European Review for Medical and Pharmacological Sciences 2016; 20: 5261-5270

Ex vivo expansion of antigen-specific $CD4^+CD25^+$ regulatory T cells from autologous naïve $CD4^+$ T cells of multiple sclerosis patients as a potential therapeutic approach

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Abstract. – OBJECTIVE: $CD4^+CD25^+$ regulatory T cells (Tregs) have been found to have a decreased effector function in patients with multiple sclerosis (MS). In this study, we co-cultured naïve $CD4^+$ T cells of MS patients with myelin basic protein $(MBP)_{85-99}$ peptide as specific antigen and allogenic B cells as antigen-presenting cells, in an attempt to generate adequate antigen-specific $CD4^+CD25+$ Tregs with normal or improved immune function.

PATIENTS AND METHODS: Naïve $CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) from patients with MS (n=5) and healthy controls (HC, n=5). Furthermore, these purified naive $CD4^+$ T cells were co-cultured with the CD40-activated B cells and MBP_{85-99} peptide to induce MBP-reactive $CD4^+CD25^{high}CD127^{low}$ Tregs. After harvesting these Tregs via a flow sorter, real-time PCR and mixed lymphocyte reaction (MLR) assay were performed to characterize cellular immune function. Supernatant interleukin (IL)-10 and transforming growth factor (TGF)-β1 protein levels were detected by an enzyme-linked immunosorbent assay (ELISA).

RESULTS: With this method, the frequency of $CD4^+CD25^{high}CD127^{low}$ Tregs in $CD4^+$ T cells was 3.5%-6%. In both MS and HC groups, there were relatively lower proliferation indices (PI) of MLR assay but higher supernatant IL-10 and TGF-β1 levels in the presence of MBP than those in the presence of other control antigens, where no significant differences were found.

CONCLUSIONS: Via the *ex vivo* culture, adequate MBP-reactive $CD4^+CD25^+$ Tregsderived from autologous naïve $CD4^+$ T cells of MS patients, were obtained and returned to normal without immune defects, and even upregulated their immunosuppressive function mostly through the elevated release of IL-10 and TGF-β1.

Key Words:
Multiple sclerosis, $CD4^+CD25^+$ regulatory T cells, Ex vivo culture.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) and occurs primarily in young adults. The disease often has a relapsing-remitting course and causes severe disability. Currently, both immunosuppressive and immunomodulatory agents are recommended options for the treatment of MS, however, the adverse effects of these medications are sometimes troublesome. Immunosuppressants, such as glucocorticoid, produce significant side effects after long-term use, while the costly expense of immunomodulators, such as interferon (IFN)-β, becomes a financial burden for some patients. Moreover, the efficacy of immunomodulators tends to decrease due to the generation of neutralizing antibodies against IFN-β over a long period.

As a master regulator of immune responses, regulatory T cells (Tregs) expressing forkhead box P3 (FoxP3) maintain homeostasis between immune activation and suppression[1,2]. Dysregulation of the immune system in a variety of autoimmune diseases results from a substantial decrease in the number or functional impairment of Tregs[3,4], which thereby leads to immune defects. Previous studies[5-7] of human Tregs have shown that blood $CD4^+CD25^{high}$ T cells were much closer to Tregs regarding the functionality, rather

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than the cells expressing medium to low levels of CD25. Other than surface marker of CD25, Tregs with suppressive activity *in vitro* also displays markedly lower surface expressions of CD127[8]. Several human investigations[9] have found that the immune function of $CD4^+CD25^{high}$ Tregs in MS patients significantly decreases as compared with normal controls. Animal researches[10] have shown that the symptoms of experimental autoimmune encephalomyelitis (EAE), an animal model of MS, in mice were remarkably alleviated after the adoptive transfer of $CD4^+CD25^+$ Tregs. On the other hand, marked deterioration of the disease was observed in EAE mice when $CD4^+CD25^+$ Tregs were removed[11], especially in the mice model of EAE induced by myelin as a self-antigen[12]. In recent years, $CD4^+CD25^+$ Tregs injections has been performed in patients with type 1 diabetes[13] and graft vs host disease[14] as an effective and safe cellular treatment. Phases 1 and 2a of clinical trials had been employed to determine the safety and efficacy of antigen-specific Tregs injection for treatment of patients with refractory Crohn's disease (CD). As a result, six of the eight patients (75%) improved significantly five to eight weeks after receiving doses of 10^6 Tregs[15]. In this way, we expect that the administration of antigen-specific $CD4^+CD25^+$ Tregs could be an efficient treatment for MS.

