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Journal

The Journal of Immunology, 192(3)

ISSN

0022-1767

Authors

Lamagna, Chrystelle
Hu, Yongmei
DeFranco, Anthony L
[et al.](#)

Publication Date

2014-02-01

DOI

10.4049/jimmunol.1301979

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This information is current as of April 3, 2014.

J Immunol 2014; 192:919-928; Prepublished online 27 December 2013;

doi: 10.4049/jimmunol.1301979

<http://www.jimmunol.org/content/192/3/919>

Supplementary Material <http://www.jimmunol.org/content/suppl/2013/12/26/jimmunol.1301979.DCSupplemental.html>

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B Cell–Specific Loss of Lyn Kinase Leads to Autoimmunity

Chrystelle Lamagna,* Yongmei Hu,* Anthony L. DeFranco,[†] and Clifford A. Lowell*

The Lyn tyrosine kinase regulates inhibitory signaling in B and myeloid cells: loss of Lyn results in a lupus-like autoimmune disease with hyperactive B cells and myeloproliferation. We have characterized the relative contribution of Lyn-regulated signaling pathways in B cells specifically to the development of autoimmunity by crossing the novel *lyn*^{flox/flox} animals with mice carrying the Cre recombinase under the control of the *Cd79a* promoter, resulting in deletion of Lyn in B cells. The specific deletion of Lyn in B cells is sufficient for the development of immune complex–mediated glomerulonephritis. The B cell–specific Lyn-deficient mice have no defects in early bone marrow B cell development but have reduced numbers of mature B cells with poor germinal centers, as well as increased numbers of plasma and B1a cells, similar to the *lyn*^{−/−} animals. Within 8 mo of life, B cell–specific Lyn mutant mice develop high titers of IgG anti-Smith Ag ribonucleoprotein and anti-dsDNA autoantibodies, which deposit in their kidneys, resulting in glomerulonephritis. B cell–specific Lyn mutant mice also develop myeloproliferation, similar to the *lyn*^{−/−} animals. The additional deletion of MyD88 in B cells, achieved by crossing *lyn*^{flox/flox}*Cd79a-cre* mice with *myd88*^{flox/flox} animals, reversed the autoimmune phenotype observed in B cell–specific Lyn-deficient mice by blocking production of class-switched pathogenic IgG autoantibodies. Our results demonstrate that B cell–intrinsic Lyn-dependent signaling pathways regulate B cell homeostasis and activation, which in concert with B cell–specific MyD88 signaling pathways can drive the development of autoimmune disease. *The Journal of Immunology*, 2014, 192: 919–928.

Lyn is a Src family tyrosine kinase expressed by hematopoietic cells. It has unique regulatory properties, as it triggers both activation and inhibitory signals (1, 2). In B lymphocytes, Lyn functions at the initial step of BCR signaling by phosphorylating tyrosines in the ITAMs of the I α /Ig β (CD79a/CD79b) BCR subunits, initiating signaling events that lead to B cell proliferation and Ab production. However, Lyn is not uniquely required for the initiation of BCR signaling, as the Src family tyrosine kinase members Fyn and Blk compensate for its deficiency (3). In contrast, Lyn has the sole capability to engage feedback inhibitory pathways by phosphorylating the ITIMs of the sialic acid-binding protein CD22 and the inhibitory Fc receptor for IgG Fc γ RIIb (4). Phosphorylation of these ITIM-containing receptors by Lyn leads to the recruitment to the membrane of SHIP-1 and Src homology region 2 domain–containing phosphatase 1 (SHP-1) that inhibit downstream BCR signaling. The function of Lyn in inhibitory signaling is dominant over its role in B cell activation. Hence, Lyn deficiency in B cells leads to enhanced BCR signaling characterized by increased calcium flux, increased activation of the MAPK pathway, and hyperproliferative responses following BCR cross-linking (5, 6).

Systemic lupus erythematosus (SLE) is a complex autoimmune disease triggered by genetic and environmental factors. It is characterized by a loss of tolerance to nuclear Ags leading to the production of autoreactive Abs, which deposit in tissues as immune complexes causing inflammation and end organ damage. In humans, polymorphisms in the *LYN* gene have been correlated with lupus disease, and a reduction in *LYN* expression in B cells has been found in patients with SLE (7, 8). *Lyn*^{−/−} mice develop an autoimmune inflammatory disease that resembles human lupus. The Lyn-deficient mice have elevated numbers of plasma cells that produce high levels of autoreactive Abs (anti-dsDNA, anti-ssRNA), leading to severe glomerulonephritis (9–12). Additionally, the *lyn*^{−/−} mice manifest considerable reduction in the numbers of mature follicular and immature transitional (T1, T2, and T3) B cells in lymphoid organs but equal numbers of newly formed immature B cells in the bone marrow (10, 13–15). The reduction in mature B cells in *lyn*^{−/−} mice is thought to be due to defects in survival (increased Bim levels) rather than defects in developmental maturation (6). Interestingly, the exaggerated BCR signaling associated with Lyn deficiency is predominant in transitional T3 and mature follicular B cell populations, but only moderate in immature T1 and T2 cells. *Lyn*^{−/−} mature follicular B cells display enhanced basal calcium signaling and ERK activation upon BCR engagement (16). In response, Lyn-deficient transitional B cells and mature follicular B cells exhibit increased expression of CD69, MHC class II (MHC II), and CXCR5 (6). *Lyn*^{−/−} mice also present defects in germinal center (GC) formation with decreased numbers of GC B cells but accumulation of splenic plasmablasts and autoantibody-producing plasma cells (9–12).

The autoimmune phenotype of *lyn*^{−/−} mice has been mainly attributed to alterations of B cell function. However, recent findings from our laboratory have shown that *lyn*^{−/−} macrophages and dendritic cells (DCs) also play an important role in the pathogenesis of lupus-like disease in the *lyn*^{−/−} model. Indeed, deletion of Lyn in myeloid cells (using mixed bone marrow chimeric mice) leads to myeloid expansion and the development of an autoimmune phenotype similar to, although not as severe as, the disease in total *lyn*^{−/−} animals (17). Additionally, the specific deletion of Lyn in

*Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA 94143; and [†]Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94143

Received for publication July 25, 2013. Accepted for publication November 23, 2013.

This work was supported by National Institutes of Health Grants AI65495 and AI68150 (to C.A.L.) and AI078869 (to A.L.D.).

