Long-Term B Cell Depletion in Murine Lupus Eliminates Autoantibody-Secreting Cells and Is Associated with Alterations in the Kidney Plasma Cell Niche

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Long-Term B Cell Depletion in Murine Lupus Eliminates Autoantibody-Secreting Cells and Is Associated with Alterations in the Kidney Plasma Cell Niche

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Autoantibodies to dsDNA, produced by autoreactive plasma cells (PCs), are a hallmark of systemic lupus erythematosus and play a key role in disease pathogenesis. Recent data suggest that autoreactive PCs accumulate not only in lymphoid tissues, but also in the inflamed kidney in lupus nephritis. We hypothesized that the variable efficacy of anti-CD20 (rituximab)–mediated B cell depletion in systemic lupus erythematosus may be related to the absence of an effect on autoreactive PCs in the kidney. In this article, we report that an enrichment of autoreactive dsDNA Ab-secreting cells (ASCs) in the kidney of lupus-prone mice (up to 40% of the ASCs) coincided with a progressive increase in splenic germinatal centers and PCs, and an increase in renal expression for PC survival factors (BAFF, a proliferation-inducing ligand, and IL-6) and PC attracting chemokines (CXCL12). Short-term treatment with anti-CD20 (4 wk) neither decreased anti-dsDNA nor IgG ASCs in different anatomical locations. However, long-term treatment (12 wk) significantly reduced both IgG- and dsDNA-specific ASCs. In addition, long-term treatment substantially decreased splenic germinatal center and PC generation, and unexpectedly reduced the expression for PC survival factors in the kidney. These results suggest that prolonged B cell depletion may alter the PC survival niche in the kidney, regulating the accumulation and maintenance of autoreactive PCs. The Journal of Immunology, 2014, 192: 000–000.

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ystemic lupus erythematosus (SLE) is prototypic autoimmune disorder characterized by dysregulation in multiple arms of the immune system and the production of hallmark autoantibodies. A central role for B cells in the pathogenesis of this disease has been well established (1–3) and includes both Ab production and Ab-independent mechanisms (4). The latter are highlighted by the abrogation of disease and reduction in activated T cells in B cell–deficient, lupus-prone mice (2), yet the maintenance of T cell abnormalities in mice with B cells incapable of secreting Ab (5). Autoantibody-independent B cell functions include Ag presentation, T cell activation and polarization, and dendritic cell (DC) modulation, which are mediated, at least in part, by the ability of B cells to produce cytokines (6, 7).

In contrast, autoantibodies produced by B cells are also critical to disease pathogenesis by both direct and indirect mechanisms. In addition to conventional roles of autoantibodies in SLE via type II (Ab dependent cytotoxicity) and type III (immune complex) mechanisms, RNA- and DNA-containing autoantigen–autoantibody complexes can play an active role in propagating the autoimmune process through TLR-mediated immune cell activation (8–11). Anti-dsDNA Abs can also directly deposit in the kidney of both SLE patients and lupus mice (12, 13), causing tissue inflammatory damage (14) and leading to end-stage renal disease if untreated.

Thus, decreasing autoantibodies may be critical in the treatment of SLE. B cell depletion (BCD) with rituximab (anti-CD20) has demonstrated efficacy in multiple autoimmune diseases including rheumatoid arthritis, multiple sclerosis, and Anti-neutrophil cytoplasmic Ab–associated vasculitis. However, the precise mechanisms by which depletion of B cells abrogates autoimmunity remain incompletely elucidated. Although several open-label studies of BCD as a targeted treatment have demonstrated clinical benefit in SLE (15–17), only a minority of patients have lasting clinical responses (18, 19). Moreover, the failure of two large randomized trials of BCD in SLE (20) highlights the need to better understand the impact of this therapy on the immune system. In particular, anti-CD20 has variable effects on autoantibodies that are produced by CD20+ plasma cells (PCs).

The variable persistence of autoantibodies after BCD could be explained by the presence of long-lived PCs and/or the ongoing generation of short-lived plasmablasts. Indeed, both long-lived and short-lived populations of Ab-secreting cells (ASCs) can contribute to chronic humoral autoimmunity in NZB/W mice (21), with up to a surprising 40% of the PCs in the spleen having a half-life of >6 mo. Long-lived PCs have also been well described to home to the bone marrow (BM) (22). Recently, autoantibody-secreting PCs were also described as enriched in the kidneys of MRL/lpr (23) and NZB/W (24) lupus-prone mice, with a high fraction appearing long-lived based on BrdU labeling (25, 26). Taken together, this suggests that long-lived PCs are a major player in SLE. Whether they are...
generated in situ in the kidney and/or home to the inflamed tissue and find survival niches is controversial.

