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Defective Early B Cell Tolerance Checkpoints in Sjögren's Syndrome Patients

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Abstract

Objective.—Central and peripheral B cell tolerance checkpoints are defective in many patients with autoimmune diseases, but the functionality of each discrete checkpoint has not been assessed in patients with Sjögren's syndrome (SS). We undertook this study to assess this functionality in SS patients.

Methods.—Using a polymerase chain reaction-based approach that allows us to clone and express, in vitro, recombinant antibodies produced by single B cells, we tested the reactivity of

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Meffre had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Meffre.

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recombinant antibodies cloned from single CD19+CD21^{low}CD10+IgM^{high}CD27[–] newly emigrant/transitional B cells and CD19+CD21+CD10–IgM+CD27[–] mature naive B cells from 5 SS patients.

Results.—We found that the frequencies of newly emigrant/transitional B cells expressing polyreactive antibodies were significantly increased in SS patients compared to those in healthy donors, revealing defective central B cell tolerance in SS patients. Frequencies of mature naive B cells expressing autoreactive antibodies were also significantly increased in SS patients, thereby illustrating an impaired peripheral B cell tolerance checkpoint in these patients.

Conclusion.—Defective counterselection of developing autoreactive B cells observed in SS patients is a feature common to many other autoimmune diseases and may favor the development of autoimmunity by allowing autoreactive B cells to present self antigens to T cells.

Sjögren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, especially the salivary and lacrimal glands, leading to dryness of the mouth and the eyes. It can occur either as a primary syndrome or as a secondary manifestation that complicates other autoimmune rheumatic conditions (1). In addition, patients with primary SS display an increased risk of non-Hodgkin's lymphoma, but the origin of lymphomas, and the mechanisms driving their malignant transformation, are poorly understood (2,3).

Both T cells and B cells play major roles in SS development. Patients with SS have altered peripheral B cell compartments characterized by fewer circulating CD27⁺ memory B cells, potentially due to their abnormal differentiation into plasma cells, resulting in increased serum IgG antibodies and soluble CD27 production (4,5). Dysregulated antibody production in most SS patients is associated with the secretion of antinuclear antibodies (ANAs) that target Ro 52/SS-A or La 48/SS-B antigens (6). Rheumatoid factors are also often detected in these patients and are associated with greater disease activity. A recent study (7) suggests defective autoreactive B cell counterselection in SS patients, as illustrated by the elevated frequency of polyreactive clones in total CD3-CD19+CD27-IgD⁺ B cells, which contain CD21^{–/low} clones previously reported to express autoreactive antibodies (8). However, the functionality of the central and peripheral B cell tolerance checkpoints, which are responsible for the elimination of developing autoreactive clones in the bone marrow and the periphery, respectively, remains to be analyzed in SS patients.

PATIENTS AND METHODS

We recruited 5 patients with primary SS diagnosed according to the American-European Consensus Group criteria (9). All samples were collected after patients provided written informed consent in accordance with protocols reviewed by the institutional review board. Patient SS201 was a 45-year-old woman who was positive for ANAs. Patients SS202, SS04, and SS05 were 67-year-old, 19-year-old, and 35-year-old women, respectively, who were positive for anti-Ro autoantibodies, while patient SS03 was a 71-year-old woman who was positive for specific anti-Ro and anti-La autoantibodies. Patients SS201 and SS03 also had lymphoma at the time of the study. Patients SS201 and SS202 both displayed an 1858T *PTPN22* allele (10).

Single-cell sorting.

Mononuclear cells from healthy donors and SS patients were enriched for B cells by magnetic separation with CD20 microbeads (Miltenyi Biotech) and stained with Pacific Blue-conjugated anti-human CD19, PerCP-Cy5.5-conjugated anti-human CD27, phycoerythrin-Cy7-conjugated anti-human CD10, allophycocyanin-conjugated anti-human CD21, and fluorescein isothiocyanate-conjugated anti-human IgM (all from BioLegend) prior to purification. Single CD19+CD21^{low}CD10+IgM^{high}CD27[–] newly emigrant/transitional B cells and CD19+CD21+CD10–IgM+CD27[–] mature naive B cells were sorted on a FACS Aria (BD Biosciences) into 96-well polymerase chain reaction (PCR) plates and immediately frozen on dry ice.

Complementary DNA synthesis, Ig gene amplification, and antibody production and purification.

RNA from single cells was reverse-transcribed in the original 96-well plate in 12.5- μ l reactions containing 100 units of Superscript II RT (Gibco BRL) for 45 minutes at 42°C. Reverse transcription-PCR reactions, primer sequences, cloning strategy, expression vectors, and in vitro antibody production and purification were as described previously (10).

