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ORIGINAL RESEARCH

Decreases in the Numbers of Peripheral Blood Regulatory T Cells, and Increases in the Levels of Memory and Activated B Cells, in Patients with Active Eosinophilic Granulomatosis and Polyangiitis

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Abstract
Purpose Eosinophilic granulomatosis with polyangiitis (EGPA), a rare disease characterized by the presence of allergic granulomatosis and necrotizing vasculitis, is often effectively treated with corticosteroids. However, relapse rates are high and, for unknown reasons, some EGPA patients suffer frequent relapses after entry into initial remission. Regulatory T (T_{reg}) cells and B cells are implicated in the development and progression of EGPA. Here, we explored the influence of T_{reg} cells and a co-stimulatory factor present on B cells on the development and course of EGPA.
Methods We studied 45 EGPA patients (19 of whom experienced frequent relapses and 26 of whom seldom relapsed) and 67 (control) patients with general asthma. We determined the counts or percentages of whole-blood cells exhibiting the following characteristics: $FOXP3^+$ cells among $CD4^+$ T_{reg} cells; CTLA-4^+ cells among $CD4^+/CD25^+$ T_{reg} cells; and $CD27^+$, $CD80^+$, $CD86^+$, or $CD95^+$ cells among $CD19^+$ B cells. We also measured serum IgG concentrations.
Results Compared with patients with asthma or seldom-relapsing EGPA, frequently relapsing EGPA patients with active disease exhibited decreased counts of T_{reg} cells and increased percentages of B cells that scored as $CD80^+$, $CD27^+$, or $CD95^+$. Patients with frequently relapsing EGPA had increased percentages of $CD27^+$ and $CD95^+$ B cells, and fewer $CD19^+$ B cells, than did patients in the other two groups. Lower $CD19^+$ B cell counts were associated with reduced T_{reg} cell counts and a lower serum IgG concentration.
Conclusion In patients with frequently relapsing EGPA, decreases in T_{reg} cell numbers and increased percentages of activated B cells may induce apoptosis of B cells.

Keywords Apo-1 · Churg-Strauss syndrome · eosinophilic granulomatosis with polyangiitis · memory B cell · regulatory T cell

Abbreviations

BD	Behçet's disease
CTLA-4	Cytotoxic T-lymphocyte antigen-4
CS	Corticosteroids
EGPA	Eosinophilic granulomatosis with polyangiitis
FFSs	Five-Factor Scores
FOXP3	Forkhead box P
MPA	Microscopic polyangiitis
T_{reg} cells	Regulatory T cells
SLE	Systemic lupus erythematosus
WG	Wegener's granulomatosis

Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA; also known as Churg-Strauss syndrome) is a rare disease characterized by the presence of allergic granulomatosis and necrotizing vasculitis. Development of the condition is preceded by peripheral blood eosinophilia and eosinophilic

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tissue infiltration [1]. EGPA is classified as a vasculitis of arteries that are small and medium in diameter, although such vasculitis is often not apparent in the initial phases of the disease [2].

Before the development of corticosteroids (CS), the mortality rate of patients with EGPA was approximately 50 % within 3 months of diagnosis [3, 4]. The 5-year survival rate was 62–79 % [3, 5–7]. The prognosis of EGPA patients, and the extent of mortality from the disease, are associated with disease severity, as assessed by using the Five-Factor Scores (FFSs) developed in 1996 [7] or 2009 [8]. In addition, myocardial involvement [4] and the presence of gastrointestinal disease [4, 9] are independent negative prognostic factors. The remission rate after initial treatment has been reported to be 81 % to 91 % in EGPA patients. This may be compared with the 30 % to 93 % remission rate in patients with Wegener's granulomatosis (WG) and the 75 % to 89 % remission rate in those with microscopic polyangiitis (MPA) [10]. However, the relapse rate is higher in EGPA patients in the first 2 years after diagnosis than in MPA patients (35 % in EGPA patients vs. 8 % in those with MPA [10]). The relapse rate in EGPA patients after 5 years or more is greater than 60 %, compared with 20 % in MPA patients [11]. Moreover, the relapse rate of EGPA patients with low FFS scores is 35 % during the first year of treatment [12]. Some patients with EGPA experience frequent relapses after initial clinical remission, and others fail to enter remission that last for extended periods. The mechanisms underlying the intractable form of EGPA remain poorly understood.

Regulatory T (T_{reg}) cells play central roles in the maintenance of both immune homeostasis and peripheral tolerance. Naturally occurring $CD4^+$ regulatory T (nT_{reg}) cells constitutively express the proteins forkhead box P (FOXP)3 and CD25. T_{reg} cells may also be identified by co-expression, on the cell surface, of cytotoxic T-lymphocyte antigen-4 (CTLA-4), CD25, and CD4. Natural T_{reg} cells suppress autoimmune T cell responses and maintain peripheral tolerance by preventing the differentiation of T_{reg} cells into effector T cells [13]. Peripheral differentiation of inducible T_{reg} (iT_{reg}) cells, which secrete IL-10, requires high-level T-cell-receptor signaling, an elevated serum concentration of TGF-β and expression of IL-10 [14]. When TGF-β mediates induction of iT_{reg} cells, CTLA-4 must bind to either of the co-stimulatory B7 family molecules CD80 or CD86 [15]. CTLA-4 and CD28 competitively bind molecules of the B7 family. Ligation of CD28 is critical for the induction of immune responses by naïve $CD4^+$ T cells. On the other hand, ligation of CTLA-4 inhibits IL-2 production, thus playing a crucial role in downregulating T cell responses and the antigen-presenting functions of cells.

B cells that can terminally differentiate into antibody-secreting plasma cells positively regulate the immune response via production of antigen-specific antibody; $CD4^+$ T cell activation is optimal in the presence of such antibody [16]. In addition, B cells can modulate T cell responses by presenting antigens to such cells, by synthesizing molecules that stimulate T cell function, and via production of cytokines [17]. For example, B cell-specific expression of co-stimulatory molecules, including CD80, CD86, and/or OX40L, is important in terms of T cell activation [18]. B cells also regulate the development of immunologic or allergic inflammation, and T cell-mediated autoimmunity, via production of IL-10 [19].

