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Phenotypic Diversity of Peripheral Blood Plasma Cells in Primary Sjögren's Syndrome

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Abstract

Production of autoantibodies is one of the main features of primary Sjögren's syndrome (pSS). Long-lived plasma cells (PC) can produce autoantibodies for prolonged period of times without being affected by immunosuppressive therapies. As of today, little is known about the long-lived PC subset and their contribution to autoimmunity. We have characterized the phenotypic and migratory properties of peripheral blood PC isolated from pSS patients (grouped by focus score, FS) and compared them to PC from rheumatoid arthritis (RA) patients and normal non-autoimmune subjects. We observed two populations of PC in all study groups, CD19+ PC and CD19− PC. Interestingly, the CD19− PC subset was most prominent in autoimmune patients (pSS and RA) compared to normal controls. Further investigation of the PC phenotype revealed that a high percentage of both CD19+ and CD19− PC isolated from pSS and RA patients did not express the CD27 marker, which is normally highly expressed on all types of PC. Differences in the expression of markers such as IgM, IgG, CD95 and CXCR3 in the group with high FS compared to FS = 1, underscore the heterogeneity of pSS patient group and demonstrate that phenotypic pattern of circulating PC associates with the severity of inflammation in the salivary glands of these patients. Our migration experiments show that addition of CXCL12 to PC *in vitro*, do not alter the migration potential of PC in any group tested. However, we observed an overall higher spontaneous migration of PC from pSS compared to both RA and normal controls.

Introduction

Primary Sjögren's syndrome (pSS) is an autoimmune disease with organ-specific and systemic manifestations [1]. It is characterized by a chronic focal inflammation of exocrine glands, especially salivary and lachrymal glands, which results in impaired saliva and tear production, thus contributing to dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). A characteristic feature of pSS is the presence of autoantibodies against Ro/SSA and La/SSB antigens, observed in 50–80% of the patients [2, 3]. A large proportion of these circulating immunoglobulins are produced by plasma cells (PC) [4] residing in the bone marrow since bone marrow stroma contain signals needed for plasma cell survival [5–10].

Autoreactive long-lived PC are a challenge for treatment strategies since they are resistant to the today's immunosuppressive therapies and capable of maintaining continuous autoantibody production even after depletion of B cells [11–13]. In recent years it has become clear that PC are more heterogenic than previously thought, presenting different phenotypes at different stages of the differentiation process (plasma blasts versus plasma cells). Additionally, PC from different compartments such as blood, tonsils and bone marrow as well as PC in different diseases can present quite different expression patterns of surface receptors [14]. Altogether, this makes it very difficult to target these cells in a specific manner and one general treatment seems not possible.

There is also the interesting matter of short-lived versus long-lived PC and how to distinguish between these subsets of PC. In general, the proliferating cells and cells produced early in the immune response (from B1 and marginal zone B cells) are thought to be short-lived IgM producing cells, whereas non-proliferating, high affinity, IgG producing cells produced by germinal centre and memory B cells are considered the precursors of long-lived PC subset (reviewed in [15]). The lifespan of PC

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can vary from a few days to months and even years depending on the intrinsic properties (such as expression of anti-apoptotic Bcl2 protein) and on their capability of finding and migrating to the sites that house the so-called plasma cell survival niches. Bone marrow is considered to be the most important compartment for the survival niches, however similar environment has also been found in the secondary lymphoid organs (spleen and lymph nodes) and at sites of inflammation [16–19]. Migration of PC to these compartments is a complex process, controlled among others by a variety of chemokines and their receptors. In particular, the expression of CXCR4 receptor on PC has been implicated as a key regulator of migration to the bone marrow [20–25], whereas CXCR3 has been shown to be important in accumulation of PC in inflamed tissue such as salivary glands of pSS patients [23].

However, it is still unknown which intrinsic or induced properties that make some plasma cells suitable for migration and long term survival. Therefore, in this study we addressed the issue of circulating plasma cells in pSS patients with FS = 1 and with pSS patients with high FS (FS ≥ 2), focusing on phenotype, survival and migration potential. In addition, we wanted to compare these observations with rheumatoid arthritis (RA) patients and a healthy control group. We detected significantly more circulating PC in pSS patients with high FS compared to pSS patients with FS = 1, RA and non-autoimmune subjects. Furthermore, high percentage of pSS PC has shown to be CD19− and CD27− PC that point out to existence of abnormal PC population in the autoimmune pSS patients but not in the non-autoimmune subjects.

Materials and Methods

Patients and controls. Heparinized whole blood (∼20 ml) was collected from 22 pSS and 19 RA patients in addition to 20 control subjects. The pSS and RA groups were consecutively enrolled outpatients at the Department of Rheumatology, Haukeland University Hospital in Bergen, Norway. All pSS patients were diagnosed according to the American–European consensus group criteria (AECC) [26]. The RA patients fulfilled the revised American College of Rheumatology criteria for rheumatoid arthritis [27]. Healthy controls were gender and aged matched and recruited from the same geographic area as the pSS and RA patients. The study was approved by the Committee of Ethics at the University of Bergen (#242.06). Clinical laboratory and histopathological characteristics of patients groups and controls are presented in Table 1. The pSS patients used in the study were divided into two groups according to focus score (FS); one group consisting of patients with FS = 1 and one group consisting of patients with FS ≥ 2.

Isolation of plasma and mononuclear cells from peripheral blood. Whole blood was carefully layered onto Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged for 30 min at 800 *g* at room temperature. The plasma was collected and stored at −70 °C until tested. The band with mononuclear cells was gently harvested and washed twice by resuspending in sterile phosphate buffer saline pH 7.4 (PBS) followed by centrifuging at 250 *g* for 10 min at room temperature.

Autoantibody assay. Plasma from pSS, RA patients and healthy controls were tested by a commercially available QUANTA Plex™ SLE Profile eight fluorescent immuno bead assay (INOVA Diagnostics, San Diego, CA, USA) against nine common autoantigens: Sm, RNP, Ro60/PSS-A, Ro52/PSS-A, La48/PSS-B, Scl-70, Jo-1, Ribosome P and chromatin. The assay was carried out as recommended by the manufacturer. The plates were analysed with a Luminex 100™ instrument (Luminex Corp., Austin, TX, USA) with Star Station software (Applied Cytometry Systems, Dinnington, UK). The autoantibody levels against Sm, RNP, Scl-70, Jo-1, Ribosome P and chromatin were low in all three groups tested, whereas significantly higher levels of autoantibodies towards Ro52 and Ro60 were observed in pSS patients compared with RA patients and control group.

