Characteristics of regulatory B10 cells in patients with rheumatoid arthritis with different disease status

ZHAOHUI ZHENG[1*], XUEYI LI[1*], XIAOYAN LI[2*], JIN DING[1], YUAN FENG[1], JINLIN MIAO[1], XING LUO[1], ZHENBIAO WU[1] and PING ZHU[1]

[1]Department of Clinical Immunology, Branch of Immune Cell Biology, State Key Discipline of Cell Biology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032; [2]Department of Endocrine and Metabolic Diseases, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068, P.R. China

Received September 1, 2014; Accepted May 13, 2015

DOI: 10.3892/mmr.2015.3927

Abstract. In the present study, the frequency and function of B10 cells in patients with rheumatoid arthritis (RA) was examined. A total of 24 healthy controls and 97 patients with RA were enrolled in the present study. Among the 75 patients with an active disease status, 51 patients received either no treatment or were treated with non-steroidal anti-inflammatory drugs (NSAIDs) only, while 24 patients underwent a disease relapse. Flow cytometry was used to assess the frequency of $CD19^+CD24^{hi}CD38^{hi}$ interleukin (IL)-10^+ cells stimulated by lipopolysaccharide plus CD40L for 48 h, followed by re-stimulation with phorbol myristate acetate and ionomycin for 5 h. The correlation of $CD19^+CD24^{hi}CD38^{hi}$IL-10^+-cell frequency with clinical/laboratory characteristics and with levels of inflammatory cytokines were assessed along with the effects of $CD19^+CD24^{hi}CD38^{hi}$ cells on the proliferation and tumor necrosis factor α expression of $CD3^+$ T cells. The median frequency of IL-10-competent cells among the $CD19^+$ B cells was significantly increased among patients with RA with active disease. However, a sub-group of patients with a high disease status that received no treatment/NSAIDs exhibited a significantly lower frequency (≤1% IL-10^+ B cells). These patients exhibited longer symptom duration, a greater number of tender and swollen joints and a higher patient global visual analogue scale and disease activity score in 28 joints-C reactive protein. Functional assays further demonstrated that B10 cells from the sub-group with ≤1% IL-10^+ B cells secreted significantly lower levels of IL-10 and exerted a significantly decreased suppressive effect on $CD3^+$ T-cell proliferation and tumor necrosis factor-α production. The frequency and functional heterogeneity of B10 cells in patients with RA at different disease stage suggested that further investigation on the underlying mechanism of the generation and function of B10 cells in patients with RA is required prior to use in clinical practice.

Correspondence to: Dr Zhenbiao Wu or Dr Ping Zhu, Department of Clinical Immunology, Branch of Immune Cell Biology, State Key Key Discipline of Cell Biology, Xijing Hospital, Fourth Military Medical University, 127 West Changle Road, Xi'an, Shaanxi 710032, P.R. China
E-mail: wuzhenbiao@fmmu.edu.cn
E-mail: zhuping@fmmu.edu.cn

*Contributed equally

Key words: rheumatoid arthritis, regulatory B10 cells, frequency, function

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease, typically characterized by symmetrical polyarthritis, joint destruction and an impaired quality of life (1). The pathogenesis of RA remains to be elucidated. Numerous factors and inflammatory cells, including monocytes, B cells, T cells, endothelial cells and fibroblasts operate in unison to initiate a chronic inflammatory process (2-5). Increasing evidence has been obtained regarding the pivotal role of B cells involved in the immune dysregulation in RA, which is supported by the clinical improvements in the patients with RA receiving B-cell depleting therapies such as rituximab, an anti-CD20 antibody (6-9). However, the absence or loss of B cells was shown to exacerbate disease symptoms in experimental autoimmune encephalomyelitis, collagen-induced arthritis (CIA) and colitis, suggesting that B cells, or specific B-cell sub-sets, are also able to negatively regulate immune responses (10-13).

Suppressor B-cells were initially observed in 1974 for their suppressive effects on delayed hypersensitivity (14). Further interest in suppressor B-cells was generated by studies demonstrating a profound inhibitory function in the inflammatory context, *in vitro* and in adoptive transfer experiments (15-20). Cumulative evidence suggested that a certain B-cell sub-set predominantly exerted regulatory functions through the production of the regulatory cytokine interleukin (IL)-10; this B-cell sub-set was therefore named as B10 cells (16). Additional types of IL-10-producing or regulatory B10 cells also exist, including transforming growth factor-β producing B-cells (12,17,21,22). Dissimilar to the $CD1d^{hi}CD5^+CD19^{hi}$ sub-set in the spleen, which was labeled as B10 cells in mice, the identification of B10 cells using membrane markers has, thus far, not been successful in humans (23). The B10 cells

were functionally identified by their ability to express cytoplasmic IL-10 following an *ex vivo* stimulation. Recently, the $CD19^+CD24^{hi}CD38^{hi}IL\text{-}10^+$ and $CD19^+CD24^{hi}CD27^+IL\text{-}10^+$ B-cell subsets were investigated in humans, such as B10 cells of autoimmunity and immune-mediated inflammation, which further facilitated the investigation of regulatory B10 cells (24-27). Contradictory to the findings in the animal models, the negative regulatory function of B10 cells was impaired in the patients with thrombocytopenia and systemic lupus erythematosus (SLE) (26,28). In the CIA model, B10 cells demonstrated their novel and effective suppressive effects as well as their potential therapeutic value in the *in vivo* treatment of severe RA, with resistance to current therapies (12). However, the frequency of B10 cells in the patients with RA and the demonstration of their similar inhibitory effects to those found in animal models require further study.

