The Anti-Citrullinated Protein Antibody immune response and its effector functions in Rheumatoid Arthritis
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Cover Page

CHAPTER 2

Synovial fluid mononuclear cells provide an environment for long-term survival of antibody-secreting cells and promote the spontaneous production of anti-citrullinated protein antibodies
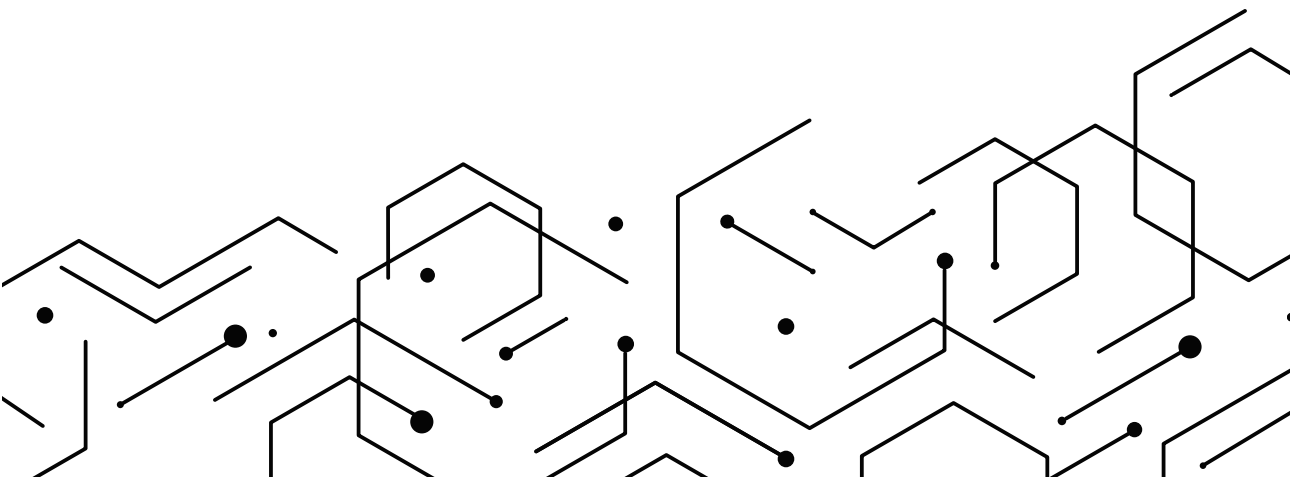
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ABSTRACT

Objectives: In rheumatoid arthritis (RA), observations point to a crucial role for (autoreactive) B cells in disease pathogenesis. Here, we studied whether cells from the synovial environment impact on the longevity of autoreactive B cell responses against citrullinated antigens.

Methods: Synovial fluid and peripheral blood mononuclear cells (SFMC/PBMC) were obtained from patients with established RA and assessed for the presence of B cell subpopulations. Cells spontaneously secreting anti-citrullinated protein antibodies (ACPA-IgG) directly ex vivo were detected by antigen-specific ELISpot. SFMC and PBMC were cultured to assess the degree of spontaneous ACPA-IgG secretion. Cells surviving for several weeks were characterised by CFSE-labelling and Ki-67 staining.

Results: Cells spontaneously secreting ACPA-IgG were readily detectable in peripheral blood and synovial fluid (SF) of ACPA-positive RA patients. SFMC showed an up to 200-fold increase in ex vivo ACPA-IgG secretion compared to PBMC despite lower numbers of B cells in SFMC. ELISpot confirmed the presence of spontaneously ACPA-IgG secreting cells, accounting for up to 50% (median 12%) of all IgG-secreting cells in SF. ACPA-IgG secretion was remarkably stable in SFMC cultures, maintained upon depletion of the CD20+ B cell compartment, and detectable for several months. CFSE labelling and Ki-67 staining confirmed the long-term survival of non-dividing plasma cells.

Conclusions: This study demonstrates a high frequency of differentiated, spontaneously ACPA-IgG secreting cells in SF. These cells are supported by SFMC for prolonged survival and autoantibody secretion, demonstrating that the synovial compartment is equipped to function as inflammatory niche for plasma cell survival.

INTRODUCTION

Many autoimmune diseases are characterised by the generation of self-reactive B cells leading to the production of pathogenic autoantibodies. Experiments in mice and evidence obtained from human bone marrow transplantation studies suggest that these autoantibodies are primarily produced by autoreactive plasma cells (PCs) residing in specified niches in the bone marrow [1-3]. However, non-dividing PCs that can survive and secrete antibodies for weeks in vitro, have also been detected in healthy human tonsils, spleen, and in the small intestine [4-6]. This has fuelled the hypothesis that also other, non-lymphoid tissues could, especially in the context of chronic autoimmune inflammation, provide an environment for long-term survival of PCs and, indeed, autoreactive PCs have been detected in kidneys of mice with lupus-nephritis [7, 8]. As these cells secrete potentially pathogenic autoantibodies, they could constitute an important if not crucial component of the inflammatory cascade that initiates and perpetuates autoimmune inflammation.