Flow cytometric analysis. Mononuclear cells isolated from peripheral blood were counted by Casy Cell Counter system (Schärfe System GmbH, Reutlingen, Germany). Cell concentration was adjusted to 1×10^6 per test and resuspended in FACS buffer (PBS containing 1% FBS, 2 mM EDTA and 0.01% azide). Cells were added FcR Blocking Reagent (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to block Fc receptors present on the surface of the lymphocytes. Surface antigens were labelled by incubation with monoclonal antibodies for 15 min followed by incubation with BD Cytofix buffer for 30 min. All incubations were performed at room temperature in the dark. After fixation, cells were washed and resuspended in FACS buffer. For intracellular Bcl2 labelling, following surface staining, cells were incubated with BD Cytofix/Cytoperm buffer for 20 min at 4 °C in the dark. After fixation and permeabilization, cells were incubated with monoclonal anti-Bcl2 antibody, washed and resuspended in FACS buffer. Cells were analysed within 2 days in a six-colour FACSCanto flow cytometer (BD Bioscience, San Jose, CA, USA). Data from 2×10^5 cells were collected for each sample. The percentage as well as the mean fluorescence intensity for each surface marker was detected and further analysed by FlowJo software (Tree Star Inc., Ashland, OR, USA). Fluorescence-minus-one (FMO) controls were performed for each surface marker and used in the gating of the cells. The monoclonal detector antibodies used in this study: CD3 APC-Cy7 (clone SK7, BD Bioscience), CD19 PE-Cy7 (clone SJ25C1, BD Bioscience), CD38 PE-Cy5 (clone HIT2,

Table 1 Information on patients and healthy controls included in the study.

	pSS	RA	Controls
Number of patients/subjects	22	19	20
Mean age	59 ± 3	62 ± 3	51 ± 2
Female/male	22/0	18/1	20/0
FS[a] = 1	11	nt	nt
FS ≥ 2	11		nt
Salivary secretion (mean value ± SEM)[b]	1.5 ± 0.24	nt	nt
Salivary secretion ≤ 1.5 ml/min	24	nt	nt
Antinuclear antibodies (mean value ± SEM)[c]	3.49 ± 0.34	0.34 ± 0.08	nt
Ro/SSA (positive/negative)	14/8	0/19	nt
La/SSB (positive/negative)	9/13	0/19	nt
Rheumatoid factor (positive/negative)	5/17	9/10	nt
Cyclic citrullinated peptide antibody (positive/negative)	0/22	14/5	nt
Other antibodies (positive/negative)[d]	3/19	1/18	nt
ESR (mean value ± SEM)[e]	30.7 ± 4.04	22.8 ± 3.22	nt
CRP (mean value ± SEM)[f]	3.8 ± 0.7	8.2 ± 2.4	nt
CRP (≥10 mg/l)	4/30	6/13	nt
Medicines/biological treatment			
Anti-malarial medicine	4/18	0/19	0/20
Peroral steroids	4/18	6/13	0/20
Methotrexate	2/20	11/8	0/20
Salazopyrin	0/22	2/17	0/20
Enbrel	0/22	7/10	0/20
Rituximab (Mabthera)	0/22	3/16	0/20
Infliximab (Remicade)	0/22	1/18	0/20

[a]Focus score (FS): the number of focal infiltrates of more than 50 mononuclear cells per 4 mm^2.
[b]Mean unstimulated whole saliva secretion per 15 min (ml/15 min).
[c]Negative ANA screen <0.99, grey zone ANA screen 1.00–1.65 and positive ANA screen >1.65.
[d]Other antibodies tested: Sm, RNP, Scl-70, dsDNA, native DNA.
[e]Erythrocyte sedimentation rate (ESR): normal range in women < 50 years 1–20 mm/h, women >1–30 mm/h, men < 50 years 1–15 mm/h, and men < 50 years 1–20 mm/h.
[f]C-reactive protein (CRP) (mg/l). Normal CRP range <10 mg/l. pSS: Sjögren's syndrome, RA: rheumatoid arthritis, nt: not tested.

BD Bioscience), CD27 APC (clone L128, BD Bioscience), CD138 PE (clone B-B4, Miltenyi Biotec), Anti-Kappa $F(ab')_2$ FITC (BD Bioscience), HLA-DR FITC (clone G46-6, BD Bioscience), CD45 APC-Cy7 (clone RA3-6B2, BD Bioscience), CD95 FITC (Fas/Apo-1) (clone DX2, Southern Biotech, Birmingham, AL, USA), Bcl2 FITC (clone Sp2/0-Ag14, BD Bioscience), CXCR3 FITC (clone 49801, R&D Systems, Minneapolis, MN USA), CXCR4 APC (clone 12G5, R&D Systems), IgM FITC (clone SA-DA4, Southern Biotech) and IgG APC (clone G18-145, BD Bioscience).

Transmigration assay. Cell migration was tested in 24-well plates containing transwell inserts with a diameter of 6.5 mm and pore size of 5 μm (Corning, Germany). The optimal chemokine concentration for the assay was found to be 300 ng/ml of CXCL12 (stromal cell-derived factor-1 alpha, SDF-1α) (Immuno Tools, Friesoythe, Germany). CD38+ plasma cells were isolated from mononuclear cells by positive selection using magnetic beads (Plasma Cell Isolation Kit II, Miltenyi Biotech) and counted by Casy Cell Counter (Schärfe System GmbH). Isolated plasma cells were resuspended in serum free X-VIVO 20 medium (Cambrex Bio Science) and 100 μl of cell suspension were added to the upper well. Six hundred μl of serum free X-VIVO 20 medium with or without CXCL12 were added to the lower wells. The plates were incubated for 17 h at 37 °C in humidified, 5% CO_2 incubator. Migrated cells were counted by Casy Cell Counter.

Statistical analysis. Statistical analysis was performed using SPSS version 15.0 software. Normal distributions of the results were tested by Kolmogorov–Smirnov. When the data was not normally distributed, statistic significance was tested by non-parametric Mann–Whitney *U*-test and presented as median. When data was normally distributed, statistic significance was tested by Student's *t*-test and presented as mean ± standard error of mean (SEM). Differences were considered significant when $P < 0.05$. The Spearman Rho test was used for the examining the correlation between different parameters.

