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Research Article

Clinical relevance of circulating anti-ENA and anti-dsDNA secreting cells from SLE patients and their dependence on STAT-3 activation

Raquel de la Varga Martínez[1,2], Beatriz Rodríguez-Bayona[3], Gustavo A. Añez[4], Fermín Medina Varo[4], José J. Pérez Venegas[5], José A. Brieva[1] and Carmen Rodríguez[2]

[1] Unidad de Investigación, Hospital Universitario Puerta del Mar (HUPM), Cádiz
[2] Servicio de Inmunología, UGC de Hematología e Inmunología, HUPM, Cádiz
[3] Área de Inmunología, UGC de Análisis Clínicos, Complejo Hospitalario Universitario de Huelva, Hospital Juan Ramón Jiménez, Huelva
[4] Sección de Reumatología, UGC de Cirugía Ortopédica, Traumatología y Reumatología, HUPM, Cádiz
[5] UGC de Reumatología, Hospital de Jerez de la Frontera, Cádiz

Disturbances of plasma cell homeostasis and auto-antibody production are hallmarks of systemic lupus erythematosus. The aim of this study was to explore the presence of circulating anti-ENA and anti-dsDNA antibody-secreting cells, to determine their dependence on plasma cell-niche cytokines and to analyze their clinical value. The study was performed in SLE patients with serum anti-ENA and/or anti-dsDNA antibodies ($n = 57$). Enriched B-cell fractions and sorted antibody-secreting cells ($CD19^{low}CD38^{high}$) were obtained from blood. dsDNA- and ENA-specific antibody-secreting cells were identified as cells capable of active auto-antibody production in culture. The addition of a combination of IL-6, IL-21, BAFF, APRIL, and CXCL12 to the cultures significantly augmented auto-antibody production and antibody-secreting cell proliferation, whereas it diminished apoptosis. The effect on auto-antibody production was dependent on STAT-3 activation as it was abrogated in the presence of the JAK/STAT-3 pathway inhibitors ruxolitinib and stattic. Among patients with serum anti-dsDNA antibodies, the detection of circulating anti-dsDNA-antibody-secreting cells was associated with higher disease activity markers. In conclusion, auto-antibody production in response to plasma cell-niche cytokines that are usually at high levels in SLE patients is dependent on JAK/STAT-3 activation. Thus, patients with circulating anti-dsDNA antibody-secreting cells and active disease could potentially benefit from therapies targeting the JAK/STAT3 pathway.

Keywords: Anti-dsDNA · Anti-ENA · Antibody-secreting cells · Cytokines · Plasma cell niche · SLE · STAT-3

Additional supporting information may be found in the online version of this article at the publisher's web-site

Correspondence: Dr. Carmen Rodríguez
e-mail: carmen.rodriguez.sspa@juntadeandalucia.es

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by B-cell hyperactivation and increased autoreactive plasma cell (PC) generation. The elevated production of auto-antibodies (autoAbs) against various self-antigens is caused by different mechanisms [1–4]. They are mainly directed against nucleic acids and ribonucleoproteins. Anti-double-stranded DNA (dsDNA) autoAbs are relevant as they are highly specific, correlate with disease activity and can cause lupus nephritis [5]. Other prevalent Abs in SLE patients recognize extractable nuclear antigens (ENAs), including anti-Smith (Sm), -U1-RNP, -SSA/Ro, and -SSB/La ribonucleoproteins, among others. Consequently, antibody-secreting cells (ASCs) are now considered a relevant target for different therapies in SLE [6].

It is known that circulating ASCs form a heterogeneous compartment. Only ASCs capable of secreting high-affinity Abs migrate to specialized survival niches of the bone marrow and reactive tissues, where they become long-lived PCs in response to soluble factors and contact-mediated signals [7, 8]. Stromal cells expressing CXCL12, megakaryocytes and eosinophils producing IL-6 and a proliferation-inducing ligand (APRIL), and other soluble factors including B-cell activating factor (BAFF) have been identified as components of the ASC survival niches [9–11]. In addition, IL-21 has been shown to act directly on human early PCs and circulating plasmablasts (PBs) [11–13]. In this context, STAT-3 activation by different cytokines has recently been shown to be a critical signal for PC survival and immunoglobulin (Ig) secretion throughout their maturation [11, 13, 14].

In an autoimmune setting, ectopic lymphoid foci and inflamed tissues can assume a dual role, behaving as inductive and final homing organs and providing survival signals for PCs [15, 16]. It has been described that autoAb-producing cells circulate and accumulate in damaged organs and ectopic lymphoid tissue in various autoimmune diseases [16–21]. However, the knowledge of circulating auto-reactive ASCs and their functional capacities in SLE is limited [3, 22–24].

The objective of this work was to determine the presence of circulating anti-dsDNA and anti-ENA Ab-producing cells, to analyze their clinical value and to explore their response to relevant cytokines in plasma cell biology and the mechanisms involved in anti-dsDNA and anti-ENA Ab production in SLE patients.

Results

ASCs produce IgG, anti-ENA and anti-dsDNA Abs in cultures of blood cells from SLE patients

Circulating ASCs are commonly identified as CD20-CD19^{low} CD38^{high} cells [8, 25–27]. The presence of these cells in SLE patients was evaluated. ASCs were significantly increased in the blood of SLE patients compared to healthy controls (0.365 ± 0.054% versus 0.065 ± 0.009%, respectively; mean ± SEM) (Fig. 1A).

The spontaneous secretion of total IgG and autoAbs was measured in the supernatants of cultured non-T PBMCs. More than 90% (54 of 57) of the patients contained IgG-secreting cells in their blood. 52% of patients with positive serum tests for anti-ENA (25 of 48) and 31% of patients with positive serum tests for anti-dsDNA (8 of 26) contained specific anti-ENA and anti-dsDNA ASCs in their circulation (Supporting Information Table 1 and Supporting Information Table 2 for details in patients with anti-ENA and anti-dsDNA ASCs). Figure 1B–D shows the levels of in vitro total IgG, anti-ENA-, and anti-dsDNA Ab production for those patients with circulating ASCs. This secretory capacity was actively carried out by the cultured cells, as it could be drastically reduced by the inclusion of the protein-synthesis inhibitor cycloheximide (CHX) (Fig. 1B–D). In addition, spontaneous and active IgG, anti-ENA-, and anti-dsDNA Ab production from SLE patients' blood was also demonstrated by using highly purified ASCs (sorted CD19^{low}CD38^{high} ASCs) (Fig. 1E–G). In contrast, preparations of blood B cells depleted of ASCs (sorted CD19^{+}CD38^{-} cells) did not exhibit this secretory activity, although they were cultured at a higher cell concentration (Fig. 1H–J).