In nonautoimmune mouse models, it has been demonstrated that treatment with anti-CD20 Ab depletes mature and memory B cells but has minimal impact on PCs (27, 28). Similarly, we previously found that a short course of BCD in NZB/W mice effectively reduced the progression of nephritis without significant change in autoantibody levels or ASCs in spleen and BM (29). To understand the mechanism of action of BCD in lupus, we examined the impact of short-term versus long-term treatment of lupus-prone NZB/W F1 mice with anti-mouse CD20 Ab (anti-mCD20) on PC generation and maintenance. We show that autoreactive PCs reside in the kidney and are eliminated by prolonged anti-CD20 treatment, with an unexpected reduction in the expression of PC survival factors in the kidney. To our knowledge, this is the first demonstration that targeted BCD can alter the survival of autoreactive PCs in inflammatory sites.

Materials and Methods

Lupus-prone NZB/NZW F1 female mice were purchased from Jackson Laboratories and maintained in a pathogen-free animal facility in the University of Rochester Medical Center. Experiments were performed following the guidelines approved by the University of Rochester Committee on Animal Resources. Different age groups of mice were used to define the kinetics and mechanisms of autoreactive PC generation and maintenance, including 8-, 19-, and 39-wk-old mice.

Short-term treatment. NZB/NZW F1 female mice (24–30 wk old) with proteinuria ≥2+ (100 mg/dl) received a weekly retro-orbital injection of 300 μg anti-mCD20 Ab (IgG2a 18B12; Biogen Idec; n = 6) or control Ab IgG2a (2B8; Biogen Idec) for four times and were sacrificed 1 wk after the last treatment.

Long-term treatment. Twenty-seven-week-old NZB/NZW F1 female mice (high anti-dsDNA Ab titer: ≥400 U/ml, negative control: 80 U/ml) were injected weekly with 300 μg anti-mCD20 Ab or control IgG. One group was treated weekly, four times (n = 6) and sacrificed 8 wk later. Another group was treated weekly for 12 wk (n = 6) and sacrificed 1 wk after the last treatment. Blood samples were collected for measuring circulating autoantibodies, and proteinuria was assessed with urine dipstick (Uristix; Bayer) twice per week.

Flow cytometry analysis

Spleen and BM lymphocytes were collected in 4°C FACS buffer (3% FBS in PBS) and smashed in a cell strainer (100-mm Nylon; BD Falcon). Cold FACS buffer. Cells were finally suspended in FACS buffer and used for ELISPOT or flow cytometry. Immune cell populations were detected with fluorescein isothiocyanate–conjugated streptavidin-peridinin chlorophyll-a (SA-PB; Invitrogen); mouse GL-7 (BD Pharmingen) followed by incubation with Pacific blue–conjugated streptavidin-peridinin chlorophyll-a (SA-PB; Invitrogen); memory B cells (CD4/CD8/F4/80 IgD–CD19–IgG–CD38+)–PE-rat anti-mouse GL-7 (BD Pharmingen) followed by incubation with Pacific blue–conjugated streptavidin-peridinin chlorophyll-a (SA-PB; Invitrogen); memory B cells (CD4/CD8/F4/80 IgD–CD19–IgG–CD38+)–PE-rat anti-mouse IgD, biotin-anti-mouse IgD, FITC–rat anti-mouse IgGl/ IgG2a/b and Cy-5–rat anti-mouse CD38. A combination of PE–Cy5–rat anti-mouse CD4+ CD8+, and F4/80 were used to exclude non-B cells. Biotin-labeled Abs were detected with SA-PB (Invitrogen).

ELISPOT assay for ASCs

ELISPOT was performed as described previously (29, 30). In brief, 96-well MultiScreen plates (Millipore) were coated with poly-L-lysine and incubated with calf thymus DNA to detect anti-dsDNA–secreting cells. Plates were coated with goat anti-mouse IgG (Fc fragment specific; Jackson Immunoresearch Laboratories) to detect total IgG-secreting cells. Cell suspensions from spleen, BM, and kidney were added to individual wells, starting with 5 × 105 cells in the top row. Cells were incubated overnight at 37°C, in a 5% CO2 atmosphere. After incubation, plates were washed several times with 0.1% Tween 20 in PBS, incubated with alkaline phosphatase goat, anti-mouse IgG Ab (Southern Biotech), and finally developed with vector blue alkaline phosphatase substrate III kit (Vector Laboratories). ASCs were enumerated with an ELISPOT Scanner (Cellar Technology).

ELISA

Blood from experimental mice (control and treated groups) was collected, centrifuged, and serum was stored at −80°C until quantification of Ab levels. Anti-dsDNA, IgG concentration was determined with a commercial mouse anti-dsDNA kit from Alpha Diagnostic International according to manufacturer’s recommendations. Total IgG and IgM levels were quantified according to a previously described standard ELISA protocol (29).