Rheumatoid arthritis (RA) is characterised by the presence of antibodies against citrullinated protein antigens (ACPA). ACPA are found in the majority of patients and represent one of the earliest detectable signs of immune disturbance, frequently predating the onset of clinical symptoms [9]. In established disease, ACPA associate with a severe disease phenotype, as ACPA-positive patients show more erosive joint destruction and extra-articular disease manifestations than ACPA-negative patients [10]. As ACPA are also highly specific for RA, these observations suggest that ACPA and/or ACPA-expressing B cells could be actively involved in initiating, aggravating and/or perpetuating the disease process. Indeed, emerging evidence shows that ACPA can exert effector functions in vitro that could promote in vivo inflammation, such as the activation of complement, mast cells, monocytes, neutrophils and osteoclasts [11-15].

While these findings support a prominent role for ACPA in disease pathogenesis, little is known about the underlying autoreactive B cell response. So far, ACPA-secreting PCs were shown to reside in synovial tissue and ACPA were successfully cloned from synovial fluid (SF) B cells [16, 17]. Furthermore, enrichment of ACPA in SF has been suggested [18]. However, it remains unknown how and where the generation of ACPA-expressing B cells is initiated and maintained. We recently demonstrated that ACPA-expressing B cells circulate in the peripheral blood of patients with ACPA+ RA [19, 20]. Next to ACPA-expressing memory B cells, we could identify a population of plasmablasts/plasma cells (PBs/PCs) that actively secrete ACPA in this compartment. These cells expressed primarily IgG or IgA, and their frequency was found to correlate with ACPA serum titres.

2

The latter suggests that the circulating PB/PC population reflects an active part of the citrulline-specific B cell response and, thus, could contain valuable information on patient-specific disease activity. Until now, however, it is unknown whether these circulating PBs/PCs home to secondary lymphoid organs to compete for survival niches, or whether the synovial compartment of inflamed joints could be one of the tissues attracting these cells. As PCs can produce excessive amounts of antibodies and survive in respective niches for years, they could be involved in driving and maintaining disease-specific pathogenic processes. In the current study, we set out to phenotypically and functionally characterise ACPA-secreting PBs/PCs in the synovial compartment. We hypothesized that spontaneously ACPA-secreting PBs/PCs could be frequent in SF, and that the synovial compartment and its cellular composition could provide a microenvironment supporting the long-term survival of (auto)antibody-secreting cells.

METHODS

Patients
Peripheral blood, serum and SF samples were obtained on a cross-sectional basis from patients with ACPA-positive RA visiting the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre (LUMC), Leiden, The Netherlands. Patients fulfilled the 1987 criteria for RA at the time of diagnosis and gave written informed consent for sample acquisition. No selection based on specific treatments was performed, but no patient had previously been treated with rituximab. Permission for conduct of the study was obtained from the ethical review board of LUMC. Additional SF samples were obtained from the Department of Rheumatology at Groene Hart Ziekenhuis, Gouda, The Netherlands, as rest material from arthrocentesis on an anonymous basis.

Cell isolation and culture
Mononuclear cells were isolated from peripheral blood and SF using Ficoll-Paque gradient centrifugation (LUMC pharmacy). Synovial fluid mononuclear cells and peripheral blood mononuclear cells (SFMC and PBMC) were cultured at a density of $2x10^5$ cells/well in IMDM (Lonza) supplemented with 10% heat-inactivated FCS, penicillin/streptomycin (100U/ml), and 2mM Glutamax with no additional stimuli. Medium was replaced every 7 days. In some experiments, SFMC were depleted for CD20+ B cells by magnetic beads (Miltenyi Biotech) and/or cells were labelled with CFSE prior to culture. Also, for specific experiments, PBMC (2×10^5 cells/well) were cultured on a layer of irradiated (7000 rad) mouse fibroblast cells stably transfected with human CD40 ligand (CD40L, 5×10^3 cells/well) in the presence of BAFF (Miltenyi, 100ng/ml), interleukin (IL)-21 (Invitrogen, 50ng/

ml) and anti-IgM F(ab')$_2$-fragments (Jackson Immunoresearch Laboratories, 5μg/ml). This is indicated where appropriate.

Flow cytometry

The following antibodies were used to stain and analyse PBMC and SFMC by flow cytometry: CD3 Pacific Blue (clone UCHT1), CD14 Pacific Blue (clone M5E2), CD19 APC-Cy7 (clone SJ25C1), CD20 Alexa Fluor 700 (clone 2H7), CD27 PE-Cy7 (clone M-T271), CD38 PerCP-Cy5.5 (clone HIT2), Ki-67 PE (clone 20Raj1), CD138 FITC (MI15) (all (except CD20 (Biolegend) and Ki-67 (eBioscience) obtained from BD Biosciences) and DAPI (Molecular Probes). Proliferation was assessed by labelling cells with CFSE (Molecular Probes).

