B-Cell Subsets in the Joint Compartments of Seropositive and Seronegative Rheumatoid Arthritis (RA) and No-RA Arthritides Express Memory Markers and ZAP70 and Characterize the Aggregate Pattern Irrespectively of the Autoantibody Status

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The aim of the present study was to determine whether different subsets of B cells characterize synovial fluid (SF) or synovial tissue (ST) of seropositive or seronegative rheumatoid arthritis (RA) with respect to the peripheral blood (PB). PB, SF and ST of 14 autoantibody (AB)-positive (rheumatoid factor (RF)-IgM, RF-IgA, anti–citrullinated peptide (CCP)), 13 negative RA and 13 no-RA chronic arthritides were examined for B-cell subsets (Bm1-Bm5 and IgD-CD27 classifications), zeta-associated protein kinase-70 (ZAP70) expression on B cells and cytokine levels (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8 and monocyte chemotactic protein (MCP)-1). Synovial tissues were classified as aggregate and diffuse patterns. No differences were found in B-cell percentages or in subsets in PB and SF between AB^+ and AB^- RA and no-RA. In both AB^+ and AB^- RA (and no-RA), the percentage of $CD19^+/ZAP70^+$ was higher in SF than in PB (AB^+: $P = 0.03$; AB^-: $P = 0.01$; no-RA: $P = 0.01$). Moreover, SF of both AB^+ and AB^- RA (and no-RA) patients was characterized by a higher percentage of $IgD-CD27^+$ and $IgD-CD27^-$ B cells and lower percentage of IgD^+CD27^- ($P < 0.05$) B cells compared to PB. In SF, ZAP70 positivity is more represented in B cell $CD27^+/IgD^-/CD38^-$. The aggregate synovitis pattern was characterized by higher percentages of Bm5 cells in SF compared with the diffuse pattern ($P = 0.05$). These data suggest that no difference exists between AB^+ and AB^- in B-cell subset compartmentalization. $CD27^+/IgD^-/ZAP70^+$ memory B cells accumulate preferentially in the joints of RA, suggesting a dynamic maturation of the B cells in this compartment.

INTRODUCTION

B cells are central in several autoimmune diseases characterized by specific pathogenic autoantibodies, such as immune thrombocytopenia and autoimmune hemolytic anemia (1–3).

In systemic autoimmune chronic inflammatory diseases, the role of B cells is much more complex, and several mechanisms of action have been hypothesized to explain how B-cell depletion can play a therapeutic role. In fact, B-cell depletion has become a remarkable tool to elucidate the pathogenetic role of B cells in

such illnesses (4). In rheumatoid arthritis (RA), several studies have characterized the status of B cells and of their subsets in peripheral blood (PB), as well as in the bone marrow before and after B-cell depletion (5–7). A general consensus was reached that no real differences exist in the PB between RA patients and healthy controls at baseline (8). After B-cell depletion, the cells that have been shown to reappear first in PB are $CD38^+IgD^+$, whereas $CD27^+IgD^+$ memory B cells seem to be the subset increased by the time of B-cell recovery (9,10). In contrast

to the nearly complete depletion and further regeneration of B cells in the PB after rituximab treatment, the B-cell depletion in other sites such as lymph nodes or tertiary lymphoid tissues appears to be directly related to the response to B-cell depletion (11,12). Failure to deplete B cells in these tissues may lead to nonresponse or early relapse, probably associated with a partial or ineffective reduction in infiltrating plasmablasts or $CD138^+$ plasma cells in these tissues. In fact, as suggested by Thurlings *et al.* (12), the reduction in synovial plasma cells at 16 weeks correlated with the reduction in serum autoantibodies and predicted clinical response at 24 weeks (12).

Thus, the general conclusion has been drawn that CD27 memory B cells and plasma cells are the most important play-

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ers of the inflammatory B-cell compartment. Formal proof of the possible hypothesis that CD27 memory B cells actually are those that have been selectively segregated into the joints has not been supported by direct evidence-based data.

In this study, we addressed the issue of B-cell subset distribution in the PB of RA seropositive for rheumatoid factor (RF) and/or anti–citrullinated peptide (anti-CCP) autoantibodies (AB⁺) and seronegative (AB⁻) patients and in no-RA patients and simultaneously in the synovial compartment, to understand whether there is a compartmentalization of some subsets specifically in AB⁺ subjects. In particular, we aimed to define whether memory B cells could preferentially accumulate into the synovial cavity of AB⁺ patients and whether some subsets could present molecular characteristics of persistently activated long-term surviving memory B cells. To this end, we examined the phenotypic characteristics of the B cells in the synovial fluids and tissues of RA (AB⁺ and AB⁻) and no-RA patients, with a particular look at zeta-associated protein kinase-70 (ZAP70)⁺ B cells that we previously showed being activated and being long-term survivors (13). We provide evidence that some subsets of B cells are recruited in the synovial compartment and are similarly present in seropositive and seronegative diseases. These B cells are CD27⁺ and ZAP70⁺ and characterize RA patients with aggregate synovitis.

MATERIALS AND METHODS

Patients and Control Populations

PB, synovial fluid (SF) or synovial tissue (ST) were collected from 27 consecutive patients fulfilling the ACR 1987 revised criteria for RA (14) (18 women, mean age 54.7 ± 19.7 years) and 13 patients with knee synovitis (no-RA: 9 women, mean age 49.0 ± 15.3 years). Clinical and laboratory evaluations were performed before the synovial biopsy. None of the patients had received corticosteroids in the last 4 wks or biologics in the last 3 months. The analysis in-

cluded the number of tender and swollen joints, erythrocyte sedimentation rate, C-reactive protein, Disease Activity Score and Health Assessment Questionnaire score. Anti-CCP antibodies were tested using the second-generation commercial assay (a-CCP2; Axis-Shield Diagnostics, Dundee, UK) kit by an enzyme-linked immunosorbent assay (ELISA), with positive anti-CCP

defined as >5 units/mL. The RF isotypes for IgA and IgM were measured by ELISA, using commercially available test systems (Orgentec Diagnostika, Mainz, Germany) and considered positive above a cutoff value of 20 units/mL. Characteristics of patient populations are shown in Table 1.