The quantities of anti-ENA and anti-dsDNA Abs produced in SLE cell culture supernatants showed a limited correlation with the corresponding serum levels of these autoAbs (for anti-dsDNA Ab: $r = 0.550$, $p < 0.01$, $n = 26$ and for anti-ENA Ab: $r = 0.566$, $p < 0.001$, $n = 48$, Spearman test) (data not shown).

Circulating ASCs of SLE patients express receptors for IL-6, IL-21, CXCL12, BAFF and APRIL

The profile of receptor expression for a group of B-lineage-related factors was analyzed in the ASC fraction of SLE patients (Fig. 2A and B). Figure 2C shows representative examples of the expression of these receptors in ASCs from healthy individuals and SLE patients. Both components of the functional IL-6 receptor complex, the IL-6R subunit (CD126) and, to a lesser extent, the signal transducer gp130 (CD130), were expressed by a substantial proportion of ASCs in SLE patients. Nevertheless, the level of expression was significantly lower than the IL-6R expression exhibited by ASCs in healthy controls. In contrast, IL-21R and BAFF-R were expressed in a higher proportion of ASCs in SLE patients. The receptors for APRIL and BAFF, TACI and BCMA, and the CXCL12 receptor, CXCR4, were expressed at similar levels by ASCs from SLE patients and healthy controls.

PC-niche cytokines promote IgG and autoAb production by circulating ASCs in a STAT-3-dependent manner

Next, we evaluated whether total IgG, anti-ENA and anti-dsDNA Ab production by circulating CD19^{low}CD38^{high}-sorted ASCs from SLE patients depended on a combination of PC-niche cytokines,

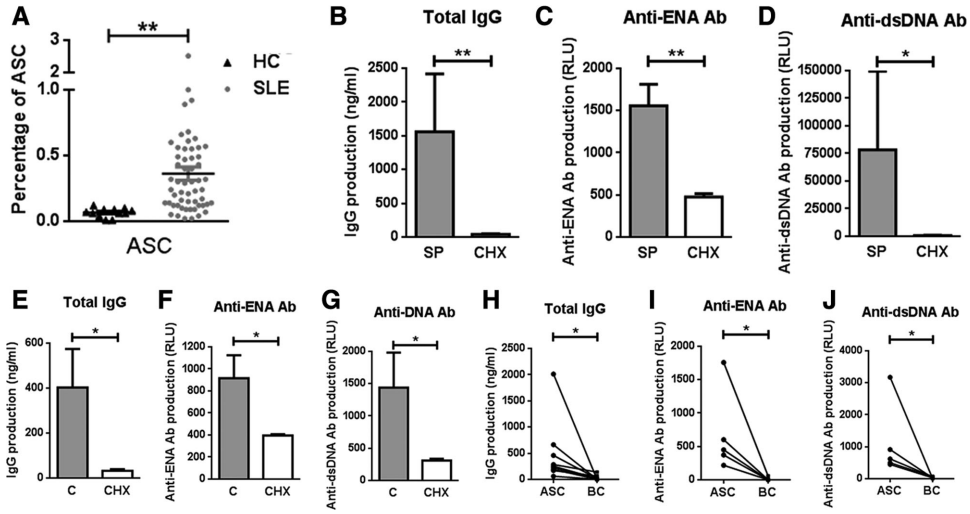

Figure 1. Circulating ASCs and spontaneous IgG and autoAb production in cell cultures from SLE patients (A) Percentage of ASCs identified by flow cytometry in non-T PBMCs as CD20-CD19^{low}CD38^{high} cells in SLE patients ($n = 57$) versus healthy individuals ($n = 12$). Each data point represents one independent sample. All data were obtained from 69 independent experiments. $^{**}p < 0.001$; Mann–Whitney U test. (B–D) IgG and auto-antibody production in cells cultures. Non-T PBMCs from SLE patients were cultured for 7 days in the absence or presence of CHX and total IgG and autoAb production were measured in the supernatants by ELISA and CLIA, respectively. Results represent the levels of IgG, anti-ENA-, and anti-dsDNA Ab production from those patients with in vitro IgG ($n = 54$, Fig. 1B) anti-ENA Ab ($n = 25$, Fig. 1C) and anti-dsDNA Ab ($n = 8$, Fig. 1D) production, respectively. Each patient sample was analyzed once. The results are expressed in ng/mL (IgG) and RLU (anti-ENA and anti-dsDNA Ab) and represent the mean + SEM of 54 (1B), 25 (1C) and 8 (1D) independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$; Wilcoxon test for paired samples. (E) IgG, (F) anti-ENA, and (G) anti-dsDNA Ab production was examined in cultures of sorted CD19^{low}CD38^{high} ASCs. ASCs from patients with serum anti-ENA or anti-dsDNA Abs were cultured in the absence or presence of CHX. IgG ($n = 11$), anti-ENA Abs ($n = 7$) and anti-dsDNA Abs ($n = 5$) were determined in the supernatants. $^{*}p < 0.05$. Wilcoxon test for paired samples. (H) IgG, (I) anti-ENA, and (J) anti-dsDNA Ab production was studied in cultures of sorted CD19^{low}CD38^{high} ASCs versus sorted CD19^{high} CD38^{neg} B-cells (BC). ASCs and BC were cultured for 7 days. IgG ($n = 9$), anti-ENA ($n = 5$) and anti-dsDNA Ab ($n = 5$) secretion was determined by ELISA and CLIA, respectively. Each patient sample was analyzed once. The background obtained in cultures with CHX was subtracted. The results are expressed in ng/ml (IgG) and RLU (anti-ENA and anti-dsDNA Ab), and represent the mean + SEM of five to ten independent experiments. $^{*}p < 0.05$; Mann–Whitney U test. ASC: antibody-secreting cell, BC: B cells, C: control, CHX: cycloheximide, RLU: relative luminescence units.

including IL-6, BAFF, APRIL, CXCL12, and IL-21 (MIX). This cytokine combination and their corresponding concentrations had been found to be optimal in previous experiments of human PC and PBs Ig secretion [11–13]. In the presence of MIX, the mean IgG production was four times higher (Fig. 3A), anti-ENA Ab levels doubled (Fig. 3B) and anti-dsDNA Ab levels tripled (Fig. 3C).