Enzyme-Linked ImmunoSpot assay

The frequency of spontaneously (ACPA-)IgG secreting cells was determined using an Enzyme-Linked ImmunoSpot (ELISpot) assay. Briefly, PVDF-based membrane plates (type MSIP) were pre-treated with ethanol and coated overnight with unlabelled antibodies to human IgG (MT91/145, Mabtech). After blocking the plate with medium for at least 2 hours, total mononuclear cells ($25 \times 10^3 - 400 \times 10^3$ cells/well) were incubated overnight on the plate directly after ex vivo isolation and without prior stimulation. IgG produced spontaneously during the incubation period was visualised with either anti-IgG-biotin (MT78/145, Mabtech), biotinylated CCP2-peptide or its arginine control variant (CArgP2), followed by incubation with extravidin-AP (Sigma) and finally development with BCIP/NBT (Sigma).

Measurement of ACPA-IgG and total IgG

Serum and culture supernatants were assessed for the presence of ACPA-IgG by ELISA based on reactivity against the CCP2-peptide. The CCP2-peptide was coupled to streptavidin-coated ELISA plates, followed by detection with polyclonal rabbit anti human IgG HRP (DAKO). Serum samples were tested at a 1:50 dilution or higher; culture supernatants were tested 1:10 diluted or undiluted. The standard was diluted to the lowest concentration at which the standard curve was consistently linear, as previously described [19]. Total IgG was assessed by coating ELISA plates with anti-Igλ light chain (clone JDC-12, BD Pharmingen), followed by detection with polyclonal rabbit anti human IgG HRP (DAKO).

Statistical analysis

Statistical analyses were performed using GraphPad Prism V.6.02. Correlations were assessed as non-parametric correlations. p Values <0.05 were considered significant.

RESULTS

Both SFMC and PBMC harbour spontaneously ACPA-IgG-secreting cells

Previously, we demonstrated the presence of spontaneously ACPA-IgG-secreting PBs/ PCs in the circulation of patients with ACPA-positive RA. To determine whether these cells would also be present in SF, we isolated paired PBMC and SFMC from individual patients followed by ex vivo culture at equal total cell density without the addition of exogenous stimuli for 7 days followed by the detection of ACPA-IgG in culture supernatants. Analysis by flow cytometry of the total mononuclear cell fraction of these and additional non-paired samples directly after isolation demonstrated that SFMC harboured, relatively, less B cells than PBMC (figure 1A, upper panel), whereas the proportion of memory B cells as well as PBs/PCs was comparable in the two compartments (figure 1A, lower panel). Despite the lower proportion of B cells, however, we detected enhanced cumulative production of ACPA-IgG in SFMC compared with PBMC culture wells at day 7 in most donors (figure 1B). This was reflected by an enhanced secretion of total, non-specific IgG in some but not all donors (figure 1C).

The frequency of ACPA-IgG-secreting cells is greatly increased in SF

The data described above suggest a higher frequency of ACPA-secreting PBs/PCs in SF. To assess whether indeed more antibody secreting cells specific for citrullinated antigens are present in SFMC as compared with PBMC, we developed an ELISpot assay to detect and enumerate ACPA-secreting cells directly upon ex vivo isolation. Importantly, cells were directly seeded and did not receive exogenous stimulation. In this system, we readily observed spontaneously IgG-secreting cells in SFMC plated at a 1×10^5 cells/ well density, but hardly any IgG-positive spots in PBMC-containing wells. PBMC plated at a higher cell density (4×10^5 cells/well) did show IgG-spots, but the frequency of citrulline-specific spots was still far lower than observed for SFMC-containing cultures (figure 2A). In fact, the abundant presence of citrulline-specific spots for SFMC allowed us to estimate the frequency of ACPA-IgG per total IgG secreting cells (figure 2B). Despite considerable variation between donors (median: 12%; IQR: 2.7-30%), we found that up to 50% of all IgG-secreting cells derived from SF had produced ACPA-IgG during overnight incubation. Importantly, hardly any spots reactive with the arginine control variant were detected and, if present, they were subtracted from the analysis. Together, these data indicate that a remarkable high frequency of differentiated, spontaneously ACPA-IgG secreting PBs/PCs is present in the SF of patients with ACPA+ RA.
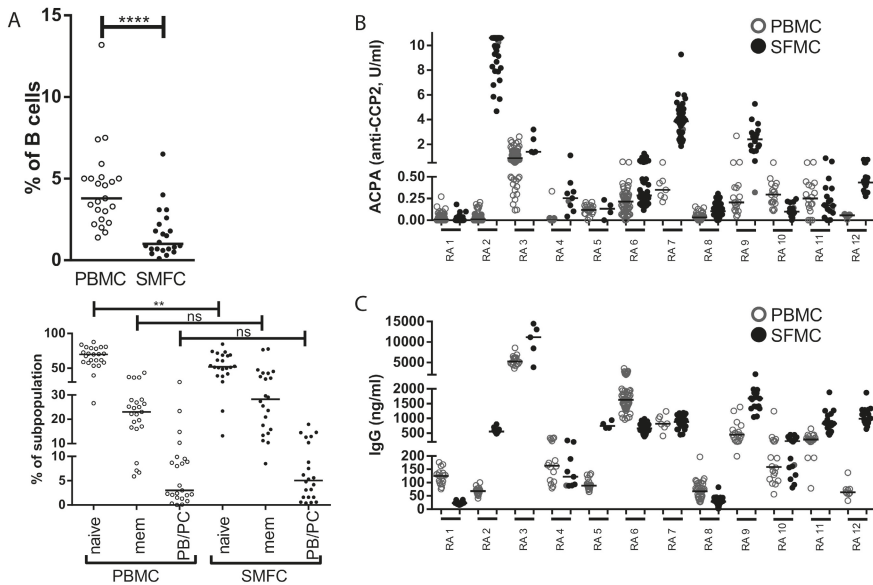
Figure 1. Synovial fluid mononuclear cells and peripheral blood mononuclear cells (SFMC and PBMC) harbour spontaneously ACPA-IgG-secreting cells. (A) The frequency of B cells in SFMC is lower than in PBMC (upper panel). Although less B cells with a naïve phenotype (defined as CD19+CD20+CD27-) were detected in SFMC, the relative distribution of the memory (CD19+CD20+CD27+) and PB/PC (CD19+CD20-CD27++) compartment was comparable (lower panel). (PBMC n=25; SMFC n=22) (B) Compared to PBMC, SFMC show enhanced spontaneous ACPA-IgG production after 7 days in most donors. Each dot represents a culture well. (C) Total IgG detected in the wells of the cultures shown in (B).
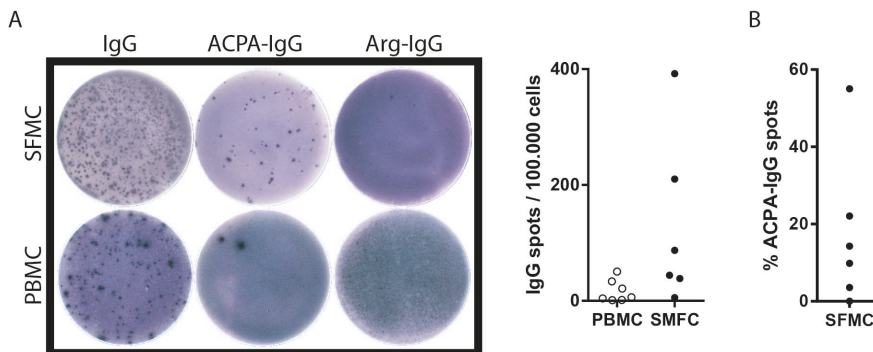

