0	1	
IIIb	8	3	
IVa	0	0	
IVb	3	0	
V	1	0	
QMGS (mean ± SD)	15.7 ± 4.9	4.1 ± 2.3	< 0.05
MMT (mean ± SD)	22.7 ± 18.1	0.2 ± 0.6	< 0.05
MG-ADL (mean ± SD)	8 ± 3.9	0.2 ± 0.5	< 0.05
MGQOL-15 (mean ± SD)	35.1 ± 12.7	5.6 ± 6.8	< 0.05
Thymectomy: histology			
Thymoma	8	2	
Thymic hyperplasia	1	0	

refractory MG patients (3.4 ± 1.3%; $P < .001$) or HCs (3.6 ± 1.5%; $P < .001$). The percentages of Treg levels were not significantly different between non-refractory MG patients and HCs. (Fig. 1F) We have observed a significant negative correlation between the percentage of Tregs and QMG score in RTX treated patients ($r = -0.370$; $P = .044$; Fig. 2). The increase of Tregs% in refractory MG was significantly correlated to the improvement of MGFA-QMG scores.

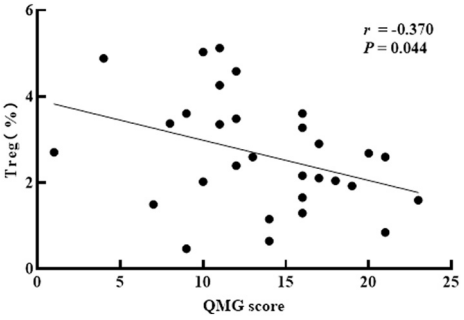

Fig. 2. Correlation between percentage of Tregs and QMG score in RTX treated MG patients. (Pearson rank test).

3.4. Effect of RTX on T and B cells in the refractory MG group

Compared with the baseline, a remarkable decrease in percentage of circulating $CD19^+$B cells was observed (−10.79% at six months, $P < .0001$) after administration of RTX in refractory MG patients (Fig. 1A). Similar results were seen in the percentage of circulating $CD27^+$ Bmems ($P < .0001$), $CD19^+CD5^+CD1d^+$ Bregs ($P < .0001$) and BAFF-R^+B cells ($P < .0001$) when compared with that of the baseline (Fig. 1B, C, E).

There were no major impacts on the mean percentage of T cells ($CD3^+$, $CD4^+$ and $CD8^+$) after administration of RTX (Supplementary Fig. 1). A significant increase in the percentage of Tregs in $CD4^+$ T cells was noticed at six months ($P < .001$) after infusion compared with that of the baseline (Fig. 1F).