Immunofluorescence analysis

Four-micrometer frozen sections were blocked for 30 min with 5% donkey serum. Slides were incubated with fluorescein isothiocyanate conjugated against IgG, IgM, and IgA (Jackson ImmunoResearch Laboratories), and FITC–peanut agglutinin (L7381; Sigma). Rabbit anti-PE (200-4199; Rockland Immunocchemicals) and Cy-3–donkey anti-rabbit (711-165-152; Jackson Immunoresearch Laboratories) were used to detect IgD+ cells and SA-Cy5 (19-4317-82; eBioScience) was used for visualization in IgG+ respectively. To detect FDC networks, T cells, and B cell follicles, we first incubated spleen sections with biotin–rat anti-mouse CD21–CD35 (clone 7E9; Biolegend), purified anti-mouse CD3 (clone M-20; Santa Cruz Biotechnology), and allophycocyanin–CD45R (B220, clone RA3-6B2; BD Pharmingen). After incubation with primary Abs, FDCs were detected with SA-Alexa Fluor 488 (S-3422; Invitrogen), and T cells were detected with Alexa Fluor 568 donkey anti-rabbit (A10157; Invitrogen). Frozen kidney sections were incubated with BR3–Fc human fusion protein, biotinylated anti-α–smooth muscle actin (anti-α–SMA; clone 1A4; Neomarkers), and FITC–rat anti-mouse IgG (BD Pharmingen). Incubation with primary Abs was followed by incubation with Cy-3 donkey anti-human IgG (Jackson Immunoresearch Laboratories; 709-166-149) to detect BAFF and SA-Cy5 (eBioScience; 19-4317-82) to visualize IgG deposition. PE–rat anti-mouse CD256 a proliferation-inducing ligand (APRIL; clone A3D8; Biolegend), FITC–anti-mouse/human CXCL12 (IC350F; R&D Systems), and purified rat anti-mouse F4/80 (MCA497GA; AbD Serotec), in combination with rabbit anti-PE (200-4199; Rockland Immunochemicals), Cy-3 donkey anti-rabbit (711-165-152; Jackson Immunoresearch Laboratories), biotin–donkey anti-rat (712-066-150; Jackson Immunoresearch Laboratories), and SA-Cy5 (19-4317-82; eBioScience) were used to detect April, CXCL12, and F4/80 macrophages. Slides were washed in PBS for 3 h and mounted with Slow Fade Gold Antifade with DAPI (S36938; Invitrogen). T cells were pseudocolored white. Representative pictures were taken with a Zeiss Axioplan Microscope and recorded with a Zeiss Axiolab camera. For morphometric analysis, all the PNA+ GCs in spleens and IgG+ deposition areas in the kidneys were outlined with the automated tool of the Carl Zeiss microscope. Because of the different size of frozen tissue sections, we calculated the percentages of area occupied by GC and IgG deposition by dividing the total area occupied by GCs or IgG signal by the total area of the tissue section.

Quantitative PCR

Ten 30-μm-thick frozen tissue slices were collected in TRizol (Invitrogen) and homogenized with a DNase and RNase free pipette tip. Five micrograms glycogen (AM9510; Ambion) was added to the sample in TRizol to increase RNA yield. RNA isolation was performed according to the TRizol protocol, and total RNA was quantified in the Nanodrop. Three micrograms total RNA was reverse transcribed using random primers and SuperScript II. cDNA concentration was adjusted to 10 ng/μl, and 50 ng cDNA was used per PCR. PCR was performed in a Step One plus ABI machine, using Applied Biosystems commercial probes for Gapdh (Mm9999991_g1), Bcl6 (Mm00477633_m1), Cxcl12 (Mm00445553_m1), April (Mm03809849_s1), Baff Mm00446347_m1), and Il6 (Mm00446190_m1), and according to the ABI protocol. To calculate changes in mRNA expression, we first normalized mRNA expression to Gapdh mRNA expression, and mRNA expression for each gene in the experimental groups was compared with the level of mRNA expression in BALB/c mice or with BALB/c mice.
Results

Progressive increase in ASCs correlates with systemic levels of circulating autoantibodies and accumulation of dsDNA-specific PCs in the inflamed kidney of SLE-prone mice

We previously demonstrated that ASCs specific for IgG or dsDNA were dramatically increased in spleen and moderately increased in BM from NZB/W mice with lupus nephritis (29). To better define the correlation between systemic levels of autoantibodies and progressive generation of ASCs in different anatomical compartments, we enumerated ASCs in spleen, BM, and kidney from NZB/W mice, collected at different ages. In 8-wk-old predisease NZB/W mice, total IgG ASCs were detected in the spleen and BM, but they were undetectable in the kidney (Fig. 1A), consistent with the findings reported in previous studies (25). In the spleen and kidney, there was a clear correlation among the increase in the number of isotype-switched PCs, the age-associated disease progression, and the levels of circulating IgG autoantibodies. In the BM, similar numbers of total IgG ASCs were detected in all groups, except in NZB/W mice with high autoantibody titers.

In contrast, dsDNA-specific ASCs were absent in the spleen, BM, and kidney at 8 wk of age (Fig. 1B). Considerable numbers of dsDNA* ASCs were first detected in the spleen (19 wk) and progressively increased in all three organs peaking at 39 wk. Confirming previous reports (26, 31), numbers of renal IgG- and dsDNA-specific ASCs increased significantly in 39-wk NZB/W F1 mice, especially those with high titers of anti-dsDNA Abs (Fig. 1A, 1B). This supports the idea that the inflamed kidney offers a unique environment for ASC generation, which is consistent with the findings reported in previous studies (25).