In the present study, the frequency of B10 cells in the peripheral blood (PB) and their correlation with clinical characteristics, laboratory characteristics and inflammatory cytokines in a total of 97 patients with RA with different disease activity and status were investigated. In addition, the frequency of B10 cells in the paired synovial fluid (SF) from 13 patients was evaluated. Finally, the IL-10 production and suppression capacities of the B10 cells on T-cells in the patients of different disease status were investigated in order to examine their potential clinical application.

Patients and methods

Patients and healthy controls. Informed consent was obtained from 97 patients (aged ≥18 years) using the revised American College of Rheumatology criteria from 1987 (29). The patients with RA with complicated infections were not enrolled in the present study. A total of 24 healthy individuals were recruited following informed consent as a control group and these patients were age and gender matched with the patients with RA. The Ethics Committee at Xijing Hospital (Xi'an, China) granted ethical approval of the present study. Demographic and clinical parameters were also collected at the same time as the blood and synovial samples. These included age, gender, disease duration/duration of flare, use of drugs, tender joint count (28 joints), swollen joint count (28 joints), erythrocyte sedimentation rate, level of C-reactive protein (CRP), patient global visual analogue scale (VAS; 0-10 cm), disease activity score in 28 joints (DAS28)-CRP scores, rheumatoid factor, anti-cyclic citrullinated peptide 2 antibodies, anti-mutated citrullinated vimentin antibody, and immunoglobulins, immunoglobulin (Ig)G, IgM, and IgM.

A total of 22 patients reached a DAS value of <2.6 for at least 6 months and were in clinical DAS28 remission. Out of the 75 patients with active disease, 51 individuals received no treatment or were only treated with non-steroidal anti-inflammatory drugs (NSAIDs), while 24 patients experienced a disease relapse. A flare was defined as any increase in the disease activity (DAS28≥2.6) and required a change in therapy (30).

B-cell surface and cytoplasmic expression analysis. Mononuclear cells in PB (PBMCs) and SF (SFMCs) were isolated by density gradient centrifugation using Ficoll-Hypaque (1.077 g/ml; Sigma-Aldrich, St. Louis, MO, USA) and were centrifuged at 400 x g for 30 min at room temperature. PBMCs and SFMC were re-suspended for 48 h prior to stimulation ($2x10^6$ cells/ml) in a RPMI-1640 medium containing 10% fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA), 4 mM L-glutamine (Invitrogen Life Technologies), 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) and 1 μg/ml CD40L (R&D Systems, Minneapolis, MN, USA). Phorbol myristate acetate (PMA; 50 ng/Ml, Sigma-Aldrich), ionomycin (1 μg/ml; Sigma-Aldrich) and GolgiPlug (Brefeldin A; BD Biosciences, San Jose, CA, USA) were added 5 h prior the end of the culture. Following harvesting, B-cell phenotypic analysis was performed using fluorescent-conjugated mouse anti-human monoclonal antibodies against human CD19-APC (cat. no. 555415), CD24-PerCP-Cy5.5 (cat. no. 561647), and CD38-FITC (cat. no. 555459) for 30 min on ice in the dark. The antibodies were purchased from BD Biosciences. The cytoplasmic IL-10 expression was analyzed according to the manufacturer's instructions, using a Cytofix/Cytoperm kit (BD Biosciences) and staining with anti-human IL-10 or an isotype-matched control. Subsequently, the cells were analyzed using flow cytometry (FACSort; BD Biosciences).

Serum cytokine concentration assays. Quantitative levels of cytokines IL-1β, IL-6, IL-21, IL-8, IL-17A and interferon (IFN)-γ were measured in the serum of the patients, following the manufacturer's instructions (BD Biosciences). In brief, six bead populations with distinct fluorescence intensities coated with capture antibodies specific for IL-1β, IL-6, IL-21, IL-8, IL-17A, and IFN-γ were mixed and added to each assay tube at room temperature followed by incubation for 3 h. Cells were then washed with wash buffer and re-suspended in a wash buffer prior to detection using flow cytometry. The data were analyzed using the BD CBA analysis software (FCAP Array v3.0; BD Biosciences). The concentration of each cytokine in the plasma was determined in reference to a standard curve.

Cell sorting and functional assays. A total of five patients with active disease and three healthy controls provided formal consent for the attainment of an additional 200-300 ml PB, which was necessary for cell sorting and functional assays. Table I details the characteristics of the five patients and the three healthy controls. PBMCs were isolated as described above. The $CD19^+CD24^{hi}CD38^{hi}$ cells were separated using a fluorescence-activated cell sorting Moflo sorter (Beckman Coulter, Inc, Brea, CA, USA) following surface staining using fluorescently-conjugated antibodies against CD24, CD19 and CD38. $CD3^+$ T cells were later enriched using MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The secretion of IL-10 by the $CD19^+CD24^{hi}CD38^{hi}$ cells was stimulated with LPS and CD40L for 48 h. Flow cytometric analysis was used to detect the cells as described above.

$CD19^+CD24^{hi}CD38^{hi}$ cells were then added to the $CD3^+$ T-cell culture in 96-well round-bottom plates at a ratio of 1:2 ($1x10^5$ cells: $2x10^5$ cells) and stimulated with CD3 monoclonal antibody (mAb; 0.5 μg/ml) for 48 h. Cultured $CD3^+$ T cells stimulated with CD3 mAb in the absence of $CD19^+CD24^{hi}CD38^{hi}$ cells served as a control. The proliferation of $CD3^+$ T cells was observed using carboxyfluorescein succinimidyl ester

Table I. Characteristics of patients with rheumatoid arthritis and healthy controls based on functional assays.

