Original Article
Restoration of immune suppressor function of regulatory B cells collected from patients with allergic rhinitis with Chinese medical formula Yupingfeng San

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Abstract: Immune dysregulation, such as defects in immune suppressor function, plays an important role in the pathogenesis of many immune disorders including allergic rhinitis (AR). Some Chinese traditional medical formulae have an immune regulatory function. This study aims to restore the immune suppressor function in regulatory B cells (Bregs) collected from AR patients with a Chinese medical formula, Yupingfeng San (YPFS). In this study, Bregs were isolated from blood samples collected from AR patients and healthy (HA) subjects. The capacity of Breg in suppressing effector T cell (Teff) proliferation was observed in an in vitro experiment to be used as an indicator of immune suppressor function of Breg. The effects of YPFS on promoting Bregs' immune suppressor functions were tested in a cell culture study. The results showed that the number of peripheral Breg in AR patients was not significantly different from that in HA subjects, while the immune suppressor function of AR Breg was compromised. Bcl2L12 expression was higher in AR Bregs than that in HA Bregs. A negative correlation was identified between expression of Bcl2L12 and IL-10 in AR Bregs. Exposure of AR Bregs to YPFS in the culture suppressed the expression of Bcl2L12 and improved their immune suppressor function. In conclusion, YPFS can restore the immune suppressor function of AR Bregs via inhibiting the expression of Bcl2L12. The data suggest that YPFS has the potential to be used in the improvement of immune dysfunction, such as AR.

Keywords: Allergy, immune regulation, B cell, Bcl2L12, Chinese medicine

Introduction

Allergic rhinitis (AR) is one of the allergic diseases, which is an adverse reaction of the immune system to innocent protein antigens [1]. It is accepted that one of the conspicuous pathological change is that plasma cells of AR patients over produce antigen specific immunoglobulin (Ig) E [2]. IgE binds to the high affinity IgE receptors on the surface of mast cells to make mast cell sensitized. Re-exposure to specific antigens triggers sensitized mast cell activation to release chemical mediators, such as histamine, to the microenvironment to initiate AR attacks [3]. However, the therapeutic efficacy of AR is unsatisfactory.

In general, the behavior of immune cells is tightly regulated by an immune regulatory system under physiological condition [4]. Regulatory T cells (Tregs) and regulatory B cells (Bregs) are the major cellular components of the immune regulatory system [5]. Upon activation, Tregs or/and Bregs release immune regulatory molecules, such as transforming growth factor (TGF)-β and interleukin (IL)-10, to restrict the ongoing immune responses within a proper range [5]. In allergic diseases, such as AR, the overproduction of antigen specific IgE, Th2 cytokines, etc., suggest that the immune regulatory system is dysfunction [5]. The number of Tregs or/and Bregs may be reduced; the immune regulatory function of Tregs or/and Bregs may be

Table 1. Clinical characteristics of the study groups

	AR	HA
Number of subjects	20	20
Age	31.6 ± 3.8	32.4 ± 3.5
Weight	56.8 ± 11.2	57.4 ± 10.3
Height	159.6 ± 13.3	157.9 ± 12.7
Gender (M/F)	10/10	10/10
AR	20 (100%)	0
AR/asthma	3 (15%)	0
AR/eczema	2 (10%)	0
Total IgE (IU/L)	586.3 ± 223.5	0.32 ± 0.25
Specific IgE (IU/L)	84.7 ± 19.6	Nd
Eosinophil count, per μl†	242 (116-295)*	81 (61-146)

AR: Patients with allergic rhinitis. HA: Healthy subjects. Values shown are means ± SD. *$P \leq 0.05$ as compared with the healthy group (*t* test). †Median (lower quartile-upper quartile).

compromised [6, 7]. Yet, the underlying mechanism is not fully understood.