Currently, polyclonal and *in vitro* antigen-specific Tregs expansions are two well-known methods that have been used to generate an adequate amount of $CD4^+CD25^+$ Tregs. The former refers to the stimulation of $CD4^+CD25^-$ T cells with anti-CD3 and -CD28 monoclonal antibodies (MoAb) or with artificial antigen-presenting cells (APCs) and interleukin (IL)-2. However, the Tregs harvested with this method are not antigen-specific and often cause a general immune suppression[16]. The latter refers to the production of $CD4^+CD25^+$ Tregs by co-culturing $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells with IL-2 and self-antigen-stimulated dendritic cells (DCs) or autologous DCs[17]. However, the difficulties of this approach include a long culture cycle and high costs. Tu et al[18] used allogenic CD40-activated B cells as APCs to induce Tregs from naïve $CD4^+$ T cells. When compared to using DCs as APCs, this method produced more $CD4^+CD25^+$ Tregs with a stronger immunoactivity. Hence, in this work, we co-cultured naïve $CD4^+$ T cells, with the MBP_{85-99} peptide as myelin antigen and allogenic B cell as APCs, in an attempt to generate adequate antigen-specific $CD4^+CD25^+$ Tregs with a normal immune function to treat MS.

Patients and Methods

Patients and Controls

Five patients with relapse-remitting MS (RRMS) were enrolled, according to McDonald diagnostic criteria of 2010[19]. They were all ethnic Han Chinese and tested positive for DR2 allele by HLA-DRBl analysis as previously described[20,21]. These patients did not receive any disease-modifying treatment (DMT) for at least 6 months. Five healthy individuals without any nervous system diseases or autoimmune diseases were included as age and sex-matched normal controls. The study was approved by the Ethics Committee of Peking University People's Hospital and all participating subjects gave their informed consent.

Sample Collection

All the heparinized blood specimens were collected between 09:00 am and 12:00 am. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood specimens by density gradient centrifugation using a standard protocol.

Separation and Culture of B Cells

Peripheral blood was obtained from healthy allogeneic donors and red blood cells (RBCs) were removed with an RBC lysis buffer (eBioscience, San Diego, CA, USA). B cells from peripheral blood mononuclear cells (PBMCs) were separated with B cells magnetic bead isolation kit (Miltenyi Biotec, Bergisch Gladbach, NRW, Germany). The purity of B cells, determined with anti-CD19 MoAb via flow cytometry, was over 90%. Purified $CD19^+$ B cells were incubated with sCD40L (1 μg/ml) (Biovision, Palo Alto, CA, USA) at 37°C and with 5% CO_2 for 5 days and activated by MBP_{85-99} peptide (ENP VVHF FKNI VTPR, synthesized by SBS Genetech Co., Ltd, Beijing, China) for 30 minutes before preparing for further co-culture with naïve $CD4^+$ T cells.

Naïve $CD4^+$ T Cells Isolation

Human naïve $CD4^+$ T cells were isolated from PBMCs of MS and control group by a magnetic bead isolation kit (Miltenyi Biotec, Bergisch Gladbach, NRW, Germany). The purity of naïve $CD4^+$ T cells, measured by using peridinin chlorophyll protein (PerCP)-labeled anti-CD4, allophycocyanin (APC)-labeled anti-CD25 and fluorescein isothiocyanate (FITC)-labeled anti-CD127 MoAb (eBioscience, San Diego, CA, USA) was 80.62%.

Induction, Expansion and Separation of CD4+CD25highCD127low Tregs

Purified naive CD4+ T cells were co-cultured with allogeneic CD40-activated B cells at a ratio of 10:1 in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) for 10 days, supplemented with 10% heat-inactivated fetal bovine serum (FBS) for 10 days in the presence of MBP_{85-99} peptide (0.2 μg/ml), soluble 4-1BBL (0.2 μg/ml, Biovision, Palo Alto, CA, USA) and rapamycin (109 nmol/L, Biovision, Palo Alto, CA, USA). The medium was changed every 5 days. Thereafter, CD4+CD25highCD127low Tregs were isolated with PerCP-labeled anti-CD4, APC-labeled anti-CD25 and FITC-labeled anti-CD127 MoAb via a flow sorter (Becton Dickinson, San Jose, CA, USA).

