Author Manuscript Author Manuscript Author Manuscript Author Manuscript

HHS Public Access
Author manuscript
Arthritis Rheum. Author manuscript; available in PMC 2015 June 24.

Published in final edited form as:
Arthritis Rheum. 2013 April ; 65(4): 1085–1096. doi:10.1002/art.37828.

Expansion of autoreactive unresponsive CD21$^{-/low}$ B cells in Sjögren's syndrome associated lymphoproliferation

D Saadoun[1,2,*], **B Terrier**[1,*], **J Bannock**[3], **T Vazquez**[1], **C Massad**[3], **I Kang**[4], **F Joly**[5], **M Rosenzwajg**[1], **D Sene**[2], **P Benech**[5], **D Klatzmann**[1], **E Meffre**[3,†], and **P Cacoub**[1,2,†]

[1]I3 “Immunology, Immunopathology, Immunotherapy” laboratory, CNRS UMR 7211 and INSERM U959; Groupe, Hospitalier Pitié-Salpétrière, Université Pierre et Marie Curie, Paris 6, Paris, France

[2]Department of Internal Medicine; Groupe, Hospitalier Pitié-Salpétrière, Université Pierre et Marie Curie, Paris 6, Paris, France

[3]Department of Immunobiology, Yale University School of Medicine, 300 George Street, New Haven, Connecticut 06511, USA

[4]Department of Rheumatology, Yale University School of Medicine, 300 George Street, New Haven, Connecticut 06511, USA

[5]P3S post-genomic plateform, Groupe, Hospitalier Pitié-Salpétrière, Université Pierre et Marie Curie, Paris 6, Paris, France; Prediguard, Marseille, France

Abstract

Background—Primary Sjögren's syndrome (pSS) is the autoimmune disease associated with the higher risk of developing non-Hodgkin lymphoma.

Objective—To determine the nature of B cells driving lymphoproliferation in pSS.

Methods—B cell subsets and function were analyzed in peripheral blood from 66 adult patients with pSS [including 14 patients with B-cell lymphoproliferative disorder (LPD)] and 30 healthy donors, using flow cytometry, calcium mobilization, and gene array analysis. We tested by ELISA the reactivity of recombinant antibodies isolated from single B cells from pSS-LPD.

Results—We report here the expansion of an unusual CD21$^{-/low}$ B-cell population which correlates with lymphoproliferation in pSS patients. A majority of CD21$^{-/low}$ B cells from pSS patients expressed autoreactive antibodies, which recognized nuclear and cytoplasmic structures. These B cells belonged to the memory compartment because their immunoglobulin genes were mutated. They were unable to induce calcium flux, become activated, or proliferate in response to

Correspondence: David Saadoun, MD, PhD, Department of Internal Medicine and Laboratory I3 “Immunology, Immunopathology, Immunotherapy”, UMR CNRS 7211, INSERM U959, Groupe Hospitalier Pitié-Salpetrière, Université Pierre et Marie Curie, Paris 6, Paris, France. Tel: + 33 1 42 17 80 08; Fax: + 33 1 42 17 80 33. david.saadoun@psl.aphp.fr.
*Co-first authors
†These authors are co-authors.

Author contributions: D.S., B.T., J.B., T.V., C.M., F.J., W.C., M.R., P.B., D.K., E.M., and P.C. devised and performed experiments; D.S., D.Se., and P.C. provided patient referrals; D.S., B.T., F.J., E.M., and P.C. interpreted results; and D.S., B.T., E.M., and P.C. designed the research and wrote the paper.

Conflict of interest disclosures: The authors declare no competing financial interests.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

B-cell receptor and/or CD40 triggering, suggesting that these autoreactive B cells may be anergic. However, CD21$^{-/low}$ B cells from pSS remained responsive to TLR stimulation. Gene array analyses of CD21$^{-/low}$ B cells revealed molecules specifically expressed in these B cells and that are likely to induce their unresponsive stage.

Conclusion—pSS patients who display high frequencies of autoreactive and unresponsive CD21$^{-/low}$ B cells are susceptible for developing lymphoproliferation. These cells remain in peripheral blood controlled by functional anergy instead of being eliminated, and chronic antigenic stimulation through TLR stimulation may create a favorable environment for breaking tolerance and activating these cells.

Keywords

Sjögren's syndrome; CD21$^{-/low}$ B cells; lymphoproliferation; autoimmunity; anergy

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease primarily characterized by chronic inflammation of the exocrine glands, in particular the salivary and lachrymal glands. Extraglandular manifestations occur in many patients and may involve almost any organ. B-lymphocyte hyperactivity in pSS is manifested by the presence of anti-SS-A and anti-SS-B antibodies, rheumatoid factor, cryoglobulins, and hypergammaglobulinemia. The threat that hangs over patients with pSS is the development of a lymphoma. Unfortunately, we have thus far no sufficient data to deal with such pertinent concerns. Prolonged B-cell survival and excessive B-cell activity, probably related to increased production of B-cell activating factor (BAFF) (1-3), may lead to lymphomas occurring in 5% of Sjögren's syndrome (SS) patients (4, 5). Significant predictors of lymphoproliferative disease in pSS include parotid, lymph node and/or splenic enlargement, monoclonal gammapathy, hypogammaglobulinemia, mixed cryoglobulinemia, palpable purpura, CD4^{+} T cell lymphopenia and/or reduced levels of C4 (6-9).

It is proposed that the first event of lymphomagenesis in Sjögren's syndrome is the chronic stimulation of polyclonal B cells secreting autoreactive antibodies, such as rheumatoid factor. Such autoreactive B cells may become monoclonal, leading to the occurrence of lymphoproliferations. The following step would be a chromosomal abnormality, which would confer to these cells low grade B cell lymphoma comportment (10). The non-random utilization of VH and VL by Sjögren's syndrome associated lymphoma B cells (11, 12) and the demonstration that these lymphoma B cells may display rheumatoid factor activity (13) support the hypothesis that these lymphomas grow through an auto-antigen driven process.

We report here that an unusual CD21$^{-/low}$ B cell population correlates with the lymphoproliferative status in pSS patients. Because CD21 augments B-cell receptor (BCR)-mediated signaling as part of the B-cell coreceptor complex, its down-regulation may confer a state of anergy to these cells, as has been demonstrated among CD21$^{-/low}$ B cells in patients with rheumatoid arthritis (RA), common variable immunodeficiency (CVID) or hepatitis C associated cryoglobulinemia patients (14-16). These CD21$^{-/low}$ B cells are enriched in autoreactive clones that are unresponsive to BCR stimulation, suggesting that

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

these cells are controlled by the tolerizing mechanism of functional anergy. Gene array analyses of CD21$^{-/low}$ B cells revealed molecules specifically expressed in these B cells and that are likely to induce their unresponsive stage. These B cells belonged to the memory compartment because their immunoglobulin genes were mutated as reported in hepatitis C associated cryoglobulinemia patients (15, 16). Taken together, our data suggest that the induction of an unresponsive program in autoreactive B cells may represent risks to develop B cell lymphoproliferation in pSS.

Materials and methods

Study subjects

We recruited 66 patients with primary Sjögren syndrome (pSS) according to the European-American consensus criteria (17) (59 women and 7 men; mean ± SD age: 55.9 ± 14.5 years) including 14 patients with B-cell lymphoproliferative disorder (LPD). B-cell lymphoproliferation was defined by an overt B-cell non-Hodgkin lymphoma (B-NHL) according to the WHO classification (18) or as the presence of type II mixed cryoglobulinemia (7, 8) sometimes associated with lymph node or splenic enlargement. Seven patients had B-NHL including 4 marginal zone lymphomas, 1 lymphocytic and 2 undetermined. Among the 7 remaining patients with type II mixed cryoglobulinemia, 2 had lymph node enlargement and 2 had splenomegaly. Blood samples from 30 healthy donors (HD) were obtained from Etablissement Français du Sang (Hôpital Pitié-Salpêtrière). The study was performed according to the Declaration of Helsinki. All study subjects provided informed consent, in accordance with the Institutional Review Board.

