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TLR7 activation of age-associated B cells mediates disease in a mouse model of primary Sjögren's disease

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Abstract

Primary Sjögren's disease (pSD, also referred to as Sjögren's syndrome) is an autoimmune disease that primarily occurs in women. In addition to exocrine gland dysfunction, pSD patients exhibit B cell hyperactivity. B cell-intrinsic TLR7 activation is integral to the pathogenesis of SLE, a disease that shares similarities with pSD. The role of TLR7-mediated B cell activation in pSD, however, remains poorly understood. We hypothesized that age-associated B cells (ABCs) were expanded in pSD and that TLR7-stimulated ABC subsets exhibited pathogenic features characteristic of disease. Our data revealed that ABC expansion and TLR7 expression were enhanced in a pSD mouse model in a Myd88-dependent manner. Splenocytes from pSD mice showed enhanced sensitivity to TLR7 agonism as compared to those derived from controls. Sort-purified marginal zone (MZ) B cells and ABCs from pSD mice showed enhanced inflammatory cytokine secretion and were enriched for anti-nuclear autoantibodies following TLR7 agonism. Finally, IgG from pSD patient sera showed elevated anti-nuclear autoantibodies, many of which were secreted preferentially by TLR7-stimulated murine MZ B cells and ABCs. Thus, these data indicate pSD B cells are hyper-responsive to TLR7 agonism and TLR7-activated B cells contribute to pSD through cytokine and autoantibody production. Thus, therapeutics that target TLR7 signaling cascades in B cells may have utility in pSD patients.

Summary sentence:

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Authorship

JMK conceived of the work, wrote the manuscript, and performed experiments. AP, EMK, and JMK critically edited the manuscript and performed experiments. CZ performed the autoantigen arrays and normalized the data. JCM and GY critically edited the manuscript and analyzed the autoantigen array.

Conflict of interest disclosure

None of the authors have any conflicts of interest related to this work.

B cell TLR7 expression and ABCs are increased in a pSD model and TLR7-stimulated ABCs derived from pSD females show pathogenic potential.

Keywords

Autoantibodies; NOD.B10; Autoimmunity; T-bet; ABC; RNA binding proteins

1. Introduction

Primary Sjögren's disease (pSD, also referred to as Sjögren's syndrome) is a chronic systemic autoimmune disease with a strong female predilection[1]. pSD is primarily characterized by the loss of exocrine function and immune hyperactivity[2]. Many pSD patients suffer from diverse disease manifestations, including salivary hypofunction, decreased tear production, interstitial lung disease and nephritis[2]. Abnormalities in peripheral blood are also observed, such as hypergammaglobulinemia and hypocomplementemia[2–4]. Currently, the etiology of pSD remains poorly understood, and as a result, treatments are only palliative in nature.

Prior studies performed by our group demonstrated that Myd88-mediated signaling cascades are crucial for specific pSD disease manifestations[5–7]. Myd88 is a ubiquitously-expressed cytosolic adaptor that controls both innate and adaptive immune cells[8–11]. Myd88 is crucial for activation of numerous IL-1R family members and TLRs, including TLR7. Several lines of evidence demonstrate B cell-intrinsic TLR7 mediates autoimmunity, and this is well characterized in Systemic Lupus Erythematosus (SLE), an autoimmune disease that shares similarities with pSD[12–14]. Previous work by our group and others suggests that TLR7 activation also mediates key aspects of pSD in both mouse models and pSD patients[15–23].

There is considerable evidence that B cells are integral to pSD pathogenesis. Indeed, patients display elevated autoantibodies and have heightened risk of B cell lymphoma[24, 25]. Data suggest loss of tolerance in the B cell compartment is an early disease event, as autoantibodies are reported years to decades before the onset of other pSD disease manifestations[26, 27]. GWAS studies also implicate B cells in the pathogenesis of pSD, as SNPs in genes that encode signaling intermediates associated with B cell activation are altered in pSD patients as compared to healthy controls[28, 29]. In fact, a recent elegant study identified a molecular subset of pSD patients that showed evidence of B cell hyperactivity in the periphery[30].

While B cells clearly contribute to pSD, the specific B cell subsets that mediate pathology in the context of pSD remain incompletely understood. Studies in lupus models and patients with SLE demonstrate a key role for age-associated B cells (ABCs) in disease[31, 32]. ABCs promote germinal center expansion, drive autoantibody production, and mediate kidney and lung damage in lupus[33, 34]. Importantly, ABC accumulation in lupus is mediated by TLR7 activation[34–36]. While there are a few studies that describe expansion of a subset analogous to murine ABCs in pSD patients[37, 38], the role of ABCs in pSD remains largely unexplored. Recent work from our group revealed that treatment of pre-disease pSD mice with the TLR7 agonist, Imiquimod (Imq), accelerates disease and drives expansion of splenic ABCs[18].

Moreover, transcriptomic studies demonstrate that a subset of B cells that shares phenotypic similarities with murine ABCs is expanded in salivary tissue derived from pSD patients[39, 40]. In the current study, we hypothesized that ABCs with pathogenic potential are expanded in pSD mice that develop disease spontaneously as compared to healthy controls. Moreover, we predict that this pathogenicity relies, at least in part, on ABC-intrinsic TLR7 activation.

We used a well-characterized mouse model, termed NOD.B10Sn-*H2*^{b}/J (NOD.B10), to examine TLR7-mediated activation of ABCs in pSD[41, 42]. NOD.B10 mice exhibit many characteristic features of disease seen in pSD patients. For example, NOD.B10 females display loss of salivary flow, and salivary and lacrimal inflammation in addition to interstitial nephritis and elevated anti-nuclear autoantibodies (ANAs) in sera[2, 3, 41, 42]. We found that ABCs were expanded in NOD.B10 mice with clinical and advanced stage disease and this expansion relied, in part, on the expression of Myd88 in the hematopoietic compartment. Expression of TLR7 was also elevated in pre-disease NOD.B10 mice and TLR7 expression was highly enriched in both the marginal zone (MZ) and ABC subsets. Splenocytes derived from NOD.B10 females with clinical disease were hypersensitive to stimulation with a TLR7 agonist. MZ B cells and ABCs from NOD.B10 mice were enriched in secretion of distinct pro-inflammatory cytokines and ANAs, including those directed against RNA binding proteins (RBPs). Finally, sera from pSD patients showed similar autoantibody profiles as those observed for TLR7-stimulated murine MZ and ABC subsets, suggesting activation of analogous B cell subsets in pSD patients. Altogether, our data demonstrate that TLR7-responsive B cell populations induce pathogenic B cell activation that likely has clinical significance in the context of pSD.

2. Methods

2.1 Mice.

NOD.B10-Sn*H2*^{b}/J (NOD.B10) (stock #002591) and C57BL/10SnJ mice (BL/10) (stock #000666) were obtained from Jackson Laboratories. NOD.B10$^{Myd88fl/fl}$ and NOD.B10$^{Myd88\Delta}$ mice were described previously[6]. All animals included in this study were female. Mice were euthanized at the clinical disease stage (26 weeks (wks) of age) or at the advanced disease stage (at least 52 wks of age). Each strain was bred and maintained in at the University at Buffalo Laboratory Animal Facility in accordance with NIH and IACUC guidelines.

2.2 Collection of tissue and sera.

Following euthanasia, spleens and cervical lymph nodes (cLNs) were harvested and single cell suspensions were generated by mechanical dispersion. RBCs were lysed using ACK lysis buffer. Blood was collected by cardiac puncture and maintained at room temperature for 2 hours. It was then centrifuged at 4,000 g for 20 minutes. Serum was removed and stored at −20°C for further analyses.