Then, the effect of STAT-3 blockade on IgG and autoAb production was explored. We found that when STAT-3 and JAK1/JAK2 were inhibited by stattic and ruxolitinib, respectively, in cultures

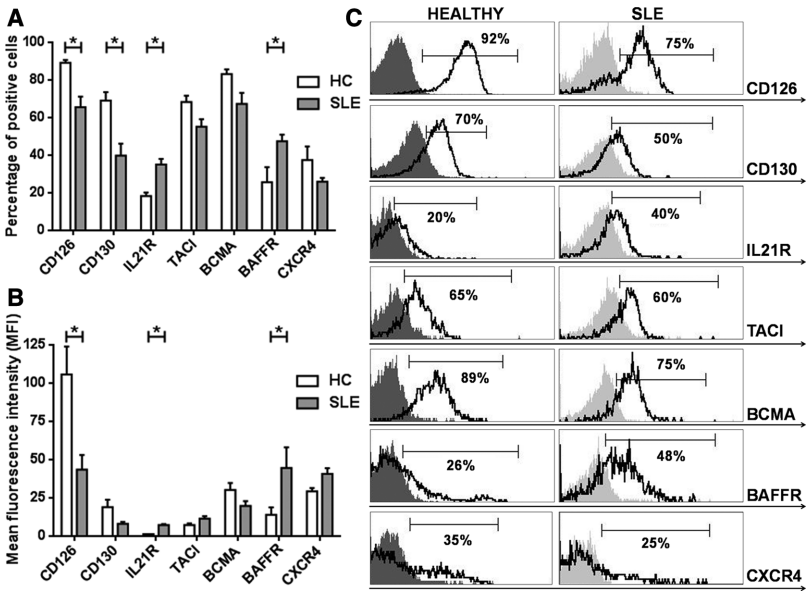

Figure 2. Cytokine receptor expression on blood CD19^{low}CD38^{high} cells (ASCs) from SLE patients. A, B) The percentage (A) and mean fluorescence intensity (B) of CD126, CD130, IL21-R, TACI, BCMA, BAFF-R, and CXCR4 expression were determined by flow cytometry on CD19^{low}CD38^{high} ASCs from the non-T-cell fraction in SLE patients ($n = 7$) and healthy individuals (HC) ($n = 7$). Each sample was analyzed once. The results are expressed as the mean+SEM of seven independent experiments for each group. $^{*}p < 0.05$; Mann–Whitney U test. C) Histograms are from a single experiment representative of seven experiments with one patient sample per experiment. Isotype controls are shown as shaded histograms.

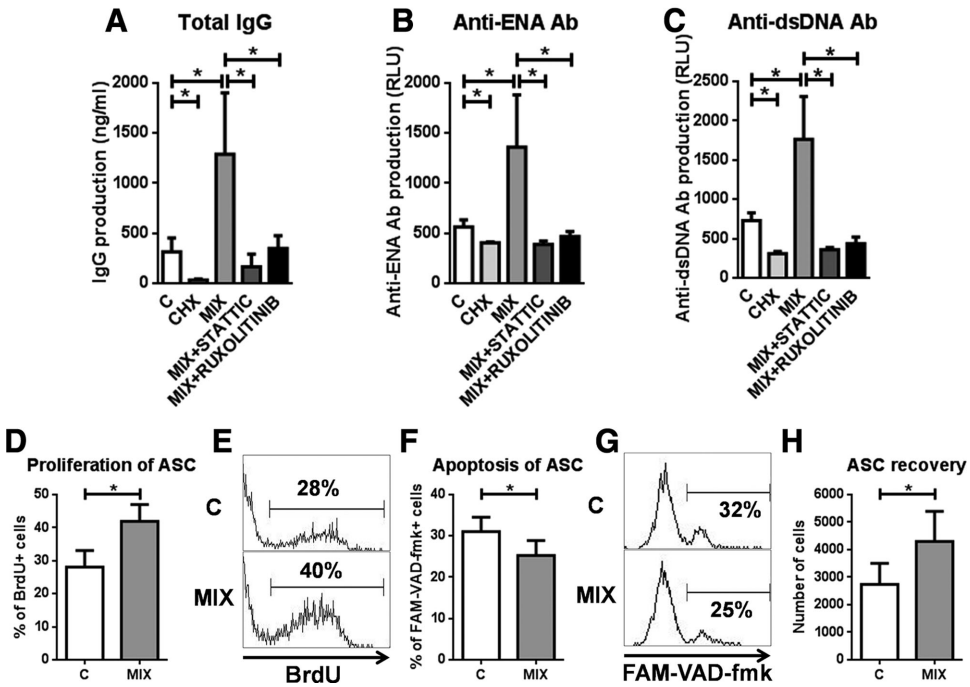

Figure 3. (A–C) Effect of MIX and STAT-3/JAK1-2 blockade on IgG (A), anti-ENA Ab (B) and anti-dsDNA Ab production (C) by blood ASCs from SLE patients. (A–C) Sorted $CD19^{low}CD38^{high}$ cells from patients with serum anti-ENA or anti-dsDNA Abs were cultured with and without MIX (IL-6, IL-21, BAFF, CXCL12 and mega-APRIL), CHX, stattic and ruxolitinib, as indicated. IgG ($n = 6$), anti-ENA ($n = 6$) and anti-dsDNA Ab ($n = 5$) production in the supernatants was determined by ELISA (for IgG) and CLIA (for anti-ENA and anti-dsDNA Ab). (D, E) Non-T PBMCs were cultured in the presence of BrdU. Proliferating ASCs were identified by flow cytometry as $CD19^{low}CD38^{high}$BrdU+cells ($n = 10$). Histograms from a single experiment representative of ten experiments with one patient sample per experiment are shown in E. F, G) Apoptotic $CD19^{low}CD38^{high}$ASCs were detected by flow cytometry after culture of non-T PBMCs by staining with FAM-VAD-fmk ($n = 6$). Histograms from a single experiment representative of six experiments with one patient sample per experiment are shown in G. H) The number of viable ASCs recovered in the cultures is indicated in H ($n = 6$). Each patient sample was analyzed once. The results are expressed in ng/mL (IgG), RLU (anti-ENA and anti-dsDNA Ab) (A–C), percentages of cells (D-F), number of cells (H) and represent the mean + SEM of the above indicated number of independent experiments (A-D, F, H). *$p = <0.05$.Wilcoxon test for paired samples was used for all comparisons; ASC: antibody-secreting cell, C: control, CHX: cycloheximide, RLU: relative luminescence units.

of MIX-induced sorted ASCs, IgG (Fig. 3A), anti-ENA Ab (Fig. 3B) and anti-dsDNA Ab (Fig. 3C) production was abrogated.

The effects of MIX on the rate of apoptosis and proliferation of $CD19^{low}CD38^{high}$ ASCs contained in non-T PBMC cultures from SLE patients were further assessed. Figures 3D and G show that ASCs in SLE patients had a high percentage of proliferating $BrdU^+$ cells (28.0 ± 4.9%, mean ± SEM), and the addition of MIX to the cultures significantly augmented this figure (41.8 ± 5.0%, mean ± SEM). In addition, MIX diminished the percentage of FAM-VAD-fmk-positive apoptotic ASCs in SLE patients (Fig. 3E and H). As a confirmation of these data, the recovery of ASCs after the culture period was higher in MIX-stimulated cultures than in control cultures (Fig. 3F).