Abnormalities in the proportions or features of B cells have been reported in several diseases involving the immune system. First, the counts of $CD25^+/CD19^+$ and $CD86^+$ B cells increase in patients in remission from WG or MPA [20]. Second, in WG patients, mutations in genes encoding immunoglobulins are evident in cells that resemble memory B cells [21]. Third, the counts of $CD80^+/CD19^+$ B cells increase in patients with Behçet's disease (BD) [22]. Fourth, counts of $CD19^{hi}/CD27^+$ memory B cells are higher in patients with systemic lupus erythematosus (SLE) [23, 24], or in those in whom anti-neutrophil cytoplasmic autoantibodies are associated with the development of small vessel vasculitis [23], than in healthy controls. Finally, patients with Sjögren's syndrome have lower peripheral blood $CD27^+/CD19^+$ memory B cell counts than do controls [25]. In addition, an increase in the concentration of serum B-lymphocyte-activating factor in WG patients has been associated with enhanced disease activity and a fall in serum IgG concentration [26].

The Fas receptor, also termed CD95 (APO-1/Fas), is a key initiator of apoptotic programmed cell death in a variety of cell types [27]. Interaction of CD95 with specific ligands is a major trigger of such death. The viability of this system is thus essential for prevention of lymphoproliferative disorders and inhibition of the development of autoimmunity [28]. Increases in serum soluble CD95 concentrations have been reported in patients with active EGPA [29], SLE [30], or BD [31].

In a previous study, we reported that enhanced disease activity in patients with EGPA was associated with decreased levels of iT_{reg} cells producing IL-10 [32]. To further explore whether T_{reg} cells played a role in development of EGPA, we compared the phenotypes and functions of nT_{reg} cells, iT_{reg} cells, and B cells, in EGPA patients who relapsed frequently or seldom, and in patients with general asthma.

Methods

Patients

Between January 2008 and June 2011, we recruited 45 patients with EGPA and 67 control adult patients with general asthma but without EGPA. All patients were

recruited at the Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara, Kanagawa, Japan. All 67 adult patients with general asthma were diagnosed by using the criteria of the Global Initiative for Asthma; the severity of asthma was also rated with the aid of these guidelines [33]. EGPA patients were defined according to the classification criteria of the American College of Rheumatology [34]. All EGPA patients were allocated to one of two groups—those who relapsed but did so infrequently, and those suffering frequent relapses—by reference to the numbers of relapses recorded each year. A patient was considered to be in remission if the disease was inactive in that patient and no clinical sign or symptom of active vasculitis was evident. A relapse was defined as the presence of active disease combined with the recurrence, after initial remission, of vasculitis symptoms (with or without an increase in the percentage of eosinophils among white blood cells). Patients who relapsed required resumption of immunosuppressive therapy or prescription of increased doses of immunosuppressant. Patients were placed in the group that experienced frequent relapses if such patients relapsed at least once every 2 years after a period of initial remission; all other patients were considered to suffer infrequent relapses.

Multiple mononeuritis, a measure of motor nerve dysfunction, was evaluated by using the manual muscle test; responses were scored (from zero to five) on the Medical Research Council scale. Sensory nerve dysfunction was evaluated upon clinical examination. Lung involvement was considered present when any of consolidation, ground grass opacity, nodules within such opacity, interlobular septal thickening, bronchial wall thickening, lymph node enlargement, pleural effusion evident upon high-resolution computer tomography, or eosinophil infiltration detected by lung biopsy was present. The heart was considered to be involved when any of chest pain, chest discomfort, back pain, palpitations, abnormal signs on cardiac echocardiography, Holter electrocardiographic abnormalities, elevated B-type natriuretic peptide levels, or [^{123}I]-meta-iodobenzylguanidine myocardial imaging abnormalities of the myocardium was evident [35]. Gastrointestinal involvement was indicated by the presence of symptoms of epigastralgia, abdominal pain, diarrhea, constipation, or positive endoscopic signs, combined with confirmation of eosinophil infiltration by biopsy. Skin involvement was defined as the presence of purpura, erythema, livedo, an ulcer, or acrocyanosis when a nodule, accompanied by eosinophilic infiltration, was additionally detected by biopsy. Central nervous system involvement was defined as the presence of headache, visual disorder, abnormal visual sensation, cerebral infarction, bleeding, or cranial nerve dysfunction. Renal involvement was defined by any of the presence of eosinophils in urine, glomerular nephritis, nephrosis, renal dysfunction (i.e., the creatinine level was elevated to over 20 % of the baseline figure), or proteinuria (>0.5 g per day). Disease severity in all EGPA patients was evaluated by using the FFS 1996 [7] or FFS 2009 [8]. The organs compromised by asthma and sinusitis were not included when the total number of involved organs was enumerated. Disease activity was assessed at onset, and at first relapse, by using the Birmingham Vasculitis Activity Score (BVAS) [36]. The BVAS evaluates symptoms and signs within nine categories (systemic; cutaneous; mucous membranes and eyes; ear, nose, and throat; chest; heart and vessels; gastrointestinal tract; renal system; and nervous system). The maximum number of possible points in each category is 7; the maximum score is thus 63.

The eosinophil counts in whole blood; the serum IgG concentration; the counts of iT_{reg} cells (IL-10-producing cells among $CD4^+/CD25^+$ T cells) and nT_{reg} cells ($FOXP3^+$ cells among $CD4^+$ T cells or CTLA-4^+ cells among $CD4^+/CD25^+$ T cells); the counts of $CD19^+$ B cells among whole-blood lymphocytes; and the percentages of $CD80^+$, $CD86^+$, $CD27^+$, or $CD95^+$ cells among $CD19^+$ B cells were measured in all patients. All EGPA patients entered clinical remission at least once. Blood samples were examined at first relapse (thus in the active disease state) and at remission (thus when the disease was inactive) in EGPA patients who relapsed both frequently and seldom, and in control general asthma patients at a time when such patients were not exhibiting disease exacerbation. The Ethics Committee of our Hospital approved the study, and written informed consent was obtained from each patient.