2.3 Culture and stimulation of stimulation of splenocytes.

Splenocytes (5×10^{6}) were cultured in RPMI 1640 containing 2% heat-inactivated FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured in media alone, in media containing Imq (0.04 or 0.625 μg/mL) (InvivoGen), in media containing anti-IgM/IgG Fab (10 μg/mL) (SouthernBiotech), or in the presence of both anti-IgM/IgG Fab and Imq (10 μg/mL and 0.625 μg/mL, respectively). Cultures were performed in 1 mL of media in 24-well plates for 24 hours or 6 days as indicated.

2.4 Culture and stimulation of sort-purified B cell subsets.

For cytokine multiplex arrays, sort-purified splenic FO (B220+ CD23+ CD21$^{lo/-}$), MZ (B220+ CD23− CD21+), or ABCs (B220+ CD11c+ CD11b+) were cultured in 2% RPMI in the presence or absence of Imq for 72 hours (1.5×10^5 cells each). Supernatants were harvested and a 16-plex cytokine array was performed (Quansys Biosciences). For autoantigen arrays, sort-purified splenic FO B cells (6×10^6 cells), MZ B cells (4.5×10^6 cells), or ABCs (5.0×10^5 cells) were cultured in 2% RPMI containing Imq (0.625 μg/mL) for 6 days. All cells were cultured in 96-well round bottom plates in 200 μL of media. Supernatants were harvested and stored at −20°C prior to analysis. We confirmed experimentally that the amount of IgG produced by each population was sufficient for autoantigen array analysis (data not shown). The autoantigen array was performed by the UT Southwestern Microarray core.

2.5 Flow cytometry and FACS.

Flow cytometry was performed as previously described. Briefly, cells were incubated with Fc block (CD16/32, clone 2.4G2, BD Biosciences) and treated wits antibodies directed against the following markers as indicated: B220 (clone RA3–6B2, BD Biosciences), CD23 (clone B3B4, Biolegend), CD21/35 (clone 7G6, BD Biosciences), T-bet (clone 4B10, BD Biosciences), CD11c (clone HL3, BD Biosciences), CD11b (clone M1/70, BD Biosciences), CD4 (clone GK1.5, BD Biosciences), and TLR7 (clone A94B10, BD Biosciences). Data were acquired using a BD Biosciences Fortessa and analyzed using FlowJo software.

For cell sorting experiments, splenocytes were pooled from 2 – 3 NOD.B10 females that were at least 12 months of age. Following dissociation and RBC lysis, cells were fluorescently labeled and sorted using the following panels: FO (B220+ CD23+ CD21$^{lo/-}$), MZ (B220+ CD23− CD21+), and ABCs (B220+ CD11b+ CD11c+). Cells were sorted using a BD Biosciences FACSAria.

2.6 ELISAs.

Antibody ELISAs were performed to quantify total IgG and IgG2c on serially diluted samples (Bethyl Laboratories and Stem Cell Technologies). IFNα (PBL Assay Science), IL-6 and IFNγ ELISAs (Invitrogen) were also performed. ELISAs were performed in accordance with manufacturer instructions and all samples were analyzed in duplicate.

2.7 Patient samples.

Patient samples were acquired from the Sjögren’s International Collaborative Clinical Alliance (SICCA) Biorepository[43, 44]. Patient demographics and clinical information are shown in Table 1. All pSD patients were females who displayed anti-Ro/SSA autoantibodies, had a focus score of at least 1, and displayed hyposalivation (n = 15, average

age = 52.2 years, range = 32 – 76 years). Non-SD controls were matched by age, sex, and ethnicity (n = 15, average age = 51.7 years, range = 30 – 79 years). All non-SD controls had a negative minor salivary gland biopsy and normal salivary production. Sera were received on dry ice and were stored at −80°C. All samples were shipped to UT Southwestern for autoantigen array analysis.

2.8 Statistics.

Mann-Whitney test at level 0.05 and ANOVA tests were performed where indicated using Prism software. *Post-hoc* analysis was done using Tukey’s multiple comparisons test. Autoantigen array data were analyzed using previously described methods for both murine and human samples[18]. For murine studies, autoantibodies from sort-purified ABC or MZ subsets were compared to those derived from FO B cells. Briefly, for MZ versus FO B cells we performed the two-sample two-sided t-test for all autoantigens, while for ABCs versus FO B cells we performed two-sample one-sided t-tests (H_1: $\mu_{ABC} > \mu_{F0}$) as the ABC subset was highly variable and *a priori* we were most interested in autoantigens that were enriched in the ABC subset as compared to the FO subset. For the p-values from each comparison we used the *p. adjust* R function in the R Stats package[45] to adjust the p-values in order to control the false discovery rate (FDR). The method proposed by Benjamini and Hochberg was used to control the FDR[46]. An autoantigen was deemed significant if the corresponding adjusted p-value was less than 0.10. The autoantigen array data are deposited in the Gene Expression Omnibus (GEO) database under the following accession numbers: GSE236254 (human) and GSE236255 (murine).

3. Results

3.1. ABCs are expanded in pSD mice in a Myd88-dependent manner.

Since the ABC population is integral to lupus pathogenesis and an ABC-like population is elevated in pSD patients[34, 37–39, 47], we first sought to determine if ABCs were expanded in pSD mice with increasing age. To this end, we harvested spleens from NOD.B10 females at a pre-disease (3 months old), clinical disease (6 – 7 months old) or advanced disease time point (at least 12 months old), as previously described[41]. Spleens were also collected from age- and sex-matched BL/10 controls. Flow cytometry was performed to quantify T-bet+ and T-bet+ CD11c+ ABC subsets. NOD.B10 mice displayed an elevated percentage of T-bet+ ABCs at the pre-disease (3-month-old), clinical disease (6-month-old) and advanced disease stages (12+ months of age) compared to age-matched controls (p = 0.001, p = 0.0009, and p = 0.0002, respectively) (Figure 1A).

We performed similar analyses to examine T-bet+ CD11c+ ABCs in pSD mice. Our data revealed that this population was also expanded with age in pSD females, as NOD.B10 mice at the clinical and advanced disease stage displayed elevated percentages of this ABC population as compared to healthy controls at each time point examined (p = 0.0002, and 0.01, respectively) (Figure 1B).

To determine if these changes relied on Myd88 expression in immune cells, we harvested spleens from NOD.B10 females that lacked Myd88 in the hematopoietic compartment

(NOD.B10$^{Myd88\Delta}$ strain). NOD.B10$^{Myd88fl/fl}$ mice were used as Myd88-sufficient controls, as previously published[6]. NOD.B10$^{Myd88fl/fl}$ females at the advanced disease stage displayed expansion of both T-bet+ and T-bet+ CD11c+ ABCs as compared to age- and sex-matched NOD.B10$^{Myd88\Delta}$ females ($p = 0.04$ and $p = 0.001$, respectively) (Figure 1C and D). These data indicate that ABCs increase with age in both strains and are expanded in pSD mice. This expansion relies, at least in part, on the expression of Myd88 in immune cells.