Our recent studies indicate that B cells in AR patients express high levels of Bcl2L12 that is associated with the reduction of IL-10 expression in B cells [8]. Bcl2L12 is a member of the Bcl2 family and is one of the anti-apoptotic proteins [9]. Early works indicate that Bcl2L12 plays a role in the pathogenesis of cancer by conferring cancer cell apoptosis resistance [9, 10]. Our work show that Bcl2L12 is also involved in promoting Th2 polarization [8]; it may interfere with immune regulation.

There are many attempts to improve the compromised immune regulatory condition. One of them is the specific immunotherapy (SIT), in which small doses of specific antigens are introduced into the body of AR patients [11]. The underlying mechanism of SIT is to generate immune regulatory cells [11]. However, the efficacy of SIT needs to be further improved. It is reported that administration of Chinese medical formulae can greatly improve AR clinical symptoms. One of the formulae is Yupingfeng San (YPFS) [12]; this formula can attenuate the expression of IL-33 [13] and regulates innate immunity [14]. However, the underlying mechanism is unclear. Based on the information above, we hypothesize that YPFS may improve AR by improving the immune regulatory function. To test the hypothesis, we collected blood samples from AR patients. The correlation between expression of Bcl2L12 and IL-10 in Bregs was assessed. The effects of YPFS on improving the Bregs' immune suppressor functions were evaluated.

Materials and methods

Reagents

Anti-IL-10 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-Bcl2L12 antibodies were purchased from abcam (Cambridge, MA). The IL-10 ELISA kit was purchased from BioMart (Beijing, China). Fluorochrome-labeled antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). PMA (Phorbol myristate acetate) and ionomycin were purchased from Sigma Aldrich (St. Louis., MO). Materials and reagents for RT-qPCR and Western blotting were purchased from Invitrogen (Carlsbad, CA). Immune cell isolation reagent kits were purchased from Miltenyi Biotech (San Diego, CA).

Human subjects

The using human tissue in the present study was approved by the Human Ethics Committee at Shenzhen University (SZUe#2015008). A written informed consent was obtained from each human subject. Perennial AR patients at the remission stage were recruited at Shenzhen University Affiliated ENT Hospital. The diagnosis and management of AR were carried out by our physicians following published procedures [15]. AR patients had paroxysmal sneezing, nasal itch and profound nasal secretions. All recruited AR patients had high levels of mite antigen specific IgE antibody. A group of age- and gender-matched healthy (HA) subjects were also recruited into this study at the same hospital. The demographic data of human subjects are presented in **Table 1**. Patients had any one of the following conditions were excluded: Autoimmune diseases; severe organ diseases; nasal surgery, cancer and under medication with immune suppressors for any reasons.

Isolation of Bregs from peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples were collected from each human subject through the ulnar vein puncture. PBMCs were isolated from the blood samples by gradient density centrifugation.

Following published procedures [16] with modification, CD73$^-$ CD19$^+$ CD25$^+$ CD71$^+$ Bregs were isolated from PBMCs by magnetic cell sorting (MACS) with commercial reagent kits following the manufacturer's instructions. The purity of isolated Breg was greater than 95% as assessed by flow cytometry. Cell viability was more than 99% as assessed by Trypan blue exclusion assay.

Cell culture

Cells were cultured in RPMI1640 medium supplemented with antibiotics (penicillin: 100 U/ml and streptomycin: 0.1 mg/ml), fetal calf serum (10%) and glutamine (2 mM). The medium was changed in 2-3 days. The cell viability was greater than 99% as assessed by Trypan blue exclusion assay. To prevent B cell apoptosis, CD40 ligand was added to the culture at 5 μg/ml.

Preparation of YPFS

Following published procedures [17, 18], we purchased the crude drugs of YPFS from Beijing Tongrentang Drug Store. Astragalus membranaceus (60 g), Atractylodes macrocephala (20 g), and Saposhnikoviae radix (20 g) were extracted by boiling the drugs with water for two times (1:6 at the first time and 1:4 in the second time, w/v), each time 40 min. The liquid was concentrated in an electric thermostatic water bath, dried in a vacuum oven, and then, the solid extract was crushed into powder and stored at 4°C until use.