Results

Plasma cells phenotype

Mononuclear cells were isolated from peripheral blood and analysed by flow cytometry. Plasma cells (PC) were

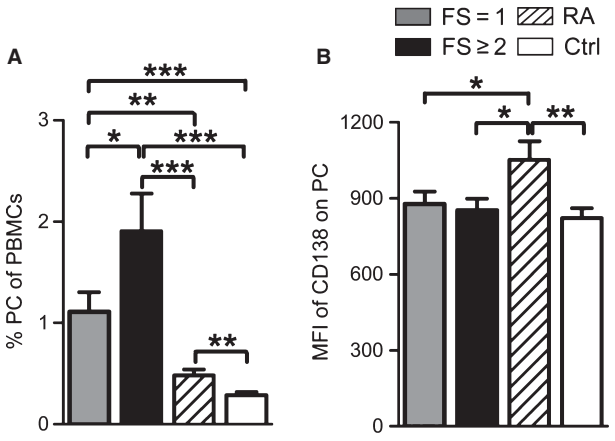

Figure 1 High levels of peripheral blood plasma cells (PC) in primary Sjögren's syndrome (pSS) patients. (A) Percentage of CD38+CD138+ PC of peripheral blood mononuclear cells (PBMCs) in pSS patients with FS = 1, pSS patients with FS ≥ 2, in rheumatoid arthritis (RA) patients and normal controls (Ctrl). (B) The density (mean fluorescence intensity, MFI) of the CD138 molecules on the surface of the PC population. Results are presented as mean ± SEM. Statistical analyses were determined by Kolmogorov–Smirnov and Student's *t*-test (**P* < 0.05, ***P* < 0.005, ****P* < 0.001).

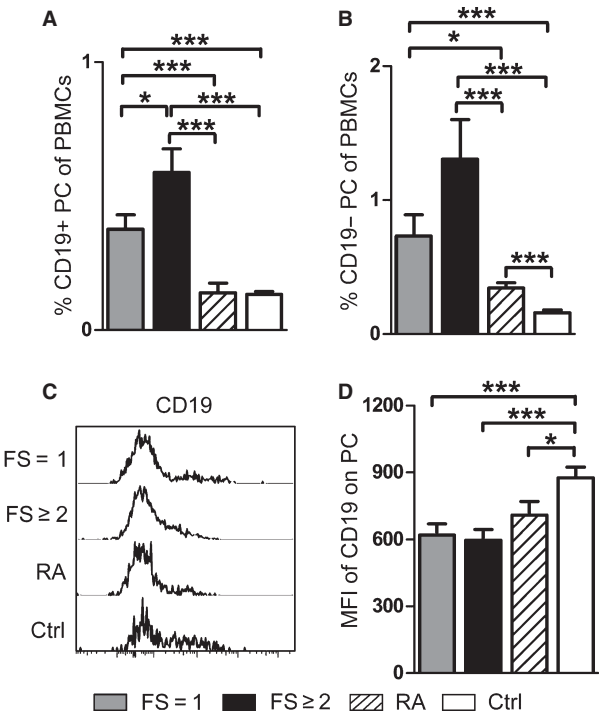

Figure 2 High levels of CD19+ and CD19- peripheral blood plasma cells (PC) in primary Sjögren's syndrome (pSS) patients. Percentage of CD19+ (A) and CD19− (B) PC of peripheral blood mononuclear cells (PBMCs) in pSS patients with FS = 1, pSS patients with FS ≥ 2, in rheumatoid arthritis (RA) patients and normal controls (Ctrl). (C) Representative histograms for the distribution of CD19 on PC from one pSS patient with FS = 1, one pSS patient with FS ≥ 2, one RA patient and one normal control (Ctrl). (D) Mean fluorescence intensity (MFI) of the CD19 molecules on the surface of the CD19+ PC population. Results are presented as mean ± SEM ($n \geq 10$). Statistical analyses were determined by Kolmogorov–Smirnov and Student's *t*-test (**P* < 0.05, ***P* < 0.005, ****P* < 0.001).

identified by expression of surface markers CD38 and CD138, markers predominantly expressed on the mature, long-lived PC subset. The CD38+ CD138+ PC constituted approximately 1.2%, 1.9%, 0.5% and 0.3% of the peripheral blood lymphocytes in pSS patients with FS = 1, pSS patients with FS ≥ 2, RA and normal subjects, respectively (Fig. 1A). Thus, pSS patients with high FS have significantly more circulating PC than pSS patients with FS = 1. Since, CD138 is an indicator of the final differentiation stage of PC and is highly expressed on the long-lived cells found in the bone marrow; in addition to investigating the percentage of CD138+ cells we examined the density [mean fluorescence intensity (MFI)] of CD138 on the surface of PC isolated from our autoimmune and non-autoimmune individuals. Interestingly, PC from RA patients were more densely covered with the CD138 (MFI = 1051) than PC from pSS patients with FS = 1 and FS ≥ 2 (MFI = 878 and MFI = 853) and PC from the control group (MFI = 821) (Fig. 1B), thus showing a more mature state of PC development.

As the CD19 molecule is highly expressed on the proliferating early PC but reduced on the non-proliferating long-lived PC found in the bone marrow, we further examined the expression of CD19 molecule on CD38+ CD138+ plasma cells. Both CD19+ and CD19− PC populations were detected in all study groups. The percentages of CD19+ PC were significantly higher in the pSS patients with high FS compared to pSS with FS = 1, RA and normal subjects (Fig. 2A).

Interestingly, CD19− cells dominated PC population in our autoimmune patients (pSS and RA groups), and the percentages of CD19− PC were significantly higher in pSS and RA patients than seen in normal subjects (Fig. 2B). In spite of low quantities, it was PC from normal subjects that were more densely covered with CD19 molecule than PC from the three autoimmune groups (Fig. 2C and D), thus showing a more mature PC phenotype in autoimmune subjects.

To identify the phenotype of CD19+ and CD19− PC in more details, we have studied the expression of CD27 (highly expressed on all types of PC), CD45 (highly expressed on early PC but reduced on more mature, long-lived PC) and HLA-DR (highly expressed on early PC but reduced on more mature, long-lived PC) surface markers. Representative histograms are shown in Fig. 3A. In normal controls 85% of CD19+ PC expressed CD27, compared to only 59%, 58% and 58% in pSS patients with FS = 1, with FS ≥ 2 and RA patients, respectively (Fig. 3B). A reduction in CD27 expression was also observed on CD19− PC subsets from the three autoimmune groups compared to normal controls.