Phenotypic analysis

Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation. Phenotypic analyses were performed with anti–human monoclonal antibodies: anti–human CD10, C19, CD20, CD21, CD22, CD44, CD69, CD86, HLA-DR, IgM, and Annexin V were obtained from Beckman Coulter; anti–human CD1c, CD25, CD27, CD38, CD84, CD95, BR3, Kappa light chain and IgD were obtained from BD Biosciences. FACS analyses were performed on a Navios flow cytometer using CXP analysis software (Beckman Coulter). CD19^{+} B lymphocytes counts (cells/μl) were established from fresh blood samples using CYTO-STAT tetraCHROME kits with Flowcount fluorescents beads as an internal standard and tetra CXP software with a Navios cytometer according to the manufacturer's instructions (Beckman Coulter, Villepinte, France).

B cell enrichment and cell sorting

CD27^{-} B cells were enriched from total PBMCs by negative magnetic bead selection using a cocktail of biotinylated antibodies against CD2, CD14, CD16, CD23, CD27, CD36, CD43 and Glycophorin A, followed by anti-Biotin MicroBeads (Naïve B cell isolation kit II, Miltenyi Biotec, Paris). The purity of CD27^{-} B cells was typically >95%. For proliferation assays, CD27^{-} B cells were separated into CD21^{+} and CD21$^{-/low}$ fractions using a one-step magnetic bead-based selection process. Cells were fractionated by CD21 with anti-CD21-PE

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(BD Biosciences), followed by anti-PE Microbeads (Miltenyi Biotec). The purity of $CD21^{+}$ and $CD21^{-/low}$ fractions was typically >85%.

For gene expression profile and single cell sorting, PBMCs were sorted by staining the cells with monoclonal anti-human antibodies against CD45-VioBlue, anti-CD27-PE (Miltenyi Biotec), CD21-FITC, CD19-ECD, and CD10-APC (Beckman Coulter). For single-cell PCR, $CD10^{-}CD27^{-}CD21^{-/low}CD19^{+}$ B cells from pSS patients were sorted on a FACSAria (Becton Dickinson) into 96-well PCR plates.

B cell activation, survival and proliferation

Enriched $CD27^{-}$ B cells, which contained $CD21^{+}$ and $CD21^{-/low}$ B cells from pSS patients, were plated at 500,000 cells per well in a 48-well plate in RPMI 10% serum and 20 μg/mL polyclonal $F(ab)'_2$ goat anti-human IgM (Jackson Immunoresearch), 1 μg/mL anti-human CD40 (Invitrogen), and/or 1 μg/mL CpG (Invivogen) for 48 hours. To assess B cell activation and survival, cells were stained with anti–human monoclonal antibodies: CD19, CD21, CD25, CD27, CD40, CD69 and CD95. The proportions of apoptotic and dead cells were assessed by flow cytometry to measure binding with annexin V using Annexin V-PE and 7-AAD (BD Pharmingen). To assess B cell proliferation, $CD21^{+}$ and $CD21^{-/low}$ MZ B cells were plated at 1×10^5 cells per well of a 96-well flat-bottom plate with various combinations of the following reagents: 20 μg/mL polyclonal $F(ab)'_2$ goat anti-human IgM; 1 μg/mL anti-CD40; 1 μg/mL CpG; 1 μg/mL of TLR3 agonist (name, company?) and 1 μg/mL of TLR7 agonist (name, company?). Cells were incubated for 48 hours and pulsed for 8 hours with tritiated thymidine.

Calcium Mobilization

Cells were resuspended in RPMI 1640 (GIBCO, Invitrogen) and stained with anti-CD21-PE, anti-CD19-ECD (Beckman Coulter), and anti-CD27-APC (BD Biosciences) antibodies for 30 min at 4°C. Cells were washed twice in RPMI and resuspended at 1×10^6 cells/mL. Cells were loaded with Fluo-4 AM (Invitrogen) at a final concentration of 5μM in the presence of 0.2% Pluronic F-127 (Sigma) for 30 min at room temperature. Cells were washed twice in RPMI-SVF 5% and resuspended at 1×10^6 cells/mL. [Ca2+]i was monitored over time by flow cytometry on gated $CD19^{+}CD27^{-}CD21^{+}$ and $CD19^{+}CD27^{-}CD21^{-/low}$ B cells. Baselines were read for 30 seconds, after which the cells were removed and stimulated with 20 μg/mL of $F(ab)'_2$ anti-IgM (Jackson ImmunoResearch Laboratories) then Ionomycin at 1mg/mL (Sigma-Aldrich).

Antibody production, ELISAs, and Immunofluorescence assays

Cloning strategy, expression vectors, and antibody reactivity against specific antigens were as described (19). Highly polyreactive ED38 was used as positive control in HEp-2 reactivity and polyreactivity enzyme-linked immunosorbent assays (ELISAs) (19). Antibodies were considered polyreactive when they recognized at least 2 and usually all of the 3 analyzed antigens that include double-stranded DNA (dsDNA), insulin, and lipopolysaccharide (LPS). All recombinant antibodies were also tested for rheumatoid factor reactivity (anti-IgG) as previously described (20). For indirect immunofluorescence assays, HEp-2 cell–coated slides (Bion Enterprises Ltd) were incubated in a moist chamber at room

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

temperature with purified recombinant antibodies at 50 to 100 μg/mL. FITC-conjugated goat anti–human IgG was used as detection reagent. Pictures were taken with an Axioskop (Zeiss) using a Plan-Neofluar 40×/.75 objective and Axiovision 3.1 acquisition software.

Microarray gene expression profile analysis

RNA was extracted from 10^5-3.10^5 batch-sorted conventional $IgM^+CD27^-CD21^+CD19^+$ B cells and $IgM^+CD27^-CD21^{-/low}CD19^+$ B cells using the NucleoSpin RNA II kit (Macherey-Nagel, Hoerd, France). Each sample contained 50-200 ng of RNA, and the quality of the purified RNA was assessed with the 2100 Bioanalyzer from Agilent. Using the Illumina TotalPrep RNA Amplification Kit (Applied Biosystems), 50 ng of RNA was amplified and labeled to produce cDNA. Labeled cDNA was hybridized on whole human genome chips (HumanHT-12 v3 Expression BeadChip Kit, Illumina).

PredictSearch™, a powerful bioinformatic solution dedicated to identifying relevant correlations between genes and concepts, was used to generate functional networks. This tool is updated daily with the whole NCBI Pubmed database and seeks relevant correlations between gene-gene or gene-concept within abstracts. It also enables an oriented search for co-citations between genes with action words such as “activation” or “repression” and their semantic variants. PredictSearch™ software was also implemented with the whole set (>18000) of transcriptional signatures deposited in the NCBI GEO database and extracted with the DBF-MCL algorithm using TranscriptomeBrowser tool (21). Furthermore PredictSearch™ provides direct access to already published gene pathways.

Statistical analyses

Data are expressed as the mean plus or minus standard deviation (SD) or median. Categorical variables were compared using the Fisher's exact or Chi-square tests, and continuous variables were compared using the t-test or Mann-Whitney U test when appropriate. All tests were 2-sided at a 0.05 significance level. Graphing and statistical analyses were performed using Prism software (GraphPad Software, Inc.).