Address correspondence and reprint requests to Dr. Clifford A. Lowell, University of California, San Francisco, Department of Laboratory Medicine, 513 Parnassus Avenue, Box 0451, San Francisco, CA 94143. E-mail address: clifford.lowell@ucsf.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional dendritic cell; DC, dendritic cell; *f/f*, flox/flox; GC, germinal center; MHC II, MHC class II; pDC, plasmacytoid dendritic cell; SFK, Src family kinase; SHP-1, Src homology region 2 domain–containing phosphatase 1; SLE, systemic lupus erythematosus; sm/RNP, Smith Ag ribonucleoprotein; T1, transitional stage 1; T2, transitional stage 2; T3, transitional stage 3.

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DCs is sufficient for the spontaneous activation of B and T cells and the subsequent development of autoantibodies and severe nephritis in mice (18). Therefore, it remains unclear whether the altered signaling in B cells lacking Lyn kinase can drive disease in the context of wild-type myeloid cells or DCs. These studies raise the question as to the relative contributions of different immune cell types to autoimmune pathogenesis in the *lyn*^{-/-} model.

To directly assess whether dysregulated Lyn-deficient B cells initiate or drive autoimmunity, we took advantage of the novel *lyn*^{fllox/flox} (*lyn*^{flf}) transgenic mouse (18), which we crossed with mice carrying the Cre recombinase under the control of the *CD79a/mb1* promoter (*Cd79a-cre*) (19), resulting in deletion of Lyn in B cells. In this study we show that the specific deletion of Lyn in B cells alone is sufficient for the spontaneous activation of B cells, the development of autoreactive Abs, and glomerulonephritis in mice. B cell-specific Lyn-deficient mice exhibited an increase in plasma cell formation as well as a reduction in splenic GCs. The additional deletion of MyD88 in B cells reversed the autoimmune phenotype observed in B cell-specific Lyn-deficient mice by blocking the development of pathogenic IgG autoantibodies. Thus, we demonstrate that the absence of Lyn inhibitory signaling in B cells alone is sufficient to drive autoimmunity.

Materials and Methods

Mice

Lyn^{-/-}, *lyn*^{flf}, *myd88*^{flf}, and *Cd79a-cre* mice were previously described (18–20). All animals were backcrossed at least nine generations onto the C57BL/6 background and kept in a specific pathogen-free facility at the University of California, San Francisco. Animal experiments were done in compliance with the Animal Welfare Act and Regulations, the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, the Public Health Service Policy on the Humane Care and Use of Laboratory Animals, and the University of California, San Francisco Policies and Guidelines.

All experiments were done comparing *lyn*^{flf}*Cd79a-cre*^{+/+}, *lyn*^{+/+} *Cd79a-cre*^{Tg/+}, and C57BL/6 animals to *lyn*^{flf}*Cd79a-cre*^{Tg/+} (hereafter *B-lyn*^{-/-}) and *lyn*^{-/-}. The groups *lyn*^{flf}*Cd79a-cre*^{+/+}, *lyn*^{+/+} *Cd79a-cre*^{Tg/+}, and C57BL/6 showed similar results (data not shown). For clarity, the data from these three groups were pooled and defined as the control group.

Flow cytometry

Single-cell suspensions were prepared from spleens as previously described (17), blocked with anti-CD16/32 (clone 2.4G2) and murine IgG (Sigma-Aldrich), and stained with the following: FITC-, R-phycoerythrin-, allophycocyanin-, allophycocyanin-Cy7- or allophycocyanin-Alexa Fluor 780-, PE-Cy7-, or biotin-conjugated Abs; CD11c (clone HL3), CD19 (ID3), CD21 (7G6), CD95/Fas (Jo2), CD117/c-Kit (2B8), CD138 (281-2), GL7 (GL7), Gr-1 (RB6-8C5), IgD^b (Igh-5b), Ly6C (AL-21), and MHC II (I-A^b, AF6-120.1) from BD Pharmingen; B220 (RA3-6B2), CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD11b (M1/70), CD23 (B3B4), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD86 (GL1), CD93 (AA4.1), IgM (II/41), and TCRβ (H57-597) from eBioscience; 7/4 from Abcam; F4/80 from Serotec; and IgM Fab from Jackson ImmunoResearch Laboratories. The rabbit polyclonal Ab anti-mouse Lyn was previously described (17). The gating strategies used to identify the different myeloid, dendritic, and T cell populations by FACS have been previously reported (18). The gating strategies used to identify B cell populations are shown in Supplemental Fig. 2. DAPI (0.1 μg/ml; Sigma-Aldrich) staining was used to exclude dead cells.

Flow cytometry data were collected on a Fortessa flow cytometer (Becton Dickinson) from the Flow Cytometry Center at University of California, San Francisco and analyzed using FlowJo software (Tree Star).

Analysis of calcium flux

For measurements of intracellular-free calcium levels, splenocytes were loaded with Indo1-AM (Invitrogen) and then labeled for CD93, CD23, IgM Fab, and B220. Cells were resuspended in RPMI 1640 supplemented with 1% BSA and 20 mM HEPES and warmed to 37°C for 3 min, and analysis was initiated with flow cytometry. After the baseline was established for

30–45 s, cells were stimulated with 15 μg/ml goat anti-mouse IgM F(ab')₂ (Jackson ImmunoResearch Laboratories). The median intracellular calcium concentration as indicated by the ratio of fluorescence 405 nm emission to 530 nm emission was measured over time by flow cytometry. Propidium iodide staining was used to exclude dead cells.

Histology

Kidneys were processed and stained with H&E by the University of California, San Francisco Pathology Core. The presence of nephritis was determined on H&E-stained sections. The severities of the disease in the glomerular and interstitial compartments were arbitrarily graded as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Morphological analysis of glomerulonephritis involved assessment of glomerular hypercellularity, glomerular size, and presence of glomerular sclerosis. Morphological analysis of interstitial nephritis involved the assessment of infiltration of mononuclear cells and loss of normal architecture. Kidneys were snap frozen in OCT and 5-μm sections were stained for C3 or IgG as previously described (17).

Quantification of serum Igs, autoantibodies, and cytokines

Serum levels of BAFF, total IgG and IgM, anti-dsDNA IgG and IgM, and anti-Smith Ag ribonucleoprotein (sm/RNP) IgG and IgM were determined by ELISA as previously described (17). Serum levels of specific IgG isotypes against dsDNA and sm/RNP were determined by ELISA using SouthernBiotech reagents. Cytokine levels in serum were quantified using Milliplex MAP mouse cytokine/chemokine kit from Millipore, following the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using GraphPad Prism. Statistical differences between three groups or more were calculated with a one-way ANOVA test and a Bonferroni multiple comparison post-hoc test. Only significant differences ($p \leq 0.05$) are indicated.