RNA Preparation and cDNA Synthesis

Total RNA was isolated using RNeasy® Mini Kit (Qiagen, Hilden, Germany), following standard protocol. Reverse transcription was performed with SUPERSCRIPT III TRANSCRIPT reagents (Invitrogen, Carlsbad, CA, USA) using random primer and primer containing 50 μM oligio (dt). The reaction was performed at 25°C for 5 min, 50°C for 60 min and 70°C for 15 min on a 9700 GeneAmp PCR system (Applied Biosystems, Norwalk, CT, USA).

Real-time PCR

PCR were performed in a total reaction volume of 20 µl containing 1 µl cDNA template, 2.5 mM $MgCl_2$, 250 µM each of dNTPs, 250 nM of each primer, 0.3 µl SYBR (20x) and 1 U of Taq polymerase (Invitrogen, Carlsbad, CA, USA). Primers were synthesized by Invitrogen, USA, using previously published sequences[22-25] (Table I). Real-time PCR was performed on an ABI 7500 fast Sequence Detector. All amplifications were preformed in replicates of three or more. Amplifications were performed at 95°C for 2 minutes and 40 cycles of 10 seconds at 95°C and 30 seconds at 60°C. The mRNA expression levels of each gene were calculated by using the $2^{-\Delta\Delta CT}$ (comparative threshold cycle, or C_T) method, as detailed by the manufacturer (Technical Bulletin 2; Applied Biosystems).

Mixed Lymphocyte Reaction (MLR) Assay

Human PBMCs from MS patients and healthy subjects were obtained by the removal of RBC with an RBC lysis buffer (eBioscience, San Diego, CA, USA). CD4+CD25- T cells were separated from the PBMCs using PerCP-labeled anti-CD4 and APC-labeled anti-CD25 MoAb via a flow sorter. 2×10^4 carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA)-labeled CD4+CD25- T cells suspension/well were added to 96-well plate in duplicate, with the addition of 2×10^3 30 Gy irradiated autologous PBMCs as APCs, in the presence of MBP_{85-99}, anti-CD3 and anti-CD28 MoAb (eBioscience, San Diego, CA, USA) at a final concentration of 0.2 μg/ml, 3 μg/ml at 37°C, 5% CO_2 for 3 days. Meanwhile, the cell suspensions were disposed with 2×10^4 isolated CD4+CD25highCD127low Tregs. Cells were harvested on the third days and proliferation indices (PI) of CFSE-labeled CD4+CD25- T cells were determined by using flow cytometry. Data were analyzed with Modfit software (Becton Dickinson, Franklin Lakes, NJ, USA).

Detection of Supernatant IL-10 and TGF-β1 Protein Levels

In parallel with the MLR assay, supernatant levels of IL-10 and transforming growth factor (TGF)-β1 were measured with an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Standard curves were produced and IL-10 and TGF-β1 protein levels of the samples were calculated using the standard substance.

Statistical Analysis

The data are presented as mean±standard deviation of the mean value. All data were analyzed with one-way ANOVA and Student-Newman-Keul's post hoc analysis using SPSS for Windows version 13.0 (SPSS Inc. Chicago, IL, USA). A p-value of less than 0.05 was considered to be statistically significant.

Results

Frequency of Induced MBP-reactive CD4+CD25highCD127low Tregs

MBP-reactive CD4+CD25highCD127low Tregs were induced and expanded from naïve CD4+ T cells after being co-cultured with sCD40L-treated B cells and MBP_{85-99} peptide. Induced Tregs of MS patients andHC were acquired with a flow sorter by using anti-CD4, anti-CD25 and anti-CD127 MoAb (Figure 1). As a result, the frequency of CD4+CD25highCD127low Tregs in CD4+ T cells was 3.5%-6% (Table II).