Enzyme-linked immunosorbent assays and immunofluorescence assays (IFAs).

Antibody reactivity analysis was performed as described previously with the highly polyreactive ED38 antibody as positive control for HEp-2 reactivity and polyreactivity assays (10). Antibodies were considered polyreactive when they recognized all 3 distinct antigens: double stranded DNA, insulin, and lipopolysaccharide. For indirect IFAs, HEp-2 cell-coated slides (Bion Enterprises) were incubated in a moist chamber at room temperature with purified recombinant antibodies at 50–100 μ g/ml according to the manufacturer's instructions.

Statistical analysis.

Statistical analysis was performed using GraphPad Prism software, version 5.0. Differences between groups of research subjects were analyzed for statistical significance with nonparametric Mann-Whitney tests. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Central and peripheral B cell tolerance checkpoints are defective in many patients with autoimmune diseases (10), but the functionality of each discrete checkpoint has not been assessed in SS patients. We therefore tested the reactivity of recombinant antibodies cloned from single CD19+CD21^{low}CD10+IgM^{high}CD27[–] newly emigrant/transitional B cells and CD19+CD21+CD10–IgM+CD27[–] mature naive B cells from 5 patients with primary SS who were positive for ANAs. While immunoglobulin heavy-chain gene segment usage, third complementarity-determining region length, and positively charged amino acid content were similar between newly emigrant/transitional B cells from healthy donors and those from SS patients, we found that the frequencies of newly emigrant/transitional B cells expressing polyreactive antibodies were significantly increased in SS patients compared to those in

healthy donors ($P=0.0003$), revealing a defective central B cell tolerance in this autoimmune disease (Figures 1A and B; also see Supplementary Figure 1A and Supplementary Tables 1–5, available on the *Arthritis & Rheumatology* web site at <http://onlinelibrary.wiley.com/doi/10.1002/art.40215/abstract>). In addition, the proportion of antinuclear clones in newly emigrant/ transitional B cells from SS patients was also significantly increased compared to the proportion in healthy donors, further demonstrating the impaired removal of developing autoreactive B cells in the bone marrow of these patients ($P = 0.0050$) (Figures 1C and D).

The frequencies of mature naive B cells expressing HEp-2-reactive antibodies were also significantly increased in SS patients, thereby illustrating a defective peripheral B cell tolerance checkpoint in these patients (36.8–66.7% in SS patients versus 16.0–26.3% in healthy donors; $P = 0.0003$) (Figures 2A and B; also see Supplementary Tables 6–10, <http://onlinelibrary.wiley.com/doi/10.1002/art.40215/abstract>). The abnormal accumulation of autoreactive clones in the mature naive B cell compartment of SS patients was further evidenced by the increased frequency of B cells expressing polyreactive antibodies compared to healthy donors ($P = 0.0003$) (Figure 2C). Immunoglobulin heavy-chain variable gene segment usage appeared altered in mature naive B cells from SS patients, while other antibody repertoire features seemed unaffected in this disease (see Supplementary Figure 1B, <http://onlinelibrary.wiley.com/doi/10.1002/art.40215/abstract>).

Antinuclear clones were not enriched in mature naive B cells from SS patients (Figures 2D and E). However, we found that many naive B cells from SS patients expressed antibodies that can bind Ro 52/SS-A, one of the major SS antigens targeted by autoimmune reactions (see Supplementary Figure 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.40215/abstract>). Most clones recognizing Ro 52/SS-A were polyreactive and therefore emerged as a consequence of defective central and peripheral B cell tolerance checkpoints in SS patients (see Supplementary Figure 2). Of note, mature naive B cells from patient SS03 contained an expanded B cell clone that expressed mutated heavy- and light-chain genes that corresponded to stimulated autoreactive B cells potentially derived from CD21^{low} B cells (Meffre E, et al: unpublished observations) (see Supplementary Tables 6 and 11, <http://onlinelibrary.wiley.com/doi/10.1002/art.40215/abstract>). These clonal cells were therefore excluded from the analysis of mature naive B cells that express unmutated Ig genes. We conclude that SS patients have defective central and peripheral B cell tolerance checkpoints, resulting in the accumulation of autoreactive B cells in their blood, a feature common to many autoimmune diseases (10–12) (Figure 3).