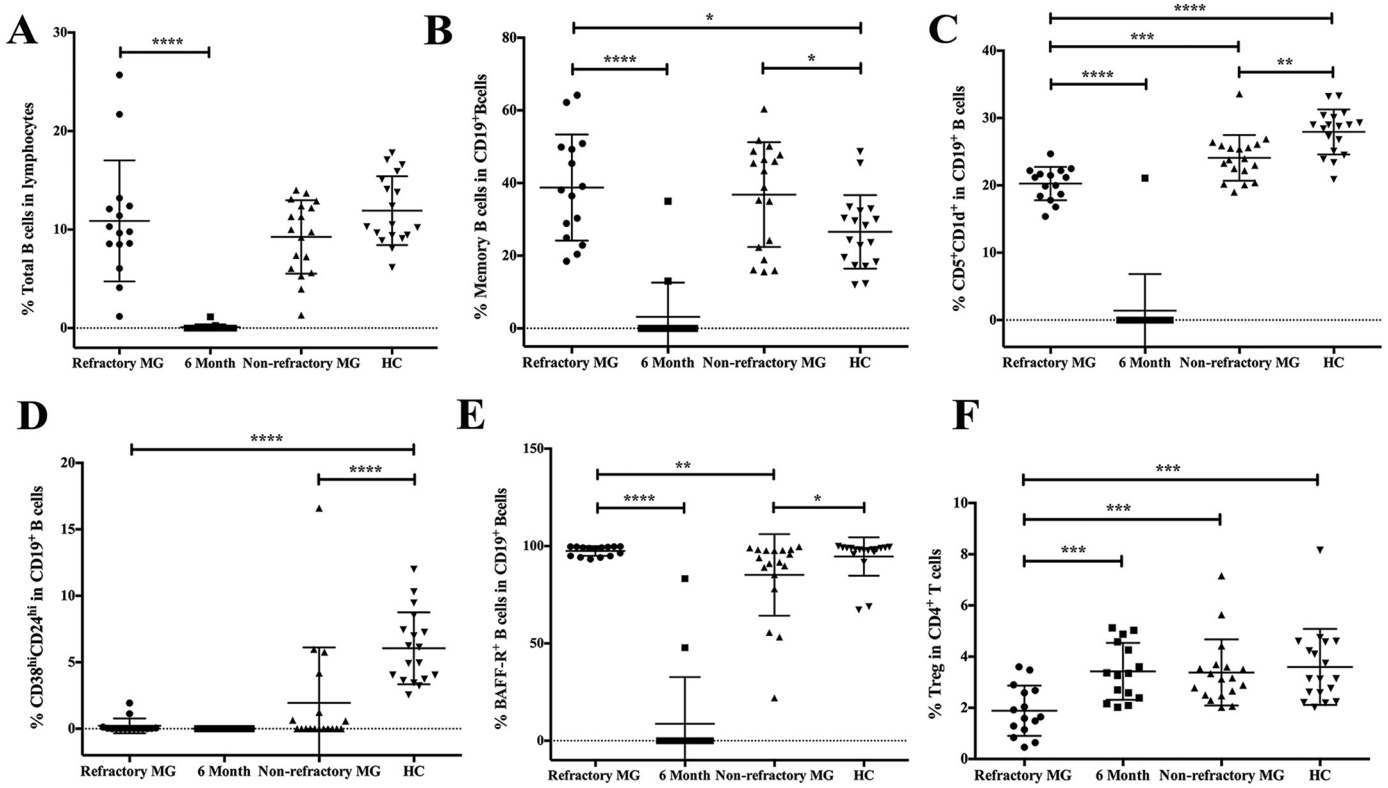

Fig. 1. The frequencies of lymphocytes in enrolled three different groups and their changes pre- and post-RTX treatment in refractory MG patients. (A) the $CD19^+$B cells % out of total lymphocytes; (B) the $CD27^+$ Bmem % out of total B cells; (C) the $CD19^+CD5^+CD1d^+$Breg % out of total B cell; (D) the $CD19^+CD24^+CD38^+$Breg % out of total B cell; (E) the BAFF-R^+B cells % out of total B cells; (F) the Treg % out of the $CD4^+$T cells. Horizontal bars indicate refractory MG group before and after 6 months of RTX treatment, non-refractory MG group and healthy controls group. Vertical bars indicate the percentages of cell subsets. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

Table 3
Changes of clinical scores and immunological indicators in refractory MG, non-refractory MG and healthy controls.