Figure 2. Enhanced frequency of anti-citrullinated protein antibodies (ACPA)-IgG-secreting cells in synovial fluid mononuclear cells (SFMC). (A) Representative ELISpot for total IgG, CCP2-reactive IgG and the control variant (IgG reactive with the arginine variant of CCP2) of a representative SFMC and PBMC sample (left). The right panel summarizes the quantity of IgG spots per 100.000 cells for PBMC and SFMC. Each dot is one donor. (B) Percentage ACPA-IgG specific spots of total IgG spots in the SFMC population. The overnight spontaneous production of IgG by PBMC and, as a consequence, the frequency of ACPA-IgG specific spots in PBMC samples was too low for a comparable analysis.

SFMC promote the long-term survival of ACPA-IgG secreting PBs/PCs

PCs can survive in specified niches in the bone marrow for many years. In these niches, PCs are non-dividing and critically depend on direct contact with surrounding stromal cells [3]. Upon isolation, however, PCs rapidly die in in vitro cultures unless they are seeded on a layer of tissue-derived stromal cells [21]. Indeed, both bone marrow derived stromal cells and fibroblast-like synoviocytes (FLS) derived from RA or osteoarthritis synovium can prolong in vitro PC survival by several days [22-27]. It is unclear, however, whether PCs can only survive in specialised tissues or whether prolonged survival can also be observed in other compartments. Given our observation of a high frequency of autoreactive PBs/PCs in SF, we investigated whether the cellular and molecular composition of SF could provide a microenvironment favouring survival of (autoreactive) PCs. To this end, we seeded total SFMC and, when possible, paired PBMC (SFMC n=12, of which n=5 paired) and cultured this heterogeneous population without external stimuli for several weeks. Medium was refreshed completely every week and supernatants were assessed for the presence of ACPA-IgG. We hypothesized to find proliferation and differentiation of pre-existing PB into PC within the first days of culture followed by either survival or rapid cell-death. Interestingly, we observed spontaneous production of ACPA-IgG for several weeks in both PBMC and SFMC cultures (figure 3A). This secretion of ACPA-IgG was maintained even if the CD20$^+$ B cell compartment had been depleted prior to culture (figure S2 and 2.S3). While ACPA production declined in cultured PBMC towards week 8, SFMC cultures, maintained continuous production that was, in some donors, remarkably