Effects of glucocorticoids on B-cell subpopulations in patients with IgG4-related disease

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Received on July 5, 2018; accepted in revised form on September 24, 2018.

Clin Exp Rheumatol 2019; 37 (Suppl. 118): S159-S166.

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Key words: IgG4-related disease, B cells, plasmablasts, corticosteroid, glucocorticoid, treatment

Funding: this work was in part supported by "Fondazione Italiana per la Ricerca sull'Artrite" (FIRA Onlus 2014) and by the "Collegio Ghislieri" (Pavia, Italy). Competing interests: none declared.

ABSTRACT

Objective. Glucocorticoids induce prompt clinical improvement in patients with IgG4-related disease (IgG4-RD) but their mechanisms of action in this specific condition are not fully understood. B lymphocytes appear central to IgG4-RD pathogenesis because B-cell depletion with rituximab leads to swift clinical responses. In the present work we aim to assess the effects of glucocorticoids on B-cell subpopulations in patients with IgG4-RD.

Methods. Fifty patients with active untreated IgG4-RD and 20 healthy controls were enrolled in the present study. Flow cytometry analysis for total circulating CD19⁺ and CD20⁺ cells, naïve B cells, memory B cells, plasmablasts, and plasma cells was performed at baseline in all patients, and after 6 months of glucocorticoid treatment in 30 patients. Correlation studies with biomarkers of disease activity were also performed.

Results. At baseline, patients with IgG4-RD showed reduced $CD19^+$ and $CD20^+$ B cells compared to healthy controls, but increased circulating plasmablasts and plasma cells. Circulating plasmablasts and plasma cells correlated with clinical and serological biomarkers of IgG4-RD activity. Glucocorticoid-induced disease remission was accompanied by a reduction of naïve B cell count, an increase of memory B cells, and by a depletion of circulating plasmablasts and plasma cells. $CD19^+$ and $CD20^+$ B cells, were not affected by glucocorticoids.

Conclusion. The efficacy of glucocorticoids in IgG4-RD is associated with selective effects on different B-cell subpopulations. Further studies are warranted to fully understand possible perturbations of the naïve and memory B-cell compartments in patients with IgG4-RD.

Introduction

IgG4-related disease (IgG4-RD) is an increasingly recognised systemic fibroinflammatory condition so named because of the characteristic findings of abundant IgG4+ plasma cells in affected tissues (1). IgG4-RD is clinically characterised by elevated serum IgG4 concentration and by tumour-like lesions with a relapsing-remitting course that promptly respond to glucocorticoid treatment (2). Originally described in the setting of chronic "type 1 autoimmune pancreatitis", IgG4-RD is now known to include a broad spectrum of disorders considered unrelated entities for decades, such as retroperitoneal fibrosis, Mikulicz's disease, and hypertrophic pachymeningitis among others (3-5).

Given its recent recognition as a disease entity, the pathogenesis of IgG4-RD is still far from being fully understood. Several evidences now suggest that B lymphocytes play a central pathogenic role, above and beyond the typical finding of IgG4+ plasma cells infiltrating affected organs. Circulating plasmablasts, the precursors of tissue resident antibody secreting plasma cells, are for instance, oligoclonally expanded in IgG4-RD patients, a finding that strongly supports an antigen driven activation of the Bcell compartment (6). In addition, B-cell depletion with rituximab, an anti CD20 monoclonal antibody, leads to dramatic clinical improvement in patients with IgG4-RD (7). Circulating plasmablasts also disappear together with rituximabinduced disease remission and increase again when disease flares, indicating a close relationship between this B-lymphocyte subpopulation and IgG4-RD clinical activity (8-11).

Additional clues are, however, necessary to confirm the pathogenic relevance of B lymphocytes/plasmablasts in IgG4-RD. For instance, it would be compelling to discriminate which B-cell

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subpopulations selectively decrease when disease remission is achieved by means of equally effective non B-cell specific agents. Rituximab, in fact, is known to globally deplete B lymphocytes counts by targeting the transmembrane protein CD20, which is expressed from late pro-B cells through memory cells, but not on plasmablasts, and plasma cells, and its effects on pathogenic B cells are indistinguishable from those on bystander clones (12). Glucocorticoids, on the other hand, are not supposed to drastically modify the total number of circulating CD20⁺ B cells, thereby offering a diverse angle to observe the variations of putative pathogenic B lymphocyte subsets (13). In addition, despite representing the treatment of choice to induce IgG4-RD remission, the mechanisms of action of corticosteroids in this specific condition remain poorly characterised (14).