Subject	Age (years)	Gender	Therapy	IL-10$^+$ B cells (%)
Patient with active disease				
1	18	F	No	0.55
2	58	F	No	0.69
3	29	F	No	4.26
4	46	M	NSAID	4.58
5	37	F	No	7.13
Healthy control subject				
1	23	M		1.59
2	51	F		0.98
3	46	F		2.11

NSAID, non-steroidal anti-inflammatory drugs; IL, interleukin; F, female; M, male.

(CFSE; Molecular Probes, Eugene, OR, USA) staining, which was quantified by flow cytometric analysis. ELISA was used to detect the cytokine tumor necrosis factor (TNF)-α in the supernatant using an IFN-γ high sensitivity ELISA (cat. no. BMS228HS; eBioscience, San Diego, CA, USA).

Statistical analysis. Statistical analyses were performed using SPSS 10.0 statistical software (SPSS, Inc., Chicago, IL, USA). For all the comparisons described in the present study Mann-Whitney U-test or t-test were used. P<0.05 was considered to indicate a statistically significant difference. Correlations were investigated using Spearman's rank correlation coefficient.

Results

Heterogeneity in the frequency of B10 cells with a CD19$^+$CD24^{hi}CD38^{hi} phenotype in patients with RA. The median frequency of the IL-10-competent CD19$^+$ B cells from the patients with RA with active disease was significantly increased following a 48-h stimulation with LPS plus CD40L and subsequent 5-h re-stimulation with PMA + ionomycin (Fig. 1A) as compared with that in the healthy controls [median (% range), 3.38 (0.26-9.74) vs. 1.78 (0.86-4.11); P<0.0001] and patients with RA in remission [median (% range), 3.38 (0.26-9.74) vs. 1.39 (0.69-4.04); P<0.0001]. Although IL-10-competent CD19$^+$ CD21^{hi} CD38^{hi} B-cells were enhanced in patients with RA with active disease, the increases were not as significant in patients with low disease status [median (% range), 3.84 (1.99-5.12)], moderate disease status [median (% range), 3.19 (1.44-9.44)] and high disease status [median (% range), 3.53 (0.26-8.28] (Fig. 1B). However, regardless of the disease status, the frequency of IL-10$^+$ B cells was higher in the SF as compared with that in the paired PB samples (Fig. 1C). An additional analysis was conducted on patients that received no treatment or were treated only with NSAIDs, and patients with RA with a disease relapse and an active disease status (Fig. 1D). Individuals that received no treatment/NSAIDs exhibited a lower level of IL-10$^+$ B cells [median (% range), 3.21 (0.26-9.74)] as compared with that in patients experiencing a disease relapse [median (% range), 4.01 (1.88-8.28)]; however, the difference was not statistically significant (P=0.10). Of note, 13 individuals (presented under the dashed line in Fig. 1B and D), which received no treatment/NSAIDs and who had a high disease status, exhibited a significantly lower IL-10$^+$ B-cell frequency as compared with that in the other 38 patients who received no treatment/NSAIDs [median (% range), 0.55 (0.26-1.00] vs. 3.8 (1.44-9.74); P<0.0001]. Similarly, the 24 patients with a disease relapse exhibited a higher IL-10$^+$ B-cell frequency compared with those with <1% IL-10$^+$ B cells among the patients with no treatment/NSAIDs [median (% range), 4.01 (1.88-8.28) vs. 0.55 (0.26-1.00); P<0.0001] (Fig. 1E). In addition, the individuals with ≤1% IL-10$^+$ B cells exhibited a longer symptom duration, a greater number of tender and swollen joints, and a higher patient global visual analogue scale and DAS28-CRP, respectively (Table II).

IL-10$^+$ B cells were isolated by flow cytometry for further functional assays, in which the phenotype of the IL-10$^+$ B cells was identified. As Fig. 1F demonstrates, the CD19$^+$CD24^{hi}CD38^{hi} cells predominantly produced IL-10 following the treatment described above. Thus, regulatory B10 cells predominantly represent a small sub-set of cells within the CD19$^+$CD24^{hi}CD38^{hi} B-cell sub-population, which was consistent with the findings of previous studies (26,27).

B10 cell frequency is negatively correlated with symptom duration, but not with pro-inflammatory cytokines in patients with active RA. The correlation of clinical characteristics of patients with active RA with the frequency of IL-10$^+$ B cells was further analyzed in order to investigate the effect of clinical characteristics on the frequency of IL-10-producing B cells. As shown in Table III, IL-10$^+$ B cells exhibited a negative association with the symptom duration (r=-0.44, P<0.0001) and the number of swollen joints (r=-0.24, P=0.036), while no association with other clinical characteristics was identified.