All the patients were informed of the objective of the study and gave their consent

Table 1. Demographical, immunological and pathological characteristics of subjects that underwent synovial biopsies.

Patient	Sex	Age (years)	Disease	Anti-CCP/ RF-IgM/RF-IgA	Disease Activity Score	IHC
1	F	73	RA	–/–/–	2.8	Diffuse
2	M	78	RA	–/–/–	3.6	Diffuse
3	F	32	RA	+/–/–	3.4	Diffuse
4	M	78	RA	–/–/–	5.5	Diffuse
5	F	76	RA	–/–/–	7.4	Diffuse
6	M	43	RA	–/–/–	4.2	Diffuse
7	F	69	RA	–/–/–	3.8	Diffuse
8	F	67	RA	+/+/–	5.6	Diffuse
9	F	40	RA	+/–/–	4.1	Diffuse
10	F	73	RA	+/–/–	2.6	Diffuse
11	M	63	RA	+/+/+	2.9	Diffuse
12	F	47	RA	+/+/+	3.9	Diffuse
13	F	19	RA	+/–/–	2.8	Diffuse
14	F	59	RA	+/–/–	4.5	Aggregate
15	F	58	RA	–/–/–	6.1	Aggregate
16	M	38	RA	–/+/–	1.2	Aggregate
17	F	77	RA	+/–/–	5.6	Aggregate
18	F	71	RA	+/–/–	4.9	Aggregate
19	F	67	RA	+/–/–	2.3	Aggregate
20	M	35	RA	–/–/–	3.1	Aggregate
21	M	68	RA	+/–/–	5.6	Aggregate
22	F	51	RA	–/–/–	2.9	Aggregate
23	F	24	RA	–/–/–	4.2	Aggregate
24	M	22	RA	–/–/–	2.8	Aggregate
25	M	25	RA	–/–/–	1.7	Aggregate
26	F	77	RA	+/+/–	3.5	Aggregate
27	F	47	RA	–/–/–	6.4	Aggregate
28	F	70	Mono–arthritis	–/–/–	—	Diffuse
29	M	51	Mono-arthritis	–/–/–	—	Diffuse
30	F	57	Mono-arthritis	–/–/–	—	Diffuse
31	F	71	Mono-arthritis	–/+/–	—	Diffuse
32	F	27	Mono-arthritis	–/–/–	—	Diffuse
33	F	56	Mono-arthritis	–/–/–	—	Diffuse
34	M	54	Mono-arthritis	–/–/–	—	Aggregate
35	F	26	Mono-arthritis	–/–/–	—	Aggregate
36	F	66	Oligo-arthritis	–/–/–	—	Diffuse
37	F	40	Oligo-arthritis	–/–/–	—	Aggregate
38	M	47	Psoriatic arthritis	–/–/–	—	Diffuse
39	M	39	Sarcoidosys	–/–/–	—	Diffuse
40	F	33	Still's disease	–/–/–	—	Aggregate

according to the Declaration of Helsinki. Approval for the study was obtained from the Institutional Review Board.

Cytokine Testing

All samples were tested for interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1. Cytokines were measured using a bead-based plex assay (Bender MedSystems, Vienna, Austria). The analytes bound to the antibodies linked to the fluorescent beads and then a biotin-conjugated second antibody mixture was added, followed by streptavidin-phycoerythrin, which permitted the emission of fluorescent signals. After incubation, samples were read with the flow cytometer (Navios; Beckman Coulter, Marseille, France). For calculation of the results, BMS FlowCytomix Pro software was used. Quantitative levels of cytokines were determined by comparison to standard curves, and they were reported as pg/mL. The detection limit of the assay was 4.2 pg/mL for IL-1β, 1.2 pg/mL for IL-6, 0.5 pg/mL for IL-8, 3.2 pg/mL for TNF-α and 18.2 pg/mL for MCP-1.

Synovial Biopsies

All patients underwent ultrasound-guided synovial biopsy of an actively inflamed joint (knee), performed under local anesthesia using a 14-G needle (Precisa 1410, HS Hospital Service Spa, Latina, Italy), to obtain at least six samples from different sites in the joint during each procedure.

Specimens from synovial biopsy were fixed with phosphate-buffered formalin, embedded in paraffin, sectioned at 3–4 μm and stained with hematoxylin and eosin or used for immunohistochemical analysis. All tissue samples were reviewed by two pathologists (F Morassi and V Arena) who were unaware of any clinical, serological or immunohistological findings (15).

Hematoxylin and eosin sections of tissue samples were analyzed for the organizational structure of the inflammatory infiltrate with attention to the topographical arrangement of T cells, B cells and macrophages to define the histological pattern of synovitis as either diffuse or aggregate. An aggregate pattern was defined as the presence of inflammatory cells with a 20-cell diameter focus at least, if less, the pattern was considered as diffuse (16,17). The definition of aggregates includes both the aggregate and germinal center (GC)-like infiltrates (defined by the presence of follicular dendritic cells), since one of the aims of the study was to assess the degree of differentiation toward GC reactions to evaluate if the aggregate infiltrates have a biological or clinical relevance. The specific pattern was evaluated in two different tissue sections 50 μm apart to further minimize sampling error (Supplemental Table 1).