3.2 B cell TLR7 expression is increased in pre-disease pSD mice and is highly enriched in the MZ and ABC subsets

To begin to examine the Myd88-mediated signals that may contribute to ABC expansion in pSD, we next focused on TLR7, because this Myd88-dependent TLR is crucial for ABC expansion in other models and is integral to lupus pathogenesis[14, 32, 34, 48]. The percentage of splenic TLR7-expressing B cells was elevated in NOD.B10 mice with advanced disease as compared to 3-month-old BL/10 controls ($p < 0.0001$) (Figure 2A). Additionally, TLR7+ B cells were expanded in the spleens and cLNs of aged BL/10 mice as compared to young strain-matched controls ($p = 0.003$ and 0.002, respectively) (Figure 2A and B). To assess TLR7 function, splenocytes from pSD mice at the clinical disease stage were cultured with a low dose of Imq (0.04 µg/mL) and supernatants were harvested. Analogous experiments were performed in BL/10 controls. Splenocytes derived from NOD.B10 mice secreted elevated levels of IL-6 as compared to those from healthy controls ($p = 0.005$) (Figure 2C). We then assessed TLR7 expression in the cultured cells. Our data revealed that the percentage of B cells expressing TLR7 is increased following Imq treatment as compared to unstimulated controls in both in both BL/10 and NOD.B10 splenocytes ($p < 0.0001$ and $p = 0.01$, respectively). No differences were observed in the percentage of B cells expressing TLR7, however, between either unstimulated or Imq-treated samples from BL/10 mice as compared to their NOD.B10 counterparts (Figure 2D).

Next, we assessed TLR7 expression in FO, MZ, and ABC splenic B cells in pSD females at the clinical disease stage and age-and sex-matched BL/10 controls. TLR7 was highly expressed in both MZ B cells and CD11b+ CD11c+ ABCs derived from both BL/10 and NOD.B10 mice, with lower expression observed in the FO populations of both strains (Figure 2E and F). Of note, there were no differences observed in TLR7 expression between analogous subsets of each strain (Figure 2F).

Finally, we sought to determine if TLR7 expression was dependent on immune-intrinsic Myd88 in pSD mice. To this end, we assessed the percentage of TLR7+ B cells in aged NOD.B10$^{Myd88\Delta}$ females. Our data revealed that this population was decreased in both splenic and cLN populations as compared to age- and sex-matched NOD.B10$^{Myd88fl/fl}$ controls ($p = 0.008$ and $p = 0.03$, respectively) (Figure 2G and H). Altogether, these results demonstrate TLR7 sensitivity is heightened in pSD splenocytes. Moreover, TLR7 expression is governed by Myd88 and is enriched in both MZ and ABC subsets in both control and pSD mice.

3.3 TLR7 activation promotes ABC differentiation and leads to heightened IgG, IgG2c, and IFN production in pSD mice.

We next performed experiments to determine if TLR7 stimulation drives heightened antibody secretion and ABC skewing in pSD mice with clinical disease, and whether this is enhanced in the presence of BCR ligation. Parallel experiments were performed in age- and sex-matched BL/10 controls. We harvested splenocytes and cultured cells in either media alone, media containing Imq, media containing anti-IgM/IgG Fab (BCR), or with media containing both Imq and the BCR crosslinker. Supernatants were harvested and secretion of IgG and IgG2c was assessed. We found IgG secretion increased following Imq stimulation as compared to media alone in NOD.B10 females ($p < 0.0001$). IgG secretion was also increased when NOD.B10 splenocytes were stimulated with BCR/Imq, but addition of BCR crosslinking did not enhance IgG secretion when compared to cells treated with Imq alone. B cells derived from NOD.B10 mice showed heightened IgG secretion as compared to BL/10 controls in response to Imq and Imq/BCR treatment ($p < 0.0001$ and $p < 0.0001$, respectively) (Figure 3A). We also assessed secretion of IgG2c, because this subclass of antibody is enriched in autoimmunity and autoreactive IgG2c is preferentially secreted by ABCs in a lupus model[49, 50]. We assessed IgG2c in the 3 samples that had significant IgG levels and found that B cells from NOD.B10 females secreted high levels of IgG2c in response to stimulation with Imq in comparison to Imq-treated B cells derived from healthy controls ($p = 0.002$). Of note, concomitant Imq/BCR stimulation did not increase IgG2c production in NOD.B10 splenocytes as compared to NOD.B10 samples treated with Imq alone (Figure 3B).

Since IgG2c production is induced by IFNα and IFNγ[51–54], we assayed our culture supernatants to quantify the levels of IFN produced. Our results revealed that IFNα levels in the culture supernatants were relatively low, although IFNα levels secreted by the NOD.B10 splenocytes cultured with Imq were higher than those detected from the Imq-treated BL/10 supernatants ($p < 0.0001$) (Figure 3C). Additionally, NOD.B10 splenocytes treated with Imq exhibited higher IFNα production as compared to strain-matched samples treated with the Imq/BCR cocktail ($p = 0.02$) (Figure 3C). BL/10 splenocytes secreted negligible amounts of IFNγ, regardless of the treatment condition (Figure 3D). NOD.B10 splenocytes treated with Imq or Imq/BCR produced high levels of IFNγ as compared with their BL/10 counterparts ($p < 0.00001$ for both comparisons). Moreover, NOD.B10 splenocytes treated with Imq and Imq/BCR secreted more IFNγ as compare to strain-matched cells cultured in media alone or with the BCR agonist alone ($p < 0.0001$ for all comparisons) (Figure 3D). Treatment of NOD.B10 splenocytes with Imq and Imq/BCR yielded similar IFNγ levels, indicating that addition of the BCR crosslinker to the TLR7 agonist did not augment the production of IFNγ (Figure 3D).

Finally, we cultured cells for 48 hours as indicated above and performed flow cytometry to assess ABC differentiation, as previously described[55]. We found that the percentage of T-bet-expressing B cells was relatively low in both BL/10 and NOD.B10 splenocytes when cells were cultured in media alone. An increase in the percentage of T-bet+ B cells was noted in BL/10 cultures following stimulation with either Imq, BCR crosslinking, or Imq in conjunction with BCR stimulation in both strains as compared to cells cultured in media alone ($p < 0.0001$, $p = 0.006$ and $p = 0.002$, respectively) (Figure 3E and F). Similarly, T-bet expression increased in NOD.B10 B cells stimulated with Imq, BCR agonism, or following Imq/BCR stimulation as compared to NOD.B10 cells cultured in media alone ($p < 0.0001$, $p = 0.006$, and < 0.0001, respectively) (Figure 3E and F). In addition, NOD.B10 splenocytes stimulated with BCR crosslinking showed elevated B cell T-bet expression as compared their BL/10 counterparts ($p = 0.002$) (Figure 3E and F).

We next examined B cells that co-expressed T-bet and CD11c. We found no differences among any of the BL/10 culture conditions. NOD.B10 splenocytes stimulated with Imq/BCR showed a higher percentage of T-bet+ CD11c+ B cells as compared to BL/10 cells cultured under the same conditions ($p = 0.02$) (Figure 3G). The percentage of NOD.B10 T-bet+ CD11c+ B cells was also increased as compared to NOD.B10 cells cultured in media or with BCR crosslinking alone ($p = 0.001$ and 0.005, respectively) (Figure 3G). Taken together, these data indicate that NOD.B10 B cells secrete higher levels of IgG, IgG2c, IFNα, and IFNγ in response to Imq as compared to BL/10 controls. Moreover, TLR7 agonism promotes ABC differentiation *in vitro* in splenocytes, and this is further enhanced by Imq/BCR stimulation in NOD.B10 mice.