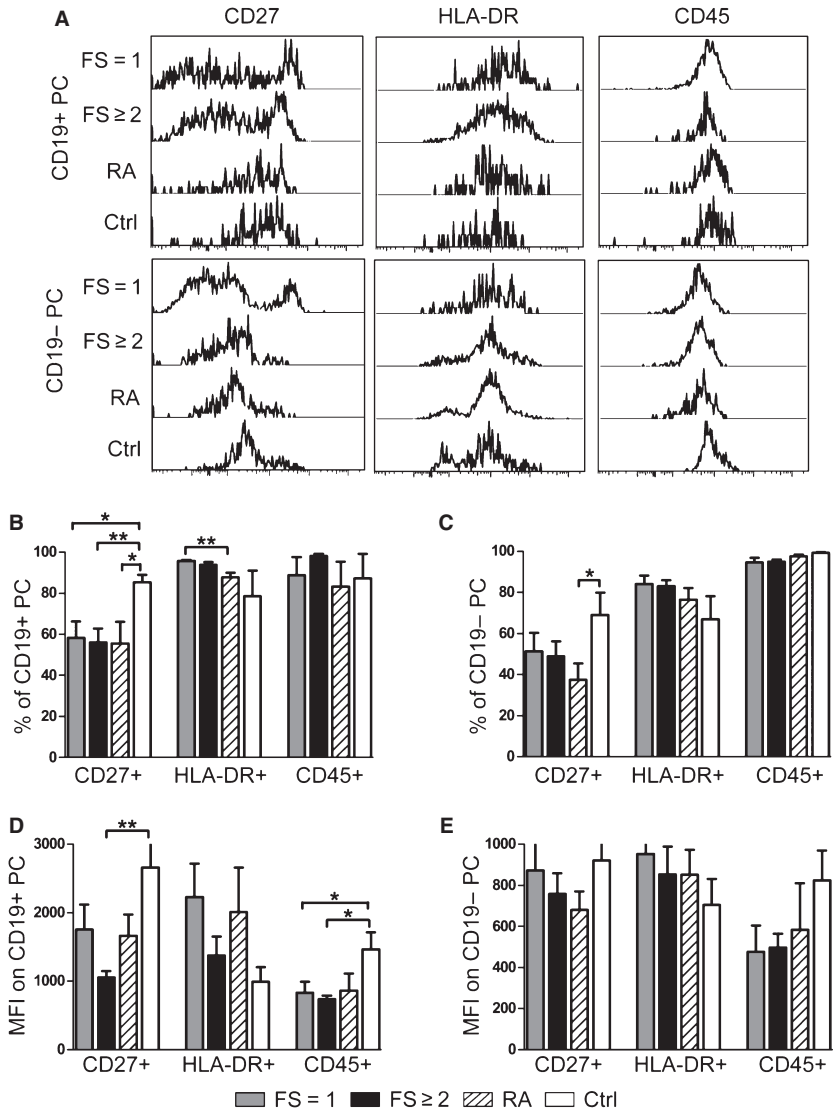

Figure 3 Expression of maturation molecules on the surface of peripheral blood plasma cells (PC). (A) Representative histograms for the distribution of CD27, HLA-DR and CD45 on CD19+ and CD19− PC from one primary Sjögren's syndrome (pSS) patient with FS = 1, one pSS patient with FS ≥ 2, one rheumatoid arthritis (RA) patient and one normal control (Ctrl). (B) Percentage of CD27+, HLA-DR+ and CD45+ cells within the CD19+ PC population from pSS patients with FS = 1, pSS patients with FS ≥ 2, RA patients and normal controls (Ctrl). (C) Percentage of CD27+, HLA-DR+ and CD45+ cells within the CD19− PC population. (D) Mean fluorescence intensity (MFI) of the CD27, HLA-DR and CD45 molecules on CD19+ PC. (E) Mean fluorescence intensity (MFI) of the CD27, HLA-DR and CD45 on CD19− PC. Results are presented as mean ± SEM. Statistical analyses were determined by Kolmogorov–Smirnov and Student's *t*-test (**P* < 0.05, ***P* < 0.005).

However, the only significant difference was detected between RA patients and normal controls (Fig. 3C).

Generally, expression of CD27 receptor is associated with terminally differentiated PC and its ligation with CD70 is important for a successful antibody production. Thus reduction in CD27 receptor on PC from autoimmune subjects could represent abnormalities in development and disturbance in antibody production by these cells.

The proportion of CD19+ and CD19− PC expressing HLA-DR and CD45 markers were high in all the groups tested (Fig. 3B and C). Interestingly, we observed differences in the density of CD45 on PC from different groups. CD19+ PC from pSS groups (FS = 1 and FS ≥ 2) showed significantly lower densities of CD45 than CD19+ PC from normal controls (Fig. 3D). We further observed a decrease in density of CD45 on CD19− PC from pSS and RA groups compared to normal subjects. However, due to variation within the groups, the differences were not significant (Fig. 3E). The reduction in CD45 receptor point to the more mature PC population in the autoimmune subjects compared with non-autoimmune controls. However, high expression of HLA-DR (a molecule important for antigen presentation by B-cells) contradicts this presumption.

Immunoglobulin isotype

In addition to diversity in the expression of the differentiation markers, the long-lived PC found in the bone marrow are mostly high affinity, IgG expressing cells, whereas the majority of short-lived PC are the IgM producing cells. Hence, we have examined expression of IgM and IgG surface immunoglobulins on CD19+ and CD19− PC in our study groups. Representative histograms are shown in Fig. 4A.