In the present work we aim to assess the effects of glucocorticoids on the B-cell compartment in patients with IgG4-RD by performing a phenotypic analysis of B-cell subpopulations at baseline and after corticosteroid-induced disease remission.

Materials and methods

Patients and treatment

Fifty patients with active untreated IgG4-RD referred to our tertiary care Centre between September 2014 and December 2016 and were consecutively included in the present prospective monocentric study. IgG4-RD was diagnosed according to the consensus statement on the pathology of IgG4-RD (15). The comprehensive diagnostic criteria for IgG4-RD were used when tissue biopsies were not available (16). Patients with isolated pancreatic involvement who did not undergo pancreatic resection were diagnosed with definite IgG4-RD according to the International consensus diagnostic criteria for autoimmune pancreatitis (17). Thirty patients were treated with oral prednisone at the initial dose of 0.6-1 mg/kg for one month and then tapered in accordance with international guidelines (14). Blood samples for serological and immunological studies, and for clinical

correlations were drawn at baseline and after 6 months of glucocorticoid treatment. Twenty healthy age- and sex-matched subjects were studied as controls for the immunological analysis on circulating B-cell subpopulations. All subjects enrolled provided written informed consent for the analyses performed. The study was conducted according to the Declaration of Helsinki and approved by the Ethical Committee of the San Raffaele Scientific Institute, Milan, Italy as a descriptive non-interventional study.

Laboratory analysis, flow cytometry, and cell sorting

Laboratory analyses included C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), total immunoglobin G (IgG), IgG1, IgG2, IgG3, and IgG4 subclasses. Flow cytometry and cell sorting were performed on fresh peripheral blood collected in EDTA tubes using a lyse-no-wash technique (ammonium chloride) and the following panel of directly conjugated antibodies: CD3-FITC, CD56-PE, CD4-ECD, CD138-PC5.5, CD27-PC7, CD20-APC, CD19-A700, CD38-A750, CD8-PB, CD45-KO (Beckman-Coulter, Brea, CA). Ten-colour flow cytometry was performed using a Navios cytometer and Navios software (Beckman-Coulter, Brea, CA). According to previous reports, naïve B cells, memory B cells, plasmablasts, and plasma cells were identified within the lymphocyte as CD19+CD20+CD27-CD38+ gate cells, CD19+CD20+CD27+CD38- cells, CD19+CD20-CD27+CD38+bright cells. and CD19+CD20-CD38+CD138+ cells, respectively (17). Total B cells were identified as either CD19+ or CD20+ cells. Plasmablasts were also sorted by MoFlo[™] XDP High Speed Cell Sorter (Beckman Coulter, Brea, CA) and directly recovered on microscope slides. Slides were stained according to the May-Grünwald-Giemsa technique and evaluated by light microscope for morphological analysis.

Disease activity and clinical assessment

IgG4-RD activity was assessed by means of the IgG4-RD responder index

(IgG4-RD RI) (18). Active disease was defined by an IgG4-RD RI \geq 3. Complete response and disease remission were defined by an IgG4-RD RI <3 in the presence or absence of concomitant corticosteroid treatment, respectively. A reduction of the IgG4-RD RI of \geq 2 points still with a total score \geq 3 was considered a partial response to treatment (18).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software 6.0 (La Jolla, CA, USA). Normal distribution of continuous variables was assessed with the D'Agostino & Pearson omnibus normality test. Normally distributed variables were compared using the Student's t-test. Non-normally distributed variables were compared using the Mann-Whitney U-test. Follow-up nonnormally distributed variables were compared through Wilcoxon Test. Nonparametric correlations were calculated using the Spearman's correlation test. Linear correlations were measured by Pearson's correlation coefficient. Receiver operating characteristic (ROC) curves were generated based on the assumption that ascending rank of plasmablast counts would increase the likelihood of an IgG4-RD diagnosis. A pvalue <0.05 was considered statistically significant. Values are presented as median and interquartile ranges (IQR), unless specified otherwise.