Anti-dsDNA IgG ASCs were undetectable in 8-wk-old NZB/W F1 female mice with high titer of dsDNA Ab (high; n = 5, >5-fold higher compared with that in 24-wk C57BL/6 mice) were compared with those with low titer of dsDNA Ab (low; n = 4, <3-fold higher compared with that in 24-wk C57BL/6 mice). To determine the kinetics of ASC development, we used 8- (n = 5) and 19-wk (n = 5) NZB/W F1 female mice. Total cells from spleen, BM, and kidney were harvested, and ELISPOT assay was performed as described in Materials and Methods. Horizontal bar represents mean (long) and SEM (short). (A) Total IgG ASCs in spleen, BM, and kidney. (B) Anti-dsDNA IgG ASCs from spleen, BM, and kidney. Anti-dsDNA IgG ASCs were undetectable in 8-wk NZB/W F1 female mice. (C) The ratio of anti-dsDNA IgG ASCs/total IgG ASCs in spleen, BM, and kidney, respectively. Left scale for spleen and BM, right scale for kidney. Significant p values (p < 0.05, unpaired t test) are as follows: *significantly different from the other groups, **significantly different from high, *significantly different from high and 8 wk, and **significantly different from corresponding group in spleen and BM.

At an early age (8 wk), IgG* PCs were rare, with the majority located inside the GC. By 19 wk, there was a modest increase in the number of IgG* PCs located either in the GC or in the extrafollicular zone. The location of IgG* PCs in close association with IgD*B220*PNA* GC B cells suggests that they were mainly derived from the GC. Finally, at 39 wk, numerous IgG* PCs were found in both the GC and the extrafollicular regions of the spleen (Fig. 2D). These results suggest that in experimental SLE, disease progression is associated with an increase in the number and size of GCs, which are likely the main source of isotype-switched PCs.
Increase in PC survival factors in lupus-prone mouse

Next, we assessed the kinetics of mRNA expression for survival factors (BAFF, APRIL, IL-6) and chemokines (CXCL12) that participate in the survival and recruitment of PCs to specialized niches in secondary lymphoid organs and inflamed tissues. Although IL-6 induces the terminal differentiation of B cells into PC and it is important for PC survival (32), IL-6 mRNA expression was stable until 39 wk, at which time a 4-fold increase was observed. APRIL mRNA expression showed a modest increase, and interestingly, CXCL12 mRNA expression progressively declined in the spleen tissue (Fig. 3A). In addition, we measured mRNA expression for Bcl-6, the master transcription factor for GC B cells and TFH cells, which are both critical players in PC production. Bcl-6 also participates in B cell proliferation, Ab class switching, and affinity maturation, and is critical for differentiation of naive B cells into PCs (33). In the spleen, we observed a modest but progressive increase in mRNA expression for Bcl-6 (data not shown).

In the inflamed kidney, IL-6 mRNA expression mirrored its expression in the spleen, dramatically increasing at 39 wk. BAFF and APRIL increased modestly in the kidney (Fig. 3B). Notably, the protein expression of IL-6, BAFF, and APRIL in the kidney was also increased in lupus compared with control mice (Fig. 3C). Although mRNA expression of CXCL12 remained constant, CXCL12 protein expression increased significantly in the kidney (Fig. 3C), following an opposite trend compared with the spleen. Overall, increased production of survival factors for PCs in both the spleen and the inflamed kidneys suggests that autoreactive PCs successfully find survival niches in both anatomical locations. A reduction in CXCL12 mRNA expression in the spleen, coupled with its increased expression in the kidney, implicates a chemokine gradient in promoting the selective movement of autoreactive PCs from the spleen to the inflamed kidney.

Short-term BCD with anti-mCD20 Ab does not impact ASCs

To better understand the impact of BCD therapy on PC kinetics and longevity, we decided to model the effects of short-term anti-CD20 treatment on PC accumulation in different anatomical compartments. Short-term therapy with anti-CD20 neither reduced the numbers of IgG- or dsDNA-specific ASCs (spleen, BM, and inflamed kidney; Fig. 4A, 4B) (29) nor the ratio of anti-dsDNA ASCs/anti-IgG ASCs (Fig. 4C). One potential explanation for the failure of short-term, anti-CD20 treatment to deplete autoreactive PCs is the lack of expression of CD20 on PCs. However, if PCs are short-lived, one would expect them to subsequently decline over time if BCD is effective. To test this possibility, we enumerated autoreactive PCs in a group of mice that received BCD therapy for 4 consecutive weeks, followed for an 8-wk resting period. Similar to short-term depletion, we did not observe any differences in the numbers of autoreactive PCs in spleen, BM, and kidney (Fig. 4; see 4/8 wk point).