Pro-inflammatory mediators, including IL-1β, IL-6, IL-21, IL-8, IL-17A, and IFN-γ, are involved in the pathology of RA (4,31). The association of these cytokines with the regulatory B10 cells was further assessed in the present study. Although IL-1β, IL-6, IL-21, IL-8, IL-17A and IFN-γ levels

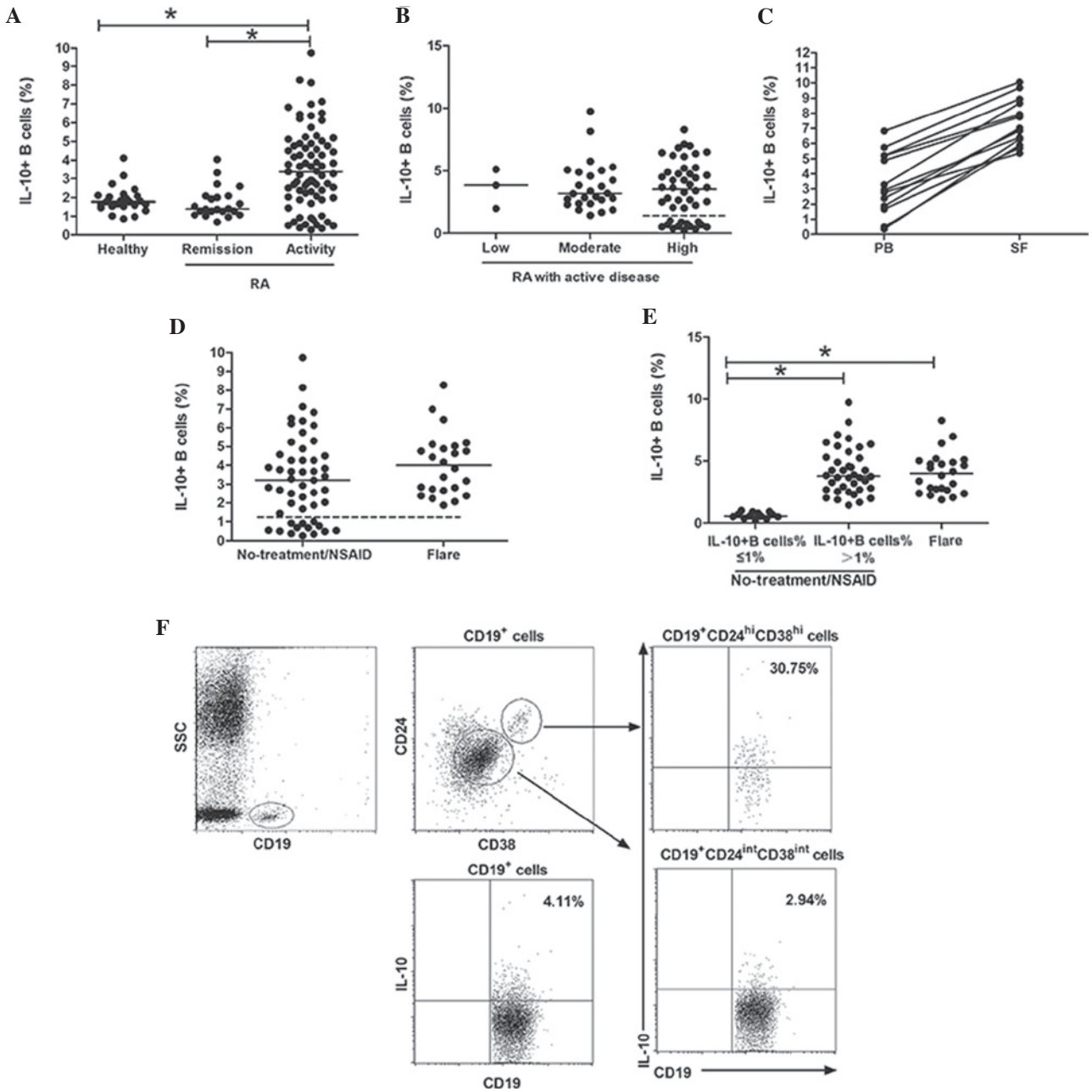

Figure 1. Frequency and phenotype of the regulatory B10 cells in the patients with RA. (A) The frequency of IL-10$^+$ B cells was significantly increased in the patients with active disease (P<0.0001) as compared with that in the healthy control group (P<0.0001). However, patients in remission exhibited a similar frequency of IL-10$^+$ B cells to that in the healthy controls (P=0.191). (B) Patients with a low, moderate and high disease status exhibited no significant differences in the frequency of IL-10$^+$ B cells. However, 13 patients with a high active disease status exhibited a low frequency of IL-10$^+$ B cells (≤1%) (under dashed line). (C) The frequency of IL-10$^+$ B cells in synovial fluid was increased as compared with that in the paired peripheral blood samples. (D) Among the patients with active disease status, patients with no treatment/NSAID exhibited a non-significant frequency of IL-10$^+$ B cells as compared with those in patients with a disease relapse. 13 patients with no treatment/NSAID (under dashed line) exhibited a low frequency of IL-10$^+$ B cells (≤1%). (E) In 13 patients with high disease status who received no treatment/NSAID, the number of IL-10$^+$ B cells was decreased as compared with that in another 38 patients with RA who received no treatment/NSAID (P<0.0001) and 24 patients with a disease relapse (P<0.0001). Horizontal lines represent the median, while highest and lowest data points represent the range. *P<0.001. (F) Representative frequencies of IL-10-producing cells. The frequency of IL-10-producing cells in total CD19$^+$ B cells was 4.11%. Using the indicated gates, IL-10 was found to be predominantly produced by the CD24^{hi}CD38^{hi} B-cell sub-population of CD19$^+$ cells. RA, rheumatoid arthritis; NSAID, non-steroidal anti-inflammatory drugs; IL, interleukin; SF, synovial fluid; PB, peripheral blood; SSC, side scatter.

were significantly increased in the serum of patients with RA with active disease as compared with those in the healthy controls, neither of these cytokines demonstrated any correlation with the B10 cells (Table III).