Immunological Analysis

Reagents

Fluorescein isothiocyanate (FITC)-bearing conjugates of mouse IgG1, anti-CD4, -CD25, -CD86, -CD80, -CD27, and -CD95; phycoerythrin (PE)-bearing conjugates of mouse IgG1, anti-CD4, and -CD25; and PE-Cy5-bearing conjugates of mouse IgG1 and an anti-CD4 antibody were purchased from BioLegend (San Diego, CA) or Invitrogen (Tokyo, Japan). PE-conjugated anti-human FOXP3 and PerCP conjugates of mouse IgG1 and an anti-CD4 antibody were the products of BD Biosciences (Rikaken Co. Ltd., Tokyo, Japan). PE-conjugated anti-human IL-10, -CTLA-4, and -CD19 were purchased from BioLegend or R&D Systems (Cosmo Bio Co. Ltd., Tokyo, Japan)

$FOXP3^+$ Cells Among $CD4^+$ T Cells; CTLA$^+$ Cells Among $CD4^+/CD25^+$ T Cells; and $CD80^+$, $CD86^+$, $CD27^+$, and $CD95^+$ Cells Among $CD19^+$ B Cells

Peripheral blood cells were assayed as described by Abdulahad et al. [37] to determine the percentages of $CD25^+$ and $FOXP3^+$ cells among $CD4^+$ T cells. In brief,

whole-blood lymphocytes were incubated with FITC-conjugated anti-CD4, -CD80, -CD86, -CD27, or -CD95, or with PE-conjugated anti-CD25 or -CD19, to identify $CD25^+$ cells among $CD4^+$ T cells and $CD80^+$, $CD86^+$, $CD27^+$, or $CD95^+$ cells among $CD19^+$ B cells. CTLA-4^+ cells among $CD4^+/CD25^+$ T cells (termed CTLA-$4^+/CD4^+/CD25^+$ T cells) were identified by incubating whole-blood lymphocytes with PE-conjugated anti-CTLA-4, FITC-conjugated anti-CD25, and PE-Cy5-conjugated anti-CD4 antibodies. $FOXP3^+$ cells among $CD4^+$ T cells (termed $FOXP3^+/CD4^+$ T cells) were identified by incubating whole-blood lymphocytes with PE-conjugated anti-FOXP3 antibody after cell permeabilization with 4 % (v/v) formaldehyde and 0.1 % (w/v) saponin. Expression of both surface and intracellular markers by $CD4^+$ T cells was analyzed by flow cytometry (FACS Calibur, Nippon Becton Dickinson, Tokyo, Japan).

Induction of Cytokine Expression and Staining of Intracellular Cytokines to Identify iT_{reg} Cells and nT_{reg} Cells

To induce cytokine expression and accumulation, peripheral blood mononuclear cells (PBMCs) were stimulated for 4 h at 37 °C with 10 μg/ml brefeldin A in the presence or absence of 20 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin [38]. We removed cells that died after such stimulation. Separated PBMCs were stimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 4 h at 37 °C and 1×10^6 PBMCs were suspended in RPMI 1640 medium supplemented with 1 ml of 10 % FCS. The percentage of dead cells among PBMCs treated with PMA, and ionomycin, was calculated by eye after addition of 40 ml microliters of trypan blue. The control was whole-blood cells diluted to the same extent in FCS-free RPMI 1640 before addition of trypan blue. The percentage of dead cells was 7.0 %±6.7 %. These dead cells stained by trypan blue could not be distinguished in the FACS. However the percentage of positive cells producing cytokines did not affect the statistical significant difference even if these dead cells were included. Surface-stained whole-blood lymphocyte samples were suspended in 0.5 ml cold 4 % (v/v) paraformaldehyde (used as a fixative) and incubated at room temperature for 10 min. Next, the cells were washed twice with phosphate-buffered saline and centrifuged at 200*g* for 7 min. Each pellet thus obtained was suspended in 2 ml serum amyloid P buffer (0.1 % [w/v] saponin, 0.05 % [w/v] NaN_3, in Hanks's balanced salt solution). Each cell suspension was again centrifuged at 200*g* for 7 min, and each cell pellet was suspended in 0.1 ml serum amyloid P buffer. Cell suspensions were diluted with phosphate-buffered saline and aliquotted into tubes containing 10^6 cells/20 μl. PE-conjugated anti-IL-10 was added to each tube. All tubes were vortexed and incubated for 35 min at room temperature in the dark. The percentage of cells generating cytokines was measured with a FACS Calibur and the data were analyzed with the aid of CELLQuest software (Nippon Becton Dickinson).

Human iT_{reg} cells were defined as $CD4^+/CD25^+$ T cells that produced either IL-10 as the dominant cytokine (these cells were termed $IL10^+/CD4^+/CD25^+$ T cells), or predominantly TGF-β [13]Human nT_{reg} cells were defined as $FOXP3^+/CD4^+$ or CTLA-$4^+/CD25^+/CD4^+$ T cells, and were identified as described above [15, 39].

Statistical Analysis

All values are expressed as means ± SDs unless otherwise specified. Statistical comparisons among groups were achieved by using two-way analysis of variance (ANOVA) employing a repeated measures algorithm, followed by *post-hoc* comparisons using the Newman-Keuls test. The two mean values obtained by this process were compared by using the Wilcoxon matched-pairs *T* test. Correlation coefficients were obtained by employing Spearman's rank correlation test. *P* values<0.05 were considered statistically significant. Statistical analysis was performed with StatView 5.0 (SAS Institute; Cary, NC).

Results

Clinical Findings and Treatment

The characteristics of patients with general asthma (mild or moderate) are shown in Table I, and those of EGPA patients in Table II. The clinical features of EGPA patients differed from those of general asthma patients. Clinically, the significant differences evident when EGPA patients who relapsed seldom and frequently were compared were the percentage of $CD4^+/CD25^+$ T cells (higher in EGPA patients experiencing fewer relapses [$P<0.05$]) and the number of recurrences of vasculitis per year (lower in EGPA patients experiencing fewer relapses); the duration of disease was not relevant in this context (Table II). With the exception of heart involvement (lower in seldom-relapsing EGPA patients [$P<0.05$]), no significant difference was evident between patients with frequently relapsing EGPA and seldom-relapsing patients when organ involvement was evaluated. Scores on the FFS 1996, FFS 2009, and BVAS were higher in patients experiencing frequent EGPA relapses than in those who relapsed infrequently (Table II). Upon relapse, the principal organs compromised in frequently relapsing patients were the heart ($N=14$) and the gastrointestinal tract ($N=9$); such patients also developed multiple mononeuritis ($N=10$) or eosinophilic tympanitis ($N=3$).