Clinical features of SLE patients with circulating anti-ENA and anti-dsDNA-specific ASCs

The relevance of the presence of a circulating phase of anti-ENA and anti-dsDNA-specific ASCs in SLE patients was explored by analyzing their relationship with the clinical and analytical features of the patients (Supporting Information Table 1). Disease activity was defined by the SLE Disease Activity Index (SLEDAI) [28]. The Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) Damage Index was assessed as a predictor of severe outcome and an indicator of morbidity [29]. Patients with serum anti-dsDNA Abs had higher disease activity indices (SLEDAI) than those with a serum anti-ENA response (Fig. 4A). In addition, patients with detectable circulating dsDNA specific ASCs had higher SLEDAI (Fig. 4B) and SLICC/ACR scores (Fig. 4C). Furthermore, patients with dsDNA-specific ASCs had lower levels of C3 and C4 (Fig. 4D and E), more proteinuria (Fig. 4F) and more elevated erythrocyte sedimentation rate (ESR) values (Fig. 4G) than patients without circulating dsDNA-secreting ASCs. No differences in these parameters were observed between patients with or without anti-ENA-specific blood ASCs (data not shown).

Discussion

The present study explores the occurrence and nature of circulating anti-dsDNA and anti-ENA Ab-producing cells in SLE patients and their requirements for autoAb production.

We observed a circulating phase of ENA-specific ASCs in more than 50% of the patients with a serological response against ENA, whereas anti-dsDNA-secreting ASCs occurred in 31% of the

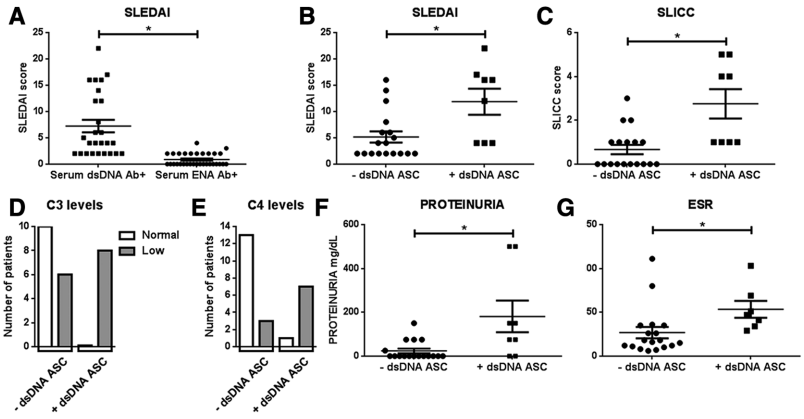

Figure 4. Correlation of circulating dsDNA ASCs with clinical data. (A) SLEDAI index in patients with serum anti-dsDNA Abs (serum dsDNA Ab^+) ($n = 26$) versus patients with only serum anti-ENA Abs (serum ENA Ab^+) ($n = 31$). (B–G) SLEDAI (B), SLICC (C) scores, complement C3 (D) and C4 (E) levels, proteinuria levels (F) and ESR values (G) in patients with circulating anti-dsDNA secreting cells (+dsDNA ASC) ($n = 8$) and without them (-dsDNA ASC) ($n = 18$). The results represent the mean ± SEM in A–C, F, G. Each patient sample was analyzed once. All data were obtained from 57 (A) and 26 (B–G) independent experiments. $^*p < 0.05$. Mann–Whitney U test. ASC: antibody-secreting cell, ESR: erythrocyte sedimentation rate, SLEDAI: SLE Disease Activity Index, SLICC/ACR: Systemic Lupus International Collaborative Clinics/American College of Rheumatology Damage Index.

corresponding patients (Supporting Information see Table 2 for details). Anti-dsDNA Ab production by blood ASCs had been previously described in SLE patients [23, 24, 30], and our results show a similar frequency of these ASCs to that observed in another study [30]. In contrast, anti-ENA ASCs in SLE have been poorly analyzed in humans [31].

It is known that normal PC maturation relies on soluble factors and contact-mediated signals provided by specialized niches that maintain survival and promote a high rate of Ab secretion [7–13]. Dysregulation of these processes seems to play a role in the pathogenesis of human autoimmune diseases [16]. In an attempt to gain knowledge of the requirements of circulating auto-reactive ASCs in SLE, we explored whether ENA- and dsDNA-specific ASCs depended on the PC-niche cytokines and factors IL-6, BAFF, APRIL, CXCL12, and IL-21. Therefore, the expression of receptors for those cytokines was assessed. It was found that CD126, BCMA and TACI were the receptors expressed at higher levels on ASCs (Fig. 2). The data regarding the expression of the BAFF and APRIL receptors on B and PCs in SLE are controversial [32, 33]. In our results, comparison with blood ASCs from healthy individuals revealed that blood ASCs from SLE patients showed significantly higher expression of IL-21R and BAFF-R and lower expression of the IL-6 receptor complex (Fig. 2). This profile of receptors exhibited by ASCs in SLE resembled that of human early PCs obtained from tonsils and lymph nodes, two organs considered inductive areas for initial PC formation [11, 12]. These data might suggest that circulating auto-reactive ASCs are at a less mature stage in SLE patients than in healthy individuals. B-cell hyperactivation [1, 34] and the marked expansion of ASCs in SLE patients (Fig. 1A) could be related to the strong generation of ASCs in inductive areas that would result in an early egress of PBs and initial PCs to the peripheral blood in SLE.

Next, we observed that ASCs, including ENA- and dsDNA-specific ASCs, responded to PC-niche cytokines as the addition of a cocktail of IL-6, APRIL, BAFF, CXCL12, and IL-21 (MIX) to cultures of sorted ASCs from SLE patients increased their total IgG, anti-ENA and anti-dsDNA Ab responses (Fig. 3A–C). This observation indicates that SLE autoAb-producing cells respond to PC-niche related cytokines, and suggests that inflamed tissues, as prominent sources of these cytokines, provide signals for ENA- and dsDNA-specific ASCs to promote a greater capacity of autoAb secretion and eventually a higher pathogenic potential. In this regard, it is known that the levels of BAFF, APRIL, IL-21, IL-6, and CXCL12 are increased in SLE patients [35–38]. This pro-survival context might favor the persistence of auto-reactive ASCs and could explain the lack of response of some SLE patients to therapies targeting CD20, as ASCs lose CD20 expression during maturation. In accordance with some reports, these patients can benefit from other B-cell directed therapies like BAFF antagonists in certain SLE patients [39, 44].