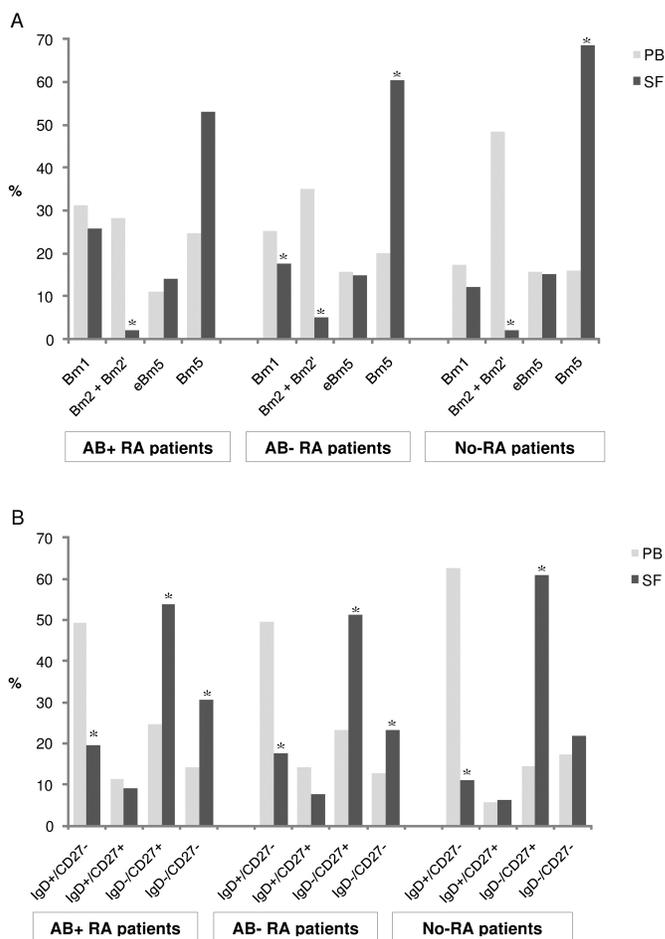For immunohistochemistry (IHC) analysis, tissue sections were deparaffinized

Table 2. B-cell phenotype distribution in PB and SF in AB$^+$ and AB$^-$ RA and no-RA patients.

B-cell subpopulation (flow cytometry)	RA patients, AB$^+$, n = 14	RA patients, AB$^-$, n = 13	P	No-RA patients, n = 13	P^a	P^b
PB						
WBC count (n/μL)	8,428 ± 3,146	8,363 ± 2,876	0.87	7,061 ± 2,037	0.21	0.23
Lymphocytes (%)	32.7 ± 12.4	28.8 ± 12.8	0.51	27.8 ± 11.1	0.48	0.71
Lymphocytes (n/μL)	2,760 ± 1,160	2,082 ± 651	0.06	2,038 ± 1,002	0.13	0.71
CD19 (%)	7.8 ± 4.5	8.4 ± 5.2	0.83	7.9 ± 4.5	0.85	0.82
Bm1 (%)	31.3 ± 12.1	25.3 ± 15.4	0.25	17.3 ± 6.7	0.08	0.32
Bm2+Bm2′ (%)	28.1 ± 20.6	35.1 ± 19.3	0.57	48.4 ± 6.2	0.11	0.25
eBm5 (%)	11.2 ± 4.8	15.8 ± 8.8	0.48	15.7 ± 6.3	0.20	1.00
Bm5 (%)	24.7 ± 22.3	20.0 ± 16.7	0.89	16.1 ± 11.0	1.00	0.57
Ratio Bm2+Bm2′/ eBm5+Bm5	2.3 ± 3.1	1.4 ± 1.0	0.89	1.6 ± 0.6	0.75	0.89
IgD$^+$/CD27$^-$ (%)	49.4 ± 24.6	49.7 ± 21.4	0.89	62.5 ± 6.0	0.75	0.39
IgD$^+$/CD27$^+$ (%)	11.5 ± 6.8	14.2 ± 14.2	0.67	5.7 ± 3.8	0.05	0.25
IgD$^-$/CD27$^+$ (%)	24.6 ± 15.4	23.2 ± 17.0	0.89	14.4 ± 6.7	0.26	0.39
IgD$^-$/CD27$^-$ (%)	14.3 ± 8.8	12.9 ± 5.6	0.89	17.4 ± 9.8	0.63	0.74
CD19$^+$/ CD27$^+$CD38$^+$ (%)	8.2 ± 3.8	11.6 ± 9.9	0.67	8.0 ± 4.4	1.00	0.67
CD19$^+$/ZAP70$^+$ (%)	7.6 ± 6.9	6.6 ± 4.6	1.00	4.0 ± 2.8	0.46	0.25
SF						
WBC count (n/μL)	14,480 ± 12,375	13,150 ± 13,085	0.67	14,920 ± 10,656	0.71	0.58
Lymphocytes (%)	26.4 ± 19.0	13.1 ± 9.1	0.07	19.4 ± 12.2	0.34	0.20
Lymphocytes (n/μL)	2,213 ± 1,609	1,092 ± 857	0.08	1,878 ± 1,278	0.58	0.14
CD19 (%)	2.2 ± 3.0	0.8 ± 0.5	0.14	0.6 ± 0.8	0.02	0.16
Bm1 (%)	25.7 ± 32.8	17.5 ± 11.1	0.67	12.2 ± 5.7	0.43	0.34
Bm2+Bm2′(%)	2.2 ± 0.8	5.0 ± 4.4	0.20	2.1 ± 1.0	0.93	0.15
eBm5 (%)	14.1 ± 11.2	14.8 ± 8.5	0.62	15.2 ± 12.3	0.62	0.75
Bm5 (%)	53.1 ± 28.6	60.4 ± 8.2	1.00	68.7 ± 14.6	0.35	0.34
Ratio Bm2+Bm2′/ eBm5+Bm5	0.03 ± 0.01	0.07 ± 0.06	0.14	0.03 ± 0.01	0.65	0.15
IgD$^+$/CD27$^-$ (%)	19.7 ± 18.3	17.6 ± 6.8	0.78	11.0 ± 3.4	0.52	0.05
IgD$^+$/CD27$^+$ (%)	9.0 ± 15.2	7.8 ± 8.0	0.48	6.3 ± 4.5	0.52	0.87
IgD$^-$/CD27$^+$ (%)	53.7 ± 12.1	51.4 ± 17.3	1.00	60.9 ± 5.4	0.41	0.52
IgD$^-$/CD27$^-$ (%) CD19$^+$/	30.7 ± 16.6	23.2 ± 11.8	0.15	21.8 ± 8.6	0.22	0.69
CD27$^+$CD38$^+$ (%)	7.6 ± 7.3	8.5 ± 7.1	0.89	10.0 ± 7.9	0.43	0.75
CD19$^+$/ZAP70$^+$ (%)	28.5 ± 27.5	20.9 ± 12.6	0.96	22.2 ± 12.6	0.96	0.83

Data are means ± SD. Kruskal-Wallis test: no significant differences between RA AB$^+$, RA AB$^-$ and no-RA patients.
^{a}Mann-Whitney test between RA AB$^+$ and no-RA patients.
^{b}Mann-Whitney test between RA AB$^-$ and no-RA patients.