stable for as long as 6 months (figure 3A-B). ELISpot performed at several time points during these cultures confirmed the presence of actively IgG secreting cells in the culture wells (not shown). As hypothesized, Ki-67 staining revealed a rapid decline of Ki-67 expression in the CD19$^+$CD27^{++}CD20$^-$ PB/PC compartment within the first 2 weeks of culture, suggesting differentiation of PBs towards non-dividing PCs (figure 4A). At later time-points, the frequency of Ki-67$^+$ cells in this compartment was low while almost all cells expressed CD138 (not shown), supporting the notion that the surviving cell population was indeed bona-fide PCs. Finally, CFSE labelling directly upon isolation was performed and cells were analysed for dilution of the dye after several weeks in culture (figure 4B and S1). Again, we observed that a fraction of cells with a PC phenotype had recently proliferated. However, we also observed a population with this phenotype that had not divided, supporting the notion that these PCs had already been present at the time of isolation and survived during weeks in culture.
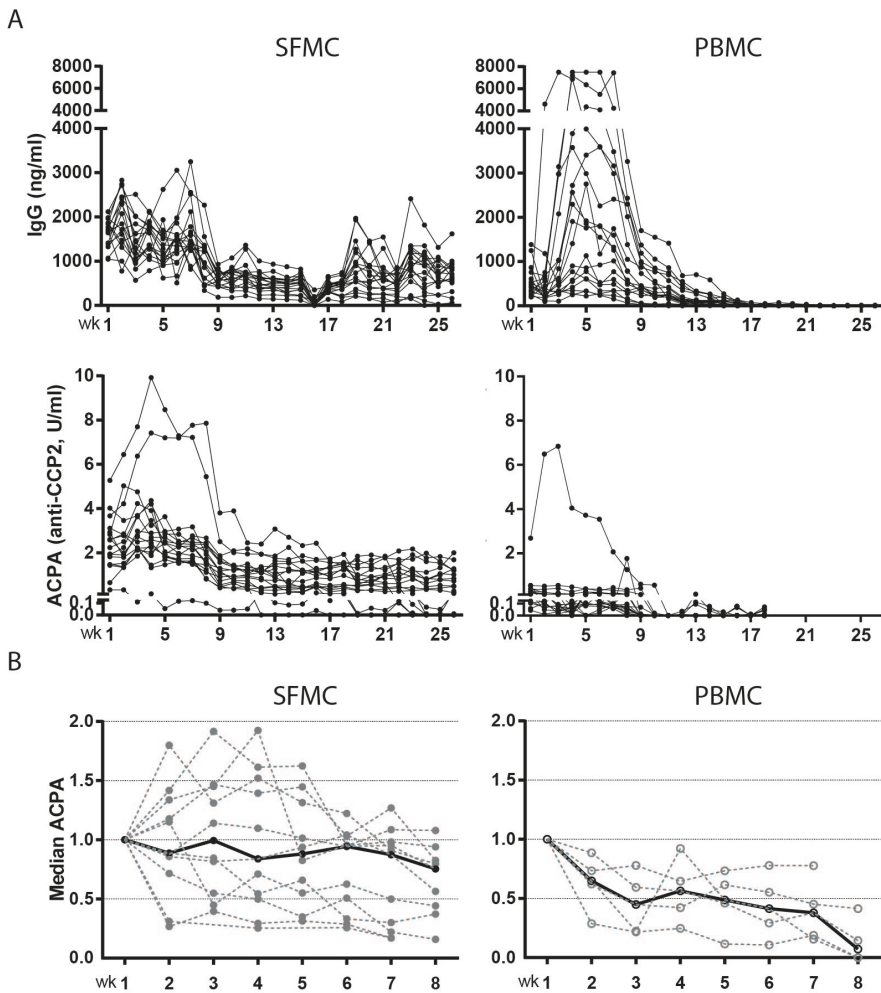
Figure 3. Spontaneous anti-citrullinated protein antibodies (ACPA)-IgG production is maintained in peripheral blood mononuclear cells (PBMC) for several weeks and in synovial fluid mononuclear cells (SFMC) for several months. (A) Weekly spontaneous total IgG and ACPA-IgG production of one representative donor (paired sample); each dot represents one well; lines connect individual wells at different time points, thereby depicting the course of (ACPA-)IgG production over time. (B) Spontaneous ACPA-IgG production during a course of 8 weeks in SFMC and PBMC. Dotted lines represent median ACPA-IgG production of all culture wells of individual donors, the solid line represents the median of all donors. To allow for comparison between donors, the median ACPA-IgG production in wells at week 1 from each donor was set to 1. After week 8, ACPA-IgG production by PBMC was below the detection level in almost all donors.
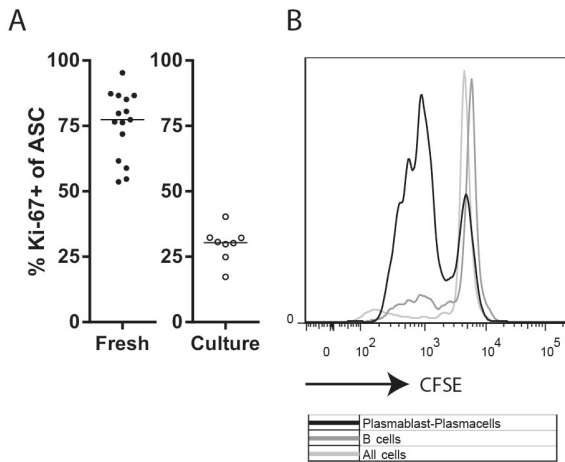
Figure 4. Antibody secreting cells (ASC) in culture show a rapid decline in Ki-67 expression and a non-dividing PC population. (A) Ki-67 expression by $CD19^+CD20^-CD27^{++}$ PB/PC upon isolation and after two weeks of culture. Every dot is one donor. (B) CFSE labelling shows that part of the cells that have a PB/PC phenotype after three weeks of culture have not divided.