It has been described in humans that STAT-3 inhibition impairs PC maturation, not only by STAT-3–activating cytokines such as IL-21 and IL-6 but also by the action of other cytokines, including BAFF, APRIL and CXCL12 [11, 14]. Therefore, STAT-3 activation appears to be necessary for enabling the function of other cytokines required for PC acquisition of long-lived status. The present results reveal that the inductive effect of MIX on IgG, anti-ENA and anti-dsDNA secretion by SLE ASCs was also inhibited when the STAT-3 pathway was blocked by inhibitors of both STAT-3 phosphorylation (stattic) and several JAK members required for upstream steps of this pathway (ruxolitinib) (Fig. 3A–C). From a clinical point of view, these results would support a rationale for using therapies blocking JAK/STAT3 pathways in SLE, such as tofacitinib (JAK1/JAK3 inhibitor) and baricitinib (JAK1/JAK2 inhibitor). In fact, Yamamoto et al. have reported that treatment of a patient with rheumatoid arthritis and SLE with tofacitinib reduced anti-DNA Abs [40]. Moreover, it has been recently reported that activation of STAT1/STAT3 in response to other cytokines such as IL-39 mediates inflammatory responses in lupus-like mice [41].

To gain deeper insight into the mechanisms responsible for the effect of MIX in the production of anti-ENA and anti-dsDNA Abs, we analyzed the rates of proliferation and apoptosis of ASCs in SLE patients. First, a high rate of spontaneous proliferation and apoptosis of ASCs was observed (Fig. 3D–H), corresponding to the early maturation stage of blood ASCs [27]. When MIX was added to the cultures, ASCs augmented their proliferation and diminished their level of apoptosis, effects that resulted in improved cell survival (Fig. 3D–H). Taken together, these results indicate that PC-niche cytokines promote the expansion (Fig. 3D and G) and survival (Fig. 3E and H) of ASCs in SLE patients, including those auto-reactive ASCs capable of producing high levels of anti-ENA

and anti-dsDNA Abs (Fig. 3B and C), and therefore, they would become putative long-lived memory auto-reactive PCs. This result agrees with those found in Sjögren's syndrome, where the salivary glands of primary Sjögren's syndrome patients express factors vital for PC survival [42]. It has been suggested that ENA- and dsDNA-specific PCs have different maturity levels and lifespans in SLE patients. The persistence of high levels of anti-ENA Abs is more consistent with a long-lived PC response. In contrast, the transitory presence of anti-dsDNA autoAbs during flares suggests that they are short-lived PCs [2, 16, 43]. The present results do not yield conclusions about this issue, although differences between anti-ENA and anti-dsDNA ASCs have been found that suggest distinctive roles in the pathogenicity, chronicity and activity of the disease. First, circulating ENA-specific ASCs were more frequent but produced autoAbs at lower levels and in a more stable way than dsDNA-specific ASCs, which exhibited an explosive release of anti-dsDNA Abs in some patients (Fig. 1C and D and Supporting Information Table 2). Second, in contrast to ENA-specific ASCs, the presence of anti-dsDNA ASCs was related to clinical parameters (Fig. 4).

The patients with circulating dsDNA-specific ASCs showed higher SLEDAI values than the patients without them (Fig. 4B), as it has also been shown by Hanaoka et al. [30]. In addition, we observed a higher SLICC/ACR organ damage index (Fig. 4C), more proteinuria (Fig. 4F), more complement consumption (Fig. 4D and E) and more elevated levels of ESR (Fig. 4G) among patients with dsDNA-specific ASCs compared to the patients without them. Therefore, the presence of circulating anti-dsDNA positive ASCs can represent a marker of more organ damage, a worse prognosis and, therefore, a predictor of a severe outcome in SLE patients. The lack of relationship between ENA-specific ASCs and clinical parameters could be due to the fact that anti-dsDNA Ab are known to be pathogenic and correlate with disease activity [5] (Fig. 4A), while anti-ENA Ab are not.

In summary, we have detected circulating anti-dsDNA and anti-ENA ASCs in SLE patients. They respond to the PC-niche cytokines that are usually at high levels in SLE patients such as IL-6, BAFF, APRIL, CXCL12, and IL-21, and depend on STAT-3 activation for survival, proliferation and the secretion of autoAbs. In addition, patients with circulating anti-dsDNA-specific ASCs showed higher markers of activity and organ damage and, hence, they represent patients with worse prognosis. These observations support the view that drugs blocking IL-6, BAFF, APRIL, CXCL12 (inhibitor agent 30D8), IL-21 (agent NNC0114-0006) and JAK-STAT pathway (tofacitinib and baricitinib) target auto-reactive PCs and offer therapeutic options for SLE [44].

Materials and methods

Patients and control populations

Heparinized blood samples were obtained from 31 normal healthy donors (16 women and 15 men; mean age 40 ± 2 years, range 26–59), 10 patients with RA and 57 SLE patients (50 women and 7 men; mean age 43 ± 2 years, range 13 to 72) who fulfilled the American College of Rheumatology criteria for RA and SLE, respectively. Only SLE patients with positive serum test for anti-ENA and/or anti-dsDNA were included in the study. Both, SLEDAI [28] and SLICC/ACR damage [29] index scores were calculated using the on-line calculators of The Medical Algorithms Company (www.medal.org). A summary of the clinical and analytical features of the SLE patients at the time of analysis is shown in an additional table file (see Supporting Information Table 1).

For some experiments, blood samples were obtained from healthy donors 7 day after receiving a conventional booster immunization against tetanus-diphtheria toxoids and acellular pertussis (Boostrix; GlaxoSmithKline Biologicals SA, Rixensart, Belgium).

Patients and healthy donors were informed of the objective of the study and gave their consent according to the Declaration of Helsinki. Approval for this study was obtained from the Institutional Review Board (Comité Ético, Hospital Universitario Puerta del Mar).

Reagents

The following monoclonal Abs (mAbs) were used: peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5)-labelled anti-CD19 (clone SJ25C1) and anti-CD38; allophycocyanin (APC)-labelled anti-CD27 and anti-CD19; fluorescein isothiocyanate (FITC)-labelled anti-CD20, phycoerythrin (PE)-labeled anti-CD126, anti-CD130, anti-IL21R, anti-TACI, anti-BAFFR and anti-CXCR4; and the appropriate labeled antibodies used as negative controls (Becton Dickinson; San Jose, CA, USA); goat PE-anti-BCMA polyclonal Ab and isotype matched controls (R&D Systems, Minneapolis, MN). Magnetic microbeads bound to anti-CD27 and MS and LS columns for immunomagnetic selection were provided by Miltenyi Biotec (Auburn, CA). IL-6, IL-21, BAFF and CXCL12 were purchased from PeproTech (London, U.K.). Mega-APRIL was provided by AdipoGen (Liestal, Switzerland). Unconjugated and peroxidase-conjugated anti-human IgG Abs used for ELISA were purchased from BioSource International (Camarillo, CA). STAT3 inhibitor V, stattic, and the JAK 1, 2 inhibitor ruxolitinib were purchased from SelleckBio (Houston, TX). The BrdU Flow Kit (BD Pharmingen) was used to detect proliferating cells. FAM-VAD-fmk (carboxyfluorescein-Val-Ala-Asp OMe-fluoromethyl ketone) was provided by SM Biochemicals (Anaheim, CA).