3.4 TLR7 agonism mediates production of distinct pro-inflammatory cytokines in MZ B cells and ABCs.

To examine the functional significance of TLR7 activation of splenic B cell subsets in the context of pSD, we sort-purified FO, MZ, and ABC subsets from the spleens of NOD.B10 females with advanced disease. Of note, ABCs represent a heterogeneous group of B cells that have been identified as either T-bet+, CD11c+, both T-bet+ and CD11c+, or both CD11b+ and CD11c+[31, 32, 56]. Recent studies using lupus mice revealed that CD11c expression was a better predictor of T-bet positivity than either cells that expressed CD11b or those that were negative for CD21/35 or CD23[47]. This observation was most consistent among B cells that expressed the highest levels of T-bet[47]. Therefore, we conducted functional assays using CD11b+ CD11c+ cells, as this strategy to sort-purify ABCs is validated by rigorous studies[34, 47].

We cultured sort-purified cells in the presence or absence of Imq, harvested the supernatants and assessed cytokine production by multiplex array (Figure 4). FO B cells did not show enhanced secretion of any of the mediators examined following TLR7 ligation. MZ B cells showed much greater TLR7 sensitivity as compared to FO, as increased secretion of TNFα, MIP-1α, IL-6, and IL-10 was observed in response to Imq as compared to supernatant collected from MZ B cells cultured in media alone ($p = 0.03$, $p = 0.003$, $p < 0.0001$, and $p < 0.0001$ respectively) (Figure 4A, B, D and E). Of note, MZ B cells stimulated with the TLR7 agonist secreted higher levels of IL-6 as compared to both TLR7-stimulated FO and ABC B cell subsets ($p = 0.002$ and 0.008 respectively) (Figure 4D). Additionally, MZ B cells stimulated with Imq secreted high levels of IL-10 as compared to TLR7-stimulated FO B cells ($p < 0.0001$) (Figure 4E).

Finally, we analyzed ABCs and found that this subset was also highly responsive to TLR7 agonism. Similar to MZ B cells, TLR7-stimulated ABCs secreted significant levels of TNFα, MIP-1α, and IL-10 as compared to ABCs cultured in media alone ($p < 0.0001$, p = 0.002, and p = 0.01 respectively) (Figure 4A, B, and E). Of note, TLR7-stimulated ABCs secreted RANTES (CCL5), and RANTES levels were significantly higher than those observed in FO and MZ stimulated with Imq and in unstimulated ABCs (p = 0.02, p = 0.03, and p = 0.02 respectively) (Figure 4C). Finally, ABCs produced higher levels of TNFα as compared to Imq-stimulated FO and MZ B cells (p < 0.0001 and p = 0.0001, respectively) (Figure 4A). Thus, MZ cells and ABCs preferentially secrete numerous pro-inflammatory cytokines in response to TLR7 agonism in pSD.

3.5 Specific ANAs are enriched in the MZ and ABC repertoires of pSD mice following TLR7 agonism.

To assess TLR7-mediated autoreactive IgG, FO, MZ, and ABC subsets were sort-purified and cells were cultured in media containing Imq for 6 days. Autoantigen arrays were performed on supernatants collected from the Imq-stimulated cultures. We focused our analyses on ANAs, as these are elevated in pSD patients[44]. ANA-specific IgG was enhanced in both the MZ and ABC subsets as compared to the FO (Figure 5 and Suppl. Figure 1). Of note, IgG derived from pSD MZ B cells was enriched for binding to numerous RBPs as compared to the FO subset, including U1-snRNP A (p = 0.002), U1-snRNP C (p = 0.004), La/SSB (p = 0.02), and Sm/RNP (p = 0.02) (Figure 5B). In addition, the MZ B cell repertoire was enriched for reactivity against PM/Scl-100 (p = 0.005), PM/Scl-75 (p = 0.002), Ku (p70/p80) (p = 0.004), PL-7 (p = 0.005), Nup-62 (p = 0.007), Jo-1 (p = 0.01), GP210 (p = 0.01), SP100 (p = 0.01), SRP54 (p = 0.01), nucleolin (p = 0.02), PL-12 (p = 0.02), DFS70 (p = 0.02), genomic DNA (p = 0.02), ssDNA (p = 0.04), CENP-A (p = 0.04), dsDNA (p = 0.04), histone 2A (p = 0.06), and Mi-2 (p = 0.09) (Figure 5B).

We also analyzed the ABC supernatants and observed enhanced reactivity to numerous ANAs as compared to the FO subset (Figure 5A). These data demonstrate that the ABC repertoire shows a high degree of anti-nuclear autoantigen reactivity and select autoantibody specificities are enriched in the ABC subset, specifically those directed against nucleolin (p = 0.005), genomic DNA (p = 0.01), histones (histone 2A (p = 0.01), histone 2B (p = 0.02), histone H1 (p = 0.03) and histone H3 (p = 0.05), nucleosome (p = 0.03), Ku (p70/p80) (p = 0.04), PML/Scl-100 (p = 0.05), PML/Scl-75 (p = 0.05), SPR54 (p = 0.06), ssDNA (p = 0.06), PL-12 (p = 0.06), Jo-1 (p = 0.06), DFS70 (p = 0.06), dsDNA (p = 0.07), Nup62 (p = 0.09), and CENP-B (p = 0.09) (Figure 5C). Additionally, the data show that reactivity to select RBPs (Sm (p = 0.06), SmD (p = 0.06), and SmD1 (p = 0.06)) are preferentially enriched in the ABC compartment (Figure 5C). Altogether, these findings indicate that TLR7-stimulated MZ B cells and ABCs secrete numerous autoantibodies with relevance to pSD.

3.6 Sera from patients with pSD are enriched for autoantibodies that are preferentially secreted by the Imq-treated MZ and ABC subsets.

To determine if sera from pSD patients showed similarities in ANA-specific IgG reactivity as compared to the cultured B cells subsets, we acquired serum samples from pSD patients (n = 15) and age, sex, and ethnicity matched non-SD controls (n = 15). We performed autoantigen arrays on these samples. As above, we focused our analyses on ANAs. We found that 25 ANA-specific IgGs were significantly enriched in pSD patients as compared to non-SD controls (p values are shown in Suppl. Table 1) (Figure 6A and B and Suppl. Figure 2). To determine whether autoantibodies that were increased in pSD patients were also enriched in murine TLR7-stimulated B cell subsets, we compared our human data to that from our pSD model (Figure 6C and Supplemental Table 2). Of note, 3 autoantibodies that were assessed in the murine autoantigen arrays were not included on the human one (SmD1, DFS70, and genomic DNA), so these were excluded from our comparisons. Our data revealed that 60% of the autoantibodies that were detected by both murine and human arrays were enriched in both the pSD patient sera (n = 12/20) and the MZ repertoire. Additionally, 12 autoantigens (60%) were recognized by autoantibodies derived from the ABC subset (Figure 6C). These data suggest that TLR7-mediated activation of specific B cell subsets shapes the pSD patient autoantibody repertoire, and TLR7-dependent signals could be a significant driver of these autoantibodies in pSD patients.

4. Discussion

Data from the current study revealed that ABCs are expanded in pSD mice, and these cells carry pathogenic potential in the context of pSD. Seminal studies in healthy mice and those with lupus demonstrate that ABC expansion is mediated by B cell-intrinsic TLR7 activation[34–36]. Similarly, our work shows that TLR7 activation contributes to activation of MZ B cells and ABCs in the context of pSD as evidence by cytokine secretion and autoantibody production. Of relevance to the human disease, numerous autoantibodies induced by TLR7 in MZ and ABC subsets were also elevated in pSD female patients. Thus, our data indicate that TLR7-driven B cell activation contributes to pathology in the context of pSD, and this is mediated, at least in part, by ABC expansion and activation.