Figure 1. PB and SF B-cell subpopulations in RA (AB⁺ and AB⁻) and no-RA patients. B-cell analysis was done by flow cytometry. A region was defined around the CD45⁺ cells, and total B cells were identified on the basis of the expression of the cell surface marker CD19. The B-cell subpopulations were classified according to IgD and CD38 expression (Bm1-Bm5 classification (A)) or IgD and CD27 expression (B) (19,20). As shown in the plots, naive B cells accumulate preferentially in the PB, whereas memory subsets localize in the synovial compartment. The distribution of B-cell subsets was similar in seropositive as well as in seronegative RA patients and in the RA cohort compared with no-RA cohort, both in blood and in the synovial compartments. *$P < 0.05$, SF versus PB.

and rehydrated through graded ethanol solutions. The sections were incubated with antibodies directed against CD20, CD3, CD27, CD68, CD38 and CD138 (Immunotech; Beckman Coulter, France). The chromogen used for the detection was 3,3′-diaminobenzidine/H_2O_2 (DAB). Pathologists skilled in IHC (F Morassi and V Arena) performed a manual cell count in three different high-magnification fields (high-power field [HPF] 400×); the mean number of the three counts was recorded.

Isolation of PB Mononuclear Cells and Flow Cytometry Analysis

Fresh PB or SF samples were processed within a few hours after sample collection. A total of 100 μL PB containing approximately 5×10^5 white blood cells (WBCs) or 5×10^5 mononuclear cells derived from SF (by Ficoll-Hipaque [Cederlane, Ontario, Canada] density gradient centrifugation) in 100 μL phosphate-buffered saline (PBS) was first incubated in the dark at room temperature for 20 min with antihuman

antibodies specific for CD19 (ECD), CD3 (PC5), CD56 (PC5), CD38 (fluorescein isothiocyanate [FITC] or phycoerythrin [PE]), CD5 (FITC), CD23 (FITC), CD27 (PC5) or FITC-conjugated IgD (Beckman Coulter, France). The cells were fixed and permeabilized using the IntraPrep kit (Beckman Coulter, France) according to the manufacturer's instructions and incubated with anti–ZAP70-PE monoclonal antibody for 30 min (clone SBZAP; Beckman Coulter, Fullerton, CA, USA). The expression of ZAP70 (SBZAP, conjugated with PE) in CD19⁺ cells was measured according to the gating strategy published by Crespo *et al.* (18). The first gate was set on lymphocytes defined by forward- and side-scatter characteristics. B cells were defined as CD56⁻ and CD3⁻ and CD19⁺ cells. The cutoff for ZAP70 expression on B cells was set at 98% of ZAP70 positivity of T-NK cells. All cell samples were analyzed on a properly compensated EPICS XL and from April 2010 with an eight-color Navios flow cytometer (CD45-APC750, CD19 APC-A700, CD3-ECD, CD56 ECD, IgD-FITC, CD38-APC, CD27-PC7 and ZAP70 PE), and data were analyzed with the Kaluza program (Beckman Coulter, France).

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA) and Prism software (GraphPad, San Diego, CA, USA). Categorical and quantitative variables were described as frequencies, percentage and mean ± SD. Data on demographic and clinical features were compared between patients by the nonparametric Mann-Whitney U test or χ^2 test, as appropriate. The Wilcoxon test was used to compare B-cell subtype distribution and cytokine levels between plasma and synovial fluid. Spearman rank correlation was used to evaluate the relationship between different parameters of disease. A P value <0.05 was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

A
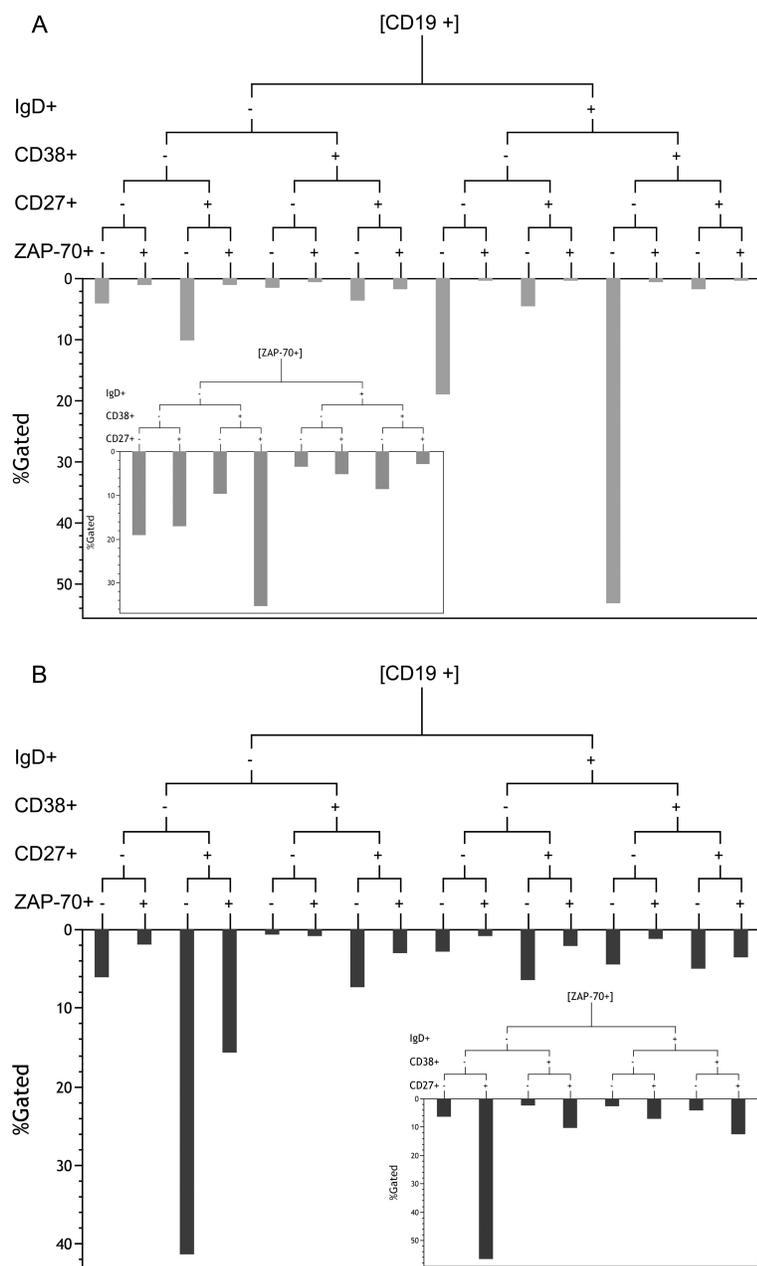
[CD19 +]