Results

Clinical and serological features of the cohort of patients with IgG4-RD

Fifty patients (35 males and 15 females) with active untreated IgG4-RD were included in the study (Table I). The median age at diagnosis was 64 years (range 53–72 years). Twenty patients (40%) reported a history of atopy. Twenty-four patients (48%) were classified as definite cases of IgG4-RD, 1 patient (2%) as probable IgG4-RD, and 25 patients (50%) as possible IgG4-RD. Twenty-four patients (48%) presented with multiple organ involvement. The pancreas was the most commonly affected organ (28 cases (56%)) followed by the salivary and lacrimal glands (9

Table I.	Clinical,	serological,	and imm	unological	features	of the	patients'	cohort.
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	Total IgG4-RD patients	IgG4-RD patients	Healthy controls
	(n=50)	treated with GC (n=30)	(n=20)
Age at disease onset median (years)	64	70	54
Male, n° (%)	35 (70)	22 (73)	12 (60)
Serum IgG4 (<135 mg/dL), median (IQR)	310 (189-474)	310 (206-427)	
ESR (0-20 mm/hr), median (IQR)	14 (9-33)	18 (10-35)	
CRP (<6 mg/L), median (IQR)	5 (2-5.2)	5 (2-6)	
IgG4-RD RI (0-3), median (IQR)	6 (6-9.75)	6 (6-9)	
CD19 ⁺ B cells (cells/mL), median (IQR)	156500 (96500-218000)	166000 (102500-219250)	236000 (200000-299000)
Naive B cells (cells/mL), median (IQR)	12760 (8775-27318)	15120 (8895-27318)	23810 (17930-54020)
Memory B cells (cells/mL), median (IQR)	25910 (10890-48840)	26065 (12410-50203)	37170 (21900-57190)
Plasmablasts (cells/mL), median (IQR)	2815 (1385-5738)	2825 (1083-6250)	340 (170-600)
Plasma cells (cells/mL), median (IQR)	0 (0-312)	135 (0-356)	0
Organ involvement			
Pancreas	28 (56%)	20 (66%)	
Salivary glands	9 (18%)	2 (6.6%)	
Lacrimal glands	9 (18%)	2 (6.6%)	
Aorta and retroperitoneum	8 (16%)	6 (20%)	
Lymph nodes	7 (14%)	5 (16.6%)	
Biliary tree	6 (12%)	5 (16.6%)	
Orbit	5 (10%)	0 (0%)	
Hard palate	4 (8%)	0 (0%)	
Nasal sinuses	4 (8%)	0 (0%)	
Meninges	3 (6%)	1 (3%)	
Lung	3 (6%)	2 (6.6%)	
Skin	1 (2%)	0 (0%)	
Testicle	1 (2%)	0 (0%)	

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; IgG4-RD RI : IgG4-related disease responder index (IgG4-RD RI); GC: glucocorticoids.

cases each (18%)), aorta and retroperitoneum (8 cases (16%)), lymph nodes (7 cases (14%)), biliary tree (6 cases (12%)), orbit (5 cases (10%)), nasal sinus and hard palate (4 cases each (8%)), lungs and pachymeninges (3 cases each (6%)), testicles and skin (1 case each (2%)). The median value of the IgG4-RD RI at disease onset was 6 (range 6-9). CRP and ESR were elevated in 12 (24%) and 29 (58%) patients, respectively. Serum IgE were measured in 38 subjects and were elevated in 29 (76%), with a median value of 251 IU/ mL (101-504 IU/mL; normal <100 IU/ mL). Serum IgG4 level was increased in 46 patients (92%), with a median value of 310 mg/dL (189-473 mg/dL; normal 3-135 mg/dL).