Results

pSS patients display an unusual expansion of peripheral $CD27^-CD21^{-/low}$ B cells

We first examined B cell subpopulations in pSS patients. We found an increase of the $CD27^-$B cell subset in pSS and pSS-LPD patients compared to HD (78.1% and 76.8% vs. 64.6% of cells among $CD19^+$ B cell subset, $P<0.001$ and $P<0.01$ respectively) (Figures 1A and 1B) but not in absolute number in which $CD27^-$ B cells were decreased in pSS and pSS-LPD (108.4 and 96.4 cells/μL vs. 129.2 cells/μL, $P<0.0001$ for both) (Figures 1A and 1B). The analysis of CD21 expression on B cells from pSS and pSS-LPD patients and HD showed an increased of $CD27^-CD21^{-/low}$ B cells both in percentage-wise (medians) and in absolute numbers in pSS and pSS-LPD patients compared to HD (8.7% and 25.9% vs. 3.1% of cells among $CD19^+$ B cell subset, $P<0.0001$ for both; and 12.8 and 42.7 cells/μL vs. 6.2 cells/μL, $P<0.0001$ for both) (Figures 1C and 1D).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

$CD27^{-}CD21^{-/low}$ B cells were significantly higher in pSS-LPD patients compared to pSS patients without LPD (P<0.001). The expression of IgD and IgM was analyzed on $CD27^{-}CD21^{+}$ and $CD27^{-}CD21^{-/low}$ B cells from pSS patients. $CD27^{-}CD21^{-/low}$ B cells displayed decreased expression of IgD and IgM compared to conventional $CD27^{-}CD21^{+}$ B cells. While $CD27^{-}CD21^{-/low}$ B cells comprised IgD^{+} and IgD^{-} cells, 92.5% of cells were positive for surface IgM (Figure 1E). $CD27^{-}CD21^{-/low}$ B cells expressed similar MFI intensity of IgD than $CD27^{-}CD21^{+}$ B cells. The proportion of IgD+ cells was lower in CD27-CD21-low cells as compared to $CD27^{-}CD21^{+}$ B cells (Figure 1E). In addition, $CD27^{-}CD21^{-/low}$ B cells expressed lower levels of surface IgM (MFI 27.9 vs. 46.6, P<0.001) when compared with $CD27^{-}CD21^{+}$ B cells from the same patients (Figure 1F). Four pSS-LPD patients had a low C4 complement level but there were no correlation between $CD27^{-}CD21^{-/low}$ B cells expansion and complement deficiency. We conclude that $CD27^{-}CD21^{-/low}$ B cells are expanded in pSS patients, especially those displaying lymphoproliferation features.

$CD27^{-}CD21^{-/low}$ cells display a unique B cell phenotype

In order to provide a more precise definition of the phenotype of this expanded population, $CD27^{-}CD21^{-/low}$ B cells were screened by flow cytometry for characteristic B cell markers, maturity markers and activation/proliferation markers, allowing a comparison with conventional $CD27^{-}CD21^{+}$ B cells from the same patients. $CD27^{-}CD21^{-/low}$ B cells expressed higher levels of CD19 and CD20 when compared with $CD27^{-}CD21^{+}$ B cells from the same patients, whereas they expressed lower levels of BAFF receptor (BAFF-R) and κ light-chain (Figure 1G). $CD27^{-}CD21^{-/low}$ B cells did not express markers found on immature B cells such as the B cell progenitor marker CD10 and, like mature cells, expressed only low levels of the development marker CD38. These markers are commonly used to distinguish new emigrants immature/transitional B cells (22). Furthermore, the $CD27^{-}CD21^{-/low}$ and conventional $CD27^{-}CD21^{+}$ B cell populations were both positive for the BCR-associated regulator CD22 that is expressed on mature B cells (23), and for other molecules found only on mature circulating B cells including CD40 and CD44. Lastly, analysis of $CD27^{-}CD21^{-/low}$ B cells for signs of recent activation in vivo demonstrated an increased expression of CD69, CD86 and CD95 compared to conventional $CD27^{-}CD21^{+}$ B cells of the same patients (Figure 1G). Furthermore, $CD27^{-}CD21^{-/low}$ B cells were not proliferating as assessed by the Ki67 expression. Hence, $CD27^{-}CD21^{-/low}$ B cells show a midly activated B cell phenotype potentially associated to chronic stimulation.

$CD21^{-/low}$ B cells in pSS express highly autoreactive antibodies

$CD27^{-}CD21^{-/low}$ B cells with a similar phenotype have been reported in several autoimmune.diseases (14, 15, 24, 25). $CD27^{-}CD21^{-/low}$ B cells from RA and CVID patients expressed mostly unmutated IgMs whereas those from patients with hepatitis C virus associated with mixed cryoglobulinemia harbor mutated Ig genes (14-16). To assess if $CD27^{-}CD21^{-/low}$ B cells from pSS patients belong to the naïve or memory B cell fraction, we analyzed the immunoglobulin repertoire and reactivity of these B cells using a single B cell cloning approach (19). We found that most $CD27^{-}CD21^{-/low}$ B cells from 4 pSS patients expressed diverse IgM antibodies, the majority of which were mutated suggesting that these B cells belonged to the memory compartment (Tables S1, S2, S3 and S4). In addition,

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

somatic hypermutations were mostly located in Ig CDRs as in conventional memory B cells, suggesting that $CD27^{-}CD21^{-/low}$ B cells were antigen selected (Supplemental Figure 1) (Reference Wardemann, Immunity IgG reactivity and SHM in CDR3s). Many $CD27^{-}CD21^{-/low}$ B cells expressed HEp-2-reactive and polyreactive antibodies (Figure 2, A and B), including some with rheumatoid factor (anti-IgG) reactivity (Figure 2 C). $CD27^{-}CD21^{-/low}$ B cells often expressed antibodies recognizing cytoplasmic and to a lesser extent nuclear structures with diverse staining patterns (Figure 2, D and E). We conclude that $CD27^{-}CD21^{-/low}$ B cells from pSS patients are enriched in autoreactive B cell clones that may have been selected by self-antigens..

$CD27^{-}CD21^{-/low}$ B cells reveal defects in BCR and CD40-mediated activation

We analyzed the function of $CD27^{-}CD21^{–/low}$ B cells after BCR (using $F(ab)'_2$ anti-IgM), CD40 (using CD40L) and/or TLR9 (using CpG) triggering by studying the expression of CD25, CD69, CD40 and CD95 on $CD27^{-}CD21^{–/low}$ and conventional $CD27^{-}CD21^{+}$ B cells from pSS patients (Figure 3). BCR, CD40, and/or TLR3, TLR7 and TLR9 triggering induced the expression of CD25, CD69, CD40 on $CD27^{-}CD21^{+}$ B cells from pSS patients, but only resulted in a slight expression of CD95. In contrast, BCR and/or CD40 triggering of $CD27^{-}CD21^{–/low}$ B cells from the same patients failed to properly induce the expression of CD25, CD69 and CD40, while the use of an alternative pathway through TLR3, TLR7 and TLR9 triggering strongly induced the expression of CD25 and CD69 compared with $CD27^{-}CD21^{+}$ B cells. In addition, $CD27^{-}CD21^{–/low}$ B cells exhibited increased CD95 expression after BCR, CD40, or TLR3, TLR7 and TLR9 stimulation compared with $CD27^{-}CD21^{+}$ B cells. Thus, $CD27^{-}CD21^{–/low}$ and $CD27^{-}CD21^{+}$ B cells display distinct B-cell responses after stimulation, and $CD27^{-}CD21^{-/low}$ B cells reveal defects in BCR and CD40-mediated activation but not after TLR triggering (Figure 3).

We next analyzed the ability of $CD27^{-}CD21^{–/low}$ B cells upon BCR stimulation to induce intracellular calcium flux compared with conventional $CD27^{-}CD21^{+}$ B cells. In contrast to $CD27^{-}CD21^{+}$ B cells from pSS patients, BCR triggering induced only a weak elevation of intracellular calcium concentration in response to BCR stimulation in $CD27^{-}CD21^{-/low}$ B cells. Hence, $CD27^{-}CD21^{-/low}$ B cells do not exhibit proper calcium flux after BCR stimulation (Figure 4).