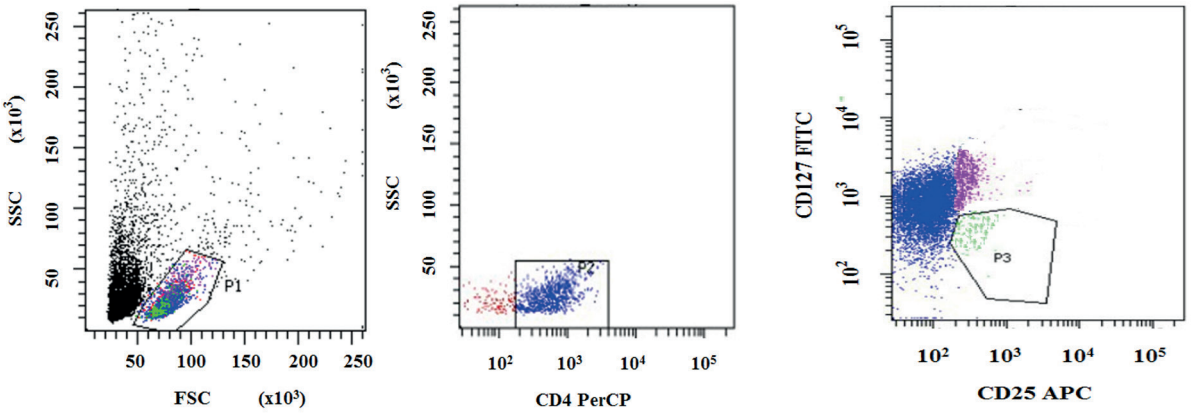

Figure 1. $CD4^+CD25^{high}CD127^{low}$ Tregs were isolated with PerCP-labeled anti-CD4, APC-labeled anti-CD25 and FITC-labeandled anti-CD127 monoclonal antibodies by a flow sorter. Region 1 (P1) is selected to set mononuclear cells gate according to forward light scatter (FSC) and side light scatter (SSC) properties and region 2 (P2) is further selected to set $CD4^+$ T cells gate. Region 3 (P3) is used to set third gate to separate $CD4^+CD25^{high}CD127^{low}$ Tregs. Control staining with isotype control antibodies is utilized as the control to define the gate.

Quantification of Foxp3, 4-1BB, Bcl-2 and Bcl-xl mRNA Expression

No significant difference in Foxp3, 4-1BB, Bcl-2 and Bcl-xl mRNA expression was found in isolated $CD4^+CD25^{high}CD127^{low}$ Tregs between MS patients and healthy controls (Table III).

MLR assay

With the addition of Tregs, MLR assay was performed via flow cytometry for analysis of PI (Figure 2A-C). In both MS and HC group, there were relatively lower PI in the presence of MBP (1.26±0.22 and 1.28±0.17) than those in the presence of anti-CD3/CD28 MoAb (2.03±0.53 and 2.12±0.46, $p<0.05$ and $p<0.05$) or those in the absence of antigen (1.39±0.14 and 1.38±0.13, $p>0.05$ and $p>0.05$), but no significant differences were found between these two groups (Figure 2D).

Supernatant IL-10 and TGF-β1 Protein Levels

Supernatant IL-10 and TGF-β1 levels were calculated using a standard curve. In both groups, there were increased supernatant IL-10 (Figure 3A) and TGF-β1(Figure 3B) levels in the presence of MBP (IL-10: 2.32+0.12 pg/ml and 2.26+0.08 pg/ml; TGF-β1: 266.75+48.97 pg/ml and 248.75+30.47 pg/ml) as compared with those in the presence of anti-CD3/CD28 MoAb (1.63+0.08 pg/ml and 1.62+0.11 pg/ml, $p<0.05$ and $p<0.05$; 181.25+25.17 pg/ml and 170.5+29.87 pg/ml, $p<0.05$ and $p<0.05$) or those in the absence of antigen (1.58+0.09 pg/ml and 1.62+0.12 pg/ml, $p<0.05$ and $p<0.05$; 167.75+22.65 pg/ml and 159.75+36.54 pg/ml, $p<0.05$ and $p<0.05$), but non-significant differences were found between these two groups.

Discussion

Unlike the cellular property in rodents, human studies demonstrated that only $CD4^+$ T cells with a high expression of CD25, i.e. $CD4^+CD25^{high}$, instead of $CD4^+$ T cells with a moderate or low expression of CD25, exhibited immunosuppressive capabilities[14]. Also, Tregs with suppressive activity in vitro display remarkably lower surface expression of CD127[8]. As for the immunosuppressive mechanism of Tregs, *in vitro* studies[26-30]

Table I. Primers used in this study.