Both type I and type II IFNs play a critical role in autoimmunity[54, 57–59], and are implicated in the etiopathogenesis of pSD in mice and humans[30, 60, 61]. TLR7-activated NOD.B10 splenocytes secreted both IFNα and IFNγ, although IFNγ levels were much higher than those observed for IFNα (Figure 3C and D). Recent work in a lupus model revealed that TLR7-driven IFNγ production was essential for the generation of germinal center B cells and antibody secreting cells[59]. Of direct relevance to our studies, concomitant activation of TLR7, IFNγR, and BCR drives expression of T-bet in B cells, leading to the differentiation and proliferation of the ABC subset[36, 62, 63]. In addition, IFNγ upregulates T-bet in B cells and mediates IgG2c secretion[64]. Approximately 50% of the cells that comprise the ABC subset express T-bet[47], and activated ABCs secrete IgG2a/c antibodies preferentially[64]. Consistent with these studies, we found that TLR7 agonism increased the percentage of NOD.B10 B cells co-expressing T-bet and CD11c following stimulation with Imq/BCR as compared to BL/10 B cells cultured under analogous conditions (Figure 3G). Moreover, TLR7 stimulation of splenocytes from pSD mice resulted in significant secretion IgG2c (Figure 3B). Thus, results from the present study, in conjunction with our prior work[18], indicate that TLR7 agonism induces heightened ABC differentiation and activation in the context of pSD, and IFNγ likely plays a key role in ABC-mediated pathology.

Prior studies in healthy mice revealed that TLR7-stimulated MZ B cells and ABCs are a significant source of inflammatory cytokines as compared to FO B cells[65–67]. Our work corroborates and extends these findings, as MZ B cells and ABCs treated with Imq produced numerous pro-inflammatory cytokines as compared to the FO subset (Figures 4), and these cytokines are both implicated in both exocrine-specific and systemic pSD manifestations[20, 68–75]. It is important to point out that while there are likely inherent differences in cell viability and proliferation among the B cells subsets examined in Figure 4, strong evidence from both healthy and autoimmune mice demonstrates that MZ B cells and ABCs are hyperresponsive to TLR7 agonism as compared to the FO subset[65, 76]. Therefore, it is likely that the differences in cytokine secretion among the subsets is derived primarily from underlying differences in TLR7 sensitivity in the B cell subsets examined, and is not simply reflective of altered cell viability or proliferation. While these cytokines are elevated locally and systemically in pSD, further studies are needed to determine whether MZ and ABC-like B cells are a significant source of these mediators in pSD patients.

Of relevance to the current study, recent work in a lupus mouse model found that females displayed significant ABC expansion that was absent in males. When *TLR7* was overexpressed in males, however, this sex bias was abrogated and males developed even more severe disease than that observed in females[34]. It is important to note that a striking female disease predilection is observed in pSD patients, and this is female sex bias is among the highest observed for all autoimmune diseases[1]. While the reasons for this remain incompletely understood, it is interesting to speculate that dysregulated TLR7 expression in immune cell populations may mediate pSD. TLR7 is expressed on the X chromosome, and females inactivate one of the TLR7 alleles through a complex process called X chromosome inactivation (XCI)[77]. Several recent studies demonstrate that certain genes, including *TLR7*, may fail to undergo proper XCI in immune cells[78], and this likely contributes to the female disease predilection observed in lupus patients[79, 80]. Of note, transcriptional profiling studies revealed that *TLR7* was overexpressed in CD19+ B cells derived from pSD female patients as compared to those derived from healthy sex-matched controls[20]. Work herein corroborates studies in pSD patients[17], although further studies are needed to determine whether immune-intrinsic TLR7 activation underlies the female disease predilection observed in pSD.

Imq-treated MZ B cells and ABCs represented a significant source of select ANA-specific IgGs, including those directed against numerous RBPs (Figure 5). Of note, RBPs form complexes with RNA resulting in the formation of ribonuclear protein particles, including small nuclear ribonuclear particles (snRNPs)[81]. Generation of anti-RBP autoantibodies may be of clinical consequence in pSD because autoantibodies that target RBPs can activate both BCR and TLR7 signaling, culminating in TLR7-dependent B cell activation that results in chronic inflammation characterized by secretion of proinflammatory cytokines and autoantibodies[82–84].

Anti-Sm and anti-RNP autoantibodies are RBPs that correlate with disease severity and predict risk of flares in patients with SLE[85–89], and RNA-binding autoantibodies are generated in a TLR7-dependent manner in lupus models[14, 48]. It is likely that antibodies directed against RNA-associated proteins contribute to pSD pathogenesis as well, through induction of TLR7-mediated B cell activation. Previous work from our group provides evidence for this disease mechanism in pSD, as sera from pre-disease NOD.B10 females treated with a TLR7 agonist were enriched in IgG autoantibodies with specificity for

RBPs[18]. Interestingly, autoantigen array studies conducted on pSD patient sera also revealed enrichment of antibodies with specificity for RBPs (Figure 6). While several of these, such as anti-Ro and -La, are well-characterized and even used diagnostically in pSD patients[44, 90], others, including Sm, SmD, and U1-snRNP C, are less studied in the context of pSD and likely have clinical significance.

A previous study found that while autoantibodies with RNP reactivity were relatively uncommon in pSD patients, those with these autoantibodies were more likely to exhibit hypergammaglobulinemia and pulmonary involvement as compared to anti-RNP-negative pSD patients[91]. Of note, of the patients who displayed anti-RNP positivity, 30% of these individuals also had anti-Sm autoantibodies, and none of these individuals were diagnosed with SLE[91]. More recent work found pSD patients with African ancestry exhibited more severe disease and B cell hyperactivity characterized by a higher prevalence of anti-RNP autoantibodies as compared to Caucasian pSD patients, suggesting these autoantibodies may be preferentially enriched among pSD patients from certain racial and ethnic backgrounds[92].

While the way in which anti-RNP antibodies are generated in pSD is poorly understood, there are a few studies that have identified putative disease mechanisms with relevance to pSD. First, epitope spreading induced by the autoantigen La can result in the generation of autoantibodies that display reactivity for U1-RNP[93]. Second, prior Epstein Barr Virus (EBV) infection may result in the loss of tolerance to the Sm antigen, as the dominant epitope of SmD is highly homologous to the EBV encoded protein EBV nuclear antigen I (EBNA I)[94]. Interestingly, mice immunized with EBNA I peptide develop anti-SmD antibodies[94]. A corroborative study found that immunization of mice with a Ro 60 peptide or the cross-reactive EBNA I peptide resulted in the generation of autoantibodies that had specificity for other Ro epitopes as well as spliceosomal components[95]. These findings are relevant to pSD, as EBV infection is implicated in pSD pathogenesis and a recent study found that greater than 90% of anti-Ro- and/or La-positive pSD patients displayed anti-EBNA I antibodies[96]. These were also detected in healthy controls, however, so the clinical significance of this finding remains unclear[97]. Nonetheless, these data suggest that epitope spreading and molecular mimicry contribute to the generation of autoantibodies with specificity for snRNPs in pSD.