Results

Generation of B cell-specific Lyn-deficient mice

To investigate the specific role of Lyn in B cells, we crossed mice carrying the *lyn*^{flf} allele with *Cd79a-cre* transgenic mice (18, 19). The efficiency of deletion of the *lyn*^{flf} allele by Cre recombination under the control of the *Cd79a* promoter was determined by intracellular detection of Lyn followed by flow cytometry using peripheral blood leukocytes from 2-mo-old mice (Supplemental Fig. 1). In the control mice, Lyn was expressed in B and myeloid cells, but not T cells. Lyn expression in the B cells of *lyn*^{flf}*Cd79a-cre*⁺ mice (*B-lyn*^{-/-} mice hereafter) was reduced to the background levels seen in the total *lyn*^{-/-} animals. We found no reduction in Lyn expression in peripheral blood myeloid cells in *B-lyn*^{-/-} mice, demonstrating the specificity of Lyn deletion in B cells of *B-lyn*^{-/-} mice.

Lyn inhibitory signaling in B cells is required to maintain B cell homeostasis and activation

Previous studies have reported that *lyn*^{-/-} mice exhibit alterations in their B cell compartment that correlate with their autoimmune phenotype (10–12, 16). We tested whether deletion of Lyn in B cells alone was sufficient to affect the B cell lineage. To this end we analyzed by flow cytometry the expression of cell surface markers indicative of developmental and activation status of B cells in the spleens of *B-lyn*^{-/-} mice, compared with *lyn*^{-/-} and control mice (gating strategies are shown in Supplemental Fig. 2). Similar to *lyn*^{-/-}, aged *B-lyn*^{-/-} animals had significantly decreased numbers of CD19⁺ and GC B cells, with increased numbers of Ab-producing CD19^{lo/-}CD138^{hi} plasma cells and B1 cells in the spleens (Fig. 1A–C, 1E). In the peritoneal cavity, B1 cells were expanded in number (control, $1.82 \times 10^5 \pm 0.66$; *lyn*^{-/-}, $6.7 \times 10^5 \pm 0.75$, $p \leq 0.001$ versus control; *B-lyn*^{-/-}, $17.29 \times 10^5 \pm 0.29$, $p \leq 0.001$ versus control, $*p \leq 0.05$ versus *lyn*^{-/-}), with a reciprocal reduction in peritoneal B2 cells (Fig. 1F). The B1 cells in

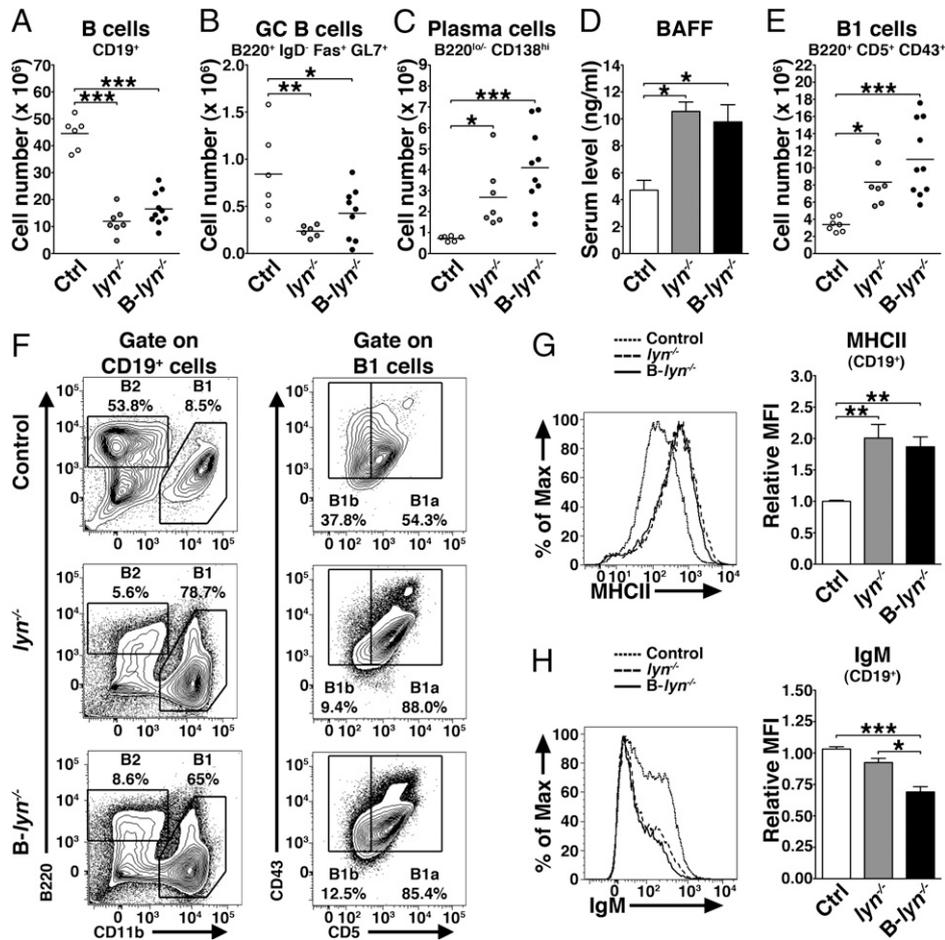


FIGURE 1. Lyn inhibitory signal in B cells controls B cell homeostasis. (**A–C**) Absolute numbers of total B cells (**A**), GC B cells (**B**), and plasma cells (**C**) in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. Data represent mean of independent experiments. Each dot represents an individual mouse. (**D**) BAFF levels in the serum of control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice (8 mo old) were determined by ELISA. Bars represent means \pm SEM from 6 to 10 mice per group. (**E**) Absolute numbers of B1 cells in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. Data represent means of independent experiments. Each dot represents an individual mouse. (**F**) Representative FACS contours showing the percentages of B1 and B2 cells in the peritoneal cavity of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. B1 cells were determined as CD19⁺CD11b⁺B220^{low}CD43⁺ cells and further defined as B1a (CD5⁺) or B1b (CD5⁻) subsets. B2 cells were determined as CD19⁺B220⁺CD11b⁻ cells. (**G**) Representative FACS histogram showing the expression level of MHC II by splenic B cells from 8-mo-old mice (*left panel*). Mean fluorescence intensity (relative to control) of MHC II expressed by B cells in the spleens of 8-mo-old mice (*right panel*). Bars represent means \pm SEM of independent experiments from 6 to 10 mice per group. (**H**) Representative FACS histogram showing the expression level of IgM by splenic B cells from 8-mo-old mice (*left panel*). Mean fluorescence intensity (relative to control) of IgM expressed by B cells in the spleens of 8-mo-old mice (*right panel*). Bars represent means \pm SEM of independent experiments from 6 to 10 mice per group. (**A–H**) **p* \leq 0.05, ***p* \leq 0.01, ****p* \leq 0.001 (one-way ANOVA).