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)
FoxP3	CAGCACATTCCCAGAGTTCCTC	GCGTGTGAACCAGTGG TAGATC
4-1BB	TCATTGCAGGATCCTTGTAGTAAC-	GAGAGGTCGGCTGGAGATGGTC
Bcl-2	AGCTGCACCTGACGCCCTT	CGCTCTCCACACACATGACCC
Bcl-xl	TGCGTGGAAAGCGTAGACAA	ATTCAGGTAAGTGGCCATCCAA
β-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG

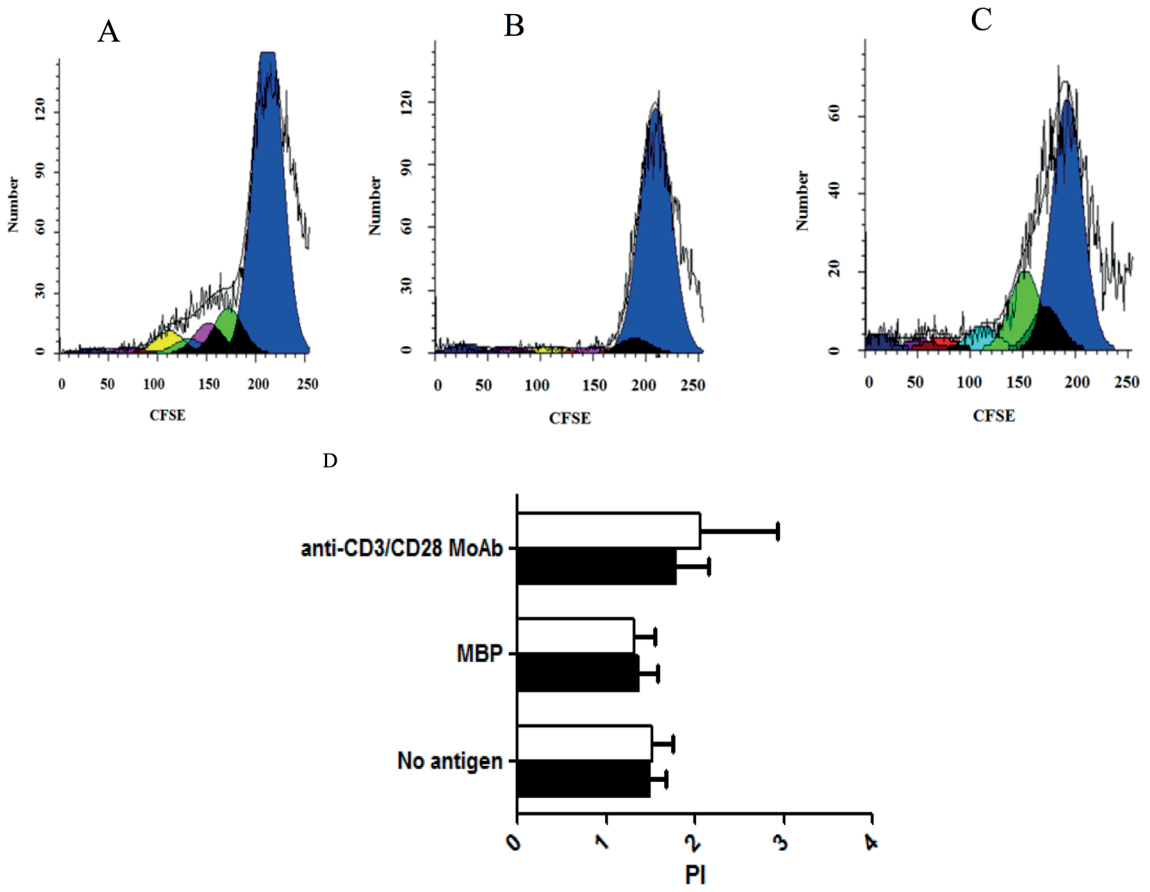

Figure 2. Immunosuppressive capability of the induced $CD4^+CD25^+$ Tregs was determined with mixed lymphocyte reaction (MLR) assay. Briefly, MLR assay was done with 2×10^4 purified carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD4^+CD25^-$ T cells/well plus 2 x 10^3 30Gy irradiated autologous PBMCs in the presence of 2×10^4 isolated $CD4^+CD25^{high}CD127^{low}$ Tregs using ***(A)*** no antigen, ***(B)*** $MBP_{85\text{-}99}$ (0.2 μg/ml) and ***(C)*** anti-CD3/CD28 monoclonal antibodies (MoAb) (3 μg/ml). Proliferation indices (PI) of CFSE-labeled $CD4^+CD25^-$ T cells were determined by using flow cytometry for further comparative analysis (D). ■ = patients with MS (n=5); □ = healthy control subjects (n=5). Bars represent the level of PI. Error bars show the standard deviations.