Cell preparation and cell sorting

PBMC were isolated by density gradient centrifugation on Hystopaque-1077 (Sigma-Aldrich), and non-T PBMCs were subsequently obtained as previously described [25]. CD27+ cells were purified from the non-T-cell fraction using an immunomagnetic cell selection technique to enrich the ASC subset [11]. Next,

$CD19^{low}CD38^{high}$ ASCs and $CD19^{+high}CD38^{-}$ B lymphocytes were purified by sorting on a FACSAria flow cytometer (BD) (Supporting Information Figure 1A). The post-sort purity was >95%.

Cell culture and functional analysis

Cell cultures were set up in medium consisting of RPMI 1640 supplemented with 10% FCS, 10 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (from Life Technologies BRL Life Technologies, Paisley, U.K.) in 24- or 96-well flat-bottom culture plates (Nunc, Roskilde, Denmark) in a final volume of 1 mL and 250 μL, respectively. Apoptotic ASCs were by determined by flow cytometry (FC), labelling active caspases with the fluorochrome-labeled inhibitor FAM-VAD-fmk [12]. Briefly, 2.5×10^6 non-T PBMCs were cultured in 1 mL of culture medium for 48 h, and FAM-VAD-fmk (1 μM) was added and incubated for 1 h at 37°C. The cells were then labeled with PE-anti-CD19 and PerCP-Cy5.5-anti-CD38 mAbs and extensively washed before the analysis. Proliferation assays were carried out on the non-T PBMC fraction (2.5×10^6 cells/ml), which was cultured for 24 h in the presence of BrdU (10 μM) during the last 18 h of the culture period. FC analysis of proliferating ASCs was performed by gating on $CD19^{low}CD38^{high}$ cells.

Four-color FC analysis of labeled cells was performed with a FACSCalibur cytometer using CellQuest software (Becton Dickinson; San Jose, CA, USA). For phenotypic analysis, at least 2×10^3 cells were collected in the ASC gate.

For IgG anti-ENA Ab, IgG anti-dsDNA Ab and total IgG production experiments, the non-T PBMC fraction was cultured at 1.25×10^5 cells/250 μL for 7 days, as indicated. Purified $CD19^{low}CD38^{high}$ ASCs were cultured at 1×10^4 cells/250 μL for 7 days. Sorted $CD19^{+high}CD38^{-}$ B lymphocytes were cultured at 5×10^4 cells/250 μL for 7 days.

The final concentrations of the cytokines, growth factors and inhibitors used in this study were as follows: CHX (10 μg/mL), IL-6 (5 ng/mL); IL-21 (50 ng/mL); BAFF and CXCL12 (100 ng/mL); mega-APRIL (1 μg/mL); STAT3 inhibitor V, Stattic, and the JAK 1, 2 inhibitor, ruxolitinib (1 μM).

IgG and autoantibody detection

Total IgG production and anti-TT IgG in the cultures were determined by in-house ELISAs [25, 26].

Indirect immunofluorescence (IIF) on HEp-2 (Delta Biologicals SRL, Pomezia, Italy) and *Crithidia luciliae* (Inova, Werfen) substrates was used to determine anti-nuclear and anti-dsDNA antibodies in the patients' serum samples, respectively.

Individual ENA and other antinuclear specificities, including U1- RNP, Sm, SSA/Ro 52 kDa, SSA/Ro 60 kDa, SSB/La, Scl-70/topoisomerase-I, Jo-1, Ribosomal P-proteins, CENP-B, PmScl, and histones, were determined by immunodot assay (Euroline anti-nuclear antibodies Profile 3, Euroimmun Lübeck, Germany).

IgG anti-ENA and anti-dsDNA Abs in the sera and undiluted supernatants from cell cultures were evaluated by a chemiluminescence technique (CLIA) using QUANTA-Flash ENA-7® and QUANTA-Flash® dsDNA, respectively (Inova, Werfen). ENA-7® uses immunobeads coupled to a mixture of nuclear antigens (Sm, U1RNP, SSA/Ro 60 kDa, SSA/Ro 52 kDa, SSB/La, Jo-1, Scl-70). The autoAb levels in the supernatants were reported in relative luminescence units (RLU). CLIA was shown to be sensitive and specific for detecting autoAb secreted into the cell cultures. An additional table file and an additional figure file show this in more detail (see Supporting Information Table 2 and Supporting Information Fig. 1). First, when the autoAb production in the cell cultures was very high ($> 3 \times 10^4$ RLU), anti-ENA and anti-dsDNA Abs contained in the culture supernatants could also be demonstrated by means of IIF on HEp-2 and *Crithidia luciliae* substrates, respectively (Supporting Information Figure 1B,C). Also, the autoAb level present in supernatants from non-T PBMC cultures from 10 healthy controls (see Supporting Information Table 2) did not exceed the autoAb background using culture medium alone (287 ± 20 and 345 ± 14 RLU for anti-ENA and anti-dsDNA Abs, respectively, mean ± 3 SD, $n = 10$, data not shown). In addition, supernatants from non-T PBMC cultures from SLE patients with positive serum tests for anti-ENA Abs, but not for anti-dsDNA Abs, showed only anti-ENA production and vice versa (Supporting Information Table 2). Furthermore, non-T PBMC cultures from 10 patients diagnosed with rheumatoid arthritis and with serum anti-citrullinated peptide Abs (ACPA) did not produce either anti-ENA or anti-dsDNA Abs (Supporting Information Table 2). As an additional control, three healthy individuals were immunized with tetanus-diphtheria toxoids and acellular pertussis and, after 7 days, when a high number of specific and non-specific Ig-secreting cells are released into the circulation [25, 26], $CD19^{low}CD38^{high}$ ASCs were sorted and cultured in the absence of stimulation, and anti-ENA, anti-dsDNA and anti-tetanus toxoid (TT) Abs were quantified in the culture supernatants. Although an anti-TT response was developed in the cultures, neither anti-dsDNA nor anti-ENA Abs were produced (Supporting Information Table 2).

Statistical analysis

Significant differences were established by Mann–Whitney U test or Wilcoxon test for unpaired or paired samples, respectively. Correlations were examined by Spearman's rank correlation test. *P*-values lower than 0.05 were considered statistically significant.