Assessment of Breg number in PBMCs by flow cytometry

PBMCs were prepared as described above and stained with fluorochrome-labeled antibodies of CD19, CD25, CD71 and CD73 or isotype IgG for 30 min at 4°C. The cells were analyzed with a flow cytometer (FACSCanto II). Results were analyzed with flowjo software. Data of isotype IgG staining were used as gating reference.

Evaluation of Breg immune suppressor function

Bregs were isolated from PBMCs as described above. Effector CD4$^+$ CD25$^-$ T cells (Teffs) were isolated from blood samples collected from HA subjects by MACS and labeled with CFSE (carboxyfluorescein succinimidyl ester). Bregs and Teffs were cocultured at a ratio of 1:5 in the presence of PMA (50 ng/ml) and ionomycin (100 ng/ml) for 3 days. The cells were analyzed with a flow cytometer. The frequency of proliferating Teffs was determined and used as an indicator of Bregs' immune suppressor function.

Real-time quantitative RT-PCR (RT-qPCR)

Bregs were collected from relevant experiments. Total RNA was extracted from Bregs with TRIzol reagents. cDNA was synthesized with the RNA with a reverse transcription kit following the manufacturer's instructions. The samples were then amplified in a qPCR device (CFX96 Touch Real-Time PCR Detection System) with the SYBR Green Master Mix and the presence of relevant primers, including IL-10 (gccaagccttgtctgagatg and aagaaatcgatgacagcgcc) and Bcl2L12 (cttctatgctttggtggccc and caagttcaagtccacgggtg). The results are presented as fold change against the housekeeping gene β-actin.

Western blotting

Total proteins were extracted from Bregs collected from relevant experiments. The proteins were fractioned with SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto a PVDF membrane. After blocking with skim milk (5%) for 30 min, the membrane was incubated with the primary antibodies of interest overnight at 4°C and followed by incubating with peroxidase-labeled secondary antibodies for 2 h at room temperature. Washing with TBST (Tris-buffered saline containing 0.1% Tween 20) was carried out after each time of incubation. Immunoblots on the membrane were developed with enhanced chemiluminescence and photographed with an imaging device.

Enzyme-linked immunosorbent assay (ELISA)

IL-10 levels in culture supernatant were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