Function of B10 cell heterogeneity in patients with RA. CD19$^+$CD24^{hi}CD38^{hi} cells were isolated from five patients with RA with active disease (the frequency of IL-10$^+$ B cells was ≤1% in two patients and 1% in three patients) and three healthy controls (Table I). Following 48-h treatment with LPS plus CD40 L, the CD19$^+$CD24^{hi}CD38^{hi} cells isolated from patients in whom the IL-10$^+$ B-cell frequency was ≤1% secreted significantly lower levels of IL-10 compared with those from the healthy controls (6.24±2.31 vs. 13.64±3.77; P<0.001) and

Table II. Demographic and clinical characteristics of patients with RA with active disease.

	RA with no treatment/NSAID			
Variable	IL-10$^+$B cells % ≤1%	IL-10$^+$B cells % >1%	RA with flare	Significance
Number (F/M)	13 (12/1)	38 (32/6)	24 (20/4)	
Age (years)	54 (18-64)	43 (21-75)	54 (18-70)	Ns
Symptom duration[a] (years)	15 (6-40)	2 (0.16-10)[b]	0.56 (0.08-4)[c]	P<0.001[b,c]
Tender joints	23 (12-28)	11.5 (4-28)[b]	10 (3-28)[c]	P<0.001[b,c]
Swollen joints	13 (4-22)	4 (0-24)[b]	3 (1-21)[c]	P<0.001[b,c]
VAS global (cm)	9 (6-10)	5 (1-10)	5 (2-10)	P<0.001[b,c]
CRP (mg/dl)	3.8 (1.2-10.8)	2.6 (0.8-19.5)	3.6 (0.25-16.9)	Ns
ESR (mm/h)	81 (35-140)	61 (19-110)	63 (15-108)	Ns
DAS28-CRP	7.36 (5.3-8.16)	5.50 (3.05-8.05)[b]	5.24 (3.20-8.45)[c]	P<0.001[b,c]
RF (IU/ml)	373 (0-7,630)	278 (0-3,680)	220 (0-3,190)	Ns
Anti-CCP2 (RU/ml)	140 (0-1,291)	218.5 (0-1,750)	193.5 (30-1,800)	Ns
Anti-MCV (RU/ml)	106 (0-1,300)	117 (0-1,340)	87.5 (0-1,200)	Ns
IgG (mg/dl)	1480 (719-3,040)	500 (737-2,460)	1535 (925-1,980)	Ns
IgM (mg/dl)	143 (69-275)	180 (75.6-343)	192 (75-262)	Ns
IgA (mg/dl)	140 (94-291)	127.5 (75-535)	123 (57-394)	Ns

Values are expressed as the median (range). [a]Symptom duration; for RA with no treatment/NSAID mean disease duration; for RA with flare mean duration after disease relapse. [b]P<0.05 and [c]P<0.01, compared with the group of IL-10$^+$ B cells ≤1%. NSAID, non-steroidal anti-inflammatory drugs; VAS global, patient global visual analogue scale; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS2, disease activity score in 28 joints; RF, rheumatoid factor; anti-CCP2, anti-cyclic citrullinated peptide 2 antibody; anti-MCV, anti-mutated citrullinated vimentin; RA, rheumatoid arthritis; IL, interleukin; Ig, immunoglobulin; Ns, not significant; F, female; M, male.

patients with >1% IL-10$^+$ B cells (6.24±2.31 vs. 15.57±4.40; P<0.001) (Fig. 2A).

CFSE labeling of the respective cells was used to assess the effect of B10 cells on the proliferation of CD3$^+$ T cells. This dye was retained in the cytoplasm and was diluted with each cell division. As shown in Fig. 2B, the proliferation of CD3$^+$ T cells was significantly inhibited in the presence of CD19$^+$CD24^{hi}CD38^{hi} cells isolated from healthy controls and patients with >1% IL-10$^+$ B cells as compared with CD3$^+$ T cells stimulated with CD3 mAb only. By contrast, the growth inhibitory function of CD19$^+$CD24^{hi}CD38^{hi} cells from patients with ≤1% IL-10$^+$B cells was observed to be decreased (Fig. 2B).

Furthermore, the effect of CD19$^+$CD24^{hi}CD38^{hi} cells on the TNF-α production by CD3$^+$ T cells was investigated. CD19$^+$CD24^{hi}CD38^{hi} cells isolated from patients with ≤1% IL-10$^+$ B cells did not exert any inhibitory effects on the TNF-α production by CD3$^+$ T cells in comparison to CD3$^+$ T cells stimulated by CD3 mAb (125.37±22.14 vs. 138.19±30.74; P=0.437). However, CD19$^+$CD24^{hi}CD38^{hi} isolated from healthy controls (125.37±22.14 vs. 74.33±20.08; P=0.015) and patients with >1% IL-10$^+$ B cells (125.37±22.14 vs. 69.50±19.31; P=0.012) exerted significant inhibitory effects on the TNF-α production by CD3$^+$ T cells compared with those of patients with ≤1% B10 cells, respectively (Fig. 2C).

Discussion

In the present study, the heterogeneity in the frequency of IL-10-competent CD19$^+$ B cells in patients with RA with different disease status was demonstrated, which was in parallel with their inhibitory effect on CD3$^+$ T cells. The median frequency of B10 cells was significantly higher in RA patients with an active disease as compared with that in the healthy controls and patients with RA in remission.