	Group A refractory MG					Group B non- refractory MG			Healthy controls		
	Baseline		6 month post-rituximab								
	N	mean ± SD	N	mean ± SD	P[a]	N	mean ± SD	P[b]	N	mean ± SD	P[c]
QMGS	15	15.7 ± 4.9	15	11.2 ± 4.4	0.013	18	4.1 ± 2.3	–	–	–	–
MMT	15	22.7 ± 18.1	15	6.9 ± 6.5	0.004	18	0.2 ± 0.6	–	–	–	–
MG-ADL	15	8 ± 3.9	15	3.6 ± 3.0	0.002	18	0.2 ± 0.5	–	–	–	–
MGQOL-15	15	35.1 ± 12.7	15	23.2 ± 13.1	0.018	18	5.6 ± 6.8	–	–	–	–
CD19+ B cell,%	15	10.9 ± 6.1	15	0.1 ± 0.3	< 0.0001	18	9.3 ± 3.7	0.74	18	11.9 ± 3.5	0.29
CD27 + memoryB cell, %	15	38.8 ± 14.6	15	3.2 ± 9.4	< 0.0001	18	36.8 ± 14.4	0.676	18	26.6 ± 10.1	0.012
BAFFR,%	15	97.5 ± 2.5	15	8.7 ± 24.0	< 0.0001	18	85.2 ± 20.9	0.003	18	94.6 ± 9.8	0.426
(CD5 + CD1d+)Breg,%	15	20.3 ± 2.5	15	1.4 ± 5.4	< 0.0001	18	24.1 ± 3.4	< 0.001	18	28.0 ± 3.3	< 0.0001
(CD24 + CD38+)Breg,%	15	0.2 ± 0.6	15	0	0.134	18	1.9 ± 4.2	0.562	18	6.1 ± 2.7	< 0.0001
Treg,%	15	1.9 ± 1.0	15	3.4 ± 1.1	< 0.001	18	3.4 ± 1.3	< 0.001	18	3.6 ± 1.5	< 0.001
CD3+ T cell,%	15	77.5 ± 8.8	15	78.5 ± 12.9	0.801	18	63 ± 12.4	0.001	18	72.5 ± 7.5	0.091
CD4 + T cell,%	15	43.5 ± 8.0	15	43.3 ± 10.5	0.960	18	33.8 ± 12.1	0.011	18	38.8 ± 6.8	0.091
CD8 + T cell,%	15	29.8 ± 10.7	15	30.5 ± 10.2	0.856	18	25.2 ± 9.3	0.199	18	25.9 ± 7.9	0.236
AChR-ab(nmol/L)	14	8.3 ± 3.5	14	6.9 ± 3.7	0.016	17	5.6 ± 2.0	0.005			

[a] Comparison of clinical score and immunological features in15 refractory MG patients, before (Baseline) and after six months of treatment with rituximab.
[b] Comparison between patients with refractory and non-refractory myasthenia gravis, before rituximab treatment.
[c] Comparison between refractory MG patients and healthy controls, before rituximab treatment.

3.5. Efficacy of RTX on the refractory MG group

Fifteen (Nos. 1–15) refractory MG (14 females and one male) patients were treated with low-dose RTX (Tables 1, 3). Of these, ten patients experienced clinical improvements and two patients had minimal residual manifestations at six months after low-dose RTX treatment.

Three patients remained clinically unchanged, but in two out of the three (Nos. 9 and 15) patients, other medications were able to be reduced. The Nos. 9 and 15 patients stopped tacrolimus and their prednisone dose was reduced by 34% and 17%, respectively. Doses of prednisone were reduced by 40% ($P = .001$) at six months in all of the 15 refractory MG patients.

Changes in the QMGS, MMT, MG-ADL, and MG-QOL-15 during low-dose RTX treatment are shown in Fig. 3. At six months, the subjects demonstrated significant improvement in QMGS, MMT, MG-ADL, and MG-QOL-15 changes, which were − 4.53, −15.74, −4.40, and − 11.87, respectively ($P = .013$, $P = .004$, $P = .002$, $P = .018$). The median values for these four disease indicators were 15.7 ± 4.9, 22.7 ± 18.1, 8 ± 3.9, and 35.1 ± 12.7 prior to the beginning of the study and 11.2 ± 4.4, 6.9 ± 6.5, 3.6 ± 3.0, and 23.2 ± 13.1 at the final visit. In the subgroup of refractory MG patients, we also observed a significant improvement in clinical scores and reduction of steroid doses—in eight patients with thymoma and seven patients with non-thymoma—after low-dose RTX treatment. (Table 4).

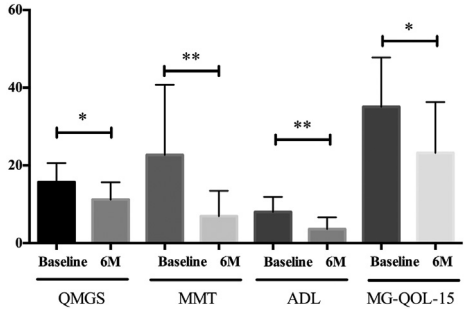

Fig. 3. The changes of quantitative myasthenia gravis (MG) score (QMGS), manual muscle testing (MMT), MG-related activities of daily living (MG-ADL), and MG-specific quality-of-life (MG-QOL) in refractory generalized MG patients after 6 months of RTX treatment. Horizontal bars indicate the different time points and vertical bars indicate the clinical status. *$P < .05$; **$P < .01$, vs. baseline.