Statistical analysis

Difference between data from two groups was determined by Student *t* test. Analysis of vari-
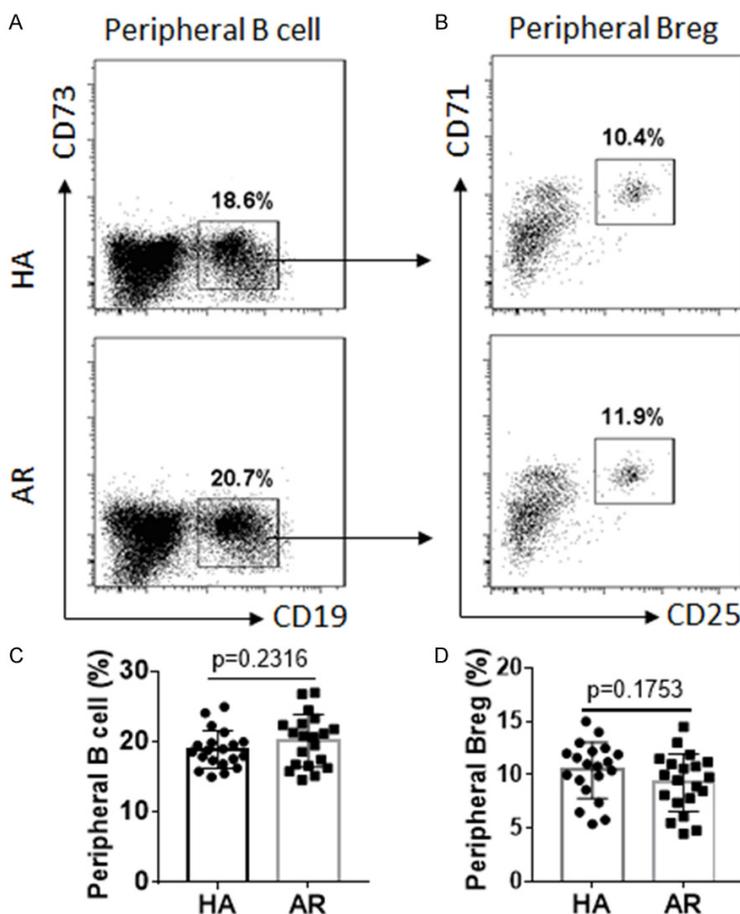
Figure 1. Assessment of peripheral Breg number in HA subjects and AR patients. Peripheral blood samples were collected from AR patients (n=20) and HA subjects (n=20). PBMCs were isolated from the samples and analyzed by flow cytometry. A. The gated dot plots show frequency of peripheral B cells. B. Each dot represents frequency of B cell in one sample; the bars show summarized data of peripheral B cells of AR and HA subjects. C. The gated dot plots show frequency of peripheral Bregs. D. Each dot represents frequency of Breg in one sample; the bars show summarized data of peripheral Bregs of AR and HA subjects. Data of bars are presented as mean ± SEM.

ance (ANOVA) followed by Dunnett's t test or SNK test was used in multiple comparisons. $P < 0.05$ was considered statistical significance.

Results

The immune suppressor function of Breg is compromised in AR patients

To understand the status of Bregs in AR patients, we collected blood samples from 20 AR patients and 20 HA subjects. Peripheral blood mononuclear cells (PBMCs) were isolated from the samples. The frequency of $CD73^- CD19^+$ $CD25^+ CD71^+$ Bregs [16] in PBMCs were assessed by flow cytometry. The results showed

that the frequency of AR Bregs was not significantly different from that of HA Bregs (**Figure 1**). The results suggest that AR condition does not affect the development of Breg in peripheral system. Thus, the immune dysregulation in AR patients may be attributed to compromise of immune suppressor function. To test this, Bregs were isolated from blood samples and co-cultured with naive effector T cells (Teffs) isolated from HA subjects in the presence of cell activators. As analyzed by flow cytometry, the immune suppressor function of AR Bregs on Teff proliferation was significantly weaker than that of HA Bregs (**Figure 2**). The results demonstrate that the immune suppressor function of AR Breg is compromised.

AR Bregs express less IL-10 than HA Bregs

We next assessed the expression of IL-10 in Bregs collected from AR patients and HA subjects. Bregs were isolated from peripheral blood samples and analyzed by RT-qPCR and Western blotting. The results showed that AR Bregs expressed less IL-10 than that in HA Bregs (**Figure 3A**, **3B**). The Bregs were also cultured overnight in the presence of activators. Levels of IL-10 in the culture supernatant were determined by ELISA. The results showed that AR Bregs released much less IL-10 than that released by HA Bregs (**Figure 3C**). The data indicate that AR Bregs only produce low levels of IL-10.