Compared with EGPA patients in the seldom-relapsing group, a higher proportion of frequently relapsing EGPA patients was prescribed immunosuppressants during the course of disease or to maintain remission. In addition, the

Table I Characteristics of patients with general asthma

	Mild N=23	Moderate N=30	Severe N=14	P value
Age (years) (mean ± SD)	49.7±16.1	48.8±13.2	57.9±10.1	$< 0.05^{\dagger *}$
Gender (M/F)	9/14	10/20	8/6	$NS^{\dagger\dagger}$
Atopic/nonatopic disease	14/9	21/9	9/5	$NS^{\dagger\dagger}$
Age at onset of asthma (years) (mean ± SD)	37.8±17.6	30.3±16.1	29.4±20.2	$NS^{\dagger}$
Duration of asthma (years) (mean ± SD)	11.9±10.2	18.5±12.0	28.5±19.1	$< 0.05^{\dagger *}$, $< 0.01^{\dagger **}$
Daily ICS dose (CFC-BDP [μg equivalent]) (mean ± SD)	17.4±57.6	560.0±199.3	1,542.9±663.0	$< 0.01^{\dagger *}$, **, ***
Duration of ICS therapy (years) (mean ± SD)	4.6±3.0	8.4±4.0	11.1±5.7	$< 0.05^{\dagger *}$, $< 0.01^{\dagger **}$, ***

ICS inhaled corticosteroid; *NS* not significant

† Two-way ANOVA employing a repeated measures test to explore the significance of differences between any two groups

†† Chi-squared testing did not disclose the existence of a significant difference between the frequencies observed in the two groups that were compared

*Severe vs. moderate

**Severe vs. mild

***Mild vs. moderate

A P value<0.05 was considered statistically significant

prednisolone (PSL) maintenance dose prescribed for frequently relapsing EGPA patients was significantly higher than that given to seldom-relapsing patients (Table III). The peripheral blood eosinophil counts in seldom-relapsing EGPA patients with active disease were higher than when the disease was inactive (P<0.01). However, the peripheral blood eosinophil counts in frequently relapsing EGPA patients with active disease did not differ from those of patients with inactive disease (Table IV). The peripheral blood lymphocyte counts in EGTA patients experiencing frequent relapses were lower than those of patients with general asthma or seldom-relapsing EGPA (P<0.01).

nT_{reg} and iT_{reg} Whole Blood Cell Counts in Asthma Patients Without Symptoms, and in EGPA Patients Who Frequently or Seldom Relapsed, in Both the Active and the Inactive Disease State

$FOXP^+/CD4^+$ T cell counts were significantly lower in frequently relapsing EGPA patients with active disease than in seldom-relapsing EGPA patients, and in frequently relapsing EGPA patients with inactive disease than in other groups. $FOXP^+/CD4^+$ T cell counts in all EGPA patients were lower than those of control asthma patients. The counts of $CTLA\text{-}4^+/CD4^+/CD25^+$ T cells were significantly higher in frequently and seldom-relapsing EGPA patients with inactive disease than in EGPA patients with active disease. The counts of $CD4^+/CD25^+$ T cells producing IL-10 were significantly higher in seldom-relapsing EGPA patients with inactive disease than in control asthmatics and frequently relapsing EGPA patients (Table IV).

Counts and Percentages of $CD19^+$ B Cells (Among Whole-Blood Lymphocytes) that Also Expressed One or More of $CD80^+$, $CD86^+$, $CD27^+$, and $CD95^+$

The $CD19^+$ B cell count was lower in frequently relapsing EGPA patients with either active or inactive disease than in patients with seldom-relapsing EGPA and control asthmatic patients. The percentages of $CD19^+$ B cells expressing $CD80^+$ or $CD95^+$ were significantly higher in patients with frequently relapsing EGPA (either active or inactive) than in those with seldom-relapsing EGPA or in the control asthma group. The percentage of $CD19^+$ B cells expressing $CD86^+$ was significantly higher in patients with frequently relapsing EGPA (either active or inactive) than in the control asthma group. The percentage of $CD19^+$ B cells that were $CD27^+$ was significantly higher in EGPA patients (both frequently and seldom-relapsing) with active disease than in patients with inactive EGPA or those with general asthma (Table IV). However, the counts of $CD19^+$ B cells expressing $CD80^+$, $CD86^+$, CD27, or $CD95^+$ did not differ significantly among groups, because the overall count of $CD19^+$ B cells was lower in patients with frequently relapsing EGPA than in the other groups.

Correlations Between the Counts of $FOXP3^+/CD4^+$ T Cells and $CTLA\text{-}4^+/CD4^+/CD25^+$ T Cells, the Percentage of $CD19^+$ B Cells Expressing $CD80^+$, and the Count of $CD19^+$ B Cells in Peripheral Blood

In EGPA patients who relapsed either seldom or frequently, the overall number of $FOXP3^+/CD4^+$ T cells was significantly (positively) correlated with the count of CTLA-

Table II Characteristics of patients with EGPA

	Seldom-relapsing EGPA N=26	Frequently relapsing EGPA N=19	P value
Age (years)	57.7±17.3	59.7±12.7	NS†
M/F	8/18	8/11	NS††
Atopic/nonatopic	12/14	7/12	NS††
Age at onset of asthma (years)	41.6±16.8	40.6±13.5	NS†
Age at onset of EGPA (years)	50.7±16.2	49.7±16.0	NS†
Duration of EGPA (years)	7.2±3.8	10.9±7.7	NS†
Total number of vasculitis recurrences	1.0±0.8	6.7±5.3	< 0.01†
Total number of vasculitis recurrences per year (number/year)	0.18±0.16	0.65±0.23	< 0.01†
WBCs ($\times 10^3$ cells/l) at onset of EGPA	13,847±5,066	15,743±6,515	NS†
Eosinophils ($\times 10^3$ cells/l)	7,202±5,192	8,819±5,950	NS†
$CD4^+/CD25^+$ T cells (%)	43.5±11.5	35.5±11.2	< 0.05†
MPO-ANCA positive/negative ratio	9/17	5/13	NS††
Clinical manifestations of EGPA:			
Number of organs involved	5.0±1.8	5.7±1.8	NS†
Five-factor score (1996 scale)	1.1±0.9	1.9±0.8	< 0.01†
Five-factor score (2009 scale)	1.5±1.0	2.2±1.0	< 0.05†
Additional organ involvement:			
Asthma (yes/no)	26/0	19/0	NS††
Sinusitis (yes/no)	22/3	19/0	NS††
Lung (yes/no)	21/5	11/5	NS††
Multiple mononeuritis (yes/no)	25/1	19/0	NS††
Minimum MMT score:	3.0±1.0	2.9±1.0	NS†
Heart (yes/no)	15/11	17/2	< 0.05††
Gastrointestinal (yes/no)	19/7	17/2	NS††
Renal (yes/no)	9/15	7/10	NS††
Skin (yes/no)	15/11	14/4	NS††
Central nervous system (yes/no)	8/18	3/16	NS††
Arthralgia (yes/no)	7/15	7/10	NS††
Myalgia (yes/no)	5/18	6/12	NS††
BVAS score at onset	30.2±8.8	38.4±8.2	< 0.01†