Long-term BCD significantly reduces ASCs with dramatic effects on autoreactive cells in kidney

In contrast with short-term BCD therapy, prolonged treatment with anti-CD20 caused a significant reduction in the number of IgG- and dsDNA-specific ASCs in the spleen (>100-fold reduction: anti-IgG ASCs, >60-fold reduction: anti-dsDNA IgG ASCs; Fig. 4A, 4B). Although IgG- and dsDNA-specific ASCs were only modestly decreased in the BM (2.7- and 5.5-fold), the reduction in autoreactive PCs was still statistically significant. However, the ratio of dsDNA-specific ASCs/IgG-specific ASCs in the spleen and BM did not significantly change (Fig. 4C). Given the enrichment of autoreactive PCs in the inflamed kidney, we also analyzed the impact of long-term BCD therapy on the numbers of autoreactive PCs in this location. Total IgG ASCs in kidney from treated mice were modestly reduced (control: 5.94 ± 0.53 × 10⁴/kidney, 12-wk treatment: 1.72 ± 0.08 × 10⁴/kidney; Fig. 4A, 4B). However, a more pronounced impact was noted on renal anti-dsDNA ASCs (15-fold reduction; control: 1.16 ± 0.06 × 10⁴/kidney versus 12-wk treatment: 0.074 ± 0.013 × 10⁴/kidney). Finally, the ratio of dsDNA-specific ASCs/IgG-specific ASCs in treated mice declined 6.8-fold compared with control group (control: 27.3 ± 3.7%; treated mice: 3.98 ± 0.73%; p = 0.022).
Correspondingly, serum anti-dsDNA Ab levels also declined with prolonged treatment (Fig. 4D; \( p = 0.0095 \) control versus 12-wk treatment). Overall, these results suggest that splenic and renal ASCs can be effectively targeted by long-term BCD therapy, but this is unlikely to be mediated by CD20 expression on their surface or the gradual disappearance of short-lived PCs. In addition, prolonged BCD suppressed the progression of nephritis (mean \( \pm \) SEM of proteinuria [0–4+] 3.17 \( \pm \) 0.19 in control \( n = 6 \) versus 1.17 \( \pm \) 0.17 in 12-wk treatment \( n = 6 ; p = 0.012 \)).

Long-term BCD therapy efficiently reduces B cells and GCs in the spleen

One mechanistic explanation for the reduction of autoreactive PCs after long-term BCD therapy is the more efficient elimination of B cells before or during differentiation in the GC. To explore this hypothesis, we analyzed the impact of long-term BCD on B cell numbers, GC differentiation, and spatial distribution by flow cytometry and immunofluorescence. In agreement with previous reports (34, 35), short-term treatment was effective in depleting B220+ B cells but had less impact on GL-7+B220+ GC B cells. The duration of BCD after short treatment was temporary because numbers of B cells returned to control levels in the group treated for 4 wk and were allowed to reconstitute for 8 wk. In sharp contrast, long-term BCD therapy had a profound effect on both B220+ B cells and B220+GL-7+ GC B cells (Fig. 5A). Detection of clusters of large IgD+B220+PNA+ cells by immunofluorescence showed that GCs disappeared or shrank substantially 12 wk after anti-CD20 treatment. Conversely, GCs were still intact in mice receiving BCD therapy for only 4 wk (Fig. 5C). Overall, these results suggest that long-term BCD therapy is required to efficiently eliminate GC B cells and interfere with the generation of GC-derived autoreactive PCs in the spleen of lupus-prone mice.
PCs decrease after long-term BCD treatment

To investigate the impact of prolonged anti-mCD20 Ab therapy on the PC population, we enumerated CD138+κL chain+ cells by flow cytometry. Only long-term BCD therapy induced a statistically significant 10-fold reduction in the number of splenic PCs. Neither short-term nor long-term BCD had a significant impact on the population of quiescent long-lived PCs in the BM (Fig. 5B).

Interactions of B cells with stromal cell populations are critical for maintaining the architecture of secondary lymphoid organs. Visual examination of spleen sections stained with Abs against FDCs (CD21-CD35), T cells, (CD3) and B cells (B220) revealed the control group contained numerous and well-organized GCs with dense CD21-CD35+ FDC networks, CD3+ T cell infiltrates, large

**FIGURE 4.** Long-term anti-mCD20 treatment significantly reduces ASCs. Twenty-four- to 28-wk NZB/NZW F1 female mice with high-titer anti-dsDNA Abs were dosed weekly with anti-mCD20 Ab or control IgG (n = 6, Ctrl). One group was treated four times weekly and sacrificed 1 wk later after last treatment (n = 5, 4 wk). One group was treated weekly four times (n = 4) and sacrificed 8 wk later (4/8 wk). Another group was treated weekly 12 times (n = 4) and sacrificed 1 wk after the last treatment (12 wk). Cells from spleen, BM, and kidney were collected, and total IgG and dsDNA IgG ASCs were determined by ELISPOT. Solid circle represents control mice (treated for 12 wk with control IgG), solid diamond represents 4 wk treated mice; solid up triangle represents 4/8 wk treated mice; solid down triangle represents 12 wk treated mice. Bars represent mean ± SEM. The p values were calculated by Mann–Whitney U test. (A) Total IgG ASCs from spleen, BM, and kidney. Significant reduction of total IgG ASCs is observed after 12 wk BCD with anti-mCD20 Ab compared with untreated control mice. (B) Anti-dsDNA IgG ASCs from spleen, BM, and kidney. Significant reduction of anti-dsDNA IgG ASCs is observed after 12 wk BCD with anti-mCD20 Ab compared with untreated control mice. (C) The percentage of anti-dsDNA IgG ASCs/total IgG ASCs in spleen (left scale), BM (left scale), and kidney (right scale), respectively. Significant reduction of dsDNA/IgG ratio is observed after anti-mCD20 Ab treatment. (D) The Ab in plasma was measured before treatment (baseline) and at end point, and the change relative to baseline was calculated. After 12 wk BCD, anti-dsDNA Ab level is significantly decreased (control versus 12 wk: p value = 0.0095; control versus 4/8 wk: p value NS).