$CD27^{-}CD21^{-/low}$ B cells are prone to dying by apoptosis and do not proliferate after BCR and CD40 triggering

We assessed apoptosis of $CD27^{-}CD21^{-/low}$ B cells by analyzing annexin V and 7AAD staining. We found that freshly isolated $CD27^{-}CD21^{-/low}$ B cells from pSS patients contained a higher frequency of annexin $V^{+}7AAD^{-}$ early apoptotic cells and annexin $V^{+}7AAD^{+}$ late apoptotic and necrotic cells compared with conventional $CD27^{-}CD21^{+}$ B cells (Supplemental Figure 2A and 2B). Compared with medium, BCR, CD40 and/or TLR9 triggering was not able to rescue $CD27^{-}CD21^{-/low}$ B cells from apoptosis. In contrast, stimulated $CD27^{-}CD21^{+}$ B cells contained lower levels of annexin V^{+} expressing cells (Supplemental Figure 2A and 2B). Thus, $CD27^{-}CD21^{-/low}$ B cells are more susceptible to apoptosis than $CD27^{-}CD21^{+}$ B cells and are poorly rescued by BCR, CD40 and/or TLR9 triggering. We next studied the proliferation induced by BCR and TLR triggering using the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

incorporation of tritiated thymidine. $CD27^{-}CD21^{-/low}$ B cells showed decreased cell proliferation compared with $CD27^{-}CD21^{+}$ B cells, mainly after BCR triggering and to a lesser extent after TLR9 stimulation (Supplemental Figure 2C). Thus, $CD27^{-}CD21^{-/low}$ B cells exhibit increased apoptosis and decreased proliferation after stimulation, features that are commonly associated with an anergic phenotype.

Gene expression profiles of $CD27^{-}CD21^{-/low}$ B cells in pSS patients reveal the selective up-regulation of an anergic pathway

In order to define gene signatures associated with expansion, phenotype and function of $CD27^{-}CD21^{-/low}$ B cells, we compared gene expression profiles expressed in $CD27^{-}CD21^{-/low}$ B cells versus conventional $CD27^{-}CD21^{+}$ B cells isolated from 4 pSS patients (Figure 5A). A total of 2510 transcripts were found to be differentially expressed between the two $CD27^{-}$ B-cell subpopulations, including 1934 up-regulated (> 1.5-fold change) and 576 down-regulated transcripts (< 0.7-fold change) in $CD27^{-}CD21^{-/low}$ B cells. Focusing on the transcriptional modulations that discriminate $CD27^{-}CD21^{-/low}$ from $CD27^{-}CD21^{+}$ B cells in the same patients, we identified transcriptional signatures that are likely to underlie the functional properties of these cells. Hierarchical clustering of differentially expressed genes between $CD27^{-}CD21^{-/low}$ and $CD27^{-}CD21^{+}$ B cells highlighted an anergy-related transcriptional signature that is specifically up-regulated in $CD27^{-}CD21^{-/low}$ B cells (Figures 5A and 5B). The gene content of this transcriptional module is enriched in genes encoding cell surface and intracellular proteins that are involved in signal transduction and in T cell co-stimulation, such as CD83, CD86/B7-2 and CD72. CBL-B, which is a potent negative regulators of intracellular signaling, is also strongly induced in the $CD27^{-}CD21^{-/low}$ versus $CD27^{-}CD21^{+}$ B cells. CD72 was shown to function as a regulator of peripheral B cell tolerance, by regulating and being associated with CBL-B, leading to the down-regulation of BCR signaling. Similarly, $CD27^{-}CD21^{-/low}$ B cells up-regulated the transcription of a whole set of immunoreceptor tyrosine–based inhibition motif (ITIM) receptor genes, which are likely to inhibit B-cell activation and proliferation, including CD72, SIGLEC, CD22 and FCRL2 genes. We also found a selective up-regulation of FCRL3, which was suggested to play a pivotal role in autoimmunity. Finally, up-regulation of RFNT1/Raftlin, the B cell-specific major raft protein that is necessary for the integrity of lipid rafts and BCR signal transduction, further highlights the related functional involvement of the genes of this transcriptional signature. Through flow cytometry, we confirmed at the protein level that $CD27^{-}CD21^{-/low}$ B cells up-regulated FCRL2, FCRL3, CD72, CD22, CD11c, and down-regulated CD1c (Figure 6). Taken together, transcriptomic analysis of $CD27^{-}CD21^{-/low}$ B cells highlights the selective up-regulation of genes that belong to an inhibitory pathway, which could explain in part the functional anergy of $CD27^{-}CD21^{-/low}$ B cells.

Discussion

We report herein on the identification of a unique autoreactive B cell population in pSS lacking CD21 expression and refractory to B-cell stimulation that correlates with the lymphoproliferative status. Unresponsive $CD21^{-/low}$ B cells expressing IgMs have been identified in RA and CVID patients as well as in patients with hepatitis C virus-associated

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

mixed cryoglobulinemia (14-16). The analysis of the immunoglobulin repertoire of CD21$^{-/low}$ B cells from pSS patients demonstrated that these B cells belonged to the memory compartment because their immunoglobulin genes were mutated. Hence, CD21$^{-/low}$ B cells from pSS patients are similar to anergic CD21$^{-/low}$ B cells from patients with hepatitis C virus-associated mixed cryoglobulinemia, which also expressed mutated IgMs (15, 16), whereas most CD21$^{-/low}$ B cells from RA and CVID patients expressing unmutated immunoglogulins likely belong to the naïve B cell compartment (14, 24). However, CD21$^{-/low}$ B cells from pSS patients differ from their counterpart in patients with hepatitis C virus-associated mixed cryoglobulinemia, in that they do not express CD27 (15, 16). Nonetheless, CD21$^{-/low}$ B cells from all patients are refractory to most stimulation, suggesting that autoreactive CD21$^{–/lo}$ B cells may use a common anergic program to be tolerized. Indeed, the phenotype of CD21$^{-/low}$ B cells revealed the modulation of the expression of many molecules that may prevent the activation of these B cells. The down-regulation of the complement receptor CR2/CD21 previously reported on mouse and human anergic B cells is likely to result in an increased BCR signaling threshold and compromise the ability of CD21$^{-/low}$ B cells to be activated (14-16, 26, 27). In addition, CD21 is able to break anergy in mouse B cells, further suggesting that its down-regulation contributes to an anergic stage in mice and humans (14-16, 26, 28, 29). In pSS, CD21$^{-/low}$ B cells were highly enriched with autoreactive clones. In addition, gene array experiments analyzing CD21$^{-/low}$ B cells from pSS patients affected the expression of genes such as CD19, CD22, CD72, and FCRL3, genes that have been found to be involved in the development of many autoimmune diseases (30-32). These genes encoded molecules belonging to important pathways leading to B cell activation, including those initiated by the BCR. The upregulated transcription of CD86, CD72, GRB2, CBL-B, and FCRL2 genes is consistent with an anergy-like phenotype. CD86 as well as CD69 and CD95 expression normally induced on activated B cells may reflect the chronic stimulation status of autoreactive CD27^{-}CD21$^{–/low}$ B cells in pSS patients. CD72 was shown to function as a regulator of peripheral B cell tolerance, by regulating and being associated with CBL-B, leading to the down-regulation of BCR signaling (33). Interestingly, CBL-B expression was shown to be a key protein required for induction of anergy in T cells (34). The upregulated transcription of immunoreceptor tyrosine–based inhibition motif (ITIM) receptor genes such as CD72, CD22, FCRL2 and SIGLEC are also likely to inhibit B-cell activation and proliferation. The validation of many gene upregulations by flow cytometry further demonstrates the functional unresponsiveness of CD27^{-}CD21$^{–/low}$ B cells in pSS. Indeed, CD21$^{-/low}$ B cells from pSS patients showed impaired calcium-mediated signaling, did not up-regulate activation markers, and did not proliferate in response to BCR triggering. CD21$^{-/low}$ B cells were also prone to die faster than their CD21^{+} counterparts, suggesting that they have a shorter half-life. In addition, the increased CD95 expression CD21$^{-/low}$ B cells could be responsible for their increased susceptibility to cell death. Most of these features are characteristic of mouse anergic B cells (35) and of more recently reported anergic human B cells (14-16).