the spleen and peritoneal cavity were CD5⁺ and therefore were B1a cells (Fig. 1E, 1F). Interestingly, *B-lyn*^{-/-} mice exhibited high levels of circulating BAFF, similar to those observed in *lyn*^{-/-} animals (Fig. 1D), which might contribute to the survival, activation, and differentiation of plasma and B1 cells in *B-lyn*^{-/-} and *lyn*^{-/-} mice. We next characterized by flow cytometry the expression of cell surface MHC II and IgM on the splenic B cells of 8-mo-old *B-lyn*^{-/-} mice, compared with *lyn*^{-/-} and control mice. We found that, similar to *lyn*^{-/-}, expression of MHC II was significantly elevated in *B-lyn*^{-/-} B cells, whereas IgM cell surface levels were significantly down-regulated (Fig. 1G, 1H). These observations likely reflect an enhanced degree of BCR signaling in vivo in both *lyn*^{-/-} and *B-lyn*^{-/-} mice (21).

Lyn^{-/-} mice have a significant reduction in mature peripheral B cells owing to compromised survival as cells pass from T1 to T2 and T2/T3 to mature stages (15, 16). We analyzed by flow cytometry the effect of B cell-specific deletion of Lyn on B cell subpopulations in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. We observed a significant decrease in the numbers

of follicular (follicular I and follicular II) and marginal zone B cells in both *lyn*^{-/-} and *B-lyn*^{-/-} animals (Fig. 2A–C). The remaining mature B cells in both strains had significantly increased MHC II expression levels, suggesting that these cells were activated (Fig. 2D–F).

To more extensively characterize the effects of Lyn deletion on B cell development, we examined the bone marrow compartment of 2-mo-old *lyn*^{-/-} and *B-lyn*^{-/-} mice. Both total *lyn*^{-/-} and *B-lyn*^{-/-} mice exhibited normal percentages of pre-B, pro-B, and immature B cells in the bone marrow (Fig. 3A), but they showed significantly decreased numbers of mature recirculating B cells as previously observed (6, 10, 22). In contrast, splenic B cell development was equivalently reduced in both *lyn*^{-/-} and *B-lyn*^{-/-} mice; both strains manifested progressively reduced numbers of splenic transitional B cells, with T3 cells being the lowest (Fig. 3B–D). The remaining splenic transitional B cell types all had significantly elevated MHC II expression (Fig. 3E–G). These results demonstrate that a Lyn-dependent mechanism, intrinsic to B cells, controls B cell homeostasis and activation.

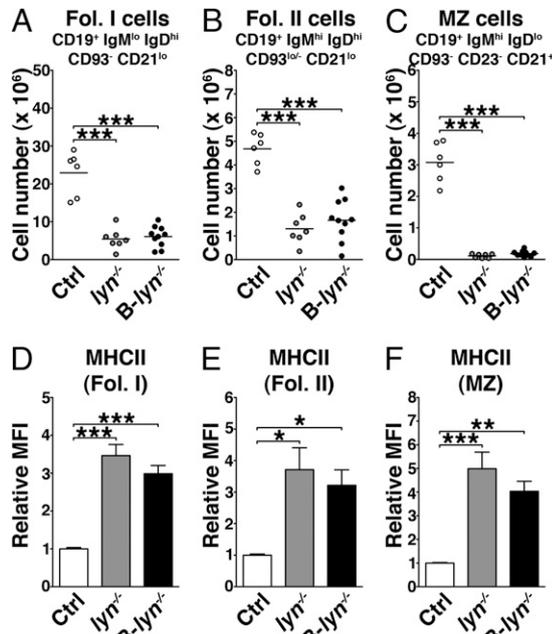


FIGURE 2. *Lyn* is required for the development of mature B cells. (A–C) Absolute numbers of follicular I [Fol. I, (A)], follicular II [Fol. II, (B)], and marginal zone (MZ) B cells (C) in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. Data represent means of independent experiments. Each dot represents an individual mouse. (D–F) Mean fluorescence intensity (relative to control) of MHC II expressed by Fol. I (D), Fol. II (E), and MZ (F) B cells in the spleens of 8-mo-old mice. Bars represent means \pm SEM of independent experiments from 6 to 10 mice per group. (A–F) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA).

B cells isolated from total *lyn*^{-/-} mice have enhanced signaling responses to BCR engagement (14, 16, 23). We asked whether the B cells in *B-lyn*^{-/-} mice manifested a similar phenotype by mea-

suring intracellular-free calcium in splenic B cells upon stimulation with 15 μ g/ml anti-IgM F(ab')₂ (Fig. 4). Similar to *lyn*^{-/-}, *B-lyn*^{-/-} follicular and T3 cells manifested increased calcium flux responses following BCR crosslinking. *Lyn* deficiency had a minor effect on calcium responses in T1 and T2 B cells in both strains of mice. These results confirm that *Lyn* expressed by mature follicular and T3 cells restrains calcium signaling upon BCR engagement.

Selective deletion of *Lyn* in B cell leads to T cell activation and myeloid cell expansion

T cells do not express *Lyn*, but it has been shown that aged *lyn*^{-/-} mice have increased numbers of activated T cells, which fuels the autoimmune disease via a feedback loop between T and myeloid cells (17, 24, 25). Specific deletion of *Lyn* in B cells led to a non-significant trend toward increased numbers of effector T cell populations in the spleen (Fig. 5A–C). Consistent with the lack of GC formation in *lyn*^{-/-} and *B-lyn*^{-/-} mice, there was no change in the numbers of ICOS⁺CXCR5⁺PD-1⁺ T follicular helper cells (among the CD4⁺CD44^{hi}CD62L⁻ T cell population; Fig. 5D).