* The percentage of $CD25^+$ cells among $CD4^+$ cells, calculated as described in Methods

BVAS Birmingham Vasculitis Activity Score; *FFS* five-factor score; *MMT* manual muscle test; *MPO-ANCA* myeloperoxidase-specific antineutrophil cytoplasmic autoantibody; *NS* not significant

† Two-way ANOVA employing repeated measures to compare data from the two groups

†† Chi-squared testing revealed no significant differences between the frequencies of the characteristics measured in the two groups

A P value<0.05 was considered statistically significant

$4^+/CD4^+/CD25^+$ T cells (Fig. 1a, d). In frequently relapsing EGPA patients, but not seldom-relapsing EGPA patients (Fig. 1b, c), the overall number of $FOXP3^+/CD4^+$ T cells was significantly (and positively) correlated with the count of $CD19^+$ B cells (Fig. 1e), and inversely correlated with the percentage of $CD80^+/CD19^+$ B cells (Fig. 1f). In patients with asthma, the overall number of $FOXP3^+/CD4^+$ T cells was significantly (positively) correlated with the count of CTLA-$4^+/CD4^+/CD25^+$ T cells, but not with the count of $CD19^+$ B cells or the percentage of $CD80^+/CD19^+$ B cells (data not shown). In addition, the percentage of $CD80^+/CD19^+$ B cells was significantly correlated with those of $CD27^+/CD19^+$ and $CD95^+/CD19^+$ B cells. Moreover, the percentage of $CD95^+/CD19^+$ B cells was significantly (inversely) correlated with the count of $CD19^+$ B cells (data not shown).

Correlations Between Serum IgG Concentration, the $CD19^+$ B Cell Count in Peripheral Blood, and the Systemic CS Concentration

The serum IgG concentration was inversely correlated with the serum PSL level measured at the time of assay in

Table III Treatment

	Seldom-relapsing EGPA patients N=26	Frequently relapsing EGPA patients N=19	P value
PSL initial dose (mg)	45.2±11.3	42.6±11.9	NS†
Steroid pulse (yes/no)	20/6	11/8	NS††
Immunosuppressant given during active disease (yes/no)	18/8	19/0	< 0.05† †
CYC/AZA/CSA/MTX/RTX	12/2/4/0/0	18/0/1/0/0	
immunosuppressant given as maintenance therapy (yes/no)	11/15	12/7	< 0.05† †
CYC/AZA/CSA/MTX/RTX	0/5/6/0/0	2/5/4/1/0	
IVIG (yes/no)	15/11	13/6	NS††
PSL given as a maintenance drug (mg) (mean ± SD)	5.9±3.6	8.3±3.1	< 0.05†

AZA azathioprine; *CSA* cyclosporin A; *CYC* cyclophosphamide; *IVIG* intravenous immunoglobulin; *MTX* methotrexate; *NS* not significant; *PSL* prednisolone; *RTX* rituximab

† Two-way ANOVA employing repeated measures of analysis was used to investigate the significance of differences between the two groups

†† Chi-squared testing revealed no significant differences between the frequencies shown by the two groups

A P value<0.05 was considered statistically significant

patients with seldom-relapsing EGPA (Fig. 2a), but not in those with frequently relapsing disease (Fig. 2d). In EGPA patients who relapsed either seldom or frequently, the serum IgG concentration was positively correlated with the $CD19^+$ B cell count in peripheral blood (Fig. 2b, e). However, in these patients, the $CD19^+$ B cell counts were not correlated with the serum PSL levels in individual patients at the time of assay (Fig. 2c, f). Both the count of $CD19^+$ B cells and the serum IgG concentration were lower in frequently relapsing, but not seldom-relapsing, EGPA patients, than in healthy controls (data not shown). This was the case whether or not the EGPA patients were treated with immunosuppressants.

Discussion

Recent studies have explored the role played by T_{reg} cells and the mechanisms of action of such cells in the initiation and progression of both human autoimmune diseases and other diseases that are thought to involve immune dysfunction; the latter conditions include multiple sclerosis and asthma [40, 41]. T_{reg} cells play critical roles in the maintenance of immune responses and T cell homeostasis. We defined nT_{reg} cells as $FOXP3^+/CD4^+$ T cells or $CTLA\text{-}4^+/CD25^{+/}/CD4^+$ T cells. However, Pan et al. [42] recently reported that $CD45RA^+/FOXP3^{low}$ cells were, in fact, resting T_{reg} cells. We confirmed that the $FOXP3^+/CD4^+$ T cell count was significantly (positively) correlated with the count of $CD45RA^-/FOXP3^{high}$ T cells (P<0.01; rs=0.95), but not with the count of $CD45RA^+/FOXP3^{low}$ T_{reg} cells (P=0.21, rs=0.19), in patients with either asthma or EGPA. In humans, differentiated $FOXP3^{hi}/CD25^{hi}/CD4^+$ nT_{reg} cells constitutively express CTLA-4 [39]. Ligation of CTLA-4 with CD80 or CD86, or both, may trigger the indoleamine 2, 3-dioxygenase (IDO) pathway in dendritic cells, in turn activating the transcription factor FOXP3 (which regulates immune functioning) and inhibiting cytokine production by dendritic cells [43]. Given the existence of such associations, we further explored whether T_{reg} and other immune cells might play disease-related roles in patients with frequently or seldom-relapsing EGPA. Our data suggest that overexpression of CD27, CD80, CD86, or CD95 on $CD19^+$ B cells may suppress differentiation of $FOXP3^+$ $CD4^+$ T_{reg} cells or $CTLA\text{-}4^+/CD4^+/CD25^+$ T_{reg} cells, or both. Subsequent decreases in $CD19^+$ B cell counts in frequently relapsing EGPA patients may in turn suppress immunoglobulin production.