**FIGURE 5.** Long-term BCD decreases PC generation by dramatically reducing B cells and GCs in the spleen. As described earlier, four groups including control mice treated with control IgG (Ctrl; n = 6), mice treated with anti-mCD20 Ab for 4 wk (4 wk; n = 8), mice with 8-wk reconstitution after 4-wk treatment with anti-mCD20 Ab (4/8 wk; n = 6), and mice treated with anti-mCD20 Ab for 12 wk (n = 4) were sacrificed. Tissue and lymphocytes were collected and analyzed by flow cytometry and immunohistochemistry. (A) Percentage of residual B cells after BCD via flow cytometry expression of B220 (upper) and GL-7 (lower) in lymphocytes in spleen. (B) Percentage of residual PCs in total cells after treatment with anti-mCD20 Ab. PCs were enumerated by flow cytometry as CD138+κL chain+ cells. Bars show mean ± SEM. Changes in the splenic architecture associated to long-term CD20 depletion. Four-micrometer spleen frozen sections were stained with Abs to detect FDCs (CD21-CD35, red), B cells (B220, green), and T cells (CD3, white). GCs and PCs were visualized with Abs against IgD (red), peanut agglutinin (green), and IgG (white). GCs are outlined with dashed yellow lines. Representative of n = 5 mice/group. The average number of IgG+ cells counted in 5–10 randomly selected (original magnification ×200) fields significantly decreased after 12-wk BCD therapy (p = 0.026).

**PCs decrease after long-term BCD treatment**

To investigate the impact of prolonged anti-mCD20 Ab therapy on the PC population, we enumerated CD138+κL chain+ cells by flow cytometry. Only long-term BCD therapy induced a statistically significant 10-fold reduction in the number of splenic PCs. Neither short-term nor long-term BCD had a significant impact on the population of quiescent long-lived PCs in the BM (Fig. 5B). Interactions of B cells with stromal cell populations are critical for maintaining the architecture of secondary lymphoid organs. Visual examination of spleen sections stained with Abs against FDCs (CD21-CD35), T cells, (CD3) and B cells (B220) revealed the control group contained numerous and well-organized GCs with dense CD21-CD35+ FDC networks, CD3+ T cell infiltrates, large
clusters of IgD- B220<sup>+</sup>PNA<sup>+</sup> GC B cells, and follicular and extrafollicular IgG<sup>+</sup> PCs. Short-term treatment with anti-CD20 depletion resulted in a reduction in the size of GCs, impaired T cell infiltration inside GC, and promoted enlargement of the T cell zone. However, GC organization and the presence of PCs were still evident after short-term BCD for 4 wk. In the group that experience reconstitution after treatment for 4 wk with anti-CD20 and 8-wk rest, there was recovery in the size and organization of B cell follicles and GC, more T cells were detected inside the GC, and the FDC networks began to acquire their classic concentric and dense distribution. Long-term treatment with anti-CD20 resulted in a remarkable shrinkage of the B cell follicles and FDC networks (Fig. 5C). In addition, there was a significant decrease in isotype-switched, IgG<sup>+</sup> PCs on morphometric analysis (IgG<sup>+</sup> PCs/×200 high-power field, mean ± SEM, control: 65.3 ± 4.8, 4 wk: 58.5 ± 2.5, 4/8 wk: 52.9 ± 4.9, 12 wk: 26.8 ± 1.5, *p* = 0.0001 for unpaired *t* test comparison between 12 wk and other groups).

**BCD increases the expression of PC survival factors in spleen but decreases expression in the inflamed kidney**

To determine whether long-term BCD therapy modulated the expression of survival factors for PCs (32, 36), we examined mRNA expression for CXCL12, IL-6, BAFF, and APRIL by quantitative PCR. Interestingly, both long-term and short-term BCD therapy increased the expression of BAFF and APRIL in the spleen (Fig. 6A). Although we were predicting that BCD would lead to an increase in available BAFF protein, an increase in BAFF and APRIL mRNA expression was unexpected. This suggests that B cells are required to modulate the production of BAFF and APRIL in the spleen and/or that increased expression of both survival factors is a compensatory mechanism to induce the rapid recovery of B cells. BCD also induced an increase in mRNA expression for CXCL12 and IL-6 in the spleen (Fig. 5A). Given that CXCL12 and IL-6 are required for PC survival (32), an increase in expression in the spleen after BCD may contribute to the maintenance of PCs in this location. In contrast with results in the spleen, we found that mRNA levels for BAFF, APRIL, and IL-6 were significantly reduced in the kidney after both short-term and long-term BCD therapy (Fig. 6B). CXCL12 mRNA expression was unchanged, although notably not increased (Fig. 6B).