There was no difference in clinical score changes and T and B cell subset changes between patients with thymoma and non-thymoma refractory MG after low-dose RTX treatment. No difference was noted in thymectomized and non-thymectomized patients regarding clinical score changes and T and B cell subset changes. (Supplemental Table 1).

No allergic reactions or other serious side effects occurred during the follow-up period. All of the 15 patients tolerated the treatment well.

3.6. The level of AChR autoantibody

The difference of AchR autoantibody levels was significantly decreased in refractory MG patients with thymoma($P < .05$) but not non-thymoma.(Table 4). Importantly, we observed a significant decrease in AChR autoantibody levels in refractory MG gruop at six months (6.9 ± 3.7, $P < .05$) after RTX infusion compared with that of the baseline (8.3 ± 3.5).

4. Discussion

Regarding refractory MG, many studies have demonstrated heterogeneity in disease course and treatment response based on patient-antibody profiles. Patients with anti-MuSK antibodies are much more likely to have refractory disease than those with anti-AChR antibodies (Joome et al., 2013). Possible factors for becoming refractory following treatment include history of thymoma/thymectomy and female sex (Baggi et al., 2013; Maggi et al., 2008). This suggests that refractory MG could have different immunopathologic mechanisms. The mechanism of abnormal T- and B-cell homeostasis and autoreactive B cells in refractory MG remains uncertain.

The balance between pathogen-induced effector function and endogenous tolerance-mediated mechanisms is critical for the host's immune response (Kyewski and Klein, 2006). So far, maintenance of this balance has been mainly attributed to Tregs, Bregs, and Bmems (Belkaid and Tarbell, 2009; Lund, 2008; Mauri and Bosma, 2012). Tregs play critical roles in maintaining immunological self-tolerance by suppressing autoreactive T cells, and the deficiency of Tregs causes autoimmune diseases such as type-1 diabetes, multiple sclerosis, Systemic Lupus Erythematosus, and MG (Buckner, 2010; Masuda et al., 2010; Nishimura et al., 2015; Ohl and Tenbrock, 2015). Many studies have demonstrated defects in Treg function and decreases in the number of CD4 + Tregs in MG and EAMG (Danikowski et al., 2017; Gertel-Lapter et al., 2013). The dysfunction of inhibitory and migratory markers on

Table 4
Comparison of clinical indicators between patients with thymoma and non-thymoma refractory myasthenia gravis .

	Thymoma				Non-Thymoma			
	N	Pre-RTX	Post-RTX mean ± SD	*P* value	N	Pre-RTX	Post-RTX mean ± SD	P value
QMGS	8	16.6 ± 4.8	12.3 ± 3.2	0.012	7	14.7 ± 5.1	10 ± 5.5	0.028
MMT	8	24.3 ± 20.9	6.3 ± 6.0	0.012	7	20.9 ± 15.6	7.7 ± 7.5	0.043
MG-ADL	8	9.6 ± 4.6	4.3 ± 3.4	0.011	7	6.1 ± 1.8	2.9 ± 2.5	0.034
MGQOL-15	8	40.0 ± 10.1	25.8 ± 13.9	0.036	7	29.4 ± 13.7	20.3 ± 12.6	0.063
Prednisone(mg)	8	7.2 ± 1.1	4.2 ± 1.9	0.018	7	5.4 ± 3.7	2.9 ± 1.4	0.028
AChR-ab(nmol/L)	8	8.4 ± 3.9	6.8 ± 4.3	0.012	6	8.3 ± 3.0	7.1 ± 3.2	0.345

Tregs are associated with the pathogenesis of MG (Danikowski, Jayaraman, 2017). During the progression of the disease, little is known about the immunological mechanism responsible for refractory MG, which is considered to be distinct from non-refractory MG (Mantegazza and Antozzi, 2018). Takafumi et al. demonstrated that the percentage of Tregs was significantly lower in the active stage than in the remission stage (Takafumi et al., 2015). In our present study, we observed that the number of Tregs was less in active refractory MG patients than non-refractory MG patients.