B

[CD19 +]

Figure 2. Schematic representation of B-cell subpopulations in PB (A) and SF (B) of a prototypic subject with RA. PB and SF B cells were stained with anti-CD45/anti-CD19/anti-CD3 and CD56/anti-CD38/anti-CD27/anti-IgD/anti-ZAP70. Flow cytometric analyses were performed on CD19-gated cells. The graphs were obtained with an eight-color flow cytometer (Navios and Kaluza software, Beckman Coulter, France).

RESULTS

Demographic, Immunological and Clinical Data of Patients

RA patients were categorized into two subgroups (AB$^+$ or AB$^-$) on the basis of the presence or absence of autoantibodies.

There were no differences in the sex profile, age and inflammatory parameters between RA and no-RA patients. Swollen joint count (SJC) was higher in the RA group than in the no-RA group (11.3 ± 10.2 versus 1.7 ± 1.1, $P = 0.01$). As shown in Table 1, positivity for autoantibodies (anti-CCP and/or RF-IgA and/or RF-IgM) was present in 14 (51.9%) of 27 RA patients and in 1 (RF-IgM positivity) of the 13 no-RA patients (7.7%, $P = 0.01$ versus RA patients).

PB and SF Mononuclear Cell Distribution in RA and No-RA Patients

No significant differences were found in WBC count and lymphocyte percentage between AB$^+$ and AB$^-$ RA patients and between AB$^+$ and/or AB$^-$ RA and no-RA patients in PB or SF (Table 2).

In PB, B-cell percentage was similar in all the groups, whereas we found a higher percentage of B cells in SF of AB$^+$ RA patients (2.2 ± 3.0%) compared with no-RA patients (0.6 ± 0.8%, $P = 0.02$) (Table 2).

Both in AB$^+$ and AB$^-$ RA patients and in no-RA patients, the percentage of lymphocytes and CD19$^+$ cells were higher in PB than in SF, whereas we found no differences in WBC count (Table 2).

PB and SF B-Cell Subset Distributions in RA and No-RA Patients

We performed a multicolor flow-cytometric analysis of PB mononuclear cells isolated from SF and paired PB samples of RA (AB$^+$ and AB$^-$) and no-RA patients, considering the expression of IgD, CD38 and CD27 as cell surface markers (Bm1-Bm5 or IgD-CD27 classifications) (19,20) as well as the expression of ZAP70 on B cells (16). Considering the Bm1-Bm5 classification, we found no differences in B-cell subset distribution between RA (AB$^+$ or AB$^-$) and no-RA both in PB and SF (Table 2). Moreover, the percentage of B cells expressing ZAP70 was similar in RA subgroups and no-RA patients both in PB and SF (Table 2).

Both in AB$^+$ and AB$^-$ RA patients, the percentage of Bm2+Bm2' was significantly lower in the SF than in the PB, with a significantly higher percentage of Bm5 (%) in SF than in PB. The obvious result of such a difference in compart-
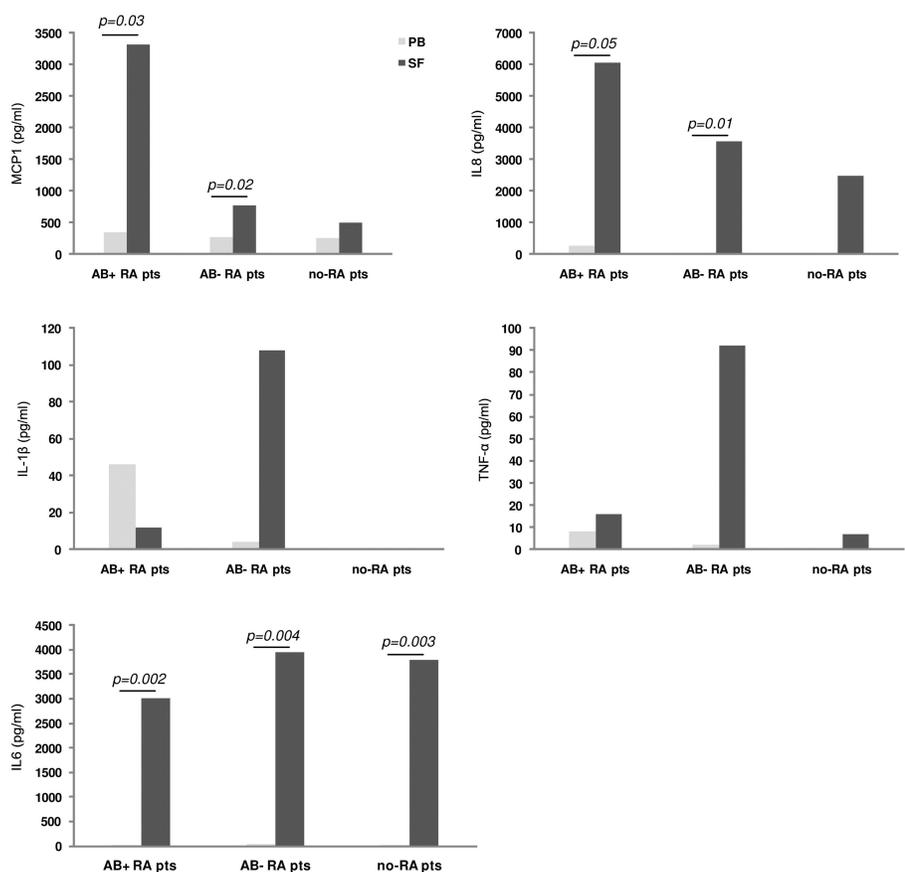
Figure 3. Mean cytokine levels in PB and SF of RA patients. The plasma and SF levels of MCP-1, IL-8, IL-1β, TNF-α and IL-6 in RA (AB⁺ and AB⁻) and no-RA patients were assessed using a bead-based assay kit.

mentalization is the significantly lower ratio of Bm2+Bm2′/eBm5+Bm5 in SF compared with PB ($P < 0.05$; Table 2).