have reported that Tregs suppress effector cells via the secretion of the inhibitory cytokine such as IL-10 and TGF-β, cell contact-dependent Treg/APCs or Treg/T cell interactions, adenosine-mediated immunosuppression involving ectoenzymes CD39/CD73 and functional modification or killing of APCs. Both 4-1BBL and rapamycin are considered to exert Tregs-related modulatory function. For 4-1BBL, it has been found to be effective in expanding Tregs cells during three weeks[31]. As for rapamycin, it has been reported

Table II. Frequency of induced $CD4^+CD25^{high}CD127^{low}$ regulatory T cells (Tregs) separated from MS patients (n=5) and healthy controls (HC) (n=5).

Subjects	Sex/Age (Year)	Frequency of Tregs (%)
MS1	F/26	3.5%
MS2	F/25	4.6%
MS3	F/66	4.2%
MS4	F/31	4.5%
MS5	F/64	6.0%
HC1	F/26	4.4%
HC2	F/35	4.5%
HC3	F/33	4.6%
HC4	F/27	4.8%
HC5	F/28	4.6%

Table III. The mRNA expression levels of FoxP3, 4-1BB, Bcl-2 and Bcl-xl gene of induced $CD4^+CD25^{high}CD127^{low}$ regulatory T cells (Tregs) from MS patient (n=5) and healthy control (n=5).

Gene	MS (n=5)	HC (n=5)	*p*-value
FoxP3	0.00039±0.00021	0.00037±0.00015	0.8660
4-1BB	0.00309±0.00092	0.00308±0.00128	0.9868
Bcl-2	0.00512±0.00106	0.00494±0.00060	0.7518
Bcl-xl	0.08884±0.00994	0.08794±0.01350	0.9072