Part of the IgG production detected could have originated from memory B cells differentiating to PCs upon autologous stimulation by bystander cells in the SF. To assess whether also memory B cells would differentiate into PCs by autologous stimulation in our PBMC cultures, and if so, to which degree, we determined the presence of anti-tetanus toxoid (TT) IgG in the supernatants as a proxy for memory B cell activation. This was performed in donors with protective serum IgG titres of anti-TT in which we expected the presence of TT-reactive memory B cells. Indeed, we observed anti-TT IgG in supernatants of stimulated PBMC but not in non-stimulated cultures of the same donors (not shown). Although this does not fully exclude that part of the IgG produced in our cultures originates from autologous stimulation of memory B cells, it suggests that memory B cell proliferation/differentiation had no major contribution to the overall IgG production in the unstimulated cultures.

Together, these data provide evidence that SFMC of patients with ACPA-positive RA contain the cellular prerequisite to form a microenvironment in which both pre-existing PCs and PBs differentiating to PCs can survive for extended periods of time.

DISCUSSION

We previously identified spontaneously ACPA-secreting PBs/PCs in peripheral blood of patients with ACPA-positive RA [19]. We suggested that this fraction of circulating B cells could reflect an active part of the citrulline-specific immune response that is constantly regenerated from the memory compartment. We now demonstrate that a remarkably high frequency of these cells is also present in SF. In addition, by comparing unstimulated cultures of PBMC and SFMC, we show that SFMC can provide a microenvironment supporting prolonged survival of ACPA-secreting PCs, and thus, could favour prolonged production of potentially pathogenic autoantibodies and/or B cell populations.

The provenance of ACPA-expressing B cells in SF is unknown. Although overall B cell counts were lower in SFMC as compared with PBMC, the frequency of spontaneously ACPA-IgG-secreting cells in this compartment was remarkably increased. Of note, we determined this frequency by antigen-specific ELISpot assessing over-night ACPA-IgG production by unstimulated cells, thus detecting only those cells that had already differentiated to antibody-secreting cells in vivo prior to isolation. The relatively high frequency of these ACPA-IgG-secreting PBs/PCs could be the result of directed migration of PBs from peripheral blood, specific retention, local generation and/or proliferation or prolonged survival due to the local microenvironment. Studies that directly compare the BCR repertoire of ACPA-expressing B cells and/or ACPA-secreting PBs/PCs in peripheral blood and SF in individual patients are missing, but high BAFF and TACI expression in RA synovitis, increased transcripts of CXCL13 and signs of local clonal B cell expansion suggest that ACPA-secreting PBs/PCs could be generated locally.[16, 28-31] Direct evidence for this assumption, however, is lacking. Importantly, we recently developed a technique to identify ACPA-expressing B cells and ACPA-secreting PBs/PCs by flow cytometry, which will allow to further investigate this particular question [20].

Irrespective of the place of generation, however, ACPA-secreting PCs secrete autoantibodies for which an increasing number of potentially pathogenic effector functions have been described. Here, we show that ACPA-IgG-secreting PCs can survive in total SFMC cultures for more than 6 months without stimulation. We observed fluctuating quantities of ACPA-IgG in the first weeks of culture, which likely reflect differentiation and proliferation of preformed PBs. However, the weekly ACPA-IgG production at later time-points was remarkably stable. Analysis by flow cytometry confirmed the presence of a non-dividing $CD19^+CD27^{++}CD20^-Ki-67^-CD138^+$ cell population in these cultures, consistent with the phenotype of terminally differentiated PCs. Although we cannot exclude that this long-term survival and microenvironment only forms in vitro, our data

suggest that APCA-IgG-secreting PCs in SF could potentially be long-lived also in vivo. Therefore, not only inflamed tissues and SFMC and, to a lesser extent, even PBMC have the ability to generate conditions in which (autoreactive) PCs are able to survive for extended periods of time.

In the present study, we have not addressed the molecular and cellular components of the SFMC-derived survival niche, but it will be important to decipher the underlying mechanisms of PC survival in order to develop targeted interventions. PCs in bone marrow survival niches depend on contact with reticular stromal cells that produce CXCL12 [32]. Also other cells, including eosinophils [33], basophils [34], monocytes/ DCs [35, 36] and osteoclasts [37], have been found to provide important PC survival signals. Next to CXCL12, IL-6 and APRIL [26, 32, 38], and the interaction of CD80/86 on monocytes/DCs with CD28 on PCs are critical for bone marrow PC survival. Different lymphatic and non-lymphatic tissues can also provide such signals, however, especially under inflammatory conditions. 'Inflammatory niches' have been identified in humans and mice in several diseases, including SLE [8, 39] and allergic rhinitis [40]. Also here, cell-contact and factors derived from stromal cells, especially synovium-derived FLS in RA, were found to be critical for PC differentiation and survival [41]. FLS express several relevant factors, such as VCAM-1 [24, 42], BAFF [43] and IL-15 [22, 23]. The expression of Bcl-x$_L$ induced in B cells upon interaction with FLS also supports PC longevity [25]. Interestingly, our experiments indicate that also the SF compartment harbours all components needed to create a PC survival niche. In fact, this capacity was maintained in the cellular fraction of SF for months in vitro, while IgG production in PBMC cultures terminated much earlier. This could be due to some degree of (epigenetic) imprinting in the non B cell fraction of SFMC, as has previously been suggested for FLS, and could play an important role in chronicity of synovial inflammation [44]. Further studies need to be performed to identify cell type(s) and soluble factors responsible for this observation. In fact, targeting the crucial components of this survival niche could be an alternative way of targeting long-lived PCs and thereby decrease persistent ACPA production in patients with RA.