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References

1 **Lipsky, P. E.**, Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. *Nat. Immunol.* 2001. **2**: 764–766.

2 **Han, S., Zhuang, H., Shumyak, S., Yang, L. and Reeves, W. H.**, Mechanisms of autoantibody production in systemic lupus erythematosus. *Front. Immunol.* 2015. **6**: 228.

3 **Tipton, C. M., Fucile, C. F., Darce, J., Chida, A., Ichikawa, T., Gregoretti, I., Schieferl, S. et al.**, Diversity, cellular origin and autoreactivity of antibody-secreting cell population expansions in acute systemic lupus erythematosus. *Nat. Immunol.* 2015. **16**: 755–765.

4 **Shen, L., Zhang, H., Caimol, M., Benike, C. J., Chakravarty, E. F., Strober, S. and Engleman, E. G.**, Invariant natural killer T cells in lupus patients promote IgG and IgG autoantibody production. *Eur. J. Immunol.* 2015. **45**: 612–623.

5 **Hahn, B. H.**, Antibodies to DNA. *N. Engl. J. Med.* 1998. **338**: 1359–1368.

6 **Hiepe, F. and Radbruch, A.**, Plasma cells as an innovative target in autoimmune disease with renal manifestations. *Nat. Rev. Nephrol.* 2016. **12**: 232–240.

7 **Cassese, G., Arce, S., Hauser, A. E., Lehnert, K., Moewes, B., Mostarac, M., Muehlinghaus, G. et al.**, Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J. Immunol.* 2003. **171**: 1684–1690.

8 **Radbruch, A., Muehlinghaus, G., Luger, E. O., Inamine, A., Smith, K. G., Dorner, T. and Hiepe, F.**, Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat. Rev. Immunol.* 2006. **6**: 741–750.

9 **Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B. I. and Nagasawa, T.**, Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* 2004. **20**: 707–718.

10 **Tokoyoda, K., Hauser, A. E., Nakayama, T. and Radbruch, A.**, Organization of immunological memory by bone marrow stroma. *Nat. Rev. Immunol.* 2010. **10**: 193–200.

11 **Rodriguez-Bayona, B., Ramos-Amaya, A., Lopez-Blanco, R., Campos-Caro, A. and Brieva, J. A.**, STAT-3 activation by differential cytokines is critical for human in vivo-generated plasma cell survival and Ig secretion. *J. Immunol.* 2013. **191**: 4996–5004.

12 **Rodriguez-Bayona, B., Ramos-Amaya, A., Bernal, J., Campos-Caro, A. and Brieva, J. A.**, Cutting edge: IL-21 derived from human follicular helper T cells acts as a survival factor for secondary lymphoid organ, but not for bone marrow, plasma cells. *J. Immunol.* 2012. **188**: 1578–1581.

13 **Ramos-Amaya, A., Rodriguez-Bayona, B., Lopez-Blanco, R., Andujar, E., Perez-Alegre, M., Campos-Caro, A. and Brieva, J. A.**, Survival of human circulating antigen-induced plasma cells is supported by plasma cell-niche cytokines and T follicular helper lymphocytes. *J. Immunol.* 2015. **194**: 1031–1038.

14 **Fornek, J. L., Tygrett, L. T., Waldschmidt, T. J., Poli, V., Rickert, R. C. and Kansas, G. S.**, Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. *Blood* 2006. **107**: 1085–1091.

15 **Cassese, G., Lindenau, S., de Boer, B., Arce, S., Hauser, A., Riemekasten, G., Berek, C. et al.**, Inflamed kidneys of NZB / W mice are a major site for the homeostasis of plasma cells. *Eur. J. Immunol.* 2001. **31**: 2726–2732.

16 **Hiepe, F., Dorner, T., Hauser, A. E., Hoyer, B. F., Mei, H. and Radbruch, A.**, Long-lived autoreactive plasma cells drive persistent autoimmune inflammation. *Nat. Rev. Rheumatol.* 2011. **7**: 170–178.

17 **Rodriguez-Bayona, B., Perez-Venegas, J. J., Rodriguez, C. and Brieva, J. A.**, CD95-Mediated control of anti-citrullinated protein/peptides antibodies (ACPA)-producing plasma cells occurring in rheumatoid arthritis inflamed joints. *Rheumatology (Oxford)* 2007. **46**: 612–616.

18 **Segundo, C., Rodriguez, C., Aguilar, M., Garcia-Poley, A., Gavilan, I., Bellas, C. and Brieva, J. A.**, Differences in thyroid-infiltrating B lymphocytes in patients with Graves' disease: relationship to autoantibody detection. *Thyroid* 2004. **14**: 337–344.

19 **Mahevas, M., Patin, P., Huetz, F., Descatoire, M., Cagnard, N., Bole-Feysot, C., Le Gallou, S. et al.**, B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells. *J. Clin. Invest.* 2013. **123**: 432–442.

20 **Espeli, M., Bokers, S., Giannico, G., Dickinson, H. A., Bardsley, V., Fogo, A. B. and Smith, K. G.**, Local renal autoantibody production in lupus nephritis. *J. Am. Soc. Nephrol.* 2011. **22**: 296–305.

21 **Kinslow, J. D., Blum, L. K., Deane, K. D., Demoruelle, M. K., Okamoto, Y., Parish, M. C., Kongpachith, S. et al.**, Elevated IgA Plasmablast Levels in Subjects at Risk of Developing Rheumatoid Arthritis. *Arthritis Rheum.* 2016. **68**: 2372–2383.

22 **Kikuchi, Y., Koarada, S., Tada, Y., Ushiyama, O., Morito, F., Suzuki, N., Ohta, A. et al.**, RP105-lacking B cells from lupus patients are responsible for the production of immunoglobulins and autoantibodies. *Arthritis Rheum.* 2002. **46**: 3259–3265.

23 **Suzuki, N., Sakane, T. and Engleman, E. G.**, Anti-DNA antibody production by CD5+ and CD5- B cells of patients with systemic lupus erythematosus. *J. Clin. Invest.* 1990. **5**: 238–247.

24 **Casali, P., Burastero, S. E., Balow, J. E. and Notkins, A. L.**, High-affinity antibodies to ssDNA are produced by CD-B cells in systemic lupus erythematosus patients. *J. Immunol.* 1989. **143**: 3476–3483.

25 **Brieva, J. A., Roldan, E., Rodriguez, C. and Navas, G.**, Human tonsil, blood and bone marrow in vivo-induced B cells capable of spontaneous and high-rate immunoglobulin secretion in vitro: differences in the requirements for factors and for adherent and bone marrow stromal cells, as well as distinctive adhesion molecule expression. *Eur. J. Immunol.* 1994. **24**: 362–366.