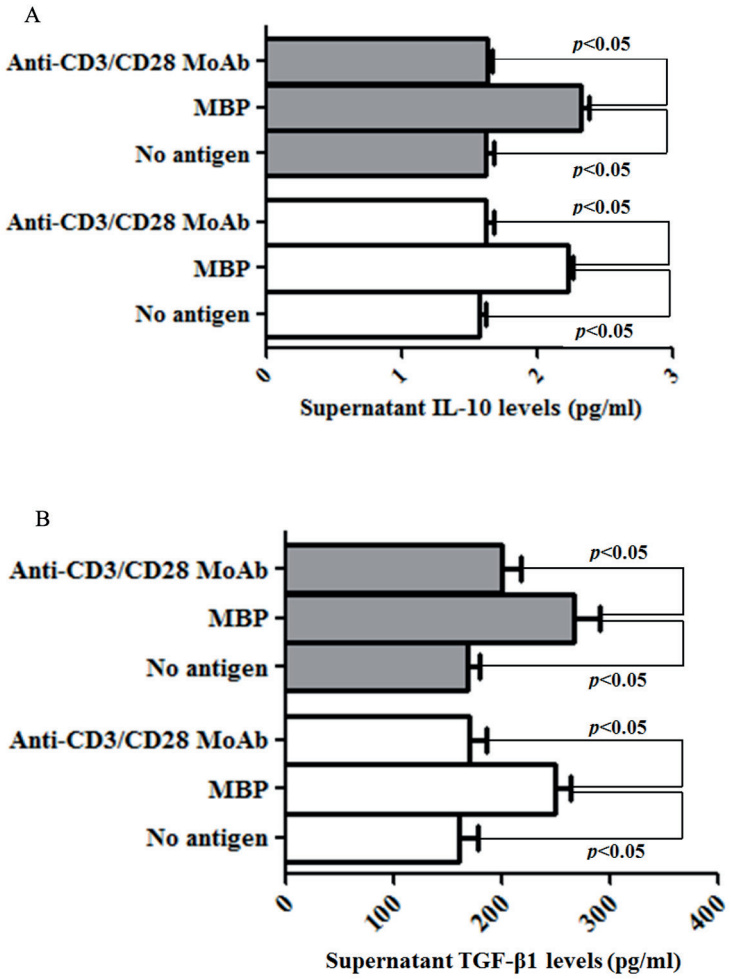

Figure 3. Comparison of supernatant *(A)* interleukin-10 (IL-10) and (B) transforming growth factor (TGF)-β1 protein levels in before-mentioned mixed lymphocyte reaction (MLR) assay using enzyme-linked immunosorbent assay (ELISA). ■ = patients with MS (n=4); □ = healthy control subjects (n=4). Bars represent the IL-10 and TGF-β1 levels. Error bars show the standard deviations.

to prompt the expansion of naturally occurring Tregs and facilitate the *de novo* generation of induced Tregs[32].

Given the existence of CD127 as a cell surface marker for further identification of Tregs, we used this marker to further characterize Tregs with a $CD4^+CD25^{high}CD127^{low}$ phenotype in our study. To exclude the possibility that the sorted Tregs originated from the amplification of already-existing Tregs in the culture system, we initially measured the proportion of Tregs in $CD4^+$ T cells purified by magnetic beads. Our findings clearly demonstrated over 98% naïve $CD4^+$ T cells and few Tregs in all the isolated cells, indicating that these sorted Tregs were almost generated via further cell differentiation of naïve $CD4^+$ T cells, rather than simple self-proliferation of already-existing Tregs. Furthermore, our study substantiates that antigen-specific $CD4^+CD25^{high}$ Tregs can be obtained from the induction of naïve $CD4^+$ T cells by the MBP_{85-99} peptide in the presence of soluble 4-1BBL and rapamycin.

Various previous investigations[9,33] have reported that when comparing the suppressive capacity of Tregs from RRMS patients with that of healthy individuals *in vitro*, the effector function of the $CD4^+CD25^{high}$ Tregs from MS patients was severely impaired. However, the underlying mechanism of functional defects remains unclear. Stasiolek et al[34] reported that the expression of 4-1BBL, a member of the tumor necrosis factor superfamily (TNFSF), was significantly reduced in plasmacytoid dendritic cells (pDCs) in MS patients. The decreased expression of 4-1BBL may hinder

the pDCs' ability to produce Tregs in the immune system. Our previous work[35] also found that the expression of 4-1BB in $CD4^+CD25^{high}$ Tregs in MS patients decreased significantly, while IFN-β treatment blocked the reduction of the 4-1BB expression. This indicated that the down-regulation of 4-1BB was involved in the impairment of Tregs immunoactivity. Supporting these studies, a study by Elpek et al[31] revealed that the activation of 4-1BB/4-1BBL costimulatory signals could promote the proliferation and survival of Tregs, especially in the presence of a high dosage of IL-2. Thereby, we added soluble 4-1BBL as one of the stimuli, to induce and acquire an adequate number of Tregs with normal immune function. Indeed, our study showed that more Tregs were induced (3.5%-6%) in MS patients, as Tregs account for only 1%-2% of human $CD4^+$ T cells. For the co-stimulatory effect of 4-1BBL(CD137L) on T cells, our previous *in vitro* study[36] showed that nonspecific T cells proliferation was significantly suppressed after treatment with an anti-4-1BBL MoAb compared with the control antibody in either MS or healthy subjects (n=5). Moreover, the FoxP3 mRNA expression and suppressive capability of these induced Tregs in MS patients displayed no significant difference as compared to HC. Interestingly, we also found that 4-1BB, anti-apoptotic Bcl-2 family member Bcl-2 and Bcl-xl mRNA levels in these induced Tregs of MS patients were not markedly distinct from those of HC. These findings, therefore, suggest that recovery of immune function and proliferation of Tregs could be largely prompted by the addition of 4-1BBL.