Lastly, we observed that autoreactive CD27^{-}CD21$^{–/low}$ B cells were functionally attenuated after BCR and/or CD40 triggering but not after TLR stimulation as reported in patients with hepatitis C virus-associated mixed cryoglobulinemia (16). Leadbetter et al. showed that activation of autoreactive RF^{+} B cells was mediated by immune complexes and requires the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

synergistic engagement of the BCR and members of the MyD88-dependent TLR family (TLR7, TLR8 and TLR9); this thus establishes a critical link between the innate and adaptive immune systems in the development of systemic autoimmune disease (36). Activation in this mode is therefore likely to be a fundamental event in the loss of peripheral B-cell tolerance in a wide variety of settings, and these data establish a critical role for endogenous TLR ligands in aberrant activation of the adaptive immune system in autoimmunity. The chronic stimulation of polyclonal B cells secreting autoreactive antibodies, such as rheumatoid factor is proposed to be the first event of lymphomagenesis in Sjögren's syndrome (10). Interestingly, pSS and hepatitis C virus-associated mixed cryoglobulinemia share increased production of B-cell activating factor (BAFF) (2, 3), and an increased risk of lymphoma, particularly mucosa-associated lymphoid tissue lymphomas (4, 5, 37). Recalling what has been observed in hepatitis C associated mixed cryoglobulinemia, the hypothesis of a viral trigger has long been suspected in pSS. Different studies reported that Epstein-Barr virus (EBV) genome and proteins were detected in salivary glands of patients with pSS (38-40). EBV encoded small RNA (EBER), a double strand RNA, complexes with SSB to induce TLR3-related secretion of type I interferon (IFN) and other proinflammatory cytokines (40). Although it is unclear at this point if infectious agents or self-antigens may favor the emergence of $CD21^{-/low}$ B cells in pSS patients, their pattern of somatic hypermutations with replacement mutations located in CDRs interacting with antigens highly suggests that these B cells followed an antigen-driven selection as reported for conventional memory B cells (Ref Wardemann Immunity IgG reactivity and repertoire). Then, these autoreactive B cells may give rise to the emergence of a preferential clone, leading to the occurrence of lymphoproliferations. The demonstration that these lymphoma B cells may display rheumatoid factor activity (13) supports the hypothesis that Sjögren's syndrome associated lymphoma B cells grow through an auto-antigen driven process. However, our sequence analysis of the immunoglobulin repertoire of $CD21^{-/low}$ B cells from the 4 pSS patients was not successful so far at identifying such monoclonal expansion, which may initially develop in the salivary gland of these patients and may not be identified in the patient's blood before later stages of the disease.

Taken together, elevated numbers of highly autoreactive unresponsive $CD21^{-/low}$ B cells remain in the blood of pSS patients instead of being eliminated, and immune reactions may create a favorable environment to break tolerance and eventually activate these $CD21^{-/low}$ B cells, potentially leading to the development of B cell lymphoproliferation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. L. Devine and C. Wang for single cell sorting. We thank Nathalie Ferry, Audrey Peres-Lascar, Catherine Blanc, Fabien Pitoiset and Cesar Prucca for technical assistance. This study was supported by the Agence Nationale pour la Recherche sur le Sida et les Hépatites (ANRS) and by Grant Number AI061093, AI071087, AI082713 and AI095848 from NIH-NIAID (to E. M.).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

References

1. Groom J, Kalled SL, Cutler AH, Olson C, Woodcock SA, Schneider P, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J Clin Invest. 2002; 109(1):59–68. [PubMed: 11781351]
2. Varin MM, Le Pottier L, Youinou P, Saulep D, Mackay F, Pers JO. B-cell tolerance breakdown in Sjogren's syndrome: focus on BAFF. Autoimmun Rev. 2010; 9(9):604–8. [PubMed: 20457281]
3. Mariette X, Gottenberg JE. Pathogenesis of Sjogren's syndrome and therapeutic consequences. Curr Opin Rheumatol. 2010; 22(5):471–7. [PubMed: 20671520]
4. Kassan SS, Thomas TL, Moutsopoulos HM, Hoover R, Kimberly RP, Budman DR, et al. Increased risk of lymphoma in sicca syndrome. Ann Intern Med. 1978; 89(6):888–92. [PubMed: 102228]
5. Baimpa E, Dahabreh IJ, Voulgarelis M, Moutsopoulos HM. Hematologic manifestations and predictors of lymphoma development in primary Sjogren syndrome: clinical and pathophysiologic aspects. Medicine (Baltimore). 2009; 88(5):284–93. [PubMed: 19745687]
6. Talal N, Sokoloff L, Barth WF. Extrasalivary lymphoid abnormalities in Sjogren's syndrome (reticulum cell sarcoma, "pseudolymphoma," macroglobulinemia). Am J Med. 1967; 43(1):50–65. [PubMed: 5006437]
7. Tzioufas AG, Boumba DS, Skopouli FN, Moutsopoulos HM. Mixed monoclonal cryoglobulinemia and monoclonal rheumatoid factor cross-reactive idiotypes as predictive factors for the development of lymphoma in primary Sjogren's syndrome. Arthritis Rheum. 1996; 39(5):767–72. [PubMed: 8639173]
8. Theander E, Henriksson G, Ljungberg O, Mandl T, Manthorpe R, Jacobsson LT. Lymphoma and other malignancies in primary Sjogren's syndrome: a cohort study on cancer incidence and lymphoma predictors. Ann Rheum Dis. 2006; 65(6):796–803. [PubMed: 16284097]
9. Skopouli FN, Dafni U, Ioannidis JP, Moutsopoulos HM. Clinical evolution, and morbidity and mortality of primary Sjogren's syndrome. Semin Arthritis Rheum. 2000; 29(5):296–304. [PubMed: 10805354]
10. Mariette X. Lymphomas complicating Sjogren's syndrome and hepatitis C virus infection may share a common pathogenesis: chronic stimulation of rheumatoid factor B cells. Ann Rheum Dis. 2001; 60(11):1007–10. [PubMed: 11602464]
11. Bahler DW, Miklos JA, Swerdlow SH. Ongoing Ig gene hypermutation in salivary gland mucosa-associated lymphoid tissue-type lymphomas. Blood. 1997; 89(9):3335–44. [PubMed: 9129040]
12. Miklos JA, Swerdlow SH, Bahler DW. Salivary gland mucosa-associated lymphoid tissue lymphoma immunoglobulin V(H) genes show frequent use of V1-69 with distinctive CDR3 features. Blood. 2000; 95(12):3878–84. [PubMed: 10845923]
13. Martin T, Weber JC, Levallois H, Labouret N, Soley A, Koenig S, et al. Salivary gland lymphomas in patients with Sjogren's syndrome may frequently develop from rheumatoid factor B cells. Arthritis Rheum. 2000; 43(4):908–16. [PubMed: 10765938]
14. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood. 2010; 115(24):5026–36. [PubMed: 20231422]
15. Charles ED, Brunetti C, Marukian S, Ritola KD, Talal AH, Marks K, et al. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. Blood. 2011; 117(20):5425–37. [PubMed: 21421840]
16. Terrier B, Joly F, Vazquez T, Benech P, Rosenzwajg M, Carpentier W, et al. Expansion of Functionally Anergic CD21-/low Marginal Zone-like B Cell Clones in Hepatitis C Virus Infection-Related Autoimmunity. J Immunol. 2011; 187(12):6550–63. [PubMed: 22084433]
17. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjogren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis. 2002; 61(6):554–8. [PubMed: 12006334]
18. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol. 1999; 17(12):3835–49. [PubMed: 10577857]

19. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. Science. 2003; 301(5638):1374–7. [PubMed: 12920303]
20. Samuels J, Ng YS, Coupillaud C, Paget D, Meffre E. Impaired early B cell tolerance in patients with rheumatoid arthritis. J Exp Med. 2005; 201(10):1659–67. [PubMed: 15897279]
21. Lopez F, Textoris J, Bergon A, Didier G, Remy E, Granjeaud S, et al. TranscriptomeBrowser: a powerful and flexible toolbox to explore productively the transcriptional landscape of the Gene Expression Omnibus database. PLoS One. 2008; 3(12):e4001. [PubMed: 19104654]
22. Meffre E, Casellas R, Nussenzweig MC. Antibody regulation of B cell development. Nat Immunol. 2000; 1(5):379–85. [PubMed: 11062496]
23. Tedder TF, Tuscano J, Sato S, Kehrl JH. CD22, a B lymphocyte-specific adhesion molecule that regulates antigen receptor signaling. Annu Rev Immunol. 1997; 15:481–504. [PubMed: 9143697]
24. Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, Driessen G, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. Proc Natl Acad Sci U S A. 2009; 106(32):13451–6. [PubMed: 19666505]
25. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol. 2004; 113(2):161–71. [PubMed: 15451473]
26. Hartley SB, Cooke MP, Fulcher DA, Harris AW, Cory S, Basten A, et al. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell. 1993; 72(3):325–35. [PubMed: 8431943]
27. Mandik L, Katsumata M, Erikson J. Effects of altered Bcl-2 expression on B lymphocyte selection. Ann N Y Acad Sci. 1997; 815:40–54. [PubMed: 9186638]
28. Lyubchenko T, Dal Porto JM, Holers VM, Cambier JC. Cutting edge: Complement (C3d)-linked antigens break B cell anergy. J Immunol. 2007; 179(5):2695–9. [PubMed: 17709481]
29. Mandik-Nayak L, Bui A, Noorchashm H, Eaton A, Erikson J. Regulation of anti-double-stranded DNA B cells in nonautoimmune mice: localization to the T-B interface of the splenic follicle. J Exp Med. 1997; 186(8):1257–67. [PubMed: 9334365]
30. Kochi Y, Yamada R, Suzuki A, Harley JB, Shirasawa S, Sawada T, et al. A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. Nat Genet. 2005; 37(5):478–85. [PubMed: 15838509]
31. Li DH, Winslow MM, Cao TM, Chen AH, Davis CR, Mellins ED, et al. Modulation of peripheral B cell tolerance by CD72 in a murine model. Arthritis Rheum. 2008; 58(10):3192–204. [PubMed: 18821699]
32. Fujimoto M, Sato S. B cell signaling and autoimmune diseases: CD19/CD22 loop as a B cell signaling device to regulate the balance of autoimmunity. J Dermatol Sci. 2007; 46(1):1–9. [PubMed: 17223015]
33. Li DH, Tung JW, Tarner IH, Snow AL, Yukinari T, Ngernmaneepothong R, et al. CD72 down-modulates BCR-induced signal transduction and diminishes survival in primary mature B lymphocytes. J Immunol. 2006; 176(9):5321–8. [PubMed: 16621999]
34. Paolino M, Thien CB, Gruber T, Hinterleitner R, Baier G, Langdon WY, et al. Essential role of E3 ubiquitin ligase activity in Cbl-b-regulated T cell functions. J Immunol. 2011; 186(4):2138–47. [PubMed: 21248250]
35. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nat Rev Immunol. 2007; 7(8):633–43. [PubMed: 17641666]
36. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. Nature. 2002; 416(6881):603–7. [PubMed: 11948342]
37. Saadoun D, Suarez F, Lefrere F, Valensi F, Mariette X, Aouba A, et al. Splenic lymphoma with villous lymphocytes, associated with type II cryoglobulinemia and HCV infection: a new entity? Blood. 2005; 105(1):74–6. [PubMed: 15353484]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

38. Mariette X, Gozlan J, Clerc D, Bisson M, Morinet F. Detection of Epstein-Barr virus DNA by in situ hybridization and polymerase chain reaction in salivary gland biopsy specimens from patients with Sjogren's syndrome. Am J Med. 1991; 90(3):286–94. [PubMed: 1848394]
39. Inoue H, Tsubota K, Ono M, Kizu Y, Mizuno F, Takada K, et al. Possible involvement of EBV-mediated alpha-fodrin cleavage for organ-specific autoantigen in Sjogren's syndrome. J Immunol. 2001; 166(9):5801–9. [PubMed: 11313424]
40. Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, Seya T, et al. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med. 2009; 206(10):2091–9. [PubMed: 19720839]

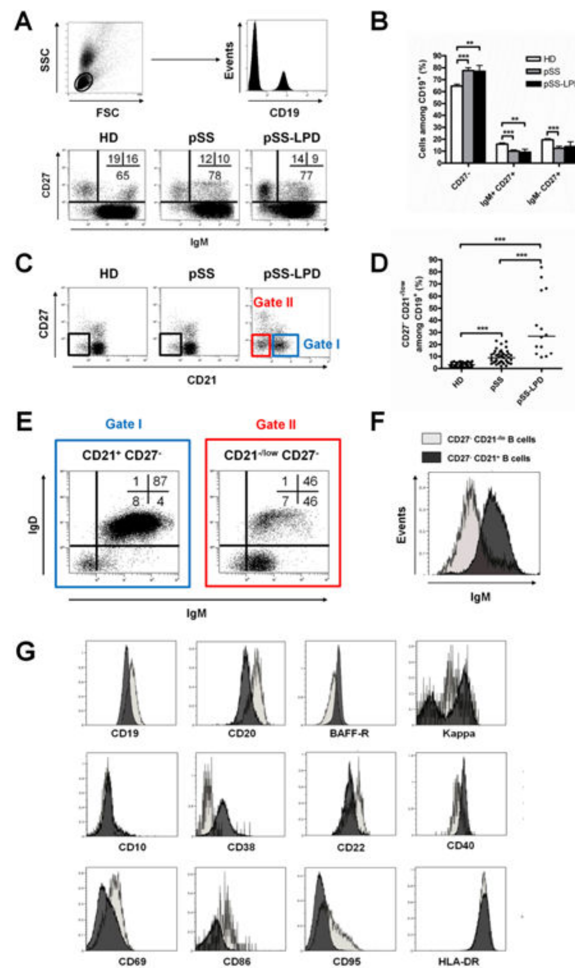

Figure 1. Expansion of an unusual $CD27^{-}CD21^{-/low}$ B cell population in pSS patients

(**A, B**) Increased proportion of $CD27^{-}$ B cells in pSS patients compared with HD. (**C, D**) Increased proportion of $CD27^{-}CD21^{-/low}$ B cells in pSS-LPD patients compared with pSS and HD (data are represented as median values in figure 1 D). (**E, F**) $CD27^{-}CD21^{-/low}$ B cells (gate II) display decreased expression of IgD and IgM compared to $CD27^{-}CD21^{+}$ B cells (gate I). (**G**) $CD27^{-}CD21^{-/low}$ cells display a unique B cell phenotype. Cells were gated on $CD27^{-}CD19^{+}$ B cells in order to delineate phenotype differences between $CD21^{-/low}$ and $CD21^{+}$ $CD27^{-}$ B cell populations for B cell markers (CD19, CD20, BAFF-R, Kappa light chain), maturity markers (CD10, CD38, CD22, CD40) and activation/proliferation markers (CD69, CD86, CD95, HLA-DR).