AR Bregs express Bcl2L12 that negatively correlates with IL-10 expression

Our recent study revealed that B cells express Bcl2L12 that suppress IL-10 expression [8]. To elucidate if the expression of Bcl2L12 is associated with the lower expression of IL-10 in Bregs, we assessed expression of Bcl2L12 in Bregs. The results showed higher expression of

A

B
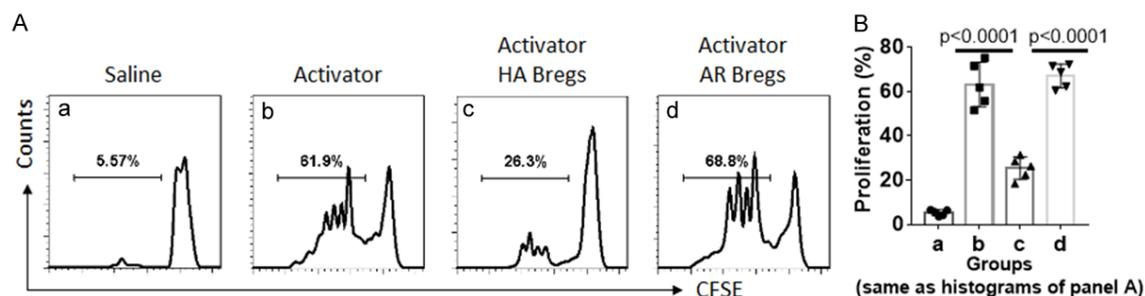
Figure 2. AR Bregs show less immune suppressor function. AR Bregs and HA Bregs were prepared. Teffs were prepared from blood samples collected from HA subjects and labeled with CFSE. Bregs were co-cultured with Teffs at a ratio of 1:5 in the presence of activators (PMA: 50 ng/ml; ionomycin: 100 ng/ml) for 3 days. The cells were analyzed by flow cytometry. (A) The gated histograms show proliferating Teffs. (B) The scatter dot plots show data of proliferating Teffs from 5 experiments. Data of bars are presented as mean ± SEM. Each dot in (B) represent data from one experiment.
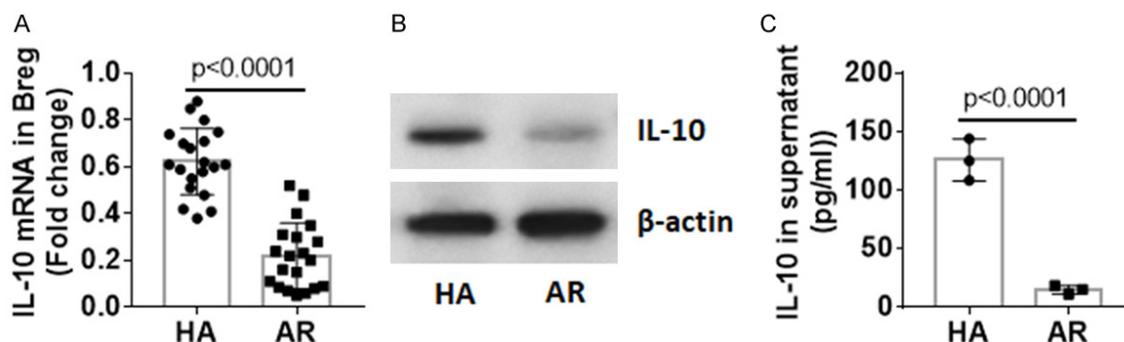
Figure 3. AR Bregs express lower IL-10. Bregs were isolated from blood samples collected from AR patients (n=20) and HA subjects (n=20). A. Scatter dot plots show IL-10 mRNA in Bregs (each dot represent data from one sample). B. Total proteins were extracted from Bregs obtained from each human subject. The proteins were pooled and analyzed by Western blotting. The immunoblots show IL-10 protein in Bregs (the data are from one experiment that represents 3 independent experiments). C. A portion of Bregs was cultured in the presence of activator (PMA/ionomycin) for 48 h. The supernatant was collected at the end of culture and analyzed by ELISA. The bars (mean ± SEM) show IL-10 in culture supernatant. Each dot represents IL-10 protein levels from one experiment.

Bcl2L12 in AR Bregs than that of HA Bregs (**Figure 4A**, **4B**). A negative correlation was identified between Bcl2L12 and IL-10 in AR Bregs (**Figure 4C**). The data imply that Bcl2L12 may interfere with the expression of IL-10 in AR Bregs.