Others have reported that $CD19^+$ B cell counts in patients with active rheumatoid arthritis, SLE, or BD are no less than those in healthy controls [22, 44]. In addition, the serum IgG concentration in WG patients is low; this is associated with reduced expression of highly soluble B-lymphocyte activating factor [26]. In our patients, the serum IgG concentration ranged from 300 to 1,700 mg/dl. More than half of all patients who were treated with 10 mg or more of PSL daily also received immunosuppressants, yet half of the latter patients had normal serum IgG concentrations. In contrast, some patients who received 5 mg or less of PSL daily, and no immunosuppressant, had low serum IgG concentrations and reduced counts of $CD19^+$ B cells. These data indicate that the $CD19^+$ B cell count was reduced in whole-blood lymphocytes of patients with frequently relapsing EGPA, but not in those with the seldom-relapsing form of the disease or with general asthma; the cell count was correlated with both the percentage of T_{reg} cells in whole-blood lymphocytes and the serum IgG concentration. Together, the data suggest that a decrease in the percentage (and hence

Table IV Counts of Treg and $CD19^+$ cells and expression levels of costimulant markers on $CD19^+$ cells in peripheral blood of EGPA patients

	Asthma			Seldom- relapsing EGPA		Comparison between patients with active and inactive disease *P* value	Frequently relapsing EGPA		Comparison between patients with active and inaction disease *P* value
	Mild	Moderate	Severe	active	inactive		active	inactive	
Eosinophils (cells/mm^3)	204±128**, ***	473±351	606±484	523±448*	175±152 **, ***	< 0.01	338±330 ***	194±109 **, ***	NS
Lymphocytes (cells/mm^3)	1,867±606	1910±355	1869±513	1,825±700*	1691±688	NS	1,185±452 *, **, ***, †, ††	1,125±380 *, **, ***, †, ††	NS
FOXP3+ among CD4 T cells (cells/mm^3)	35.7±24.8	26.9±15.8	16.9±9.1*	9.8±10.3 *, **,	39.6±24.0 **, ***	< 0.01	3.9±6.0*, **, ‡‡‡, $, ††	14.4±10.5*, ‡‡, ††	< 0.01
CTLA-4+ among CD4 + CD25+ T cells (cells/mm^3)	2.7±2.7	2.1±1.8	1.0±1.0	1.0±1.4	6.5±6.3 *, **, ***	< 0.01	0.6±1.2††	3.1±3.4††	< 0.01
IL-10+ among CD4 + CD25+ T cells (cells/mm^3)	4.7±6.2	3.4±5.7	2.0±3.6	3.2±9.0	15.1±23.3 ‡, **, ***	< 0.05	2.4±7.2††	6.1±12.1$$	NS
CD19+ B cells (cells/mm^3)	91.2±57.1	84.7±43.9	80.3±44.8	92.7±64.0	96.1±60.2	NS	48.9±75.6 ‡, ‡‡, ‡‡‡, †, ††	41.7±65.9‡, ‡‡, ‡‡‡, †, ††	NS
CD80+ among CD19+ B cells (%)	6.5±4.0	7.7±4.7	7.0±4.4	14.8±9.5 *, ‡‡, ‡‡‡	9.0±6.6	< 0.05	37.4±19.0*, **, ***, †, ††	26.6±13.4*, **, ***, †, ††	< 0.05
CD86+ among CD19+ B cells (%)	8.4±7.7	6.9±3.6	6.5±4.8	10.3±5.2	7.6±4.8	< 0.05	14.8±12.3‡, ‡‡, ‡‡‡	13.8±10.8‡, ‡‡, ‡‡‡	NS
CD27+ among CD19+ B cells (%)	28.9±15.0	20.2±15.1	21.9±14.0	45.2±16.6 *, **, ***	22.3±10.5	< 0.01	64.1±20.0*, **, ***, †, ††	28.1±13.7 †	< 0.01
CD95+ among CD19+ B cells (%)	41.2±14.3	39.6±16.9	43.0±17.4	36.2±29.4	42.6±17.6	NS	72.1±23.6*, **, ***, †, ††	73.8±12.9*, **, ***, †, ††	NS

Data are presented as means ± SDs

The mean values obtained from EGPA patients with active and inactive disease were compared by using the Wilcoxon matched-pairs *t*-test

Statistical comparisons were performed by using the Mann–Whitney *U*-test to compare data from the two groups

NS not significant

*Vs. mild asthma; $P<0.01$

**Vs. moderate asthma: $P<0.01$

***Vs. severe asthma: $P<0.01$

‡Vs. mild asthma: $P<0.05$

‡‡Vs. moderate asthma: $P<0.05$

‡‡‡Vs. severe asthma: $P<0.05$

†Vs. EGPA patients experiencing few relapses but in the active stage of disease: $P<0.01$

††Vs. EGPA patients experiencing few relapses but in the inactive stage of disease: $P<0.01$

$Vs. EGPA patients experiencing few relapses but in the active stage of disease: $P<0.05$

$$ Vs. EGPA patients experiencing few relapses but in the inactive stage of disease: $P<0.05$