* $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

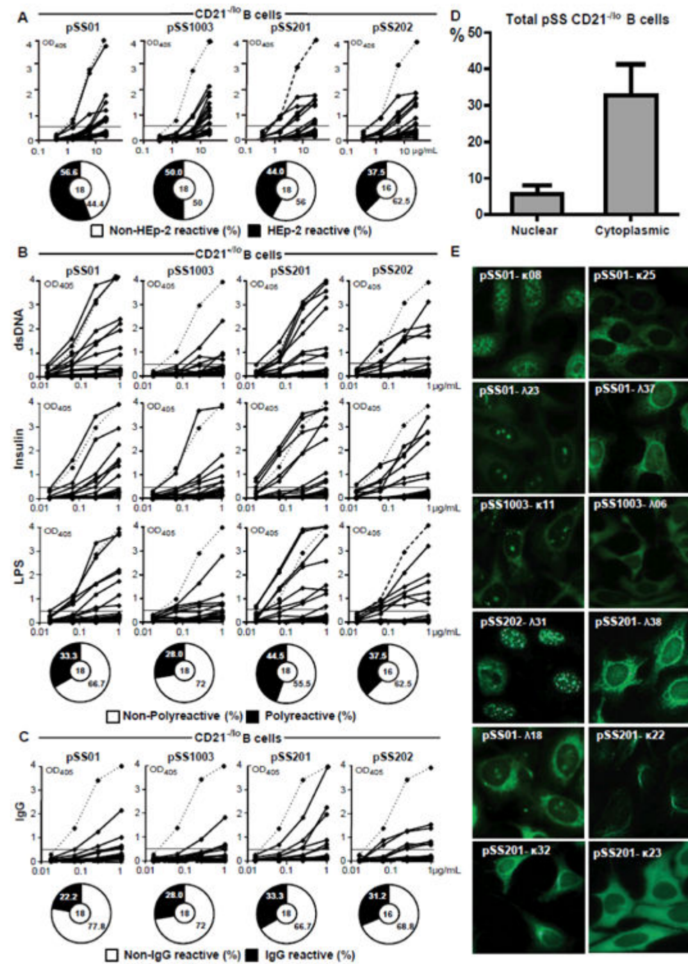

Figure 2. CD21$^{-/low}$ B cells in pSS express highly autoreactive antibodies

(**A**) Antibodies from CD27^{-}CD21$^{-/low}$ B cells from four pSS-LPD patients were tested by ELISA for anti-HEp-2 cell reactivity (**A**) and against double-stranded DNA (dsDNA), insulin and lipopolysaccharide (LPS) (**B**) and for rheumatoid factor (anti-IgG) reactivity **(C)**. CD27^{-}CD21$^{-/low}$ B cells often expressed antibodies recognizing cytoplasmic and to a lesser extent nuclear structures (**D** and **E**).

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

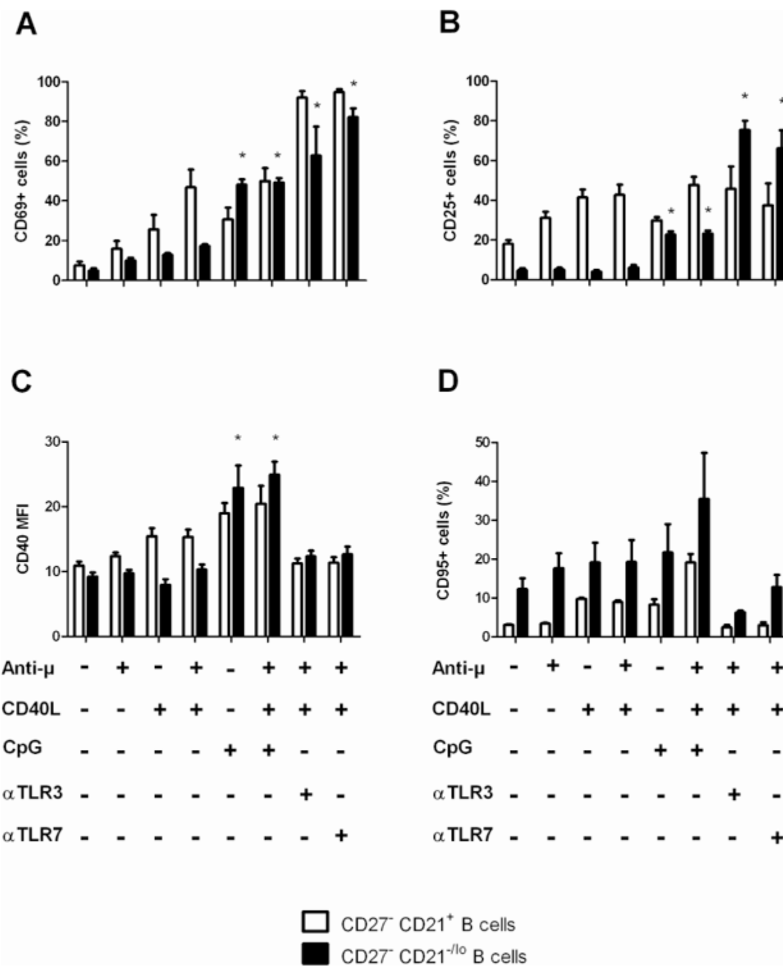

Figure 3. CD27$^-$CD21$^{-/low}$ B cells reveal defects in BCR and CD40-mediated activation

CD21$^{-/low}$ CD27$^-$ B cells failed to up-regulate CD69 (**A**), CD25 (**B**) and CD40 (**C**) expression after stimulation with F(ab′2) anti-IgM and/or recombinant human CD40L but not after stimulation with with TLR3 and TLR7 agonists and/or TLR9 agonist CpG; they also exhibited an increased CD95 expression compared with CD21$^+$ CD27$^-$ B cells (**D**). Data are representative of at least four independent experiments. * P<0.05 compared to stimulation wit anti-IgM and/or CD40L.