YPFS suppresses Bcl2L12 expression and restores IL-10 expression in AR Bregs

Previous studies indicate that YPFS can inhibit AR [17, 19]. We hypothesize that the underlying mechanism by which YPFS inhibits AR may be through suppressing Bcl2L12 and restore IL-10 in Bregs. To test this, we adopted previous experimental procedures [8] to induce expression of Bcl2L12 in B cells with or without the presence of YPFS. The results showed that YPFS did have inhibitory effects on Bcl2L12

expression in B cells in a dose-dependent manner (**Figure 5A**). The results suggest that YPFS may restore the expression of IL-10 in AR Bregs via inhibiting Bcl2L12. To test this, AR Bregs were prepared and treated with YPFS at gradient concentrations in the presence of LPS (to up regulate expression of IL-10). The results showed that exposure to YPFS significantly increased the expression of IL-10 in AR Bregs; the IL-10 was also released into the culture supernatant (**Figure 5B-D**). The data demonstrate that YPFS can restore the expression of IL-10 in AR Bregs.

YPFS restores immune suppressor function in AR Bregs

The results of **Figure 5** suggest exposure to YPFS may restore the immune suppressor func-
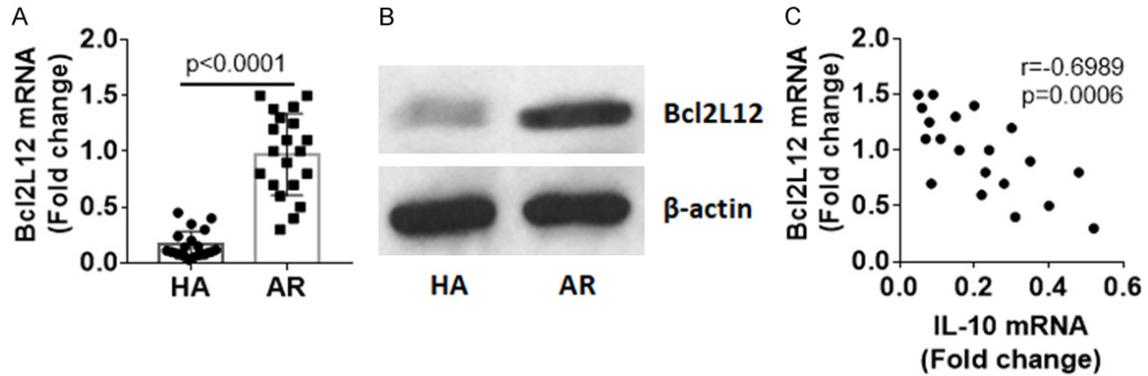
Figure 4. IL-10 expression is negatively correlated with Bcl2L12 expression in AR Bregs. Bregs were isolated from blood samples of AR patients (n=20) and HA subjects (n=20) and analyzed by RT-qPCR and Western blotting. A. Each dot represents Bcl2L12 mRNA in Bregs isolated from one human subject (data of bars are presented as mean ± SEM). B. Proteins were extracted from Bregs and pooled. The immunoblots show Bcl2L12 protein levels of one experiment which represent 3 independent experiments. C. Scatter plots show a negative correlation between expression of Bcl2L12 and IL-10 in AR Bregs.
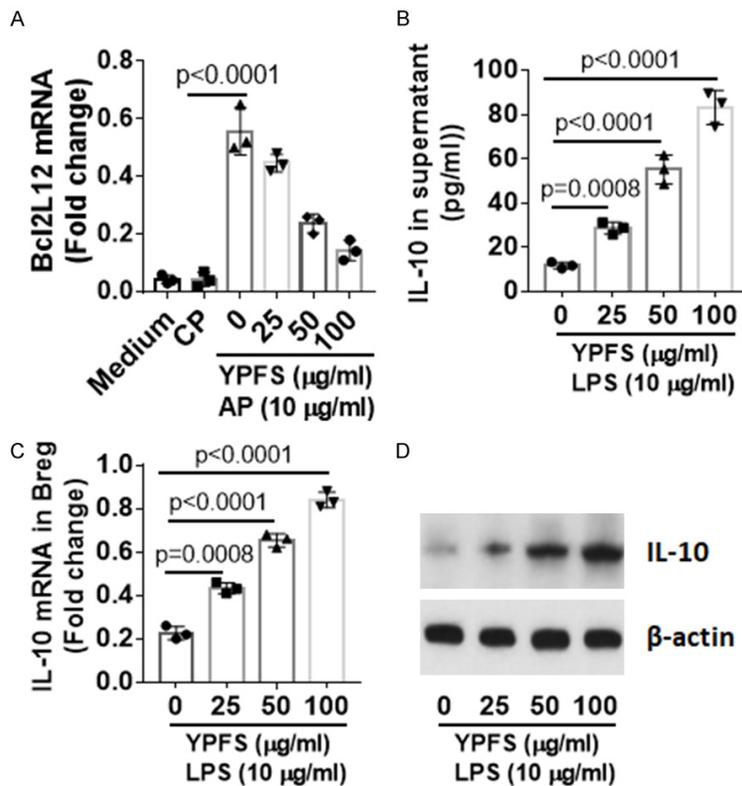
Figure 5. YPFS inhibits Bcl2L12 expression and restores IL-10 expression in AR Bregs. AR Bregs were prepared and treated with the procedures denoted on the X axis of (A and B) in the culture for 48 h. (A) Bcl2L12 mRNA levels in AR Bregs. (B) IL-10 levels in culture supernatant. (C, D) IL-10 expression in AR Bregs. Each dot in (A-C) represent data of one experiment. The immunoblots of (D) were from one experiment that represent data from 3 independent experiments. Data of bars are presented as mean ± SEM.