To more directly characterize the PC niche in the kidney and the impact of BCD, we next examined the protein expression and cellular localization of survival factors implicated in PC survival. In control mice, BAFF was expressed predominantly in glomeruli (Fig. 6C, control panel on left and magnified image) and was dramatically reduced to undetectable levels after BCD (see quantitation in Fig. 6D), correlating with the PCR data. In marked contrast, BAFF expression was unchanged in mice treated with short-term BCD therapy (Fig. 6D). APRIL was preferentially produced by stromal cells in and around inflamed glomeruli (Fig. 6C, control panel on right) and was significantly decreased with long-term BCD, but only modestly changed with short-term treatment. Both treatment groups displayed a reduction in IL-6 and CXCL12 expression (Fig. 6D). Both stromal cells and F4/80<sup>+</sup> macrophages (37) in the inflamed kidney were prime producers of CXCL12 (Fig. 6C). Notably, F4/80<sup>+</sup> macrophages were significantly decreased, but only after long-term BCD (see quantitation in Fig. 6D). These findings suggest that macrophages and stromal cells contribute to homing and survival of PCs in the inflamed kidney, and BCD alters this niche by decreasing the numbers of macrophages and the expression of key cytokines and chemokines.

**Discussion**

This study explored the generation and maintenance of autoreactive PCs in murine lupus and the impact of BCD therapy. Anti-dsDNA PCs were rapidly enriched in the kidney after generation in GC reactions in the spleen, concurrent with the development of serum autoantibodies and preceding the onset of frank nephritis. Auto-reactive PCs further accumulated in the kidney, with disease progression coincident with an increase in renal expression of key PC survival factors (BAFF, APRIL, and IL-6) and PC-attracting chemokines (CXCL12). Although short-term anti-CD20 treatment suppressed the progression of nephritis, ASCs in multiple organs were not significantly impacted even after a prolonged period elapsed between treatment and follow-up (8 wk). In marked contrast, long-term BCD with anti-CD20 (12 wk) dramatically reduced ASCs in the spleen and kidney, with particularly notable effects on autoreactive PCs in the kidney. The reduction in PCs in the kidney was accompanied by decreases in PC survival factors and PC-attracting chemokines, suggesting that BCD can alter the PC survival niche.

The accumulation of autoantibody-secreting cells and long-lived PCs in the kidneys of lupus-prone mice has been recently described (25, 26). However, our results detail for the first time, to our knowledge, the kinetics of this process and suggest a role for specific soluble factors (BAFF, APRIL, CXCL12, IL-6) in establishing a PC survival niche. Because GC B cells have not been readily detected in the kidney, it was proposed that autoreactive PCs are generated in secondary lymphoid organs such as spleen and migrate into kidney, finding survival niches (26, 31). Indeed, in our experiments, spontaneous GCs were first noted at 8 wk in the spleen of lupus-prone mice and increased further at the onset of serum autoantibody production (19 wk) and nephritis progression (39 wk). This correlated with an increase in Bcl-6 first noted at 8 wk, notable given that it is a key transcription factor for GC B cells and TFH cells, which are both critical players in GC reactions and PC production (33). Another factor that enhances B cell differentiation to PC is IL-6 (38), but this was significantly enriched in the splenic microenvironment only in older diseased mice (39 wk old). In contrast, the PC survival factor APRIL was increased even predisease onset similar to Bcl-6. Notably, in the kidney of older lupus-prone mice, the protein expression of multiple PC survival factors including BAFF, APRIL, IL-6, and CXCL12 was increased. Although we found active immune responses in the spleen of lupus-prone mice with numerous active GC reactions and ongoing PC generation, it remains possible that PCs also arise within the kidney. Indeed, a recent study in human lupus nephritis described GC and T:B aggregate in the renal tubulointerstitium. Moreover, B cell proliferation and somatic hypermutation were ongoing, implicating the kidney as a site of active immune responses and source of PCs (39).

The other notable finding in our study is the impact of long-term BCD on ASCs in the kidney, spleen, and BM. We propose two mechanisms for this effect: 1) decreased generation of new PCs via interruption of GC reactions in the spleen, and 2) disruption of the PC survival niche that contributes to the maintenance of long-lived PCs. It has been previously shown that anti-CD20 effectively depletes marginal zone, GC, and memory B cells but does not decrease long-lived Ab levels, suggesting that memory B cell subsets are not necessary for maintaining Ab levels (27, 28). However, in autoimmunity, there may be some resistance of B cell subsets to depletion in both mice (29, 34) and humans (15, 40). Our data suggest that prolonged treatment with anti-CD20 may overcome this effect. Although autoreactive ASCs decreased in all tissues with prolonged BCD, there was differential sensitivity with...
effects in the kidney more pronounced than spleen and BM. Only in the kidney was the high ratio of anti-dsDNA IgG ASC to total IgG ASCs decreased. This could be because the frequency of long-lived versus short-lived PCs varies in the different tissues. For example, in NZB/W mice, up to 40% of splenic PCs are long-lived (21), whereas this frequency is even higher in the BM (41).