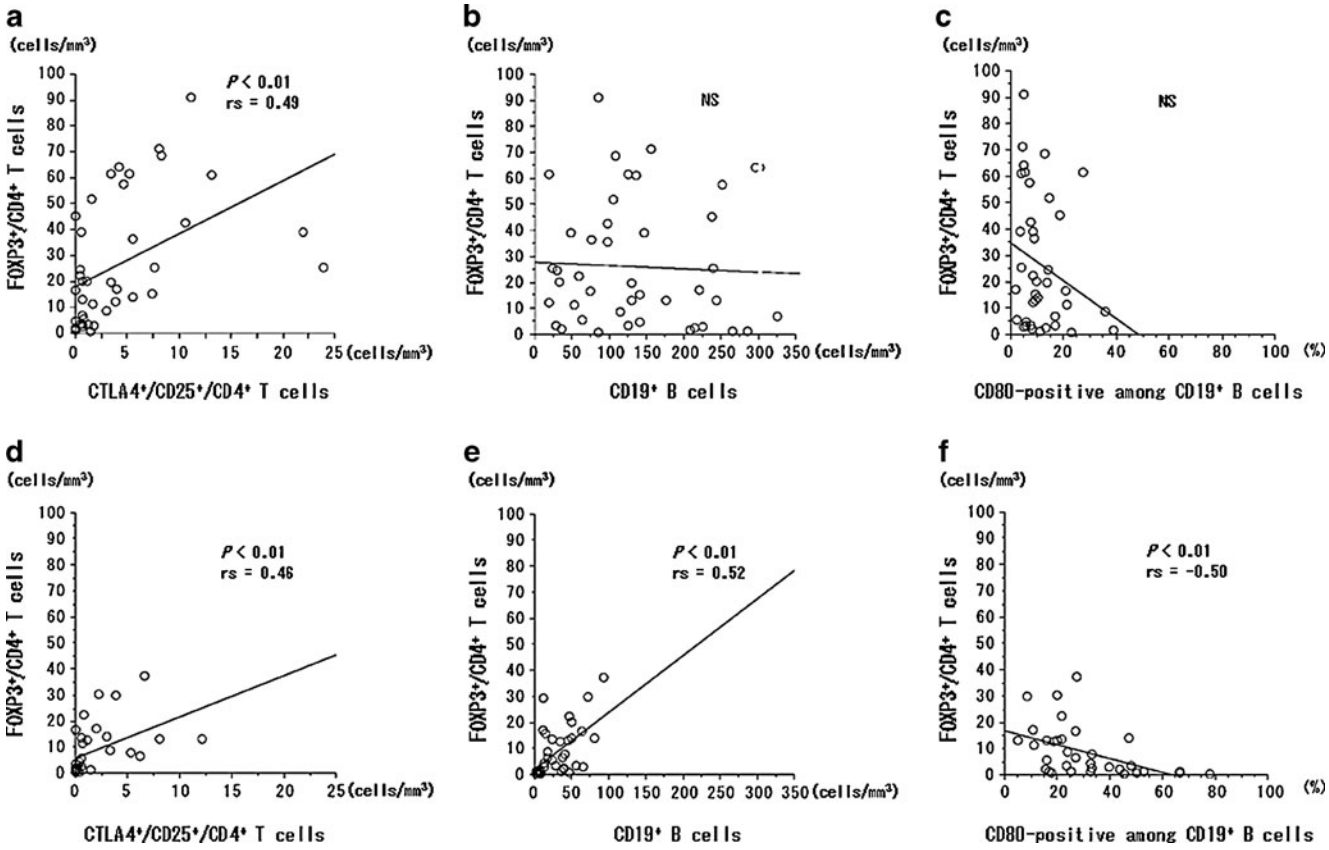

Fig. 1 Correlations between cell counts (per microliter) in peripheral blood from EGPA patients (of the seldom-relapsing group [**a**, **b**, and **c**] and the frequently relapsing group [**d**, **e**, and **f**]). $FOXP3^+/CD4^+$ T cell counts are compared with those of (**a** and **d**) $CTLA\text{-}4^+/CD25^+/CD4^+$ T cells; **b** and **e** $CD19^+$ B cells; and (**c** and **f**) $CD80^+/CD19^+$ B cells. Correlation coefficients (rs values) and *P* values were calculated by using Spearman's rank correlation test

the functionality) of T_{reg} cells reduces $CD19^+$ B cell concentrations and IgG production.

In patients with seldom-relapsing EGPA, serum IgG concentrations were decreased when high doses of PSL, with or without immunosuppressants, were prescribed. However, serum IgG concentrations returned to normal when the PSL dose was tapered to 10 mg or less per day (data not shown). In patients with frequently relapsing EGPA, the maintenance dose of CS was high and immunosuppressants were frequently used; however, the serum PSL concentration was not correlated with the $CD19^+$ B cell count. No significant differences in apoptotic B-cell counts have been found in patients with SLE or primary systemic vasculitis when patients who do or do not take immunosuppressants or steroids of various types are compared [44]. In the present work, the observed correlations between serum IgG concentration and $CD19^+$ B cell count on the one hand, and the PSL concentration on the other, did not differ significantly among patients. However, immunosuppressant treatment may decrease serum IgG concentrations. Our results suggest that the reduction in the $CD19^+$ B cell count noted in frequently relapsing EGPA patients is a consequence of the disease, and not a side-effect of treatment with CS or immunosuppressant. In contrast, antigen-presenting B cells expressing CD80 or CD86 significantly enhance T cell proliferation and differentiation. The percentage of $CD80^+/CD19^+$ B cells in BD patients with active disease is higher than that in healthy controls [22]. Similarly, the percentage of $CD80^+/CD19^+$ B cells in WG patients increases in those in remission compared with patients suffering active disease or healthy controls [20]. Moreover, interaction between CD80 or CD86 on B cells and CD28 on T cells induces arthritis in mice [18]. These findings suggest that an increase in the percentage of $CD80^+/CD19^+$ B cells suppresses differentiation of T_{reg} cells. However, T cells can trigger apoptosis of B cells via the Fas pathway [27]. In this context, the concentration of soluble CD95 is associated with the extent of disease activity in EGPA, SLE [30], and BD [29–31] patients. In frequently relapsing EGPA patients, we found that a reduction in the $CTLA\text{-}4^+/CD4^+/CD25^+$ T cell count was associated with the presence of an increased percentage of $CD27^+$ and $CD80^+$ $CD19^+$ B cells. Under such circumstances, apoptosis of $CD19^+$ B cells may be enhanced because of increased expression of CD95 by such cells. Thus, CD95 may be mechanistically involved in the diseases mentioned above. We