primed with or without YPFS. AR Bregs were cocultured with Teffs for 72 h in the presence of activators. The effects of AR Breg on suppressing proliferation of Teff was assessed by flow cytometry and used as indicators of the immune suppressor function of AR Bregs. The results showed that after exposure to activators, Teffs proliferated markedly, which was significantly suppressed by the presence of YPFS-primed AR Bregs, but not by those AR-Bregs primed by saline (**Figure 6**). The results demonstrate that exposure to YPFS restores the immune suppressor function of AR Bregs.

Discussion

The present data show that Bregs of AR patients (AR Bregs) are less capable of suppressing Teff proliferation. The fact suggests that AR Bregs have immune suppressor defects, although the frequency of AR Breg is not significantly different from that of HA Breg. The pathological feature of AR Breg is that less IL-10 is expressed by AR Bregs. The data also show that YPFS can

tion of AR Bregs. To test this, Teffs and AR Bregs were prepared. The AR Bregs were
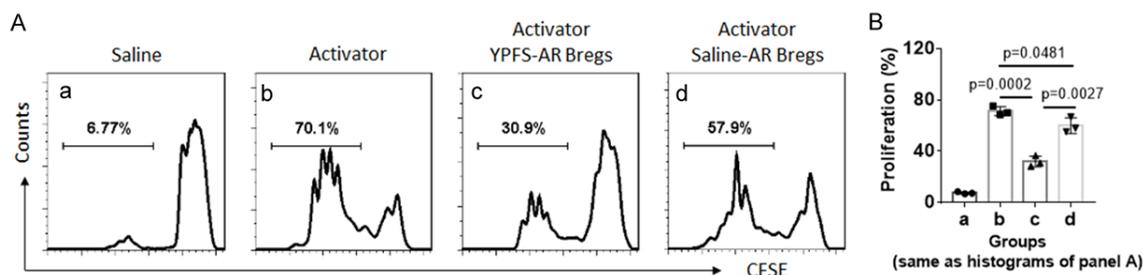
Figure 6. YPFS restores AR-Bregs' immune suppressor function. Teffs were prepared with CD4^{+} CD25^{-} T cells collected from HA subjects and labeled with CFSE. AR Bregs were isolated from blood samples collected from AR patients and primed by exposing to YPFS (100 µg/ml; YPFS-AR Bregs) or saline (saline-AR Bregs) in the culture for 48 h. Teffs and Bregs were cocultured (5:1) for 3 days in the presence of activators (PMA/ionomycine) for 3 days. The cells were then analyzed by flow cytometry. A. The gated histograms show proliferating Teffs. B. Bars show summarized frequency (mean ± SEM) of proliferating Teffs from 3 independent experiments. Each dot show data of one experiment.