As previously shown, in both AB⁺ and AB⁻ RA patients, the percentage of CD19⁺/ZAP70⁺ was higher in SF than in PB (AB⁺: $P = 0.03$; AB⁻: $P = 0.008$) (Table 2 and Figure 1).

In no-RA patients as well, we found that, in SF, the percentage of Bm2+Bm2′ was lower and the percentage of Bm5 was higher than in PB (Bm2+Bm2′: $P = 0.03$; Bm5: $P = 0.03$), data confirmed by the lower ratio of Bm2+Bm2′/eBm5+Bm5 in SF compared to PB [$P = 0.03$]. As shown in RA patients, also in no-RA patients, the percentage of CD19⁺/ZAP70⁺ was higher in SF than in PB ($P < 0.01$) (Table 2 and Figure 1).

Data of B-cell subset distribution with the Bm1-Bm5 classification was con-

firmed by the staining of B cells with IgD and CD27 antibodies. SF of both AB⁺ and AB⁻ RA patients was characterized by a higher percentage of IgD-CD27⁺ and IgD-CD27⁻ B cells and a lower percentage of IgD⁺CD27⁻ ($P < 0.05$) B cells compared with PB (Table 2 and Figure 1).

As illustrated in Figure 2 (representative RA patient), B cells derived from SF seem to be more IgD⁻, CD38⁻ and CD27⁺ than B cells from PB; moreover, in SF, ZAP70 positivity is more represented in B cells CD27⁺, IgD⁻ and CD38⁻.

Plasma and Synovial Fluid Cytokine Levels in RA and No-RA Patients

AB⁺ and AB⁻ RA patients showed comparable plasma levels of IL-8, IL-1β, IL-6, TNF-α and MCP1, but AB⁺ RA patients had significantly higher concentrations

of MCP-1 in SF (3,009.2 ± 3,477.0 pg/mL) compared with AB⁻ RA patients (802.9 ± 494.7 pg/mL, $P = 0.04$) and no-RA patients (503.8 ± 395.4 pg/mL, $P = 0.03$). Moreover, in plasma of AB⁺ RA patients, the concentration of IL-8 was significantly higher than in no-RA (280.2 ± 729.8 versus 0.1 ± 0.0 pg/mL, $P = 0.01$). No differences were found between RA and no-RA patients for the other studied cytokines, both in PB and SF (data not shown). In SF of RA patients, we found significantly higher values of IL-6 (AB⁺: $P = 0.002$; AB⁻: $P = 0.004$), MCP-1 (AB⁺: $P = 0.03$; AB⁻: $P = 0.02$) and IL-8 (AB⁺: $P = 0.05$; AB⁻: $P = 0.01$) compared with PB; moreover, in AB⁻ RA patients, we found a trend to have higher levels of TNF-α and IL-1β in SF than in PB (Figure 3).

Immunohistochemistry in Synovial Biopsies of RA and No-RA Patients

Synovial samples from RA and no-RA patients were characterized by mononuclear cell infiltrates and consisted mainly of lymphocyte- and macrophagelike cells and plasma cells. In addition, the IHC phenotype analysis showed similar counts in AB⁺ and AB⁻ RA patients ($P = 0.84$). In our samples, synovial lining layer proliferation and fibrinoid necrosis were also observed. For RA, 14 (51.9%) specimens revealed diffuse lymphocyte infiltration, and they were categorized as diffuse rheumatoid synovitis. Lymphocyte aggregates, sometimes with germinal center–like structures, were observed in 13 (48.1%) samples. Such RA synovial samples were therefore classified as an aggregate pattern of synovitis. Four of 13 no-RA synovial samples showed aggregate pattern of synovitis (30.8%, $P = 0.33$ versus RA patients).

We found no significant differences in the analysis of CD68⁺, CD3⁺, CD20⁺, CD38⁺ and CD138⁺ cells in the synovial tissue between RA and no-RA, even though the mean count of all the cells tended to be higher in RA (data not shown). CD20⁺ cells correlated significantly with CD3 ($r = 0.68$, $P = 0.01$) and CD38 ($r = 0.63$, $P = 0.04$).

Table 3. Distribution of B-cell populations in RA patients with diffuse and aggregate patterns of synovitis.