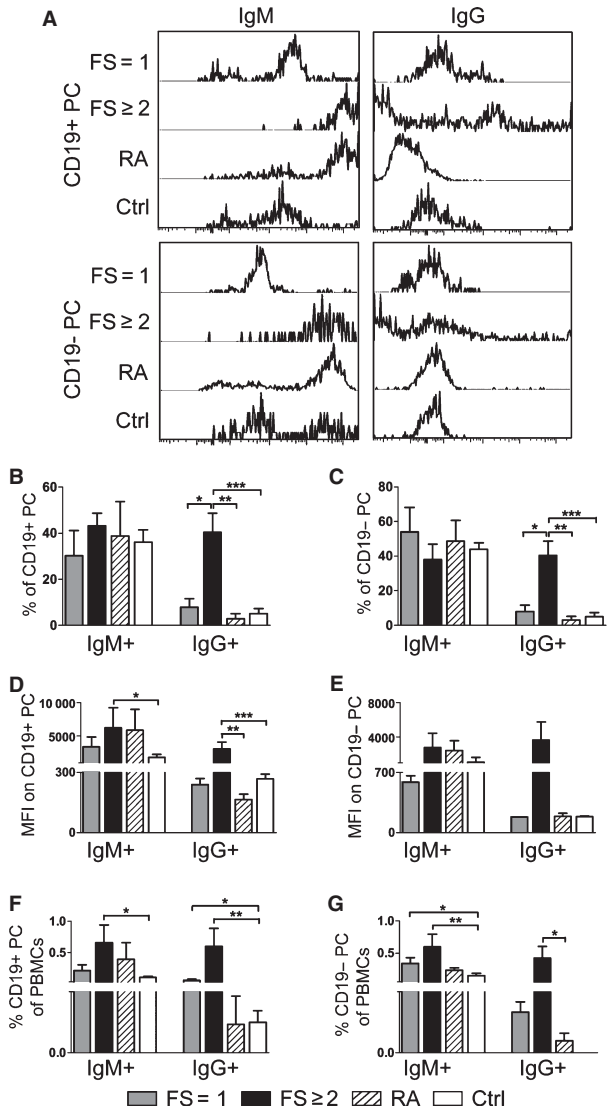

Figure 4 Immunoglobulin expression on the surface of peripheral blood plasma cells (PC). (A) Representative histograms for the distribution of IgM and IgG on CD19+ and CD19− PC from one primary Sjögren's syndrome (pSS) patient with FS = 1, one pSS patient with FS ≥ 2, one rheumatoid arthritis (RA) patient and one normal control (Ctrl). (B) Percentage of IgM+ and IgG+ cells within the CD19+ PC population from pSS patients with FS = 1, pSS patients with FS ≥ 2, RA patients and normal controls (Ctrl). (C) Percentage of IgM+ and IgG+ cells within the CD19− PC population. (D) Mean fluorescence intensity (MFI) of the IgM and IgG molecules on CD19+ PC. (E) MFI of the IgM and IgG molecules on CD19− PC. (F) Percentage of IgM+CD19+ and IgG+CD19+ PC of peripheral blood mononuclear cells (PBMCs). (G) Percentage of IgM+CD19− and IgG+CD19− PC of PBMCs. Results are presented as mean ± SEM. Statistical analyses were determined by Kolmogorov–Smirnov and Student's *t*-test (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$).

We have detected remarkable difference in the IgM distribution on both CD19+ and CD19− PC between the groups. In generally, we have observed three different PC populations: (1) IgM^{low}, (2) IgM^{medium} and (3) IgM^{high} PC. In pSS patients with FS = 1 and in normal subjects we detected IgM^{low} and IgM^{medium} CD19+ and CD19− PC. However, in pSS patients with high FS and in RA patients we detected IgM^{low} and IgM^{high} CD19+ and CD19− PC (Fig. 4A). This could indicate possible different developmental mechanism and involvement of different B cell subsets (B1 versus B2 cells) in the expansion of PC in pSS with high FS contra PC in pSS with FS = 1.

In pSS group with FS = 1, only 30% of CD19+ PC compared to 53% of CD19− PC expressed IgM. In pSS with FS ≥ 2, similar distribution of IgM+ cells was observed in CD19+ (43%) and CD19− (38%) PC (Fig. 4B and C). Investigation of the density of IgM molecules revealed a generally higher IgM MFI on CD19+ PC than CD19− PC. When comparing the groups the MFI of IgM was generally higher on PC isolated from pSS patients with high FS and from RA patients compared to pSS patients with FS = 1 and normal subjects (Fig. 4D and E).

The IgG immunoglobulin was mainly expressed on CD19+ and CD19− PC from pSS patients with FS ≥ 2, whereas, only a small percentage of CD19+ and CD19− PC from pSS patients with FS = 1, RA and normal controls expressed IgG (Fig. 4B and C). Additionally, the density of the IgG molecules was higher on the surface of PC from pSS patients with FS ≥ 2 compared to the three other groups (Fig. 4D and E). The absolute percentages of CD19+IgM+ PC, CD19-IgM+ PC, CD19+IgG+ PC and CD19-IgG+ PC of PBMCs were highest in the pSS patients with high FS (Fig. 4F and G), thus indicating a high level of PC activation in these patients.

Survival and activation

Long-lived PC have shown a high expression of anti-apoptotic protein Bcl2 and low expression of the so-called death receptor, CD95. Thus, we further examined the autoimmune and non-autoimmune PC for the expression of these markers. Representative histograms are shown in Fig. 5A.

Interestingly, in normal subjects around 30% of CD19+ PC expressed Bcl2 compared to 17%, 18% and 23% in pSS patients with FS = 1, pSS patients with FS ≥ 2 and RA patients, respectively. Additionally, the intracellular density of Bcl2 in the CD19+ PC was significantly lower in the pSS patients (FS = 1 and FS ≥ 2) compared to RA patients (Fig. 5D), indicating presence of higher anti-apoptotic signals in PC from non-autoimmune subjects. In contrast, only 8% of CD19− PC from normal controls expressed Bcl2 compared to17%, 10% and 12% in pSS patients with FS = 1, pSS patients with FS ≥ 2 and RA patients, respectively (Fig. 5B and C). The differences were however not significant. Despite of differences in the distribution of Bcl2 expression within