Although *B-lyn*^{-/-} myeloid cells express *Lyn*, we observed a significant increase in the numbers of CD11b⁺ cells in both 8-mo-old *B-lyn*^{-/-} and *lyn*^{-/-} spleens (Fig. 6A) due to increases in both the monocyte/macrophage (CD11b⁺F4/80⁺) and granulocyte (CD11b⁺7/4⁺Gr-1^{hi}) subsets (Fig. 6B, 6C). The myeloid expansion observed in the *B-lyn*^{-/-} mice resulted in significant splenomegaly and altered architecture of the lymphoid tissues at 8 mo of age, comparable to that observed in the *lyn*^{-/-} mice (Fig. 6D, Supplemental Fig. 3).

In *lyn*^{-/-} mice, conventional DCs (cDCs) and plasmacytoid DCs (pDCs) express high levels of the costimulatory molecules CD86 and MHC II, respectively, indicative of their activated state. In the spleens of 8-mo-old *B-lyn*^{-/-} mice, the numbers of cDCs and pDCs remained unchanged (Fig. 6E, 6G). However, the *Lyn*-sufficient cDCs from *B-lyn*^{-/-} mice expressed high levels of CD86 (Fig. 6F). Hence, deletion of *Lyn* in B cells alone is sufficient

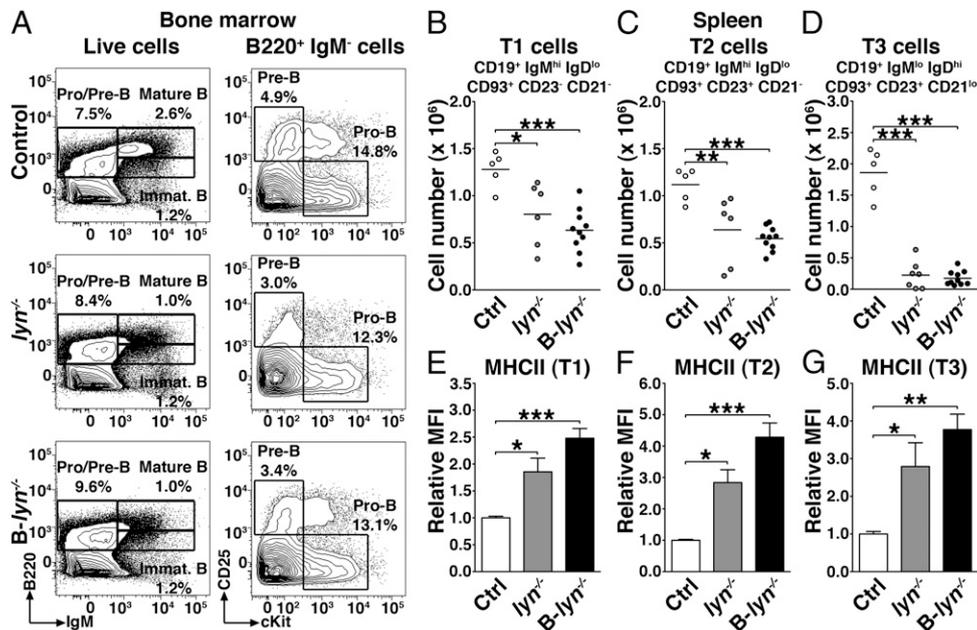


FIGURE 3. B cell-intrinsic loss of *Lyn* leads to reduction in splenic T1, T2, and T3 cells. (A) Representative contour plots showing the percentages of pro-B/pre-B and mature/immature B cells in the bone marrow of 2-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. (B–D) Absolute numbers of transitional T1 (B), T2 (C), and T3 (D) B cells in the spleens of 2-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. Data represent means of independent experiments. Each dot represents an individual mouse. (E–G) Mean fluorescence intensity (relative to control) of MHC II expressed by transitional T1 (E), T2 (F), and T3 (G) B cells in the spleens of 2-mo-old mice. Bars represent means \pm SEM of independent experiments from 5 to 10 mice per group. (B–G) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA).

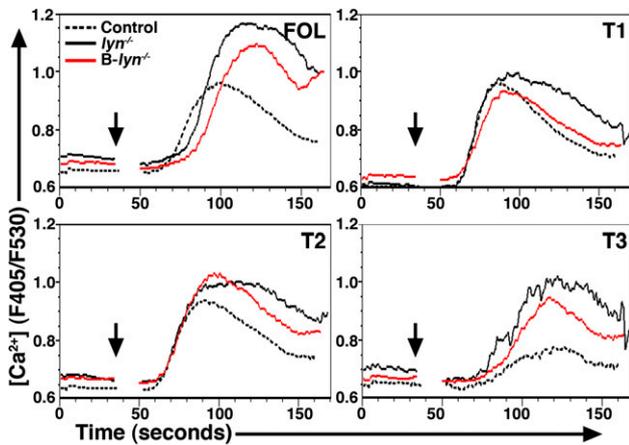


FIGURE 4. Lyn signal in follicular and T3 B cells restrains calcium signaling upon BCR engagement. Calcium release in control, $lyn^{-/-}$, and $B-lyn^{-/-}$ T1, T2, T3, and follicular splenic B cells after stimulation with 15 $\mu\text{g/ml}$ anti-IgM $\text{F}(\text{ab}')_2$. Histograms show median intracellular Ca^{2+} concentration as measured by fluorescence ratio (F405/F530). Data are representative of three independent experiments.

to induce expansion of the myeloid cell compartments and modest activation of DCs.

Specific deletion of Lyn in B cells results in the development of autoimmunity

To provide a conclusive proof that Lyn deficiency in B cells alone can lead to lupus-like autoimmune disease, $B-lyn^{-/-}$ mice were examined for the presence of circulating autoantibodies and tissue immune complexes. Consistent with the presence of exaggerated numbers of plasma cells in the spleens, $B-lyn^{-/-}$ mice showed high levels of IgM and IgG isotype anti-sm/RNP and anti-dsDNA autoreactive Abs in the serum, at levels similar to those found in $lyn^{-/-}$ animals (Fig. 7A). As a result, $B-lyn^{-/-}$ mice showed