Distribution of B-cell subpopulations in patients with active untreated IgG4-RD

At baseline, total lymphocytes counts were comparable between IgG4-RD patients and healthy subjects. Flow cytometry analysis revealed a significant CD19⁺CD20⁺ and CD20⁻ B-cell lymphopenia in IgG4-RD patients as compared to healthy controls, both in absolute counts and in percentage of total lymphocytes (p<0.05) (Fig. 1A; Supplementary Fig. 1-2).

Naïve B cells were also significantly reduced in patients with IgG4-RD compared to healthy subjects but only in absolute numbers (median, 12.760 cells/ml (IQR, 8.775–27.318 cells/ml) and 23.810 cells/ml (IQR, 17.930–54.020 cells/ml), respectively) (p<0.01) and not in percentage of CD19⁺ B cells (Suppl. Fig. 3A). The levels of circulating memory B cells were comparable between IgG4-RD patients and healthy individuals both in absolute numbers and in percentage of CD19⁺ B cells (Suppl. Fig. 3A).

Circulating CD19⁺CD20⁻CD27⁺CD-38^{+bright} plasmablasts were significantly expanded in IgG4-RD patients (median, 2.815 cells/mL; IQR, 1.385–5.738 cells/ mL) as compared to healthy controls (median, 340 cells/mL; IQR, 170–600 cells/mL) (p<0.0001) (Fig. 1A). The median percentage of plasmablasts over total CD19⁺ B cells was also significantly increased in IgG4-RD patients (median, 1.65%; IQR, 0.78–5.45%;) as compared to healthy controls (median, 0.19%; IQR 0.05–0.29 %) (*p*<0.0001) (Fig. 1A).

Circulating plasma cells were detected in 20 (40%) patients with IgG4-RD and in none of the healthy individuals, showing significantly higher levels both in absolute counts (mean, 197 cell/mL (range, 0-1332 cell/mL) and 0 cell/mL, respectively) (p<0.01) and in percentage of total CD19⁺ B cells compared to controls (mean, 0.16%) (range 0-1.27%) and 0%, respectively) (p<0.01) (Fig. 2A). All the 20 IgG4-RD patients with detectable circulating plasma cells had elevated serum IgG4 levels. No other clinical or serological features differed between IgG4-RD patients with and without detectable circulating plasma cells.

Correlation studies between

circulating plasmablasts, plasma cells, and biomarkers of IgG4-RD activity To assess a potential pathophysiological link between circulating plasmablasts/plasma cells and IgG4-RD, we performed correlation studies with the following validated clinical and sero-

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Fig. 1. Analysis of total CD19⁺ cells, circulating plasmablasts, and plasmablasts/CD19⁺ cells ratio in patients with active untreated IgG4-RD. (A) Total CD19⁺ B-cells count is reduced in patients with IgG4-RD, but circulating plasmablasts are expanded. (B) Circulating plasmablasts count and (C) the plasmablast/CD19⁺ B-cells ratio positively correlate with serum IgG4 levels, with the IgG4-RD RI, and with the number of organs involved by IgG4-RD.

logical biomarkers of disease activity: IgG4-RD RI, number of organs involved, serum IgG4 levels, ESR, and CRP. Absolute count of circulating plasmablasts as well as the plasmablast/CD19⁺ B-cell ratio at baseline showed statistically significant positive correlation with the number of organs involved, serum IgG4 levels, and IgG4-RD RI (p<0.05) (Fig. 1B-C).

The absolute count of circulating plasma cells as well as the plasma cells/ CD19⁺ B-cell ratio at baseline showed statistically significant positive correlation with serum IgG4 levels (p<0.05), but not with the number of organs involved and the IgG4-RD RI (Fig. 2B). No statistically significant correlation was found between circulating plasmablasts and plasma cells counts, and commonly used inflammatory markers, such as CRP and ESR (data not shown).