Our autoantigen array studies on sort-purified MZ B cells show that this subset is enriched in Ro52 and La/SSB autoantibodies (Figure 5), both of which are included in the ACR diagnostic criteria for pSD[44]. While the mechanisms underlying this observation are unclear at present, autoantibodies directed against RBPs, such as La/SSB, could contribute to the chronic activation of both the MZ and ABC subsets through activation of TLR7-dependent signaling cascades, as discussed above. The role of Ro52 autoantibodies in disease, however, is much less well understood. Of note, mice that lack Ro52 expression develop autoimmunity and B cell hyperactivation[98, 99]. Additionally, Ro52 autoantibodies from pSD patients neutralize Ro52 function *in vitro*, although it remains to be determined whether this ability is maintained *in vivo*[100, 101]. It is interesting to speculate that autoantibodies directed against Ro52 may act in an autocrine manner to inhibit the function of this protein in MZ B cells. Thus, concomitant TLR7 activation and inhibition of Ro52 function could be dual mechanisms that contribute to activation and even malignant transformation of MZ B cells in pSD patients, as anti-Ro52 and La/SSB autoantibodies are risk factors for lymphoma development in the context of pSD[102]. Further studies, however, are needed to determine this conclusively.

It is important to note that in the current study we only examined female pSD mice and anti-Ro-positive female patients who shared common clinical features of disease. Moreover, the pSD patients selected for the study showed relatively homogenous clinical findings. Thus, additional studies are needed to assess TLR7 signaling and ABC activation in males and in more diverse pSD patient populations.

Conclusion

In conclusion, this study provides evidence for ABC activation in pSD that is driven by TLR7 agonism. Our work provides a strong rationale for further studies to examine the role of TLR7 and ABC-mediated pathology in both males and females with pSD. These results carry clinical relevance, as blockade of TLR7-mediated B cell activation could represent a potential therapeutic approach in pSD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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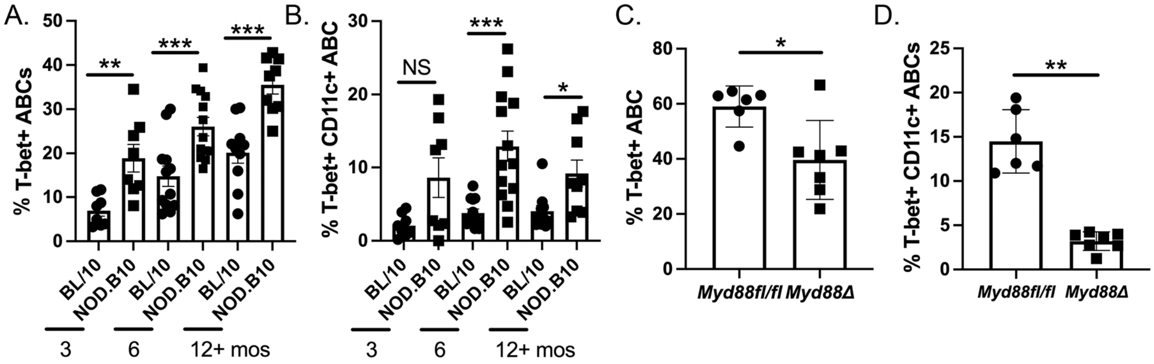

Figure 1: ABCs are expanded in aged NOD.B10 mice in a Myd88-dependent manner.

(A and B) Spleens were harvested from NOD.B10 females at 3 (n = 8), 6 (n = 13), and at least 12 months of age (n = 9) and from sex-matched BL/10 controls at 3 (n = 8), 6 (n = 12), and at least 12 months of age (n = 10) and flow cytometry was performed. (C and D) Spleens were harvested from NOD.B10$^{Myd88\Delta}$ females (12 months of age, n = 7) and age- and sex-matched NOD.B10$^{Myd88fl/fl}$ controls (n = 6). Cells were gated on ABCs (B220+, CD21−, CD23−) and expression of (A and C) T-bet+ and (B and D) T-bet+ CD11c+ cells is shown. Horizontal lines represent mean and SEM (NS, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001). Data from at least 2 independent experiments are shown.

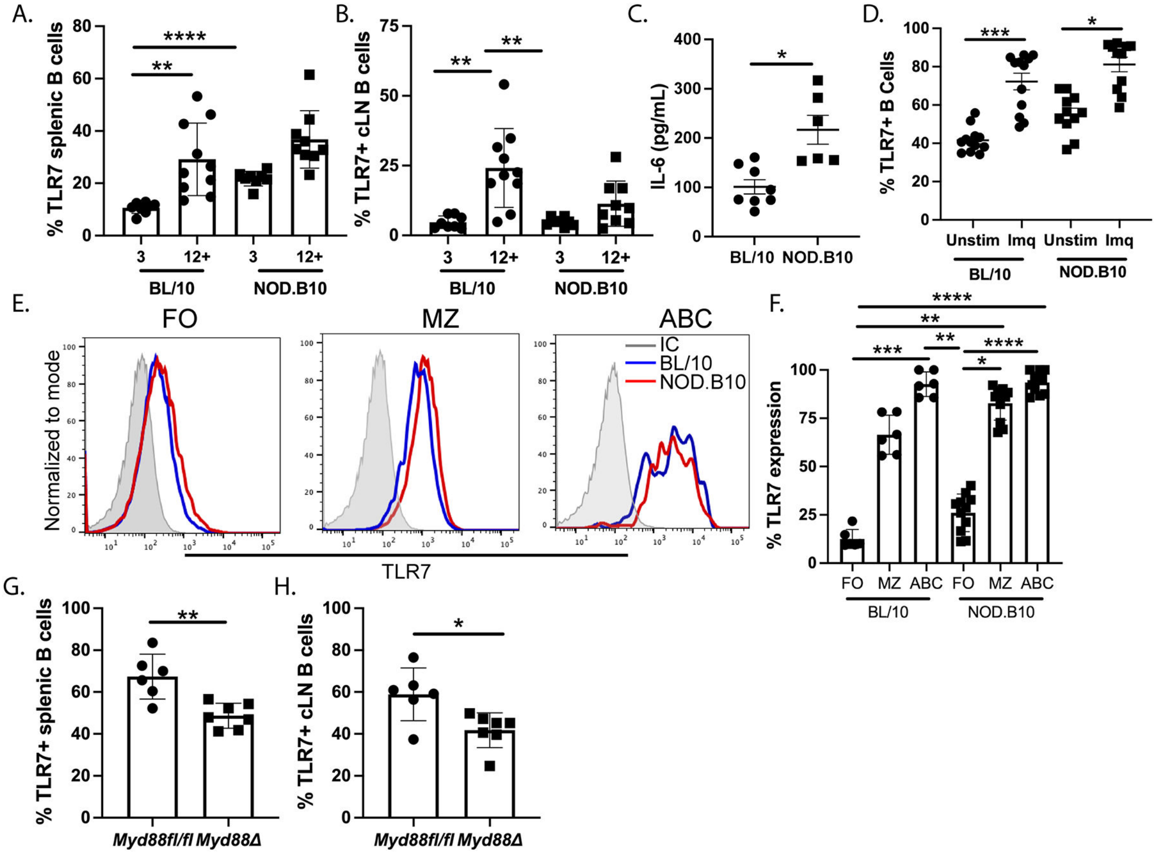

Figure 2: B cell TLR7 expression increases with disease progression in pSD and NOD.B10 splenocytes show heightened responsiveness to TLR7 agonism.