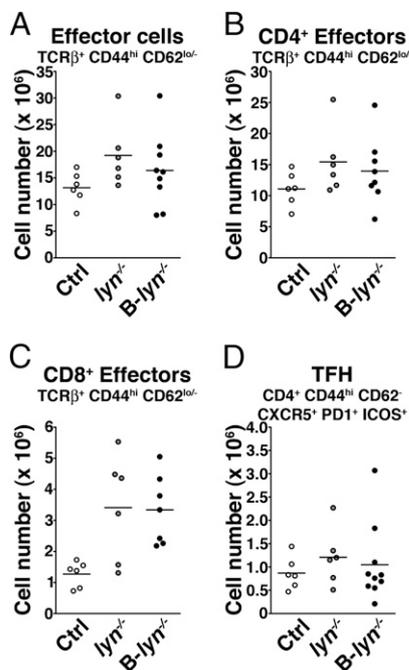


FIGURE 5. Selective deletion of Lyn in B cells leads to mild T cell activation. Absolute numbers of total (A), CD4^+ (B), CD8^+ (C) effector, and T follicular helper cells (D) in the spleens of 8-mo-old control, $lyn^{-/-}$, and $B-lyn^{-/-}$ mice. Data represent means of independent experiments. Each dot represents an individual mouse. (A–D) NS (one-way ANOVA).

enlarged kidney glomeruli, with both IgG and C3 deposition, as well as increased leukocyte infiltration representative of glomerulonephritis (Fig. 7B). The degrees of IgG and C3 deposition, as well as glomerulonephritis, were similar in $lyn^{-/-}$ versus $B-lyn^{-/-}$ animals (Table I). Hence, specific deletion of Lyn in B cells alone is sufficient to cause development of immune complex-mediated nephritis in mice.

Cytokines play an important role in the pathogenesis of SLE by stimulating immune cell activation leading to organ damage. We measured the concentration of cytokines in the sera of 6-mo-old $B-lyn^{-/-}$, $lyn^{-/-}$, and control mice by Luminex bead array (Fig. 7C). As previously observed, the $lyn^{-/-}$ mice had elevated levels of numerous cytokines such as IFN- γ , IL-6, IL-10, IL-17, IL12p40, M-CSF, and GM-CSF (26). To a lesser extent, similar changes were also observed in the $B-lyn^{-/-}$ animals (Fig. 7C). However, neither $lyn^{-/-}$ nor $B-lyn^{-/-}$ mice showed signs of exaggerated tissue inflammation other than in the kidney. Thus, we demonstrate that the absence of Lyn in B cells alone is sufficient to induce the development of glomerulonephritis in mice, resembling the process seen in the global $lyn^{-/-}$ strain.

Lack of MyD88 in Lyn-deficient B cells prevents the development of pathogenic class-switched IgG autoantibodies

MyD88-dependent TLR signals in B cells or DCs regulate the degree of IgG responses during immunization (27). We investigated whether MyD88 signals in B cells also control the anti-self Ab response in $B-lyn^{-/-}$ animals that leads to the lupus-like disease in these mice. To this end we generated mice bearing deletion of both MyD88 and Lyn in B cells (20). Double mutant $lyn^{\text{fl}}\text{myd88}^{\text{fl}}\text{Cd79a-cre}^+$ mice ($B-lyn^{-/-}\text{myd88}^{-/-}$ mice hereafter) were euthanized at 8 mo of age for analysis. MyD88 deletion in B cells of $B-lyn^{-/-}$ mice was sufficient to reverse the splenomegaly, myeloid expansion, as well as reduce the T cell activation present in $B-lyn^{-/-}$ mice (Fig. 8A–C). Indeed, the weights of $B-lyn^{-/-}\text{myd88}^{-/-}$ spleens were decreased compared with $B-lyn^{-/-}$ mice, similar to those observed in control mice (Fig. 8A). Analysis of splenocytes by flow cytometry revealed that the numbers of myeloid and total effector T cells in $B-lyn^{-/-}\text{myd88}^{-/-}$ mice were significantly diminished compared with $B-lyn^{-/-}$ mice, reaching the baseline numbers of control mice (Fig. 8B, 8C). However, deletion of MyD88 in Lyn-deficient B cells did not rescue the defect in B cell homeostasis observed in Lyn-deficient B cells. $B-lyn^{-/-}\text{myd88}^{-/-}$ mice exhibited decreased numbers of total and mature B cells, but increased numbers of plasma cells, similar to the $B-lyn^{-/-}$ animals (Fig. 8D–F). Interestingly, $B-lyn^{-/-}\text{myd88}^{-/-}$ mice still showed high levels of serum BAFF and increased numbers of B1 cells in the peritoneal cavity as observed in the $B-lyn^{-/-}$ and $lyn^{-/-}$ mice ($10 \times 10^5 \pm 1.8$ B1 cells, $p \leq 0.001$ versus control, $p \leq 0.01$ versus $lyn^{-/-}$). Our observations suggest that elevated BAFF levels might be responsible for the expansion of the B1 compartment (Fig. 8G, 8H).

We then asked whether double mutant mice developed autoimmunity by examining their serum levels of autoreactive Abs and their kidneys for signs of lupus-like disease. Although they showed similar levels of IgM Abs, $B-lyn^{-/-}\text{myd88}^{-/-}$ mice failed to develop pathogenic anti-dsDNA or anti-sm/RNP IgG Abs compared with $B-lyn^{-/-}$ mice (Fig. 9A, 9B). We further investigated the specific IgG subclasses of the autoantibodies in the serum of these mice. In the serum of $lyn^{-/-}$ and $B-lyn^{-/-}$ mice, IgG autoantibodies (anti-dsDNA and anti-sm/RNP) were mainly of the IgG2b and IgG2c subclasses, which are highly pathogenic (Fig. 9C, 9D). MyD88 deficiency in $B-lyn^{-/-}$ mice resulted in the abrogation of the IgG2b and IgG2c anti-dsDNA and anti-sm/RNP autoreactive Abs (Fig. 9C, 9D). In agreement, kidney glomeruli from $B-lyn^{-/-}\text{myd88}^{-/-}$