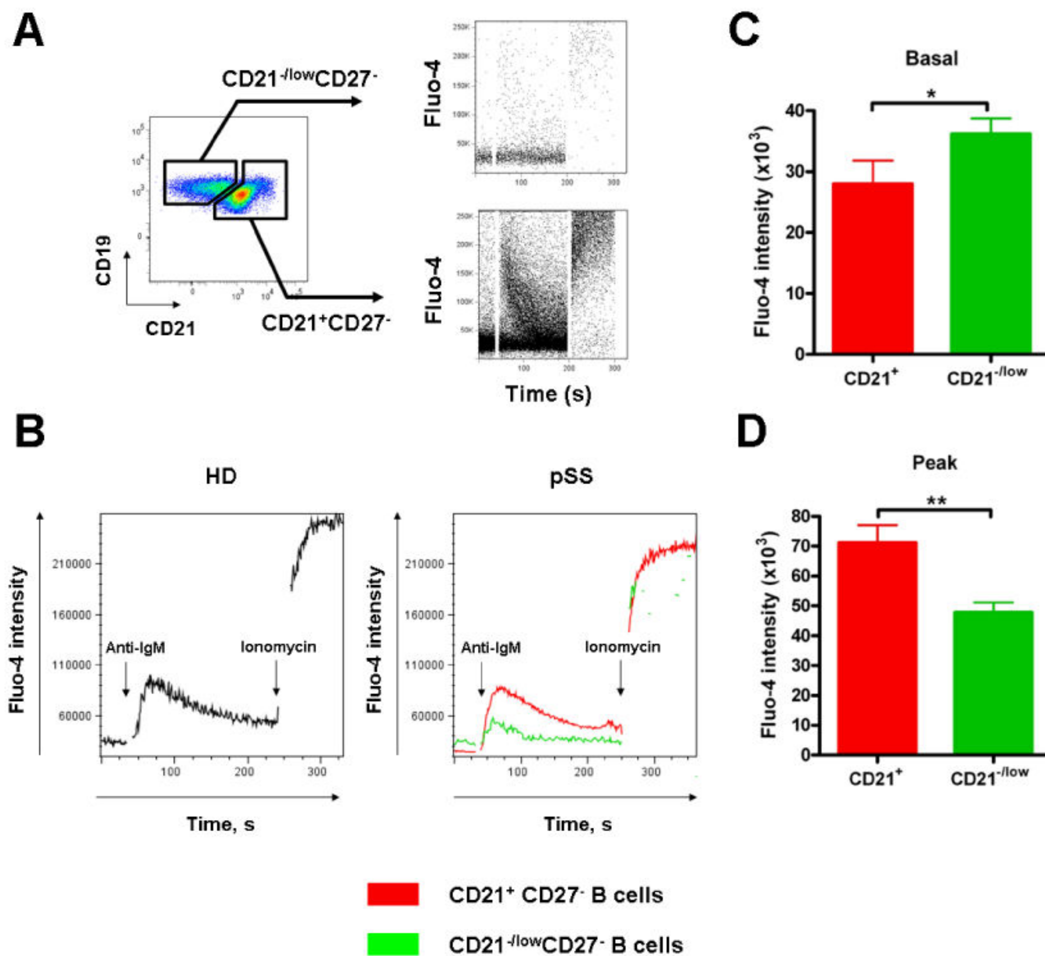

Figure 4. CD21-/low B cells fail to increase [Ca2+]i in response to BCR triggering

(**A, B**) CD21$^{-/low}$ B cells had attenuated calcium responses compared with CD21^{+} B cells after BCR cross-linking. (**C**) Slightly higher amount of mean fluorescence of Fluo-4 in the CD21$^{-/low}$ B cell population. (**D**) Higher increase of maximal normalized peak fluorescence of Fluo-4 in the CD21^{+} B cell population. Data are representative of five independent experiments. * $P<0.05$, ** $P<0.01$.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

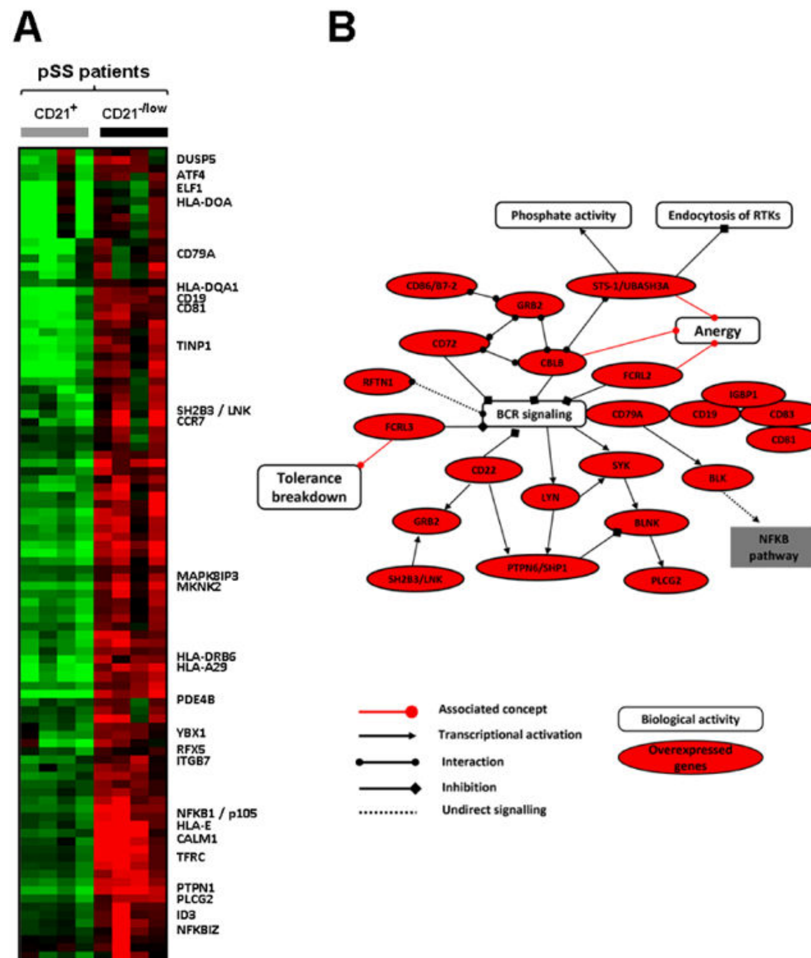

Figure 5. Gene array comparisons of CD21^{+} and CD21$^{-/low}$ CD27^{-} B cells from pSS patients using the Illumina Human Whole Genome Array

(**A**) Over-expressed transcripts differentially expressed by CD21$^{-/low}$ and CD21^{+} CD27^{-} B cells from 4 pSS patients are shown. Up- and down-regulated transcripts are indicated in red and green, respectively. (**B**) Over-expressed transcripts involved in an anergy and tolerance breakdown cluster are presented.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript

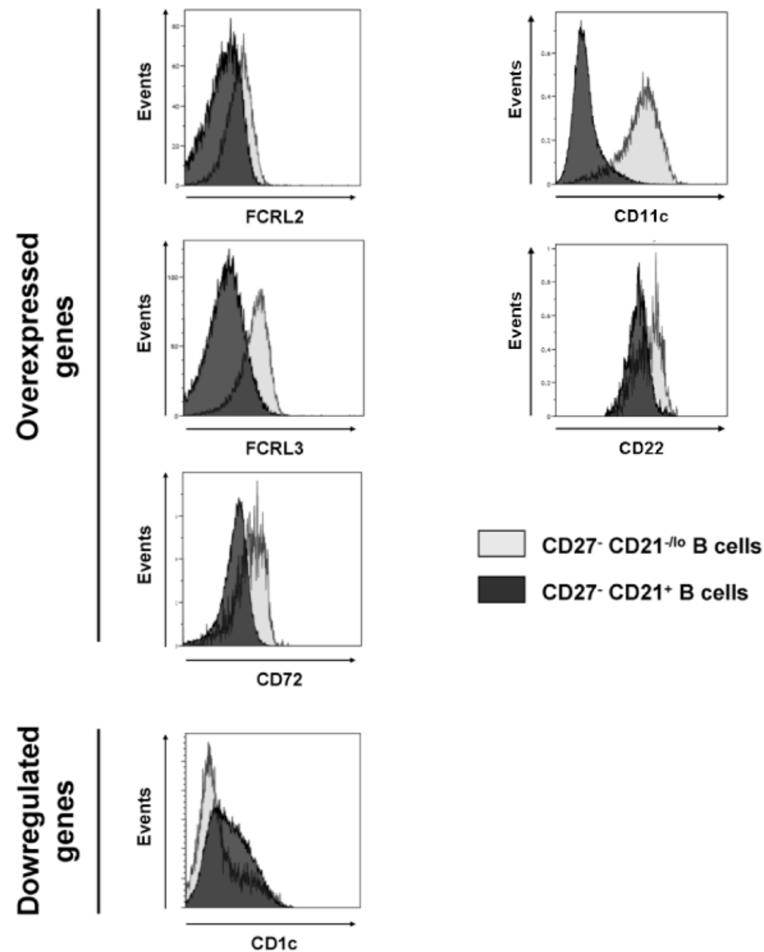

Figure 6. FACS analysis of proteins differentially expressed between CD21^{+} and CD21$^{-/low}$ CD27^{-} B cells from pSS patients

FACS plot analysis of surface markers overexpressed and downregulated in CD21$^{-/low}$ CD27^{-}B cells. FCRL2, FCRL3, CD72, CD11c and CD22 are overexpressed at the protein level in CD21$^{-/low}$ CD27^{-} B cells from pSS patients, as found in transcriptomic analysis. In contrast, CD1c marker is downregulated at the protein level in CD21$^{-/low}$ CD27^{-} B cells from pSS patients.