Diagnostic performance of circulating plasmablasts for the diagnosis of IgG4-RD

Given the expansion of circulating plasmablasts in the large majority of patients with IgG4-RD, we assessed their diagnostic performance by generating ROC curves considering both their absolute number and the ratio over total CD19⁺ B cells. A value of 1080 plasmablasts/mL demonstrated excellent performance in distinguishing between IgG4-RD patients and controls, with a sensitivity of 80%, a specificity of 100%, a positive predictive value of 100%, and a negative predictive value of 75% (area under the ROC curve (AUC) = 0.92, p < 0.05, 95% CI 0.85-0.98). Similarly, a plasmablasts/ total CD19⁺ B-cell ratio of 0.33 had a sensitivity of 90%, a specificity of 90%, a positive predictive value of 94%, and

a negative predictive value of 85% for the same comparison (AUC=0.95, *p*<0.05,95% CI 0.90–0.99) (Suppl. Fig. 3B). To evaluate whether morphological features could be used to identify circulating plasmablasts on peripheral blood smear, plasmablasts were stained after cell sorting and examined morphologically by two independent haematologists. Plasmablasts were undistinguishable from activated circulating lymphocytes on Giemsa staining as they appeared as small round cells (10 to 12 µm size) with high nuclear/cytoplasmic ratio and scanty pale dark blue cytoplasm (Fig. 2C).

Effects of glucocorticoids on B-cell subpopulations in patients with IgG4-RD

Thirty patients were treated with glucocorticoids according to international



Fig. 2. Analysis of circulating plasma cells in patients with active untreated IgG4-RD and morphological evaluation of circulating plasmablasts. (A) Plasma cells are expanded in active untreated IgG4-RD, both in absolute numbers and percentage of $CD19^+$ B cells, and (B) correlate with serum IgG4 levels. (C) Plasmablasts sorted from the peripheral blood of patients with IgG4-RD (upper box) resemble activated lymphocytes, with low cytoplasm/nucleus ratio and smaller dimensions than plasma cells and malignant plasmablasts.

guidelines and studied for variations in the B-cell compartment after 6 months of treatment (13). The remaining 20 patients were excluded from the analysis because three underwent surgical removal of the fibrotic mass with no additional immunosuppressive treatment; 7 patients had a poorly controlled diabetes and received steroidsparing agents in order to rapidly taper glucocorticoid therapy; and 10 were treated with rituximab. No clinical and serological differences were observed at baseline between patients included in the analysis and those excluded.

At 6 months, clinical improvement was observed in all patients, with an IgG4-RD RI that decreased from a median baseline value of 6 (IQR, 6–9) to 2 (IQR, 1–2.75) (paired p<0.05). Seven patients achieved partial response, 20 patients achieved complete response, and 3 patients achieved disease remission. No differences in B-cell subsets at baseline were observed between partial and complete responders. The median daily dose of prednisone at the time of follow-up analysis was 5 mg (range, 0-7.5 mg). Serum IgG4 levels decreased in 26 patients (86.6%) to a median value of 185 mg/dL (IQR, 103-228 mg/dL) (paired p<0.05 compared to baseline) and normalised in 8 patients.

Effects of corticosteroids on B-cell subpopulations are summarised in Fig. 3. Total lymphocytes, CD19+, and CD20+ B-cell counts were unaffected by glucocorticoids (Suppl. Fig. 2). Absolute numbers of naïve B cells as well as the percentage of naïve B cells over total CD19⁺ B cells significantly decreased to a median of 7205 cells/mL (IQR, 4195-12983 cells/mL) and of 4.78% (IQR 3.04-8.33%), respectively (paired p<0.0001 for both comparisons with respect to baseline), values significantly lower than that of healthy individuals (p < 0.01). Memory B cells significantly increased with disease improvement both in absolute numbers (median, 35445 cells/mL; IQR, 16015-63763 cells/mL) and percentage of CD19+ B cells compared to baseline (median 24.49%; IQR 13.33-34.18%) (paired p < 0.01 for both the comparisons), but did not differ from the levels of healthy controls. The median circulating plasmablasts count declined from 2.825 cells/mL at baseline (IQR, 1.083-6.250 cells/mL) to 270 cells/mL (IQR, 167-1.338 cells/mL), (p<0.0001), a value comparable to that of healthy controls (median, 340 cells/mL; IQR, 170-600 cells/mL) (p>0.05). The percentage of plasmablasts over total CD19⁺ B cells also significantly decreased to a median of 0.23% (IQR, 0.08-0.83%) (paired p < 0.0001). Plasma cells counts significantly decreased both in absolute number and percentage of CD19⁺ B cells to a mean value of 48 cell/mL (range, 0-423 cells/mL) and 0.06% (range 0-0.64%) (p<0.01 for both comparisons with baseline).