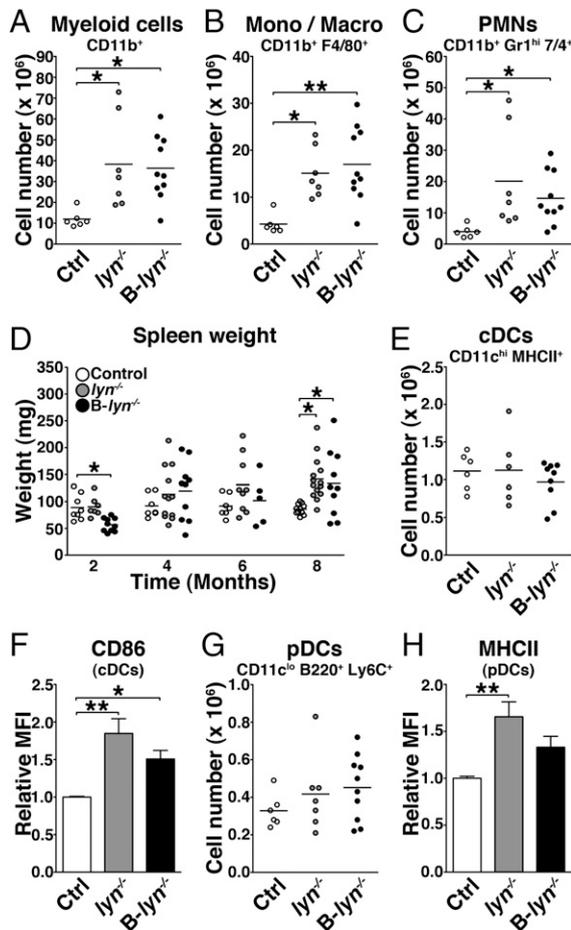


FIGURE 6. Specific deletion of Lyn in B cells leads to myeloproliferative phenotype comparable to *lyn*^{-/-}. (A–C) Absolute numbers of total myeloid cells (A), monocytes/macrophages (B), and granulocytes (C) in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. (D) Weights of spleens harvested from control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice were recorded at 2, 4, 6, and 8 mo of age. (E) Absolute numbers of cDCs in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. (F) Mean fluorescence intensity (relative to control) of CD86 expressed by cDCs in the spleens of 8-mo-old mice. Bars represent means ± SEM of independent experiments from 6 to 10 mice per group. (G) Absolute numbers of pDCs in the spleens of 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. (H) Mean fluorescence intensity (relative to control) of MHC II expressed by pDCs in the spleens of 8-mo-old mice. Bars represent means ± SEM of independent experiments from 6 to 10 mice per group. (A–E, G) Data represent means of independent experiments. Each dot represents an individual mouse. (A–H) **p* ≤ 0.05, ***p* ≤ 0.01 (one-way ANOVA).

mice were normal in size and cellularity, and they showed little or no evidence of leukocyte infiltration or C3 or IgG deposition (Fig. 9E). Thus, the loss of MyD88-dependent signals in Lyn-deficient B cells prevented class switching to pathogenic IgG autoantibodies, reversing the autoimmune phenotype observed in *B-lyn*^{-/-} mice.

Discussion

There is growing recognition that complex interactions between B, T, and DCs underlie the pathogenesis of autoimmune diseases such as SLE (28, 29). Clearly, this is the case in the Lyn kinase-deficient mouse model. Although many studies have focused on the role of this kinase as a negative regulator in B lymphocytes, it is also a major regulator of innate immune cells, including DCs, neutrophils, and mast cells (2). To determine whether loss of Lyn-mediated inhibitory signaling in B cells alone is sufficient to

initiate lupus-like autoimmunity, we have used the newly generated *lyn*^{fl/fl} mouse to delete Lyn specifically in B cells (18). The *B-lyn*^{-/-} mice displayed alterations in B cell homeostasis (low follicular, marginal zone, and GC cells) and signaling (elevated calcium responses) as seen in the global *lyn*^{-/-}, confirming that these defects are due to B cell-intrinsic alterations. Importantly, the *B-lyn*^{-/-} animals developed autoantibodies leading to glomerulonephritis accompanied by myeloproliferation and T cell activation, similar to what was seen in the global *lyn*^{-/-} strain. The disease process in the *B-lyn*^{-/-} mutants was dependent on MyD88-mediated signals in B cells, as deletion of *myd88* in the B cells of *B-lyn*^{-/-} mice (i.e., double conditional mutant mice lacking both Lyn and MyD88 in B cells alone) reversed the disease process. These results demonstrate that loss of Lyn-mediated inhibitory signaling in B cells alone is sufficient to initiate systemic autoimmunity.

It has been assumed that the myeloproliferative phenotype observed in the *lyn*^{-/-} mice was not B cell-mediated but was rather due to the hyperresponsiveness of Lyn-deficient hematopoietic progenitors and myeloid cells to growth factors such as GM-CSF, G-CSF, and IL-6 (2). In this line, *lyn*^{-/-} μ MT/ μ MT mice (lacking mature B cells) or *lyn*^{-/-} *Rag*^{-/-} mice (lacking mature B and T cells) still develop myeloproliferation indistinguishable from *lyn*^{-/-} mice (2, 30). It is thus intriguing that myeloid expansion still occurs in *B-lyn*^{-/-} mice in the presence of Lyn-sufficient myeloid cells. This observation suggests that myeloproliferation in *lyn*^{-/-} mice is not only due to intrinsic defects in myeloid progenitors and cells, but it can also be a consequence of the inflammatory effects of immune complexes produced by self-reactive B lymphocytes, as has been also observed in other models of autoimmunity (31).

The observation that MyD88 deficiency in B cells alone reversed the autoimmune process in *B-lyn*^{-/-} mice is consistent with a number of reports implicating TLR7 and TLR9 signaling in B cells as promoting the activation of B cells recognizing nuclear autoantigens, both in vitro and in vivo, in a variety of mouse models of lupus (32–35). We found that loss of MyD88-dependent signals in Lyn-deficient B cells prevented class switching to pathogenic IgG2b and IgG2c autoreactive Abs. In line with our findings, Ehlers et al. (33) have shown that TLR9/MyD88 signaling is crucial for class switching to pathogenic autoreactive Abs in the *Fc γ RIIb*^{-/-} mouse model of lupus-like disease.