26 **Gonzalez-Garcia, I., Ocana, E., Jimenez-Gomez, G., Campos-Caro, A. and Brieva, J. A.**, Immunization-induced perturbation of human blood plasma cell pool: progressive maturation, IL-6 responsiveness, and high PRDI-BF1/BLIMP1 expression are critical distinctions between antigen-specific and nonspecific plasma cells. *J. Immunol.* 2006. **176**: 4042–4050.

27 **Medina, F., Segundo, C., Campos-Caro, A., Gonzalez-Garcia, I. and Brieva, J. A.**, The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. *Blood* 2002. **99**: 2154–2161.

28 **Bombardier, C., Gladman, D. D., Urowitz, M. B., Caron, D. and Chang, C. H.**, Derivation of the SLEDAI. A disease activity index for lupus patients.

The Committee on Prognosis Studies in SLE. *Arthritis Rheum.* 1992. **35**: 630–640.

29 **Gladman, D., Ginzler, E., Goldsmith, C., Fortin, P., Liang, M., Urowitz, M., Bacon, P. et al.**, The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum.* 1996. **39**: 363–369.

30 **Hanaoka, H., Okazaki, Y., Satoh, T., Kaneko, Y., Yasuoka, H., Seta, N. and Kuwana, M.**, Circulating anti-double-stranded DNA antibody-secreting cells in patients with systemic lupus erythematosus: a novel biomarker for disease activity. *Lupus* 2012. **21**: 1284–1293.

31 **Smith, G., Skinner, R. P., Bliss, J. and Maddison, P. J.**, In vitro autoantibody synthesis by anti-La(SSB) positive SLE patients and healthy controls. *Clin. Exp. Rheumatol.* 1986. **4**: 209–216.

32 **Salazar-Camarena, D. C., Ortiz-Lazareno, P. C., Cruz, A., Oregon-Romero, E., Machado-Contreras, J. R., Munoz-Valle, J. F., Orozco-López, M. et al.**, Association of BAFF, APRIL serum levels, BAFF-R, TACI and BCMA expression on peripheral B-cell subsets with clinical manifestations in systemic lupus erythematosus. *Lupus* 2016. **25**: 582–592.

33 **Kim, J., Gross, J. A., Dillon, S. R., Min, J. K. and Elkon, K. B.**, Increased BCMA expression in lupus marks activated B cells, and BCMA receptor engagement enhances the response to TLR9 stimulation. *Autoimmunity* 2011. **44**: 69–81.

34 **Rodriguez-Bayona, B., Ramos-Amaya, A., Perez-Venegas, J. J., Rodriguez, C. and Brieva, J. A.**, Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in systemic lupus erythematosus patients. *Arthritis Res. Ther.* 2010. **12**: R108.

35 **Petri, M., Stohl, W., Chatham, W., McCune, W. J., Chevrier, M., Ryel, J., Recta, V. et al.**, Association of plasma B lymphocyte stimulator levels and disease activity in systemic lupus erythematosus. *Arthritis Rheum.* 2008. **58**: 2453–2459.

36 **Koyama, T., Tsukamoto, H., Miyagi, Y., Himeji, D., Otsuka, J., Miyagawa, H., Harada, M. et al.**, Raised serum APRIL levels in patients with systemic lupus erythematosus. *Ann. Rheum. Dis.* 2005. **64**: 1065–1067.

37 **Linker-Israeli, M., Deans, R. J., Wallace, D. J., Prehn, J., Ozeri-Chen, T. and Klinenberg, J. R.**, Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. *J. Immunol.* 1991. **147**: 117–123.

38 **Wang, A., Fairhurst, A. M., Tus, K., Subramanian, S., Liu, Y., Lin, F., Igarashi, P. et al.**, CXCR4/CXCL12 hyperexpression plays a pivotal role in the pathogenesis of lupus. *J. Immunol.* 2009. **182**: 4448–4458.

39 **Stohl, W.**, Inhibition of B cell activating factor (BAFF) in the management of systemic lupus erythematosus (SLE). *Expert Rev. Clin. Immunol.* 2017. **4**. https://doi.org/10.1080/1744666X.2017.1291343.

40 **Yamamoto, M., Yokoyama, Y., Shimizu, Y., Yajima, H., Sakurai, N., Suzuki, C., Naishiro, Y. et al.**, Tofacitinib can decrease anti-DNA antibody titers in inactive systemic lupus erythematosus complicated by rheumatoid arthritis. *Mod. Rheumatol.* 2015. **3**: 1–2.

41 **Wang, X., Wei, Y., Xiao, H., Liu, X., Zhang, Y., Han, G., Chen, G. et al.**, A novel IL-23p19/Ebi3 (IL-39) cytokine mediates inflammation in Lupus-like mice. *Eur. J. Immunol.* 2016. **46**: 1343–1350.

42 **Szyszko, E. A., Brokstad, K. A., Oijordsbakken, G., Jonsson, M. V., Jonsson, R. and Skarstein, K.**, Salivary glands of primary Sjogren's syndrome patients express factors vital for plasma cell survival. *Arthritis Res. Ther.* 2011. **13**: R2.

43 **Neves, M. and Alves, J. D.**, Factors implicated in the generation and persistence of long-lived plasma cell-mediated autoimmunity. *Autoimmun. Rev.* 2011. **10**: 375–382.

44 **Thanou, A. and Merrill, J. T.**, Treatment of systemic lupus erythematosus: new therapeutic avenues and blind alleys. *Nat. Rev. Rheumatol.* 2014. **10**: 23–34.

Abbreviations: **Ab**: antibody · **APRIL**: a proliferation-inducing ligand · **ASC**: antibody-secreting cell · **AutoAb**: auto-antibody · **BAFF**: B-cell activating factor · **CHX**: cycloheximide · **CLIA**: chemiluminescence · **dsDNA**: double-stranded DNA · **ENA**: extractable nuclear antigen · **ESR**: erythrocyte sedimentation rate · **Ig**: immunoglobulin · **IIF**: indirect immunofluorescence · **mAbs**: monoclonal Abs · **non-T**: T cell-depleted · **PB**: plasmablast · **PC**: plasma cell · **PBMC**: peripheral blood mononuclear cell · **RA**: rheumatoid arthritis · **RLU**: relative luminescence units · **SLE**: Systemic Lupus Erythematosus · **SLEDAI**: SLE Disease Activity Index · **SLICC/ACR**: The Systemic Lupus International Collaborative Clinics/American College of Rheumatology Damage Index · **TT**: anti-tetanus toxoid

Full correspondence: Dr. Carmen Rodríguez, Servicio de Inmunología. Hospital Universitario Puerta del Mar., Avda. Ana de Viya 21. 11009 Cádiz. España
Fax: 34-956-002218
e-mail: carmen.rodriguez.sspa@juntadeandalucia.es

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