In general, Tregs constitute 1%-2% of human $CD4^+$ T cells, and antigen-specific Tregs constitute even less with a proportion of 1/20000-1/2000[37]. It is essential to obtain adequate antigen-specific Tregs for the treatment of the immune-related diseases with a known target antigen, such as MS, diabetes or rheumatoid arthritis (RA). In an animal research[38], EAE model was successfully induced by active immunization with MBP_{85-99} in SJL mice or Lewis rats. Various human studies revealed that the MBP-reactive T cells in MS patients presented more active immune functions compared to those of health controls[39]. Combined with HLA-DR2, MBP_{85-99} peptide could substantially activate T cell clones from peripheral blood of MS patients. Once activated, these T cells would likely release inflammatory cytokines such as IL-2 and IFN-γ[40,41]. Hence, in this work we selected MBP_{85-99} peptide as the specific antigen, and the five healthy individuals positive for DR2 allele were recruited as normal controls since MBP-reactive T cells were also found in healthy subjects[42]. Consequently, adequate antigen-specific $CD4^+CD25^+$ Tregs in MS patients were obtained with normalized or even enhanced function since no significant difference in immunosuppressive activities or MBP-reactive Tregs frequency was found between MS and healthy control subjects when stimulated with MBP_{85-99} peptide. Also, these Tregs failed to efficiently inhibit CD3/CD28-induced $CD4^+CD25^-$ T cell proliferation, indicating a complex action mechanism of CD3/CD28 co-stimulation effecting on the immunofunction of Tregs, as one recent study[43] reported that induced Tregs generated by activation with anti-CD3/CD28 MoAb differed from those generated with antigen/APC. However, this issue needs to be further investigated.

TGF-β and IL-10 have been considered as the key cytokines secreted by $CD4^+CD25^+$ Tregs during in vitro suppression assays[27,28]. In total, there are six subtypes of TGF-β, of which TGF-β1 accounts for over 90%. Our paper showed that in both MS and HC group, there were higher levels of TGF-β1 and IL-10 in the presence of MBP than other antigen control groups, suggesting that the induced Tregs response could be antigen specific. This is noteworthy as Putheti et al[44] referred that after co-culturing with glatiramer acetate used as the first-line drug *in vitro*, these Tregs secreted remarkable levels of IL-10, suggesting that both TGF-β and IL-10 could be important for treating MS. Together with the results of MLR assay revealing upregulated immunoactivities of the Tregs in the presence of MBP, all these findings suggest that the induced MBP-reactive Tregs may inhibit activation and proliferation of T cells predominantly through the secretion of cytokines such as IL-10 and TGF-β1. In parallel with this, two distinct EAE investigations reported that reduced disease severity was closely associated with $CD4^+CD25^+$ Tregs number as well as the levels of IL-10 or TGF-β[45,46], although a meta-analysis failed to show the frequency of Treg cells as a risk factor in MS[47]. Notably, TGF-β, in turn, can induce the upregulation of Foxp3 gene expression in peripheral naïve $CD4^+$ $CD25^-Foxp3^-$ T cells, which can further develop into $CD4^+CD25^+$ $FoxP3^+$ Tregs[48]. On the other hand, considering the complexity of the action mechanism of Tregs as described before, other immunoregulatory factors such as cell-cell contact inhibition and ectoenzymes CD39/CD73 should also be taken into consideration, and further evidence is needed to clarify such factors.

Conclusions

We found that, via *ex vivo* culture, adequate MBP-reactive $CD4^+CD25^+$ Tregs derived from autologous naïve $CD4^+$ T cells of MS patients, were obtained and did return to normal without immune defects, and even upregulated their immunosuppressive function mostly through the elevated release of IL-10 and TGF-β1. To our knowledge, this is the first reported human study regarding the *in vitro* induction and expansion of function-normalized antigen-specific Tregs in MS. As such, our findings undoubtedly pave the way for a novel therapeutic method of specific cellular immunotherapy against MS.

Acknowledgements

We would like to thank Dr. Hounan Wu for her technical assistance with flow cytometry and cell sorting. We also thank Dr. Xin Yu, Mr Bowen Ren and Ms Kaitlyn Wang for their careful discussion of this work. This work was supported by grants from Peking University People's Hospital (RDB2009-31), the National Natural Science fund (NSF 81171123 and NSF 810288).

Conflicts of interest

The authors declare no conflicts of interest.

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