DISCUSSION

The impaired removal of developing autoreactive B cells was previously reported in patients with type 1 diabetes mellitus (type 1 DM), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or multiple sclerosis, and this may promote autoimmunity by increasing the probability of self antigen presentation on major histocompatibility complex class II molecules to T cells by autoreactive B cells (10–13). Patients with primary immunodeficiencies also display altered central and/or peripheral B cell tolerance checkpoints and often have autoimmune conditions (10). Analysis of these patients with rare genetic mutations has revealed that decreased signaling from receptors that recognize self

antigens at the immature B cell stage (i.e., B cell receptors and potentially Toll-like receptors) results in a defective central B cell tolerance, while abnormal Treg cell function or decreased Treg cell numbers correlates with the accumulation of autoreactive mature naive B cells in the blood (10,14).

While the origins of the defective early B cell tolerance checkpoints in patients with autoimmune diseases are not known, Meffre reported that the 1858T polymorphism in the *PTPN22* gene associated with type 1 DM, RA, and SLE was sufficient to induce autoreactive B cell counterselection defects in asymptomatic individuals that were similar to those in patients with autoimmune diseases (10). Although an association between 1858 C/T single-nucleotide *PTPN22* polymorphism and SS was not identified (15), 2 of the 5 SS patients analyzed in the current study harbored the 1858T *PTPN22* variant, which may account for their defective early B cell tolerance checkpoint (10). Therefore, polymorphisms associated with autoimmune disorders and identified by genomewide association studies, rare gene mutations as seen in patients with primary immunodeficiencies, and perhaps nongenetic factors may result in impaired early B cell tolerance checkpoints common to autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Defective central B cell tolerance checkpoint in patients with Sjögren's syndrome (SS). **A**, Antibodies from newly emigrant/transitional B cells from 1 healthy donor (HD) and 5 SS patients were tested by enzyme-linked immunosorbent assay for reactivity against the antigens double stranded DNA (dsDNA), insulin, and lipopolysaccharide (LPS). Antibodies were considered polyreactive when they recognized all 3 analyzed antigens. Irregular dotted lines indicate ED38 antibody (positive control). Horizontal dotted lines show OD_{405 nm} cutoff for positive reactivity. For each individual, the frequencies of polyreactive and nonpolyreactive clones are summarized in pie charts, with the number of antibodies tested indicated in the centers. **B** and **C**, Frequencies of polyreactive (**B**) and antinuclear (**C**) newly emigrant B cells were compared between healthy donors and SS patients. Symbols represent individual subjects; bars show the median. ** = $P = 0.01$; *** = $P = 0.001$ by Mann-Whitney test. **D**, Antinuclear (top) and nuclear nonreactive (bottom) autoantibodies expressed by newly emigrant/transitional B cells from SS patients show various patterns of HEP-2

staining. The symbols κ or λ indicate that the corresponding antibody contains a κ or λ light chain, respectively. Original magnification $\times 40$.

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Figure 2.

Defective peripheral B cell tolerance checkpoint in SS patients. **A**, Antibodies from mature naïve B cells from 1 healthy donor and 5 SS patients were tested by enzyme-linked immunosorbent assay for reactivity against HEP-2 cell lysate. Irregular dotted lines indicate ED38 antibody (positive control). Horizontal dotted lines show OD_{405 nm} cutoff for positive reactivity. For each individual, the frequencies of HEP-2-reactive and non-HEP-2-reactive clones are summarized in pie charts, with the number of antibodies tested indicated in the centers. **B-D**, Frequencies of HEP-2-reactive (**B**), polyreactive (**C**), and antinuclear (**D**) clones in mature naïve B cells from SS patients were compared with corresponding frequencies in cells from healthy donors. Symbols represent individual subjects; bars show the median. *** = $P < 0.001$ by Mann-Whitney test. In **C**, for each individual, the frequencies of polyreactive and nonpolyreactive clones are summarized in pie charts, with the number of antibodies tested indicated in the centers. **E**, Mature naïve B cells from SS

patients often expressed antibodies recognizing cytoplasmic structures, and, to a lesser extent, nuclear structures. The symbols κ or λ indicate that the corresponding antibody contains a κ or λ light chain, respectively. Original magnification $\times 40$. dsDNA = double-stranded DNA (see Figure 1 for other definitions).

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Figure 3.

Defective central and peripheral B cell tolerance checkpoints are features common to many autoimmune diseases. Frequencies of polyreactive newly emigrant/transitional B cells (**A**) and HEp-2-reactive mature naïve B cells (**B**) from SS patients were compared with those of cells from healthy donors and patients with multiple sclerosis (MS) (12), type 1 diabetes mellitus (T1D) (10), rheumatoid arthritis (RA) (10), and pediatric systemic lupus erythematosus (SLE) (11). Dashed lines indicate the mean frequency of polyreactive B cell clones in healthy donors. Symbols represent individual subjects; bars show the median. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$ versus healthy donors, by Mann-Whitney test. See Figure 1 for other definitions.