(A and B) Spleens and cLNs were harvested from NOD.B10 females at 3 (n = 8) and at least 12 months of age (n = 9) and from sex-matched BL/10 controls at 3 (n = 8) and at least 12 months of age (n = 10) and flow cytometry was performed. (C) Spleens were harvested from NOD.B10 females (6 – 7 months, n = 6) and age- and sex-matched BL/10 controls (n = 8). Cells were cultured with Imq and IL-6 ELISAs were performed on the supernatants. (D) Cultured splenocytes from NOD.B10 females (6 – 7 months of age, n = 11) and age- and sex-matched BL/10 mice (n = 12) were harvested after 24 hours and flow cytometry was performed. Cells were gated on B220 and expression of TLR7 is shown. (E) Spleens were harvested from NOD.B10 females (6 – 7 months, n = 11) and age- and sex-matched BL/10 controls (n = 6). Expression of TLR7 is shown for FO B cells (B220+ CD23+ CD21$^{lo/-}$), MZ B cells (B220+ CD23− CD21+), and ABCs (B220+ CD11b+ CD11c+) from one representation BL/10 and NOD.B10 female. Data from all animals is quantified in (F). (G and H) Spleens were harvested from NOD.B10$^{Myd88\Delta}$ females (12 months of age, n = 7) and age- and sex-matched NOD.B10$^{Myd88fl/fl}$ controls (n = 6). Cells were gated on B220 and expression of TLR7 is shown. Horizontal lines represent mean and SEM (NS, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).

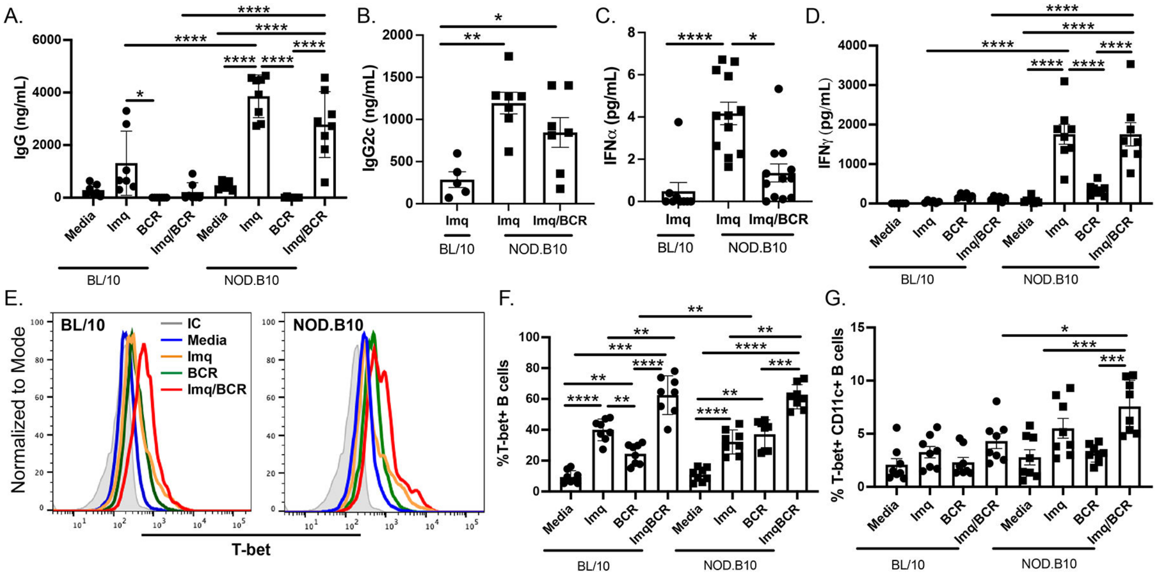

Figure 3: TLR7 activation drives heightened production of IgG, IgG2c, IFNα, and IFNγ in pSD splenocytes.

Spleens were harvested from NOD.B10 females (6 – 7 months, n = at least 7) and age- and sex-matched BL/10 controls (n = at least 5). Cells were cultured as indicated for 6 days and ELISAs were performed for (A) IgG, (B) IgG2c, and (C) IFNα and (D) IFNγ. Splenocytes were cultured for 48 hours and flow cytometry was performed. Cells were gated on B220 and expression of T-bet and CD11c was assessed. T-bet expression from one representative (E) BL/10 and NOD.B10 female is shown. The percentage of T-bet+ and T-bet+ CD11c+ ABCs from each strain and culture condition was quantified and is shown in (F) and (G), respectively. Data from at least 2 independent experiments are shown.

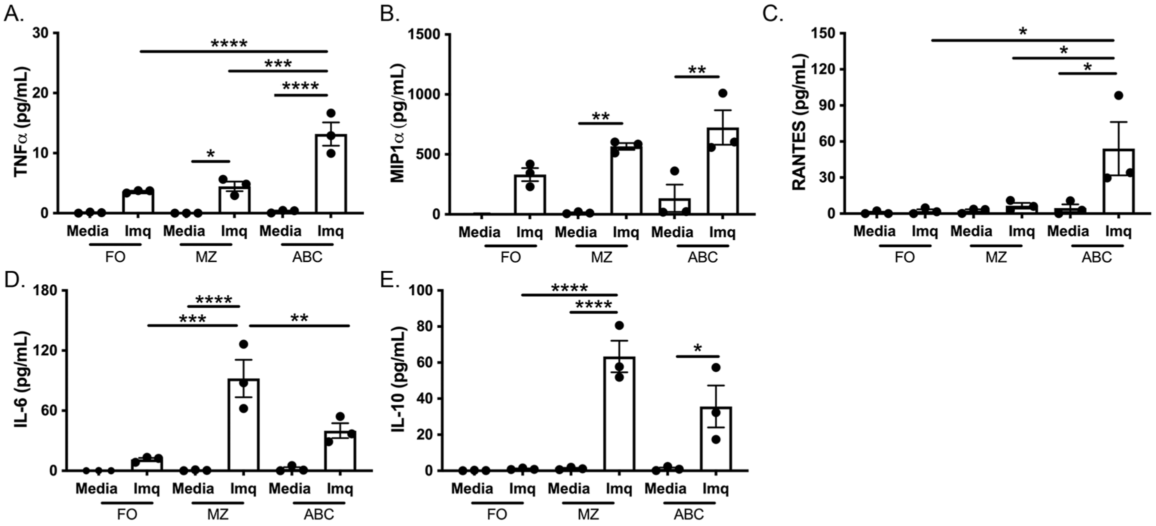

Figure 4: TLR7 mediates inflammatory cytokine production in pSD the MZ and ABC subsets.
Spleens (n = 2 or 3 pooled) were harvested from NOD.B10 females at least 12 months of age and FO (B220+ CD23+ CD21$^{lo/-}$), MZ (B220+ CD23− CD21+), and ABCs (B220+ CD11b+ CD11c+) subsets were sort-purified and cultured for 72 hours as indicated. Supernatants were harvested and cytokine multiplex arrays were performed to assess the levels of (A) TNFα, (B) MIP-1α, (C) RANTES, (D) IL-6, and (E) IL-10. Horizontal lines represent the mean and SEM (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001). Data from 3 independent experiments are shown.