In conclusion, SFMC can create an environment that promotes survival of ACPA-IgG secreting PCs and, consequently, secretion of ACPA-IgG for months. Our study sheds light on the characteristics, distribution and dynamics of the citrulline-specific immune response in RA. Our finding of a high frequency of differentiated, ACPA-secreting PBs/ PCs in SF, and the observation that SF can create an ideal environment for these cells to become long-lived, support the notion that SF and its components are active contributors to chronicity of synovial inflammation. Further studies using these spontaneously

generated niches could provide more detailed knowledge on how ACPA-secreting PCs survive in vivo and identify potential targets that could shorten the lifespan of ACPA-secreting PCs.

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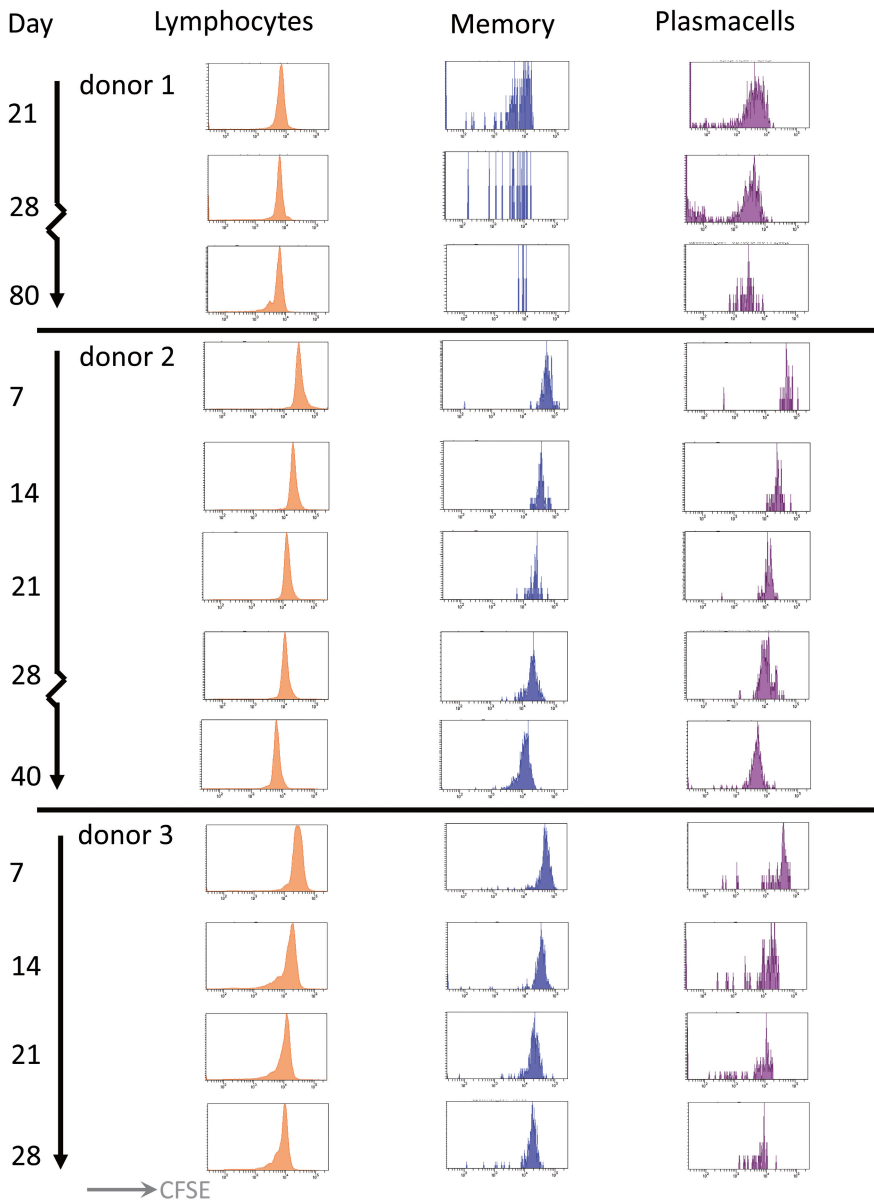
SUPPLEMENTARY FIGURES