Variables	Aggregate phenotype (PB), n = 13	Diffuse phenotype (PB), n = 14	*P*	Aggregate phenotype (SF), n = 13	Diffuse phenotype (SF), n = 14	*P*
Age (years)	53.4 ± 19.0	55.9 ± 20.9	0.59	—	—	
Disease duration (years)	10.7 ± 7.3	5.5 ± 4.6	0.05	—	—	
AB⁺ (%)	46.2	50.0	0.84	—	—	
Erythrocyte sedimentation rate (mm/first hour)	46.7 ± 24.9	42.1 ± 22.4	0.64	—	—	
C-reactive protein (mg/L)	25.9 ± 16.0	43.0 ± 53.2	1.00	—	—	
Disease Activity Score	4.1 ± 1.6	3.9 ± 1.6	0.58	—	—	
Health Assessment Questionnaire	1.5 ± 0.8	1.3 ± 0.7	0.45	—	—	
WBC count (μ/μL)	7,066 ± 2,116	9,632 ± 3,161	0.03	16,010 ± 12,740	11,620 ± 12,195	0.34
Lymphocytes (μ/μL)	2,289 ± 854	2,516 ± 1,100	0.74	2,147 ± 1,665	1,165 ± 836	0.21
Lymphocytes (%)	31.9 ± 10.8	29.8 ± 14.3	0.57	22.5 ± 18.7	17.0 ± 13.2	0.58
CD19 (μ/μL)	179 ± 162	228 ± 105	0.34	22 ± 14	9 ± 8	0.06
CD19 (%)	7.4 ± 5.7	8.7 ± 3.7	0.51	1.1 ± 0.5	2.1 ± 3.4	0.46
Bm1 (%)	29.7 ± 16.8	26.3 ± 10.6	1.00	15.0 ± 10.8	30.0 ± 34.3	0.25
Bm2+Bm2′ (%)	20.1 ± 12.8	44.6 ± 18.6	0.02	1.9 ± 0.6	7.0 ± 4.3	0.01
eBm5 (%)	16.0 ± 8.7	10.9 ± 4.7	0.25	14.9 ± 11.0	13.8 ± 8.8	0.89
Bm5 (%)	29.8 ± 22.2	13.3 ± 9.1	0.20	66.6 ± 12.1	44.6 ± 24.5	0.05
Ratio Bm2+Bm2′/eBm5+Bm5	0.9 ± 1.0	2.9 ± 2.7	0.06	0.02 ± 0.01	0.1 ± 0.1	0.01
IgD⁺/CD27⁻ (%)	37.5 ± 18.1	66.7 ± 17.9	0.02	11.6 ± 5.4	27.0 ± 16.3	0.06
IgD⁺/CD27⁺ (%)	16.4 ± 13.0	8.9 ± 7.3	0.15	7.8 ± 7.2	9.2 ± 16.7	0.09
IgD⁻/CD27⁺ (%)	31.4 ± 17.5	15.1 ± 6.6	0.12	55.1 ± 12.7	47.8 ± 18.2	0.57
IgD⁻/CD27⁻ (%)	14.7 ± 8.8	12.2 ± 4.3	0.32	25.5 ± 9.4	29.3 ± 19.8	0.78
CD19⁺/CD27⁺CD38⁺ (%)	12.9 ± 9.0	6.7 ± 4.0	0.15	9.0 ± 7.0	6.9 ± 7.2	0.39
CD19⁺/ZAP70⁺ (%)	7.1 ± 7.0	7.0 ± 4.6	0.53	22.6 ± 19.3	27.5 ± 24.8	0.89
IHC						
CD20, count	—	—		88.1 ± 80.4	24.1 ± 17.4	0.09
CD3, count	—	—		157.8 ± 46.4	72.1 ± 53.7	0.02
CD68, count	—	—		61.6 ± 40.5	35.6 ± 16.9	0.10
CD27, count	—	—		128.6 ± 89.3	34.2 ± 29.7	0.01
CD38, count	—	—		45.0 ± 27.8	32.9 ± 31.0	0.47
CD138, count	—	—		36.5 ± 32.9	21.2 ± 25.3	0.25

Data are mean ± SD.

Demographic Characteristics, Inflammatory Milieu and B-Cell Phenotypes in RA Patients with an Aggregate or Diffuse Pattern of Synovitis

In RA, patients with an aggregate pattern had a longer disease duration than patients with diffuse synovitis (*P* = 0.05) (Table 3). Regarding the B-cell distribution subsets, in patients with aggregate synovitis, the percentage of Bm2+Bm2′ was lower in PB and in SF compared with patients with diffuse phenotype (PB: *P* = 0.02; SF: *P* = 0.01). These data were confirmed by the lower percentage of IgD⁺CD27⁻ B cells in SF of aggregate synovitis compared with diffuse synovi-

tis. However, the percentage of SF Bm5 was significantly higher in aggregate synovitis (*P* = 0.05), and we found a lower SF Bm2+Bm2′/eBm5+Bm5 ratio in aggregate compared with diffuse phenotype (0.02 ± 0.01 versus 0.1 ± 0.1, respectively; *P* = 0.01) (Table 3 and Figure 4A–F). As already reported (13), the percentage of CD19⁺/ZAP70⁺ cells was higher in SF than in PB in RA patients with both a diffuse and aggregate pattern of synovitis (Figure 4G).

The IHC analysis revealed that the aggregate pattern was associated with a higher CD3 count in synovial tissue compared with the diffuse phenotype (*P* = 0.02); the presence of CD27 positivity

alone, which marks either T cells as well as B memory cells, was also statistically increased in aggregate versus diffuse type (*P* = 0.01) (Table 3).