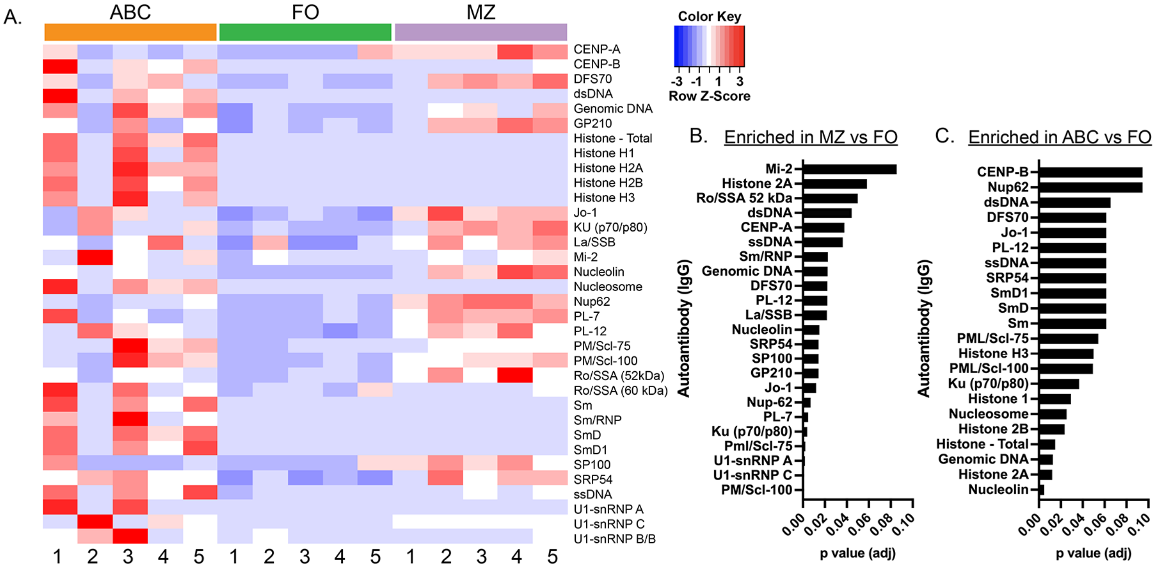

Figure 5: TLR7 drives heightened autoantibody production in MZ B cells and ABCs derived from NOD.B10 mice.

Spleens (n = 2 or 3 pooled) were harvested from NOD.B10 females at least 12 months of age and FO (B220+ CD23+ CD21$^{lo/-}$), MZ (B220+ CD23− CD21+), and ABCs (B220+ CD11b+ CD11c+) cells were sort-purified. Cells were cultured in the presence of Imq for 6 days and supernatants were harvested and an autoantigen array was performed. (A) Heatmap summarizing IgG autoantigen array data is shown. ANA-specific IgG autoantibodies that were enriched in (B) MZ B cells as compared to FO B cells and (C) ABCs as compared to FO B cells are shown. Data are from 5 independent experiments.

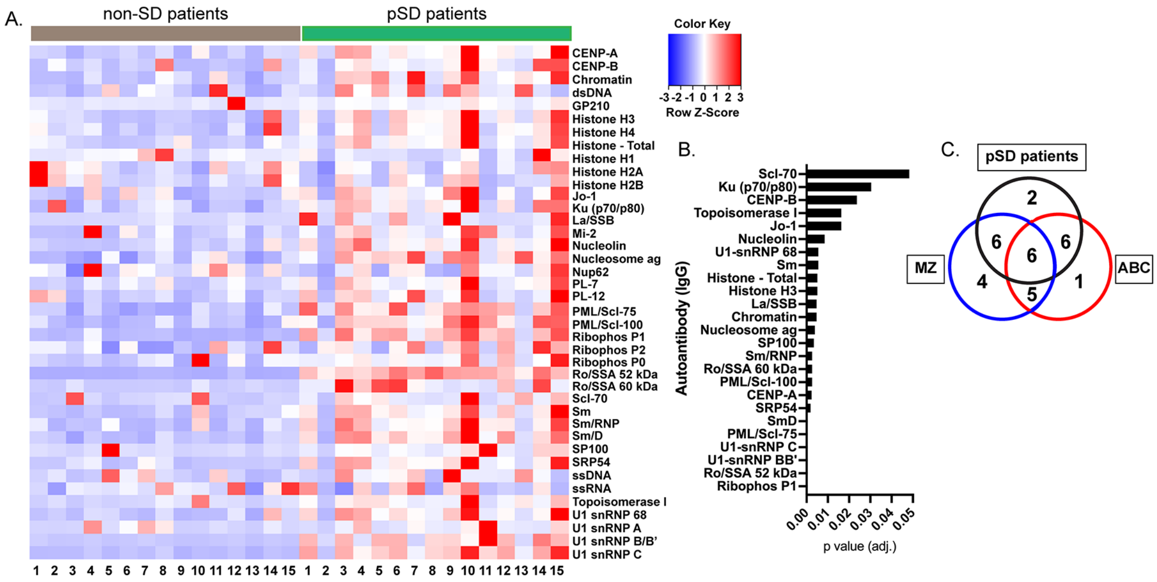

Figure 6: ANA-specific IgG from pSD patients shows heightened specificity for ANAs that are preferentially secreted by TLR7-stimulated pSD B cells.

(A) Autoantigen arrays were performed on pSD and non-pSD control sera for IgG and a heatmap of the ANA-specific IgG is shown. (B) Autoantibodies that were significantly enriched in the pSD patient sera are shown. (C) Venn diagram showing autoantibodies enriched in pSD patient sera (black circle) and TLR7-stimulated murine MZ (blue circle) and ABC subsets (red circle). Figure C only includes autoantibodies that were common to both the human and mouse arrays. Ribophos P1 = Ribophosphoprotein P1.

Table 1:

Patient demographics

pSS	Sex	Age	Ethnicity	Ro/SSA	La/SSB	OSS score	Schirmer test
1	F	35	Caucasian	Negative	Negative	2	Negative
2	F	55	Caucasian	Negative	Positive	4	Negative
3	F	47	Hispanic/Latino	Negative	Negative	2	Negative
4	F	48	Caucasian	Negative	Negative	0	Negative
5	F	59	Caucasian	Negative	Negative	2	Negative
6	F	67	Caucasian	Negative	Negative	2	Negative
7	F	49	Afro-American or African heritage	Negative	Negative	1	Negative
8	F	76	Caucasian	Negative	Negative	0	Negative
9	F	59	Asian or Pacific Islander	Negative	Negative	1	Negative
10	F	51	Asian or Pacific Islander	Negative	Negative	3	Negative
11	F	61	Asian or Pacific Islander	Negative	Negative	3	Negative
12	F	48	Caucasian/Native American	Negative	Negative	0	Negative
13	F	53	Caucasian	Negative	Negative	4	Negative
14	F	43	Caucasian	Negative	Negative	0	Negative
15	F	32	Asian or Pacific Islander	Negative	Negative	2	Negative
Non-SS							
1	F	36	Caucasian	Positive	Positive	6	Negative
2	F	55	Caucasian/Native American	Positive	Positive	11	Positive
3	F	48	Hispanic/Latino	Positive	Positive	11	Negative
4	F	48	Caucasian	Positive	Positive	6	Positive
5	F	61	Caucasian	Positive	Positive	11	Negative
6	F	68	Caucasian	Positive	Positive	10	Positive
7	F	43	Afro-American or African heritage	Positive	Negative	9	Positive
8	F	79	Caucasian	Positive	Negative	9	Positive
9	F	57	Asian or Pacific Islander	Positive	Positive	5	Positive
10	F	49	Asian or Pacific Islander	Positive	Positive	9	Positive
11	F	62	Asian or Pacific Islander	Positive	Positive	11	Negative
12	F	46	Caucasian	Positive	Positive	4	Negative
13	F	51	Caucasian	Positive	Positive	11	Positive
14	F	43	Caucasian/Native American	Positive	Negative	1	Negative
15	F	30	Asian or Pacific Islander	Positive	Negative	NP	Negative