Discussion

The prominent IgG4 signature that characterises IgG4-RD has driven major research focus on the B-cell compartment of patients with this fibrotic condition over the last decade. Several



Fig. 3. Effect of glucocorticoids on B-cells subpopulations in patients with IgG4-RD after 6 months of glucocorticoid treatment.

Naïve B-cell, circulating plasmablasts, and plasma cells significantly decrease in absolute counts and percentage of total CD19⁺ B-cells.

Memory B cells significantly increase compared to baseline values.

landmark evidences now indicate that B lymphocytes are central to the pathogenesis of IgG4-RD, including (i) the expansion of oligoclonal somatically hypermutated IgG4⁺ plasmablasts in patients' peripheral blood (7); (ii) the production of autoantibodies (20); (iii) the contribution of B cells in the establishment of tissue fibrosis (1, 9); and (iv) the prompt clinical and pathological response to B-cell depletion with rituximab (6, 9). In particular, circulating plasmablasts have been directly implicated in IgG4-RD pathogenesis based on their correlation with IgG4-RD activity; they namely increase during active disease, disappear after rituximab-induced remission, and increase again when disease relapses (8). How plasmablasts contribute to IgG4-RD is still, however, matter of debate, because their disappearance after rituximab is not a finding that supports, *per se*, a causative role for this population of activated lymphocytes. Rituximab, in fact, globally depletes circulating B cells, thus making it difficult to discriminate the effects on putative pathogenic B lymphocytes from those on bystander clones. In the present work we studied the B-cell compartment of patients with active untreated IgG4-RD and systematically assessed, for the first time, the effects of glucocorticoid treatment on different major B-cell subpopulations. Corticosteroids, in fact, are supposed to have minimal effects on total circulating B lymphocytes in the long-term, thus offering a different perspective to identify alterations of B cells eventually associated with IgG4-RD activity (13).

The most striking alterations of the peripheral B-cell compartment in our cohort of patients with active untreated IgG4-RD were (i) a global reduction of CD19⁺ and CD20⁺ B lymphocytes, and (ii) the expansion of circulating plasmablasts and plasma cells. A reduced circulating B-cell count has already been reported in other chronic inflammatory conditions where it has been associated with a state of active disease (21). These inflammatory conditions include giant-cells arteritis, polymyalgia rheumatica, and ANCA-associated vasculitides, among others (21, 22). B-cell lymphopenia has never been observed, instead, in IgG4-RD and its implications in this specific setting remain unclear. A correlation between low B-cell count and disease activity appears however unlikely because B-cell lymphopenia in our cohort of patients did not ameliorate with glucocorticoid treatment or with clinical improvement. It is, therefore, tempting to speculate that B-cell lymphopenia in IgG4-RD might rather represent a consequence of the compartmentalisation of B lymphocytes in secondary lymphoid organs and inflamed tissues, where prominent germinal center reactions and lymphoplasmacytic infiltrate are typically observed (15). On the other hand, the finding of expanded circulating plasmablasts and plasma cells confirms a pathogenic hypothesis whereby class-switched, antigen-experienced B lymphocytes have the capability to migrate into affected organs, secrete antibodies, and

act as antigen presenting cells (1). Indeed, circulating plasmablasts strongly correlated with clinical and serological parameters of disease burden, both in absolute numbers and in percentage of CD19⁺cells, representing a reliable biomarker of IgG4-RD activity.