Recent studies on B10 cells in animal models demonstrated that IL-10-competent B-cells exerted profoundly negative regulatory functions on innate and adaptive immunity, and revealed their therapeutic potential in the treatment of autoimmune diseases (15). However, studies of IL-10-competent B-cells in humans yielded diverse results, which were difficult to unify into a coherent model. In 2009, Blair *et al* (26) identified a specific sub-set of human Breg cells with a phenotype of CD19$^+$CD24^{hi}CD38^{hi}, which were previously associated with immature transitional B-cells, but were able to produce IL-10 in response to stimulation *in vitro*. In this study, CD19$^+$CD24^{hi}CD38^{hi} cells from patients with SLE secreted low levels of IL-10 and exerted defective suppressive effects on CD4$^+$ T cells as opposed to those from healthy controls. Sequentially, a deficit of this B10-cell suppressive ability was also found in patients with immune thrombocytopenia (28). A recent study revealed that patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis had diminished levels of IL-10-producing B-cells (32). All of these results indicated that B10-cell dysfunction may be implicated in a number of diseases in humans.

RA is an auto-inflammatory disease that is characterized by complex pathogenic inflammation that differs from SLE and ANCA-associated vasculitis. Although several studies (12,17,21)

Table III. Correlation of clinical characteristics of patients with active rheumatoid arthritis as well as correlation of serum cytokines with the frequency of IL-10$^+$ B cells.

Variable	Spearman	P-value (two-tailed)
Clinical characteristic		
Age	0.072	0.54
Symptom duration	-0.44	<0.0001[a]
Tender joints	-0.19	0.09
Swollen joints	-0.24	0.036[a]
VAS global	-0.14	0.22
CRP	0.08	0.47
ESR	-0.18	0.12
DAS28-CRP	-0.15	0.31
RF	-0.09	0.42
Anti-CCP2	0.07	0.57
Anti-MCV	0.18	0.13
IgG	0.13	0.28
IgM	-0.10	0.39
IgA	-0.11	0.34
Cytokine		
IL-1β	-0.28	0.35
IL-6	-0.36	0.23
IL-21	-0.33	0.28
IL-8	-0.18	0.55
IL-17A	-0.35	0.26
IFN-γ	-0.20	0.51

[a]P<0.05. Correlation analyses were determined between the clinical characteristics and the cytokines with the frequency of IL-10$^+$ B cells using Spearman's rank correlation. VAS global, patient global visual analogue scale; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score in 28 joints; RF, rheumatoid factor; anti-CCP2, anti-cyclic citrullinated peptide 2 antibody; anti-MCV, anti-mutated citrullinated vimentin; IL, interleukin; IFN, interferon; Ig, immunoglobulin.

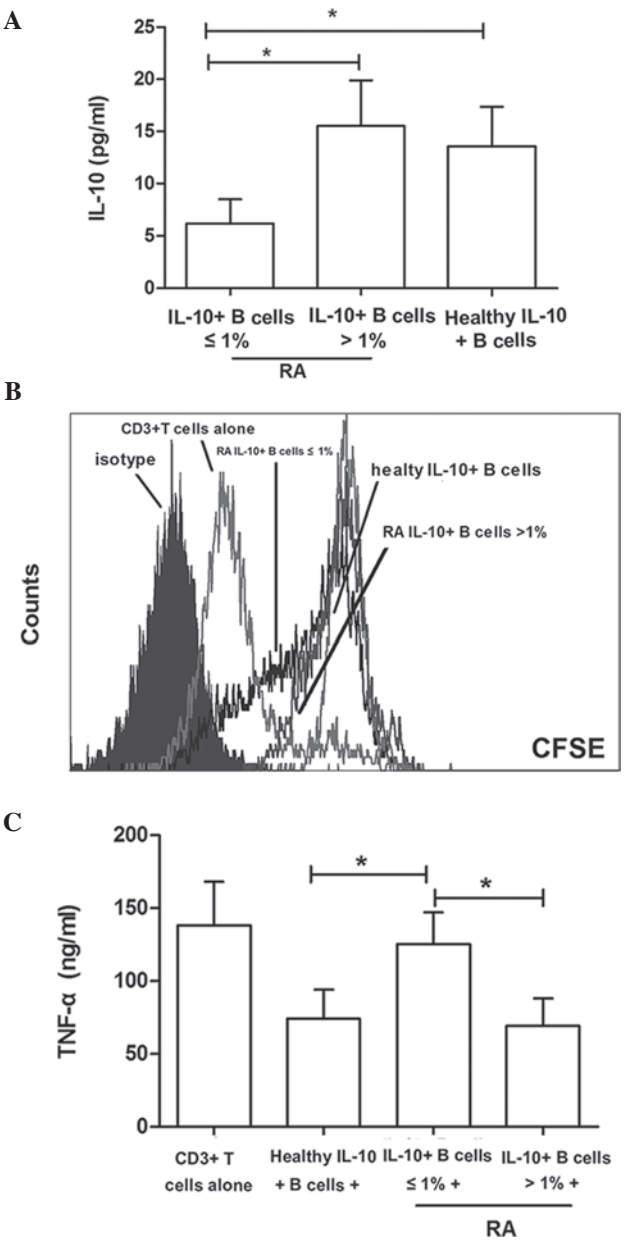

Figure 2. IL-10 producing ability of regulatory B cells in patients with RA and their function on CD3$^+$ T cells. (A) CD19$^+$CD24^{hi}CD38^{hi} cells were isolated. Cells from patients with a B10 cell count of ≤1% produced significantly lower levels of IL-10 as compared with those from the healthy controls and patients with a B10 cell count of >1%. Values are expressed as the mean ± standard deviation. *P<0.001. (B) Representative histograms of carboxyfluorescein succinimidyl ester-labeled CD3$^+$ T-cells isolated from different patients with RA. CD3$^+$ T-cells were observed to proliferate upon stimulation with CD3 mAb in the presence of CD19$^+$CD24^{hi}CD38^{hi} cells. (C) CD19$^+$CD24^{hi}CD38^{hi} cells isolated from healthy controls and patients with a B10 cell count of >1% significantly inhibited TNF-α production by CD3$^+$T cells stimulated with CD3 mAb as compared with those from patients with a B10 cell count of ≤1%. Values are expressed as the mean ± standard deviation. *P<0.05. IL, interleukin; TNF, tumor necrosis factor; RA, rheumatoid arthritis; mAb, monoclonal antibody; CFSE, carboxyfluorescein succinimidyl ester.