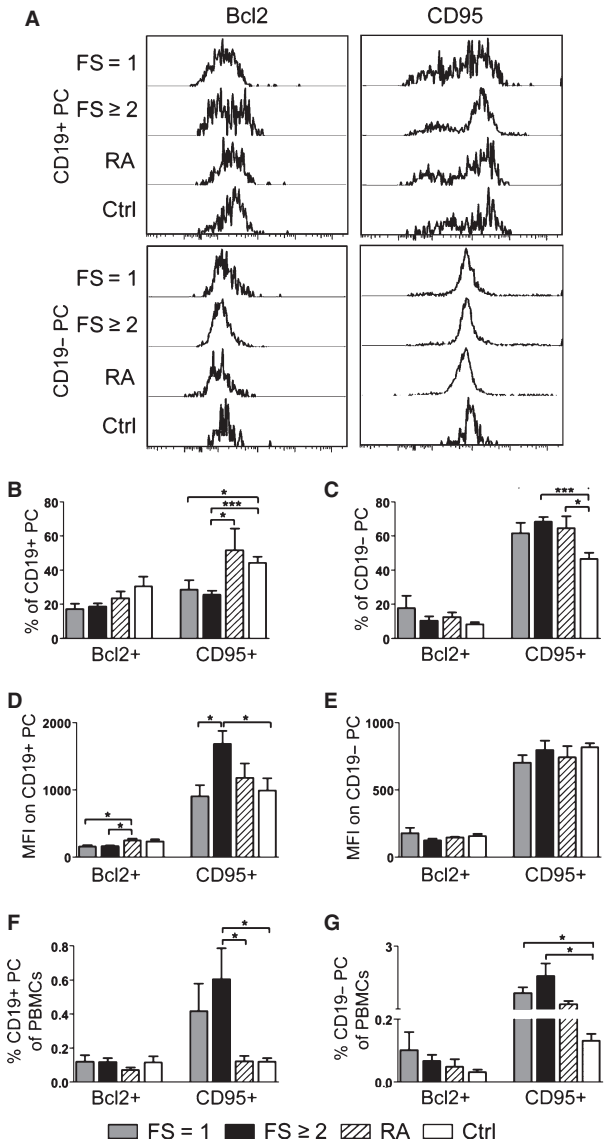

Figure 5 Expression of Bcl2 and CD95 by peripheral blood plasma cells (PC). (A) Representative histograms for the distribution of Bcl2 and CD95 on CD19+ and CD19− PC from one primary Sjögren's syndrome (pSS) patient with FS = 1, one pSS patient with FS ≥ 2, one rheumatoid arthritis (RA) patient and one normal control (Ctrl). (B) Percentage of Bcl2+ and CD95+ cells within the CD19+ PC population from pSS patients with FS = 1, pSS patients with FS ≥ 2, RA patients and normal controls (Ctrl). (C) Percentage of Bcl2+ and CD95+ cells within the CD19− PC population. (D) Mean fluorescence intensity (MFI) of the Bcl2 and CD95 molecules on CD19+ PC. (E) MFI of the Bcl2 and CD95 molecules on CD19− PC. (F) Percentage of Bcl2+CD19+ and CD95+CD19+ PC of peripheral blood mononuclear cells (PBMCs). (G) Percentage of Bcl2+CD19− and CD95+CD19− PC of PBMCs. Results are presented as mean ± SEM. Statistical analyses were determined by Kolmogorov–Smirnov and Student's *t*-test (**P* < 0.05, ****P* < 0.001).

the PC subsets, the total percentages of CD19+Bcl2+ PC and CD19-Bcl2+ PC of PBMCs were comparable between all the groups.

Significantly higher percentages of CD95 expressing cells were detected within the CD19+ PC subsets from RA and normal controls compared to the pSS patient groups (Fig. 5B). However, when investigating the CD19− PC we observed higher distribution of CD95 in all autoimmune patients compared to normal controls (Fig. 5C). In generally, the CD19+ PC were more densely covered with CD95 than CD19− cells (Fig. 5D and E), with highest CD95 MFI detected on CD19+ PC from pSS with high FS. No differences were observed in the density of CD95 on the CD19− PC between the four groups (Fig. 5E).

In spite of lower distribution of CD95 in the CD19+ PC, the absolute frequency of CD19+CD95+ PC of the PBMCs was significantly higher in the pSS patients with high FS compared to both RA and normal control group (Fig. 5F). Furthermore, the absolute frequencies of CD19-CD95+ PC of the PBMCs were significantly higher in both pSS groups (FS = 1 and FS ≥ 2) compared to normal subjects (Fig. 5G).

Expression of chemokine receptors

In order to survive, PC need particular survival signals from the environment. As a consequence they have to migrate to the sites that home the survival niches which can be found in different compartments such as bone marrow (BM), secondary lymphoid organs and at the sites of inflammation. Expression of particular chemokines will guide PC to particular places, i.e. expression of CXCR4 is important in PC homing to the BM, whereas expression of CXCR3 drives PC to the sites of inflammation such as salivary glands (SGs) of pSS patients. We examined expression of both CXCR3 and CXCR4 chemokine receptors on the surface of circulating PC. Representative histograms are shown in Fig. 6A.

The frequency of CXCR3 expression on CD19+ PC was significantly higher in normal controls compared to pSS patients with high FS and RA patients. No difference in expression of CXCR3 on CD19+ PC was observed between pSS patients with FS = 1 and pSS patients with FS ≥ 2 (Fig. 6B). When investigating the CD19− PC population, the only significant difference in the distribution of CXCR3 was found between pSS patients with FS ≥ 2 and normal subjects (Fig. 6C). There were no differences in the density of CXCR3 on CD19+ PC compared to CD19− PC, and there were no differences in the density of CXCR3 on PC between all the groups (Fig. 6D and E).

Comparable distribution pattern of CXCR4 was observed on both CD19+ and CD19− PC in all four groups (Fig. 6B and C). In generally, CD19+ PC were more densely covered with CXCR4 than CD19− PC. No differences were however detected in the densities of CXCR4 between the groups (Fig. 6D and E).