The most relevant findings within the Bcell compartment of IgG4-RD patients after 6 months of glucocorticoid treatment were: (i) a substantial stability of total B lymphocytes; (ii) a significant decline in the naïve B-cell count; (iii) and the disappearance of circulating plasmablasts and plasma cells. Corticosteroid treatment also led to a significant increase in memory B-cell counts, yet within levels comparable to healthy individuals. The stability of total B lymphocytes after glucocorticoids, indeed, represented an important pre-requisite to further analyse the variations of B-cell subsets potentially implicated in the pathophysiology of IgG4-RD. In light of this observation we were able to demonstrate that corticosteroids differentially affect naïve B cells, memory B cells, circulating plasmablasts, and plasma cells in patients with IgG4-RD. Interestingly, while circulating plasmablasts and plasma cells depletion after glucocorticoids has already been reported (23, 24), the decrease of naïve B cells represents a novel finding in the contest of IgG4-RD. Whether glucocorticoids affected the entire population of naïve B lymphocytes or a specific subset, however, is difficult to discriminate. The naïve B-cell compartment, in fact, encompasses a variety of lymphocytes subpopulations ranging from immature-transitional B cells to mature-naïve B cells, all characterised by the absence of the surface receptor CD27. Interestingly, increasing evidences have recently linked different subsets of naïve B cells to autoimmune diseases in humans (25). Translational T3 IgD+CD10^{neg}D27^{neg} B cells, for instance, have been found elevated in the peripheral blood of patients with Sjögren's syndrome (SS), a well-known mimicker of IgG4-RD (25, 26). In systemic lupus erythematosus (SLE), mature naïve B cells have been described in a state of chronic activation, and a minor population of IgD-CD27neg switched

memory B lymphocytes has been associated with autoantibody production and renal damage (25,27). CD27neg IL-10- and IgG4-producing regulatory B cells, a potential novel subset of naïve B lymphocytes, have also been observed in response to chronic allergenic stimulation and might be implicated in the pro-fibrotic processes characteristic of IgG4-RD (28). Hence, given the heterogeneity of the naïve B-cell compartment, we cannot exclude that one of these B-lymphocyte subsets might pathologically expand in IgG4-RD patients, and differentiate into plasmablasts and plasma cells. Glucocorticoids, as well as rituximab, may, in turn, target this putative pathogenic population of CD20⁺ naïve B-cell lymphocytes, thereby blocking their further maturation into antibody-secreting cells. This model would also explain the decline of circulating plasmablasts and plasma cells observed in patients with IgG4-RD together with clinical improvement. Finally, the increase of memory B cells after glucocorticoid treatment also represents an unanticipated finding in the field. Indeed, we recently demonstrated that patients showing elevation of memory B-cell counts following glucocorticoid induced disease remission are at risk of IgG4-RD relapse within 2 years (29). It is, therefore, possible that potential future relapsers within this cohort of IgG4-RD patients drive the overall increase of memory B cells observed after corticosteroid treatment. A longer follow-up is, however, necessary to confirm this hypothesis, and a more detailed flow-cytometry analysis is required to identify subsets of memory B cells that selectively expand at the time of disease remission in these patients. Our work has both points of strength and weaknesses. This is a monocentric prospective study conducted in one of the largest single centre cohorts of IgG4-RD patients, thus ensuring uniform inclusion criteria and follow-up. In addition, flow cytometry analysis was performed directly on fresh blood samples, an aspect that avoided potential modifications of the B cell compartment due to repeated freezing and thawing steps. The major weakness of our work is the limited number of IgG4-RD

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patients treated with glucocorticoids that we used for pursuing our primary aim. Indeed, although 30 patients still represent an informative cohort for a rare disease such as IgG4-RD, few outliers might have driven some of the reported results, especially with regard to correlation studies. We also recognise that a more detailed flow-cytometry analysis could have provided additional information about the modification of B-cell subsets induced by glucocorticoids in IgG4-RD patients. This study, however, was designed to address the general impact of corticosteroids on the B-cell compartment, and further characterisation of B-cell subsets of interest is currently being pursued.

In conclusion, we here report for the first time the comprehensive effects of glucocorticoids on B-cell subpopulations in IgG4-RD and add novel insights into the efficacy of corticosteroids in patients with this fibrotic condition. In particular, our work confirms that circulating plasmablasts and plasma cells expansion represents the most evident perturbation of the B-cell compartment in patients with IgG4-RD, but also unveils alterations within the population of naïve and memory B lymphocytes that might be central to IgG4-RD pathogenesis. Further examination of B-cell subsets in patients with IgG4-RD will yield to a better understanding of the immunological perturbations associated with IgG4-RD activity and, possibly, to novel targeted therapeutic strategies.

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