on CIA demonstrated the negative regulatory effects and therapeutic potential of B10 cells in RA treatment, the characteristics of B10 cells in patients with RA have not been well-investigated. In 2011, Iwata *et al* (25) reported that IL-10-producing B-cells increased in the PB of patients with RA. However, the authors did not correlate the characteristics of B10 cells in patients with RA with different disease stages as only 19 patients were recruited in the study. It was therefore critical to further assess the characteristics of B10 cells in patients with RA prior to their use in the clinical practice. Therefore, the present study recruited a total of 97 patients with RA with a range of disease statuses. The results revealed that the frequency of B10 cells significantly increased in the patients with RA with an active disease status compared with that of patients with RA in remission or in healthy controls. This suggested that the inflammatory factors increased the frequency of B10. This was confirmed by the observed higher frequency of B10 cells in the SF as compared with that in the PB of the patients with RA. The frequency of B10 cells at low, moderate and high disease severity status revealed no significant differences, which suggested that the disease status did not affect the frequency of B10 cells. B10 cells from patients with RA with an active disease status were also investigated and it was found that the frequency of B10 cells exhibited no difference between an untreated sub-group and a sub-group

undergoing a disease relapse. This indicated that the frequency of B10 cells was not affected by previous therapy. It is important to note that a small group of patients with active RA with a high disease status that received no treatment were found to exhibit a significantly decreased frequency of IL-10$^+$ B cells (≤1%). When compared with patients with >1% IL-10$^+$ B cells in the untreated group and the patients with a disease relapse, the individuals in the ≤1% IL-10$^+$ B-cell sub-group exhibited a significantly longer symptom duration, a greater number of tender and swollen joints and a higher global VAS and DAS28-CRP score for the demographic and clinical characteristics. In order to further investigate the factors that affected the frequency of B10 cells in the patients with RA, the correlation of the B10-cell frequency in the blood with the clinical characteristics was assessed. It was identified that the B-cell frequency exhibited a negative correlation with symptom duration and the number of swollen joints, which suggested that disease duration and inflammatory factors may be critical in decreasing the B10-cell frequency. As the pro-inflammatory cytokines, including IL-1β, IL-6, IL-21, IL-8, IL-17A, and IFN-γ, are considered to have an important pathogenic role in RA and were produced at increased levels in the patients with RA with an active disease status compared with those in the other groups, a correlation analysis of these cytokines with the B10-cell frequency was also performed. However, no significant correlation was identified between these inflammatory factors and B10-cell frequency. This indicated that multiple factors in the regulation of B10-cell production and other unknown inflammatory cytokines may have affected the B10-cell frequency in patients with RA.

Subsequently, the regulatory ability of B10 cells on the proliferation of CD3$^+$ T cells was assessed. It was identified that CD19$^+$CD24^{hi}CD38^{hi} cells predominantly produced IL-10, which was consistent with previous studies (25-27). Therefore, they have been termed 'B10 cells' in recent years (26). Similarly, based on the observations regarding B10-cell frequency detection, the B10 cells from patients with RA with different disease status revealed heterogenic functions. The B10 cells isolated from the RA sub-group containing ≤1% IL-10$^+$ B cells secreted significantly decreased levels of IL-10 and exerted a significantly diminished suppressive effect on CD3$^+$ T-cell proliferation and TNF-α production compared with those of healthy controls and the RA sub-group containing >1%IL-10$^+$ B10 cells. These functional studies on B10 cells further demonstrated the complexity of the mechanisms underlying B10-cell generation and function in patients with RA.

In conclusion, the present study demonstrated that the frequency and function of B10 cells displayed heterogeneity in patients with RA with a different disease status as well as healthy controls. Adoptive transfer of B10 cells is considered to be a potential therapeutic strategy for RA based on the results of previous studies on animals; however, further study is required prior to the use of B10 cells in clinical practice.

Acknowledgements

The present study was funded by a grant from the National Natural Science Foundation of China (grant no. 81001335). The authors would like to thank Mr. Yanhong Wang and Miss. Chunmei Fan of the Department of Clinical Immunology, State Key Discipline of Cell Biology, Xijing Hospital, Fourth Military Medical University (Xian, China) for their excellent technical assistance.