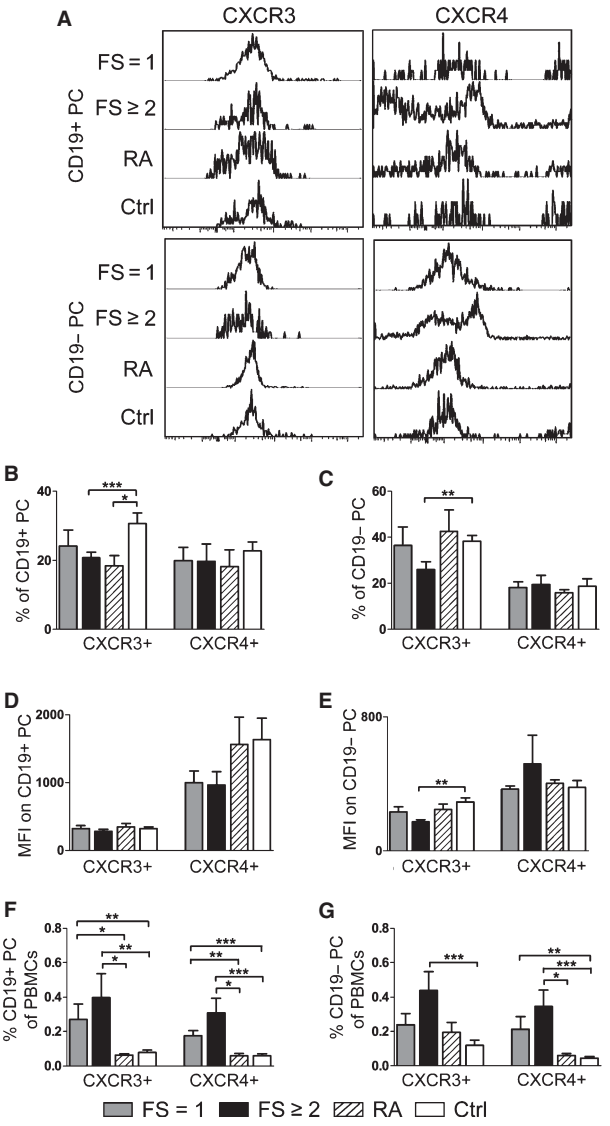

Figure 6 Expression of CXCR3 and CXCR4 chemokine receptors by peripheral blood plasma cells (PC). (A) Representative histograms for the distribution of CXCR3 and CXCR4 on CD19+ and CD19− PC from one primary Sjögren's syndrome (pSS) patient with FS = 1, one pSS patient with FS ≥ 2, one rheumatoid arthritis (RA) patient and one normal control (Ctrl). (B) Percentage of CXCR3+ and CXCR4+ cells within the CD19+ PC population from pSS patients with FS = 1, pSS patients with FS ≥ 2, RA patients and normal controls (Ctrl). (C) Percentage of CXCR3+ and CXCR4+ cells within the CD19− PC population. (D) Mean fluorescence intensity (MFI) of the CXCR3 and CXCR4 molecules on CD19+ PC. (E) MFI of the CXCR3 and CXCR4 molecules on CD19− PC. (F) Percentage of CXCR3+CD19+ and CXCR4+CD19+ PC of peripheral blood mononuclear cells (PBMCs). (G) Percentage of CXCR3+CD19− and CXCR4+CD19− PC of PBMCs. Results are presented as mean ± SEM. Statistical analyses were determined by Kolmogorov–Smirnov and Student's *t*-test (**P* < 0.05, ***P* < 0.005, ****P* < 0.001).

In spite of lower distribution of CXCR3, the absolute frequency of CD19+CXCR3+ PC of the PBMCs was significantly higher in the pSS patients with FS = 1 and FS ≥ 2 compared to RA and normal control group.

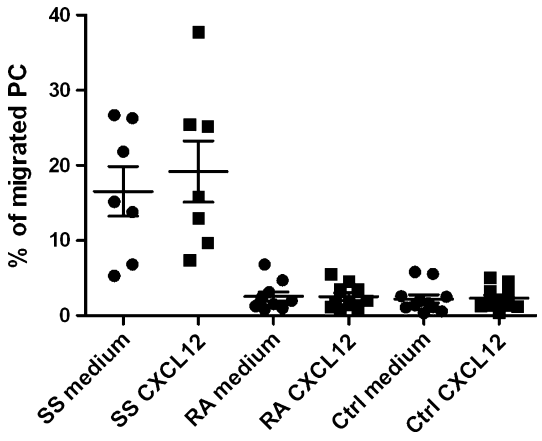

Figure 7 Transmigration of peripheral blood plasma cells (PC) from primary Sjögren's syndrome (pSS) patients, patients with rheumatoid arthritis (RA) and normal controls against CXCL12 or medium only. The results are presented as mean ± SEM.

Furthermore, the absolute frequencies of CD19-CXCR3+ PC of the PBMCs were significantly higher pSS with FS ≥ 2 compared to normal subjects (Fig. 5F and G). The absolute frequencies of CD19+CXCR4+ and CD19-CXCR4+ PC of PBMCs were significantly higher in pSS patients compared to RA and normal subjects (Fig. 5F and G). Thus an important fining is that there are higher numbers of PC that could migrate to both BM and SGs in blood of pSS patents compared to RA and normal subjects.

In vitro migration of plasma cells

Due to the generally low percentages of PC in the blood, we have used CD38 marker alone to isolate PC for the migration experiment. As a result in this part of the study we have tested the migration potential of both early PC (CD38+CD138-) and mature PC (CD38+CD138+).

The functionality of chemokine receptor CXCR4, and thus the migration potential of CD38+ PC against its CXCL12 was analysed *in vitro* by a transmigration assay (Fig. 7). There were no difference ($P > 0.05$) between the spontaneous migration of PC (medium only) and migration (chemotactic response) towards the CXCL12 chemokine in the three groups tested (Fig. 7). However, a higher percentage of PC from pSS patients migrated against both medium only and medium with CXCL12 compared with RA and normal controls. Therefore it seems that PC from pSS patients were generally more migratory than PC from RA patients and normal controls regardless of chemotactic stimulus.

Discussion

The presence of auto antibodies against the Ro/SSA and La/SSB antigens is one of the main characteristics of

primary pSS. As circulating autoantibodies have been detected in pSS patients after B cell depletion [11–13], it has been proposed that these autoantibodies are produced by a subset of long-lived PC residing in the bone marrow [7]. Investigation of this particular subset of PC has been going on for some time now [8] and more recently it has become clear that PC are more complex than previously thought. Depending on their developmental stage, they can be divided into different subsets (plasma blasts and plasma cells) that exhibit different expression of intracellular and surface markers. Therefore, in this study we have been interested in characterising PC present in the circulation of pSS patients with regards to phenotype, survival properties and migratory potential. In order to see if there is any association between the severity of inflammation seen in the target organ (salivary glands) and the type and amount of PC circulating in the blood, we have divided pSS patients into two groups according to FS; one group consisting of patients with FS = 1 and one group consisting of patients with FS ≥ 2.