increase the IL-10 expression and restore the immune suppressor function in AR Bregs by inhibiting the expression of Bcl2L12.

In this study, we observed that AR Bregs were dysfunction in suppressing Teff proliferation, while the number of Breg was not significantly reduced. Published data indicate that either the reduction of the number or the suppressor functional defects of immune regulatory cell are associated with the pathogenesis of many immune disorders. For example, the number of peripheral Bregs and IL-10-production by B cells in patients with common variable immunodeficiency is less than that in HA subjects [20]; the Bregs present less suppressor effort in IFN-γ^{+} TNF-α^{+} CD4^{+} T cell development [21]. Our data show that although the number of Breg in AR patients is not significantly reduced, their immune regulatory capacity is compromised. Since the compromise of immune regulatory function is an important event in the pathogenesis of many immune disorders [6, 7], the present data suggest that the immune suppressor defects of Breg are associated with the pathogenesis of AR.

We also found that less IL-10 was expressed by Bregs in AR patients. IL-10 is the signature regulatory cytokine in Bregs [22]. It is accepted that Th2 polarization is one of the major pathological features of AR. Dendritic cells (DCs) present specific antigens to CD4^{+} T cells is a critical step in the development of Th2 polarization, in which the major histocompatibility antigen II (MHC II) is required in the process of antigen presentation, while IL-10-producing Bregs restrict the expression of MHC II and other co-stimulatory molecules by DCs [23]. The deficiency or insufficiency of IL-10 was detected in Bregs in patients with autoimmune diseases [24]. Our data are in line with these reports that the incompetency in expressing IL-10 by AR Bregs was identified. Whether this phenomenon contributes to AR development needs to be further investigated.

Our previous reports indicate that AR B cells express Bcl2L12; the latter suppresses the expression of IL-10 in B cells. The present study further detected the expression of Bcl2L12 in AR Bregs and found a negative correlation between the expression of Bcl2L12 and IL-10 in AR Bregs. Bcl2L12 was found playing a role in cancer development by its anti-apoptosis function [25]. By its proline-rich property, Bcl2L12 binds to p53 and caspases to interfere with the functions of these molecules [25, 26]. Our recent work also showed that Bcl2L12 bound transcription factor of IL-10 in B cells to suppress the expression of IL-10, which is confirmed by the present data. Besides, the present data further show Bcl2L12 prevents the expression of IL-10 in B cells in an experimental system. The data suggest that inhibition of Bcl2L12 may facilitate the expression of IL-10 in AR Bregs.

Indeed, our data show that YPFS inhibits expression of Bcl2L12 and increases IL-10 expression in AR Bregs. YPFS can efficiently inhibit experimental airway allergy [17, 19] and has been used in the treatment of human allergic rhinitis in China [12]. The present study pro-

vides mechanistic evidence to support previous studies [12, 17, 19] that YPFS inhibits Bcl2L12 and restores IL-10 expression in AR Bregs to up regulate the immune suppressor function in AR Bregs.

In summary, the present data show that YPFS can inhibit Bcl2L12 expression and increases IL-10 expression in Bregs of AR patients.

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Disclosure of conflict of interest

None.

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