B cells not only produce autoantibodies, but actively regulate immune responses by producing cytokines. Bmems amplify humoral and cellular immune responses by secreting proinflammatory cytokines responsible for the pathogenic effects during autoimmune processes, while Bregs may promote tolerance and suppress inflammatory responses by secreting regulatory cytokines, such as IL-10 and transforming growth factor (TGF)-β (Duddy et al., 2007; Lund, 2008; Rincón-Arévalo et al., 2016). In recent years, multiple studies have shown that both the frequency and number of Bregs are decreased in MG patients, which correlates with disease severity (Guptill et al., 2015; Karim et al., 2017; Sheng et al., 2016; Sun et al., 2014). In the present study, we observed an increased frequency of $CD27^+$ Bmems out of the total CD19+ B cells in refractory and non-refractory MG patients as compared to that in HC, but no differences between those in refractory and non-refractory MG patients. The reduction trend of ($CD24^+$ $CD38^+$, $CD5^+$, $CD1d^+$) Bregs in patients with refractory MG is greater than that in patients with non-refractory MG.

B-cell-activating factor (BAFF) is essential for B-cell survival and development. Although BAFF also binds to two other cell-surface receptors, including B-cell maturation antigen (BCMA) and transmembrane activator calcium modulator and cyclophilin ligand interactor (TACI), its survival signal to B cells is mainly transduced by BAFF-R (Thompson et al., 2001). BAFF-R expression starts with immature B cells developing into transitional B cells, and BAFF-R is expressed on the majority of circulating B cells. Transitional (early naive) human B cells are most sensitive to pro-survival signals delivered by BAFF through BAFF-R prior to activation through the B-cell receptor (Becerra et al., 2015). We found here that BAFF-R expression was increased significantly in refractory MG patients compared to that in non-refractory MG patients. Our results also suggested that the percentage of Tregs was relatively reduced in the refractory MG group, reflecting that relative activation of T cells may be related to up-regulation of BAFF-R expression on $CD19^+$ B cells.

In this present study, we performed an analysis of 15 refractory MG patients (14 anti-AChR, one anti-MUSK) treated with low-dose RTX, over a six-month period. In our previous studiey, we found that one cycle of low-dose RTX could induce a clinical improvement in all of the eight refractory MG patients, reflecting by improvement of the myasthenia gravis foundation of America (MGFA)-QMGS, MMT, and ADL changes (Jing et al., 2017). With a larger sample size in this present study, the result is consistent with our previous report, showing that clinical symptoms improved in the majority (12 out of 15) patients following low-dose RTX and that conventional immunotherapies could be reduced. The QMGS, MG-ADL, MMT, and MG-QOL-15 scores were significantly improved at six months ($P < .05$). No difference was noted in thymectomized and non-thymectomized patients regarding effectiveness of RTX.

A retrospective study had showed the dose of steroids was gradually reduced in 7/8 refractory MG patients after four infusions of RTX (Singh and Goyal, 2019). Another retrospective case series study had showed eight $AChR^+$ refractory patients with steroid-sparing effect were able to taper and discontinue prednisone after multiple cycles of RTX treatment (Robeson et al., 2017). A prospective, open-label study of RTX in refractory MG had revealed significant average daily prednisone dose reduction from 25.2 ± 15.1 mg/d to 7.3 ± 7.1 mg/d after repeat cycles RTX treatment (Beecher et al., 2018). In this present study, the beneficial effect of RTX in treating MG was further evident in its reduction of steroid doses at six months (−40%, $P = .001$).

To our knowledge, adverse events related to RTX are mainly due to infections. Progressive multifocal leukoencephalopathy (PML) after RTX therapy is also a matter of concern; however, according to a recent review, the relative risk is low (Memon et al., 2018). Our present study shows that the adverse effects are low. A lower dose is desirable because of decreased cost and also potentially reduced toxicity, including reduced opportunistic infections.