The division of patients revealed that there is significantly higher percentage of PC in the peripheral blood of pSS patients with high FS compared to pSS patients with FS = 1. This increase of PC in the patients with high FS could be a result of higher production rate of these cells due to increase severity of inflammation or that pSS patients with high FS produce PC that have higher survival potential. The intrinsic factors that make some PC able to survive longer than other PC are still unknown, however, it has been stated by others that short-lived plasma cells are highly proliferative and less maturated than the long-lived subset [16]. CD138 marker has proven to be a good marker for terminally differentiated long-lived PC present in the bone marrow [14]. It is also essential in the adhesion of PC to the survival factors presented by for instance the bone marrow stroma [28]. Our results show that in spite of quantitative differences, PC from the two pSS groups are similar in term of CD138 density and they express similar amounts of CD138 as PC from the non-autoimmune subjects. Interestingly, it was PC from RA patients that were more densely covered with CD138 indicating more maturated state of these cells compared to pSS and normal subjects.

In addition to CD138, expression of the CD19 is often used in identification of PC as it is highly expressed by the circulating blood PC but reduced on the non-proliferating long-lived PC found in the bone marrow [14]. We observed two PC subsets in the blood of all four groups tested; CD19+ and CD19− PC. Our most striking finding was the two-fold higher CD19− PC subset compared to CD19+ subset in all of the autoimmune patients but not in the normal subjects. To our knowledge, presence of CD19− PC in the circulation of pSS patients has not been reported before. Lack of CD19 on the surface of PC has been extensively studied and is at present associated with malignant potential of PC (reviewed in [29]). Therefore the CD19− PC detected in our autoimmune cohort of patients could represent such an abnormal population. Furthermore, since the reduced expression of CD19 is a common feature of the long-lived PC subset found in the bone marrow and has been shown to correlate with increased lifespan of these cells [14], these cells could be the long-lived PC precursors.

Next, we examined the phenotype of both CD19+ and CD19− plasma cells in more detail by studying the expression of CD27, HLA-DR, CD45, IgM and IgG by these cells. CD27 is normally expressed by germinal centre B cells, memory B cells and PC [30–32]. In our study, we found reduced expression of CD27 on both CD19+ and CD19− PC isolated from pSS and RA patients compared to normal subjects. The loss of CD27 marker has similarly to the loss of CD19 marker been associated with myeloma cells and poor prognosis for myeloma patients [33]. However, further studies are needed to explore the role of this CD27− PC in the outcome of pSS and autoimmunity in general.

By studying the expression of HLA-DR and CD45 we wanted to determine the maturation state of the circulating PC. Interestingly, there was only a very small fraction of both CD19+ and CD19− PC that did not express these markers. Examination of densities revealed however, that the density of CD45 on PC from pSS patients with both high FS and FS = 1 was significantly lower than from healthy controls. The reduced CD45 density on PC from autoimmune patients is an important finding, since it has been shown by others that proliferation and maturation of PC correlate with expression of CD45 [34, 35]. Non-proliferative PC commonly found in the bone marrow have lower expression of CD45 [35], pointing to a connection between CD45-low PC in the peripheral blood and the establishment of long-lived PC in the bone marrow.

Isotype of PC is also proven to be important in distinguishing between the short-lived and long-lived PC. Long-lived PC are primarily class-switched, somatically mutated IgG PC that originate from germinal centres (GCs) reactions [36]. Hence we investigated the isotype of circulating PC in pSS patients. We found striking differences in the expression of IgM and IgG by PC between the two pSS patient groups. Of three different IgM expression profiles detected in our study, PC from pSS patients with FS = 1 comprised IgM^{low} and IgM^{medium} cells similarly to the normal subjects. Remarkably, PC from SS patients with high FS comprised IgM^{low} and IgM^{high} cells similarly to the RA patients. Additionally, there were a significantly higher numbers of IgG expressing plasma cells in pSS patients with FS ≥ 2 than in the FS = 1 group of patients. These results underline the heterogeneity of pSS patients as a

group and could signal a shift in the PC compartment with increasing inflammation in the target organ.

In order to determine the survival potential and activation state of the circulating PC, we chose to measure the expression of anti-apoptotic protein Bcl2 and death receptor, CD95 (Fas). In consensus with others [14] we observed generally low levels of Bcl2+ PC in peripheral blood of all patients and normal subjects. Additionally, higher percentages of CD95+ PC were detected in the blood of pSS patients compared to normal subjects. It is important to keep in mind that high expression of CD95 dose not necessarily means that the cells will die as CD95 is also an important marker for activated cells [37]. Thus we conclude that PC from pSS patients are activated cells that can be rescue from death if they migrate to the sites that home survival niches. The homing or migration of PC is a highly regulated and complex process that involves among others expression of particular chemokines by PC. In our study we examined expression of CXCR3 (which drives migration of PC to the salivary glands of pSS patients) and CXCR4 (which drives the migration of long-lived PC to the bone marrow). The percentages of CXCR3+ and CXCR4+ PC in the peripheral blood were higher in the pSS patients compared with RA and normal controls thus implying a higher migration potential of these cells to the site of inflammation and to the bone marrow. However, in spite of higher quantities of CXCR4+ PC seen in pSS patients, when investigating *in vitro* migration of PC against CXCL12 (a molecule that drives CXCR4 expressing cells to bone marrow) we observed no effect of CXCL12 on the migration of PC in any of the groups tested. PC from pSS patients showed significantly higher spontaneous migration potential than PC isolated from both RA and normal controls. Thus the migration potential can differ between the cells, due to factors not yet uncovered.

High spontaneous migration observed in pSS patients may result in more effective contact with the survival niches in the bone marrow and thus more effective survival of these cells. This is an important feature since it is known that the bone marrow plasma cells are long-lived and capable of producing high-affinity antibodies [4, 38, 39].

In conclusion, the present study shows that pSS patients are a heterogeneous group of patients that comprise PC subsets of different phenotype with changes that can be associated with increase inflammation in the target organ. To our knowledge this has not been demonstrated on circulating PC before. Furthermore, our results revealed high quantities of CD19− PC in the blood of autoimmune patients, a possible candidate for abnormal autoimmune cell population. The CD19− PC population could be used in distinguishing between the autoimmune and non-autoimmune patients but also between pSS with high and low FS. The phenotype of this subset shows some similarities to myeloma cells however, further characterization is needed as it may shed light on the complex process of B cell differentiation and its important role in the pSS pathogenesis.

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