DISCUSSION

This study aimed to investigate the distribution of B-cell subsets in blood and synovial fluids and tissues of seropositive and seronegative RA patients because it is of fundamental importance to understand how B cells behave during rheumatoid inflammation and how B-cell depletion could work in this disease. Our data show that naive B cells accumulate in the PB, and the mature-memory subsets localize in the synovial
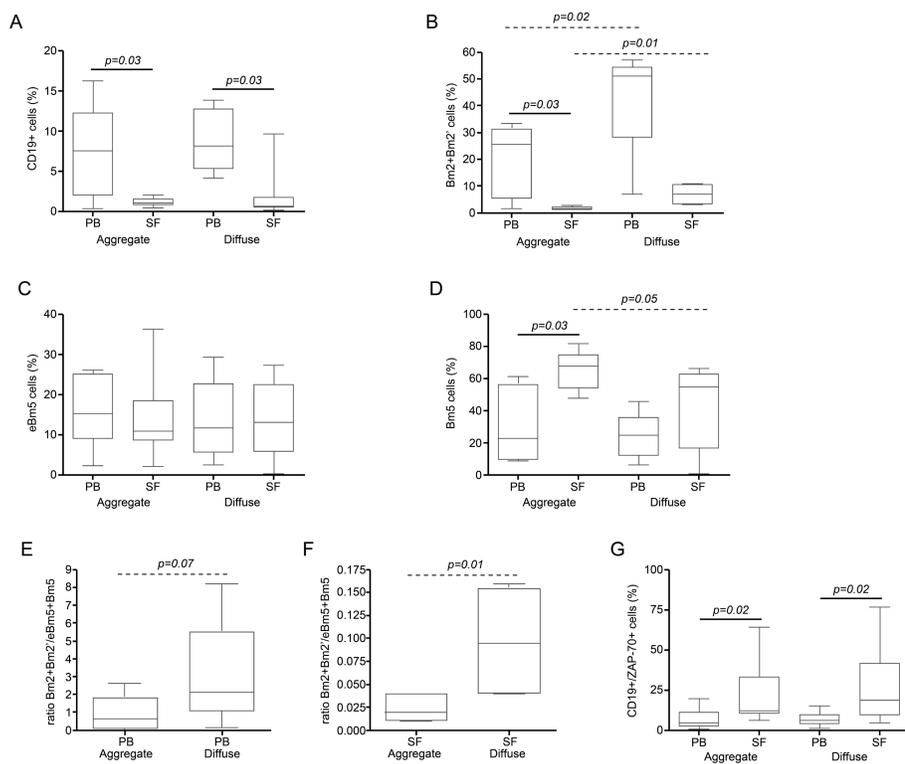
Figure 4. Distribution of B-cell subpopulations in PB and SF of RA patients with aggregate or diffuse pattern of synovitis. Plots represent the percentage of CD19⁺ cells (A), Bm2+Bm2' cells (B), eBm5 cells (C), Bm5 cells (D) and CD19⁺/ZAP70⁺ cells (G) in PB and SF of patients with aggregate or diffuse pattern of synovitis. The ratio between the percentage of Bm2+Bm2' cells and eBm5+Bm5 cells is represented in plots in E and F. The Mann-Whitney test (solid line) and the paired Wilcoxon test (dotted line) were used for comparison of unpaired and paired data. Box plots show the 10th, 25th, 50th (median), 75th and 90th percentiles of the variable.

compartment. Moreover, we found a similar distribution of the B-cell subsets in seropositive as well as in seronegative RA patients and in the RA compared to no-RA cohorts, both in blood and in the synovial compartments. We also observed a gradient of B-cell maturation between the PB and the SF, thus suggesting that a persistent inflammation probably leads to a maturation of B cells in the joint. In particular, we observed that Bm5 cells are more abundant in the synovial compartment than in the PB. These data were confirmed by the analysis of IgD/CD27. The compartmentalization of memory B cells in the joints confirmed, in a much larger cohort, what was seen previously by Möller *et al.* (21) in three longstanding RA patients. These authors emphasized that the formation of lym-

phoid structures depends not only on the expansion of switched memory B cells locally, but also on traffic circulation, suggesting the importance of monitoring B cells, both in blood and synovial compartments.

Moreover, a recent study focused on the importance of the evaluation of pre- and post-switch CD27 B cells in the synovium. The authors reported a lower percentage of IgD⁺CD27⁺ pre-switched memory cells and a higher percentage of IgD⁻CD27⁺ post-switch memory B cells in PB of longstanding RA patients when compared with healthy controls. They also reported that pre-switched IgD⁺CD27⁺ and post-switch IgD-CD27⁺ memory B cells accumulate in the synovium, particularly in patients with a long disease duration, suggesting an en-

hanced sequestration of these cells in the synovium (22).

Our data confirm the previous findings of a higher presence of IgD⁻CD27⁺ memory B cells in the synovium, but we also found a higher percentage of IgD⁻CD27⁻ B cells both in AB⁺ and AB⁻ RA patients when compared with PB. IgD⁻CD27⁻ B-cells were recently characterized in systemic lupus erythematosus (SLE) patients as memory B cells with an activated phenotype. These cells are more present in patients with disease flares, and they are correlated with disease activity; the authors suggested that IgD⁻CD27⁻ B cells might be designated to migrate to inflamed tissues (23). In our study, even if this subtype of B cells is compartmentalized only in the synovium of RA patients and more in seropositive patients, we were not able to find a direct correlation between these cells and parameters of disease activity or inflammatory status in RA. Hence, the higher presence of memory B cells, and in particular IgD⁻CD27⁻ B cells, in the synovium of RA patients appears to be an alteration that is permanent in RA patients, irrespective of disease activity. The compartmentalization of memory B cells in the synovium probably is associated with a specific recruitment *in situ* of this subgroup of cells because of the increased expression in macrophages, synovial fibroblasts and follicular dendritic cells of either chemokines or their receptors responsible for T- and B-cell recruitment (24,25).

Recent data have provided evidence that the synovial fluid of RA patients is enriched of memory B cells that express CXCR4 together with IL-8 receptors CXCR1 and CXCR2, and these cells may be recruited into the synovial membrane, where they accumulate and are responsible for the follicularlike structure formation, thus contributing to the perpetuation of chronic synovitis (22). Our study supports these observations, as we found that the levels of IL-8 and MCP-1, cytokines with proinflammatory and chemotactic functions, were significantly higher in PB of RA compared with no-RA

patients; and in RA more in the synovial fluid than in the PB, suggesting that interactions between chemokines and chemokine receptors might contribute to memory B-cell migration into the synovium.

Finally, our data show that memory B cells accumulate into the synovial fluid and show a clear-cut presence in the aggregate pattern of synovial infiltrates. Moreover, memory B cells are more ZAP70^{+}, a member in the protein-tyrosine kinase family. We recently reported an increase in ZAP70 expression in synovial CD38^{+}, CD5^{+} and CD23^{+} B-cell RA patients. These ZAP70^{+} B cells exhibited an increased survival *in vitro* compared with ZAP70^{−} B cells, and they are linked with the inflammatory and autoimmune phenotype (13). Consistent with these data, van de Sande *et al.* (26) recently suggested that the presence of lymphocyte aggregates seems to be related to the level of synovial inflammation in early-arthritis patients and can change over time.

In conclusion, we provide data showing that a subset of memory B cells characterized by CD27^{+}/IgD^{−}/ZAP70^{+} accumulate preferentially in the joints of RA patients and that there seems to be a dynamic maturation of B cells according to disease activity and disease duration. Also, no difference exists between RA AB[+] and RA AB[−]and that seropositive and seronegative differ more in terms of degree of local inflammation than in terms of subsets of B-cell involvement.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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