Figure S1: Long-term persistence of a non-dividing plasma cell population in SFMC in vitro cultures. Dilution of CFSE by lymphocytes and B cell subsets of three SFMC donors at different time points of culture. Despite decreasing signal strength and low numbers of cells, a fraction of cells with a plasma cell phenotype (CD19$^+$CD27^{++}CD38$^+$CD138$^+$) did not show dilution of CFSE even after weeks of culture.
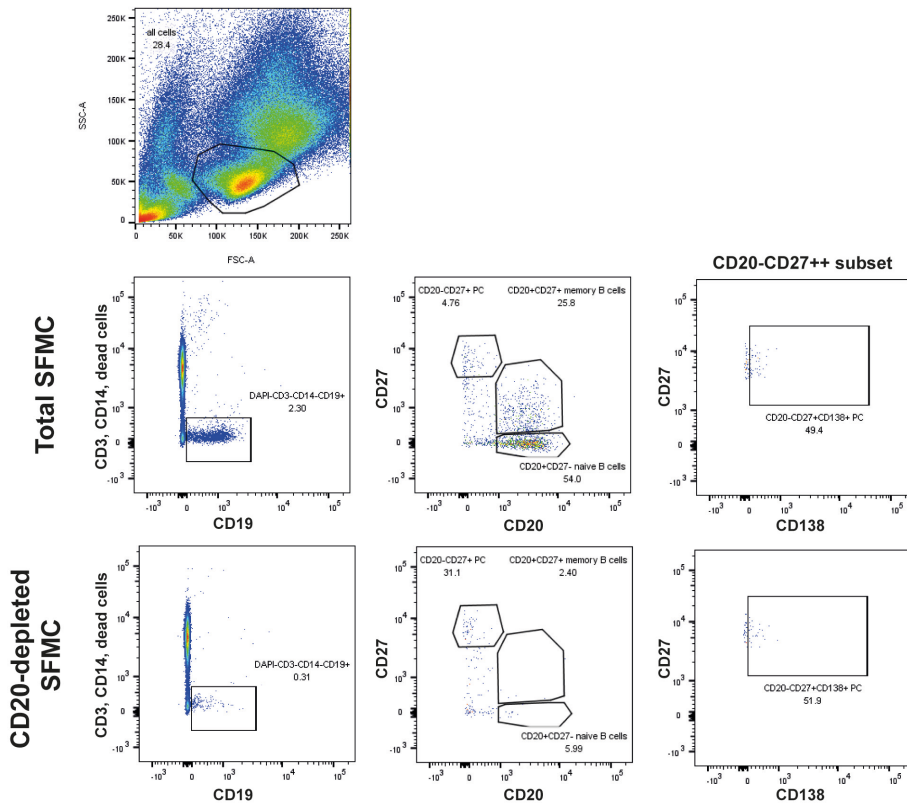
Figure S2: Depletion of CD20+ B cells from synovial fluid mononuclear cells. Depicted is one representative experiment of five. Upon depletion of CD20+B cells, a fraction of CD19+CD20- CD27++ cells remained, of which ~50% expressed CD138 (lower right panel). Gates are set based on the respective isotype control.
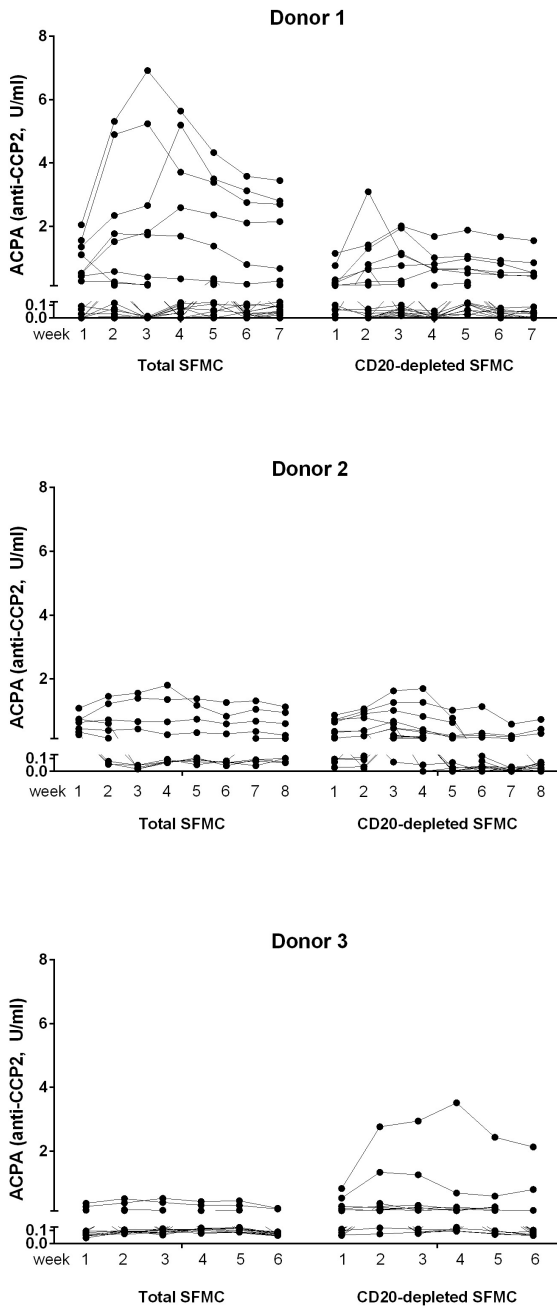
Figure S3: Spontaneous production of ACPA-IgG in cultures of total SFMC and of SFMC depleted of CD20+ B cells as measured by ELISA. Data are depicted for three donors, each dot represents one culture well.