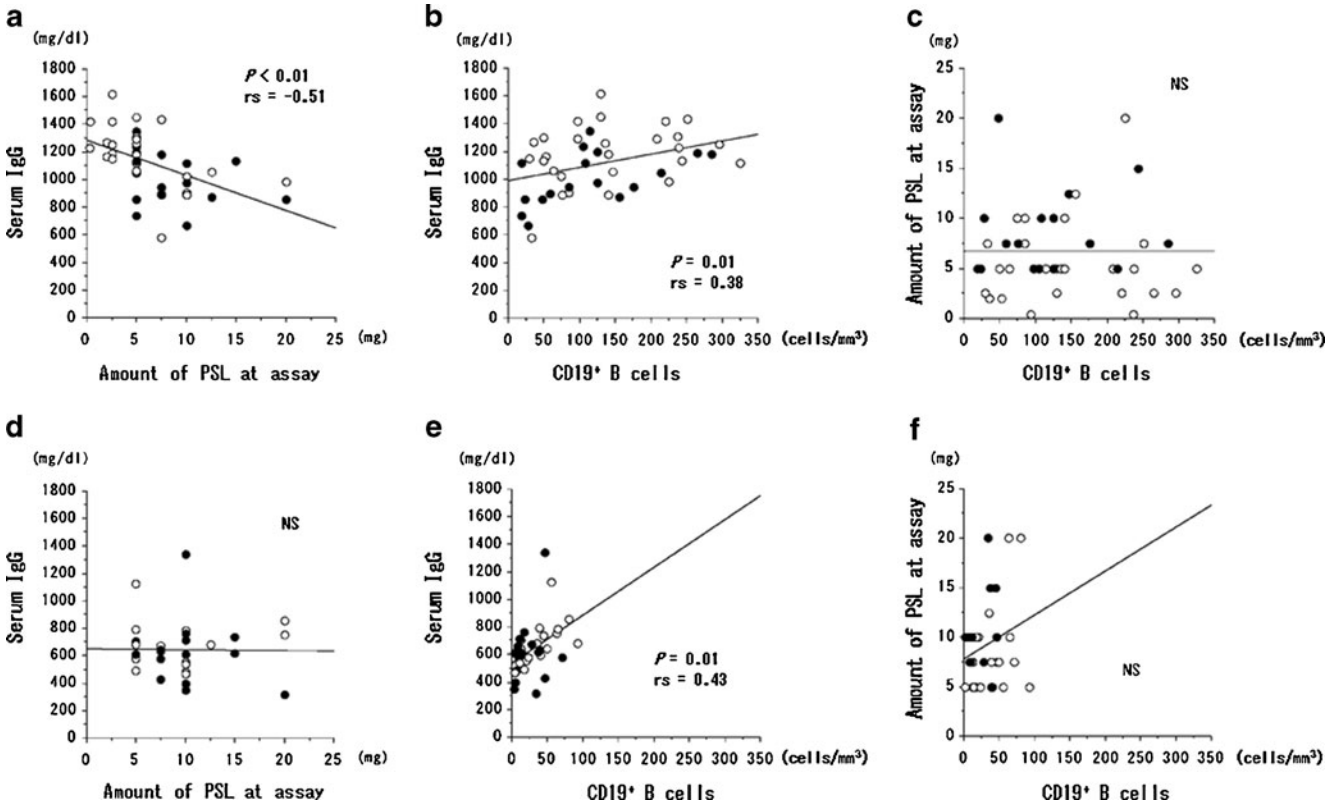

Fig. 2 Correlations, in EGPA patients with either active or inactive disease, between serum IgG concentration and (**a** and **d**) CS level (in PSL equivalents) at the time of assay; and (**b**) and E) $CD19^+$ B cell counts among whole-blood lymphocytes. **c** and **f** Correlations between $CD19^+$ B cell counts among whole-blood lymphocytes and systemic CS levels at the time of assay. Panels **a**, **b**, and **c** refer to EGPA patients with seldom-relapsing disease; panels **d**, **e**, and **f** to patients with frequently relapsing disease. *Closed circles*: EGPA patients undergoing treatment with CS and an immunosuppressant at the time of assay. *Open circles*: EGPA patients undergoing treatment with CS (only) at the time of assay. Correlation coefficients were obtained by using Spearman's rank correlation test

found that the percentage of $CD27^+CD19^+$ B cells increased in both seldom-relapsing and frequently relapsing EGPA patients only when the disease became active; the percentages were not elevated in the inactive disease state. However, the percentages of B cells expressing CD80, CD86, or CD95 were elevated in (only) frequently relapsing EGPA patients in both the active and inactive disease states. These results indicated that an increase in the percentage of $CD27^+CD19^+$ B cells did not directly affect the level of B cell apoptosis. On the other hand, the percentages of B cells expressing CD80, CD86, or CD95 did not decrease after treatment with CS and IS. The overall number of B cells fell when the percentages of B cells expressing high levels of CD80 or CD86 increased; expression of these markers in turn elevated the expression level of CD95 on B cells.

Rituximab, a drug that causes depletion of B cell numbers, has been successfully used to treat WG [45, 46] and EGPA [47, 48]. Increases in the levels of co-stimulatory molecules, including CD80 or CD86, on B cells, may be induced via reconstitution of T_{reg} cell function by rituximab.

Previously, we showed that the counts of iT_{reg} cells producing IL-10 were reduced in EGPA patients at disease onset and upon relapse, but increased when the disease was inactive [32]. Moreover, we found that the Th17 cell count was elevated in EGPA patients with active disease and upon relapse, and that this count was inversely correlated with the count of iT_{reg} cells [49] but positively correlated with the count of $CD4^+$ T cells producing IL-25 [50]. In addition, we have shown that the IDO expression level in monocytes from all EGPA patients is positively correlated with the count of $CD4^+/CD25^+$ T_{reg} cells producing IL-10 and inversely correlated with the count of Th17 cells [51]. Thus, EGPA relapse may be linked to an increase in the count of $CD4^+$ T cells producing IL-25. Such cells promote Th2-mediated inflammation and reduce iT_{reg} cell subpopulations. A reduction in IDO expression by monocytes triggers similar effects. Transient depletion of B cells after autoantigen challenge in a mouse model reduces the counts of autoantigen-specific $CD4^+$ T cells producing IFN-γ and IL-17 [52]. A reduction in the count of $FOXP3^+$ T_{reg} cells, along with an elevation in the counts of inflammatory Th1 and Th17 cells, is evident in mice lacking IL-10-producing B cells [53]. We therefore hypothesize that a decrease in the overall count of $CD19^+$ B cells in association with enhancement of the percentages of

such cells co-expressing CD27, CD80, CD86, or CD95 affects the differentiation of T_{reg} or Th17 cells.

We conclude that a decrease in T_{reg} cell numbers and increases in the percentages of activated B cells in EGPA patients with frequently relapsing, but not seldom-relapsing, disease, may induce apoptosis of B cells by increasing the expression level of CD95.

Conflict of Interest No author has any conflict of interest to disclose. The work was not funded by a grant or any other external source of financial support.

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