References

1. Drossaers-Bakker KW, de Buck M, van Zeben D, Zwinderman AH, Breedveld FC and Hazes JM: Long-term course and outcome of functional capacity in rheumatoid arthritis: The effect of disease activity and radiologic damage over time. Arthritis Rheum 42: 1854-1860, 1999.
2. Davignon JL, Hayder M, Baron M, Boyer JF, Constantin A, Apparailly F, Poupot R and Cantagrel A: Targeting monocytes/macrophages in the treatment of rheumatoid arthritis. Rheumatology (Oxford) 52: 590-598, 2013.
3. Gol-Ara M, Jadidi-Niaragh F, Sadria R, Azizi G and Mirshafiey A: The role of different subsets of regulatory T cells in immunopathogenesis of rheumatoid arthritis. Arthritis 2012: 805875, 2012.
4. Brennan FM and McInnes IB: Evidence that cytokines play a role in rheumatoid arthritis. J Clin Invest 118: 3537-3545, 2008.
5. Bugatti S, Codullo V, Caporali R and Montecucco C: B cells in rheumatoid arthritis. Autoimmun Rev 7: 137-142, 2007.
6. Leandro MJ, Cambridge G, Ehrenstein MR and Edwards JC: Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis. Arthritis Rheum 54: 613-620, 2006.
7. Mariette X, Rouanet S, Sibilia J, Combe B, Le Loët X, Tebib J, Jourdan R and Dougados M: Evaluation of low-dose rituximab for the retreatment of patients with active rheumatoid arthritis: A non-inferiority randomised controlled trial. Ann Rheum Dis 73: 1508-1514, 2014.
8. Reddy V, Croca S, Gerona D, De La Torre I, Isenberg D, McDonald V, Leandro M and Cambridge G: Serum rituximab levels and efficiency of B cell depletion: Differences between patients with rheumatoid arthritis and systemic lupus erythematosus. Rheumatology (Oxford) 52: 951-952, 2013.
9. Bredemeier M, de Oliveira FK and Rocha CM: Low-versus high-dose rituximab for rheumatoid arthritis: A systematic review and meta-analysis. Arthritis Care Res (Hoboken) 66: 228-235, 2014.
10. Goetz M, Atreya R, Ghalibafian M, Galle PR and Neurath MF: Exacerbation of ulcerative colitis after rituximab salvage therapy. Inflamm Bowel Dis 13: 1365-1368, 2007.
11. Wolf SD, Dittel BN, Hardardottir F and Janeway CA Jr: Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. J Exp Med 184: 2271-2278, 1996.
12. Mauri C, Gray D, Mushtaq N and Londei M: Prevention of arthritis by interleukin 10-producing B cells. J Exp Med 197: 489-501, 2003.
13. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR and Mauri C: Novel suppressive function of transitional 2 B cells in experimental arthritis. J Immunol 178: 7868-7878, 2007.
14. Neta R and Salvin SB: Specific suppression of delayed hypersensitivity: The possible presence of a suppressor B cell in the regulation of delayed hypersensitivity. J Immunol 113: 1716-1725, 1974.
15. Yang M, Rui K, Wang S and Lu L: Regulatory B cells in autoimmune diseases. Cell Mol Immunol 10: 122-132, 2013.
16. Mauri C and Bosma A: Immune regulatory function of B cells. Annu Rev Immunol 30: 221-241, 2012.
17. Yang M, Deng J, Liu Y, Ko KH, Wang X, Jiao Z, Wang S, Hua Z, Sun L, Srivastava G, *et al*: IL-10-producing regulatory B10 cells ameliorate collagen-induced arthritis via suppressing Th17 cell generation. Am J Pathol 180: 2375-2385, 2012.
18. Li X, Braun J and Wei B: Regulatory B cells in autoimmune diseases and mucosal immune homeostasis. Autoimmunity 44: 58-68, 2011.
19. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M and Tedder TF: Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest 118: 3420-3430, 2008.
20. Gray M, Miles K, Salter D, Gray D and Savill J: Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. Proc Natl Acad Sci USA 104: 14080-14085, 2007.
21. Lemoine S, Morva A, Youinou P and Jamin C: Regulatory B cells in autoimmune diseases: how do they work? Ann N Y Acad Sci 1173: 260-267, 2009.

22. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D and Anderton SM: B cells regulate autoimmunity by provision of IL-10. Nat Immunol 3: 944-950, 2002.
23. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M and Tedder TF: A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity 28: 639-650, 2008.
24. Zha B, Wang L, Liu X, Liu J, Chen Z, Xu J, Sheng L, Li Y and Chu Y: Decrease in proportion of CD19+CD24(hi) CD27+ B cells and impairment of their suppressive function in Graves' disease. PLoS One 7: e49835, 2012.
25. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, *et al*: Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 117: 530-541, 2011.
26. Blair PA, Noreña LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR and Mauri C: CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity 32: 129-140, 2010.
27. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, Burlingham WJ, Marks WH, Sanz I, Lechler RI, *et al*: Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest 120: 1836-1847, 2010.
28. Li X, Zhong H, Bao W, Boulad N, Evangelista J, Haider MA, Bussel J and Yazdanbakhsh K: Defective regulatory B-cell compartment in patients with immune thrombocytopenia. Blood 120: 3318-3325, 2012.
29. Arnett FC1, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, *et al*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315-324, 1988.
30. Wells GA, Tugwell P, Kraag GR, Baker PR, Groh J and Redelmeier DA: Minimum important difference between patients with rheumatoid arthritis: the patient's perspective. J Rheumatol 20: 557-560, 1993.
31. Hughes-Austin JM, Deane KD, Derber LA, Kolfenbach JR, Zerbe GO, Sokolove J, Lahey LJ, Weisman MH, Buckner JH, Mikuls TR, *et al*: Multiple cytokines and chemokines are associated with rheumatoid arthritis-related autoimmunity in first-degree relatives without rheumatoid arthritis: Studies of the Aetiology of Rheumatoid Arthritis (SERA). Ann Rheum Dis 72: 901-907, 2013.
32. Wilde B, Thewissen M, Damoiseaux J, Knippenberg S, Hilhorst M, van Paassen P, Witzke O and Cohen Tervaert JW: Regulatory B cells in ANCA-associated vasculitis. Ann Rheum Dis 72: 1416-1419, 2013.