**FIGURE 6.** Long-term anti-CD20 BCD alters PC survival in the kidney. Total RNA was isolated from spleens (A) and kidney (B) from control group (n = 8 spleen, n = 12 kidney), mice treated for 4 wk (n = 5 only spleen), 4/8 wk mice (n = 5 spleen and n = 6 kidney), and mice treated for 12 wk (n = 4 spleen and kidney). Quantitative PCR was performed as described in Fig. 3 and Materials and Methods. Levels of expression for each gene were first normalized to GAPDH and then compared with the levels of expression in the control group, according to the ΔΔCT method. Representative data from two experiments with similar results are shown. (C) Decrease in PC survival factor expression in the kidney after long-term CD20 depletion. Four-micrometer kidney frozen sections were stained with Abs to detect BAFF (red), APRIL (red), IgG (green), CXCL12 (green), and IL-6 (data not shown). Macrophages were visualized with Abs against F4/80 (white). Anti-SMA (white) was used for detecting myoepithelial cells. Glomeruli are outlined with dashed yellow lines. The square area stained with BAFF/IgG/SMA is magnified and shown in the left middle. The square area stained with APRIL/CXCL12/F4/80 is magnified and shown in the right middle. Arrow highlights a CXCL12-expressing macrophage. Asterisk highlights a stromal cell coexpressing APRIL and CXCL12. Representative of n = 5 mice/group (original magnification ×200), quantitated in (D). (D) Quantitation of F4/80+ macrophages, BAFF, APRIL, CXCL12, and IL-6 expression was performed by morphometric analysis and reveals a significant decrease after BCD. Bars show mean ± SEM. The p values are calculated by unpaired t test. ND, nondetected.
Short-lived PCs (plasmablasts) can still express low levels of CD20 (42), and thus may be directly targeted by anti-CD20. Alternatively, thorough depletion of precursor B cells and interruption of ongoing PC generation should ultimately decrease short-lived PCs.

An additional explanation for the impact of prolonged BCD on autoreactive ASCs is an alteration in the niche that affects the survival of long-lived PCs. Many factors are required for B cell differentiation to PC (IL-6), homing to the PC niche (CXCL12), and PC survival (BAFF, APRIL) (43, 44). In BAFF KO and APRIL KO mice, PCs in BM are profoundly decreased (45). In contrast, once a long-lived PC pool is established in the BM, blockade of both BAFF and APRIL, and other factors may be necessary to deplete long-lived PCs (22). Thus, blockade of BAFF or APRIL decreased autoreactive PCs in the spleen and BM of lupus-prone mice (46), but combined BAFF/APRIL blockade had a greater impact on total IgG ASCs in the BM (46) and the frequency of PCs in the spleen (47). The impact of such therapy on renal PCs has not been studied, although activated renal macrophages did decrease (46). In this study, we find that BM total IgG ASCs and anti-dsDNA ASCs were slightly, although significantly, reduced with prolonged BCD, with more pronounced effects in the spleen and even more significant effects in the kidney. Interestingly, PC survival factors including BAFF and APRIL were dramatically decreased by long-term but not short-term BCD.

A major question is how BCD alters PC survival and migration factors in the kidney. We found that macrophages producing APRIL and CXCL12 in the kidney were significantly decreased and BAFF production was abrogated with prolonged BCD, suggesting for the first time, to our knowledge, that BCD may alter the PC survival niche by impacting infiltrating and resident cells in the kidney that produce key PC survival factors and chemoattractants. This is in accord with published data demonstrating an accumulation of activated renal macrophages in the kidney in murine lupus nephritis (48), but therapeutic targeting of these pathways has not been previously demonstrated. These effects are likely critical to the beneficial impact of prolonged BCD given that PCs in the inflamed kidney in murine lupus nephritis appear to be long-lived based on phenotype and BrdU labeling (25, 26). We speculate that splenic and BM PC niches are not impacted by BCD in the same way as the kidney given that decreases in ASCs in these locations were more modest. A recent study in human immune thrombocytopenia (ITP) found that splenic PCs after BCD acquired a long-lived gene expression signature similar to BM PCs and were resistant to rituximab (49). This may be mediated by increases in BAFF and APRIL in the setting of BCD, as we have demonstrated in this article.

In summary, our experiments show that the inflamed kidney is a site for enrichment of long-lived PCs, a compartment notoriously difficult to target. Although short-term BCD with anti-CD20 had no significant effect on ASCs, long-term BCD significantly reduced ASCs in the spleen, BM, and kidney by both interrupting the ongoing generation of short-lived PCs in the spleen and altering the long-lived PC niche in the kidney. The results provide important information about BCD and the factors that regulate the survival of PCs, with critical implications for the treatment of autoimmune disease.

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Disclosures
The authors have no financial conflicts of interest.