B-cell depletion occurred in all of the 15 patients in combination with clinical assessment. In the previously reported studies, RTX significantly reduced circulating $CD20^+$ B cells for up to six months after a cycle of infusion for the treatment of B-cell lymphoma (Harrison et al., 2013). In contrast, our present study found that low-dose RTX can deplete B cells for up to six months in refractory MG patients.

RTX exerts its effect through B-cell elimination (normal and abnormal) and subsequent B-cell repopulation, resulting in an altered distribution of B-cell subsets. Rehnberg et al. (Rehnberg et al., 2009) have reported changes in B-cell subsets in rheumatoid arthritis patients treated with RTX. The authors showed that short-term treatment with RTX depletes all of the B cells in circulation and 70% of bone-marrow B cells (at one and three months). The remaining bone-marrow B cells include immature and/or transitional B cells ($CD38^{++}$, $CD24^{++}$) and $CD27^+$ IgD^- Bmems. The long-term follow-up group had a reduced number of $CD27^+$ Bmems and an increase in immature/transitional B cells (mean = 22 months). The authors also noticed that the reduction of $CD27^+$ Bmems did not prevent autoantibody production, suggesting that RTX-mediated B-cell depletion does not disrupt mechanisms regulating the formation of auto-reactive clones. Becerra et al. (Becerra et al., 2015) have shown that memory B-cell subsets are slowly regenerated and the expression of BAFF-R on all of the B-cell subsets is continuously reduced following the long-term effects of RTX treatment on thrombotic thrombocytopenic purpura (TTP) patients, reflecting the delay in the selection and differentiation of potential auto-reactive B cells and relatively low activity of disease. In the present study, the recovery of peripheral-blood B cells is rare within the six months following RTX. We still need to extend follow-up periods to further verify the imbalance of B-cell subpopulations.

In addition, there was an increased percentage of the Treg population after RTX treatment. The effect of RTX on Tregs has been studied in other autoimmune diseases, such as immune thrombocytopenia (Li

et al., 2011), lupus nephritis (Vigna-Perez et al., 2006), and RA (Cao et al., 2003). Treatment with RTX led to an increase in this subset of Tregs in all of these conditions. The correlation between changes in the percentage of Tregs and clinical improvement is still unclear. It is still possible that Treg modulation is a consequence of treatment, with no relationship with its clinical effect. However, our results are consistent with previous reports that there is an association between Treg amplification and response to RTX. Current research suggests that at least two mechanisms can be envisaged to explain the effect of RTX on T cells: (1) Indirectly, B-cell depletion could affect T-cell function by intervening in B-T cell interactions and/or Treg proliferation, and (2) directly, by depleting CD20 + T cells (Marino et al., 2018). There are several reports showing that B cells expressing IFN-γ may inhibits Treg cells differentiation (Feng et al., 2011; Lund and Randall, 2010). In a murine model of autoimmune experimental arthritis (Olalekan et al., 2015), B-cell depletion leads to an increase in the percentage of Tregs due to the increased differentiation of naive CD4+ T cells into Tregs. Furthermore, we could not find any specific trend in the percentage of T cells ($CD3^+$, $CD4^+$ and $CD8^+$) after RTX infusions.

A relatively short follow-up period,a small number of enrolled patients and not evaluating the vitality of T/B cell subsets are the main limitations of the current study. The potential effects of Rituximab and related cytokines should be the subject of further investigation.

5. Conclusions

Our investigation disclosed impacts of low-dose RTX on clinical responses in refractory MG patients for up to six months following treatment. RTX exerted its effect through B-cell elimination and subsequent B-cell and T-cell repopulation. The present study elucidated that there is indeed an immune imbalance of circulating T and B cells in refractory MG patients compared to that in non-refractory MG patients. It seems that, in refractory MG, the proportion of Bregs and Tregs with immunosuppressive effects is reduced and the expression of BAFF-R with survival and development is greater than in non-refractory MG. The increase of Tregs% in refractory MG was significantly correlated to the improvement of MGFA-QMG scores. Further studies are needed to investigate the clinical effect and mechanism of RTX-treatment regimen in patients with MG.

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Disclosures

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