B1 cells produce most of the serum IgM; expansion of B1 cell numbers and elevated IgM is often associated with autoimmunity (36). It is thought that BAFF protects B1 cells against Fc γ RIIb-mediated apoptosis and that excessive BAFF levels in mice lead to the expansion of low-affinity self-reactive B cells, including B1 cells, rather than to a dramatic breakdown in B cell tolerance (37–39). Interestingly, in the *B-lyn*^{-/-} mice, deficiency of B cell MyD88 signaling abrogated the production of pathogenic IgG autoreactive Abs, but the *B-lyn*^{-/-} *myd88*^{-/-} mice still produced high levels of IgM self-reactive Abs and high levels of serum BAFF, and they exhibited increased numbers of B1 cells in the peritoneal cavity, suggesting a similar mechanism in *lyn*^{-/-} mice. This also suggests that the main role of TLR signaling in B cells is to promote class switching of self-reactive clones, which would normally take place in GC B cells. In agreement with this notion, previous studies of mice immunized with nucleic acid-containing virus particles also demonstrated that TLR signaling in B cells can promote the GC response (27). However, *lyn*^{-/-} animals, whether immunized or not, have altered lymphoid architecture lacking clear follicles and T cell zones, and they exhibit poor splenic GC formation (9, 11, 25, 40, 41). These phenotypes were also observed in *B-lyn*^{-/-} mice. The alteration in splenic architecture

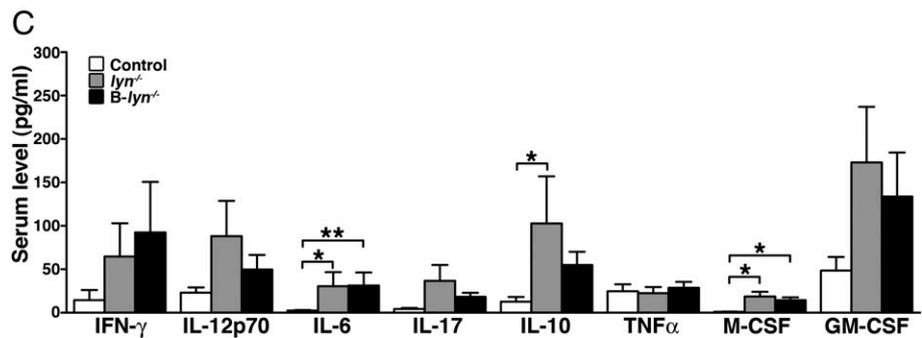
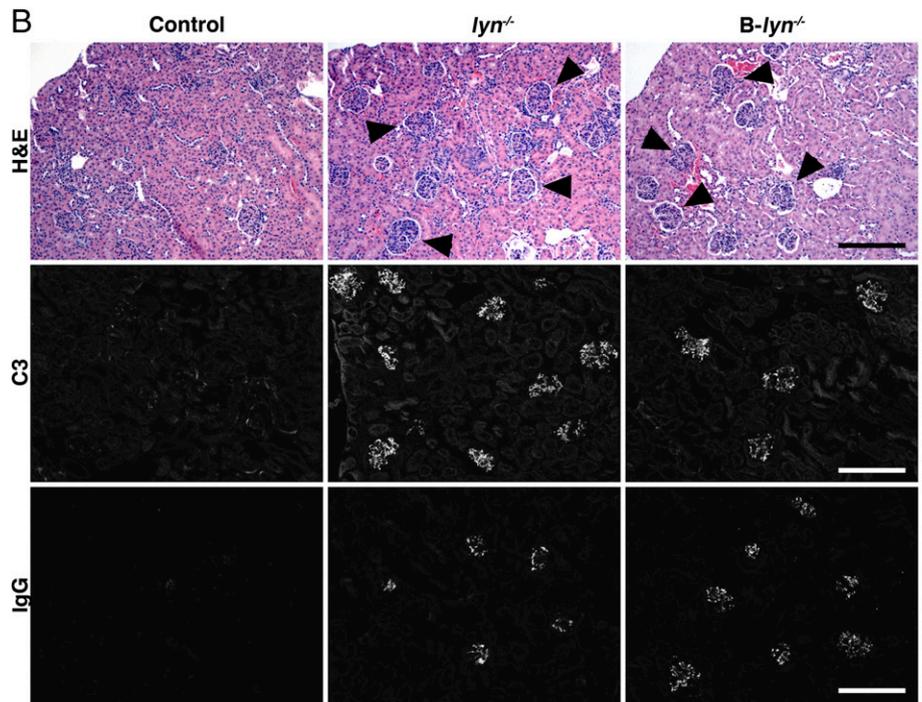
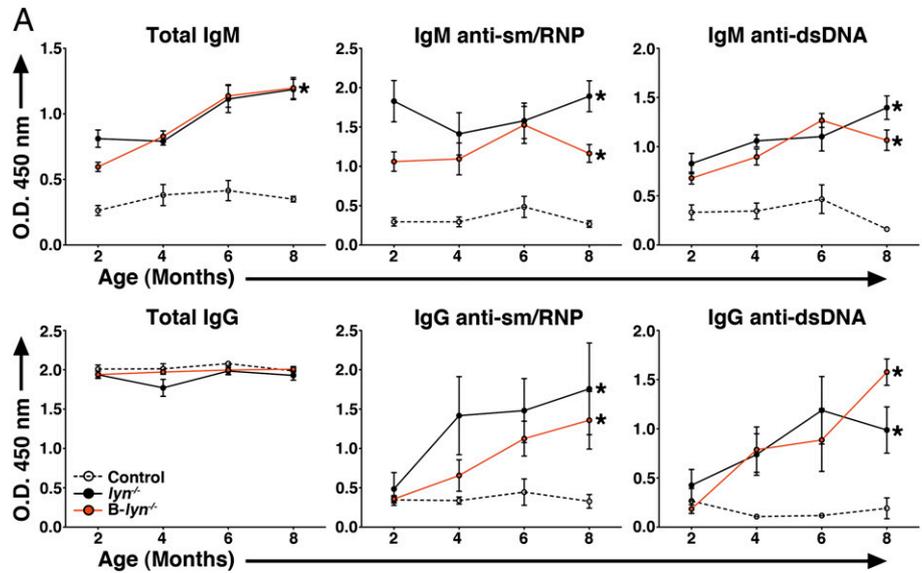


FIGURE 7. Specific deletion of Lyn in B cells is sufficient for the development of lupus-like autoimmunity but not inflammatory disease. **(A)** Detection of autoreactive IgM (upper panel) and IgG isotype (lower panel) Abs in the serum of 2-, 4-, 6-, and 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. Data represent means \pm SEM from 6 to 10 mice per group. **(B)** H&E, C3, and IgG staining of kidney sections from 8-mo-old control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice. Arrowheads indicate enlarged glomeruli. Representative images are shown. Scale bars, 50 μ m. **(C)** Serum cytokine levels comparing control, *lyn*^{-/-}, and *B-lyn*^{-/-} mice (6–8 mo old) were determined by Luminex assay. Bars represent means \pm SEM from 6 to 14 mice per group. (A and C) * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA).

may be secondary to increased extramedullary myelopoiesis in these animals. The absolute numbers of GC B cells in *lyn*^{-/-} or *B-lyn*^{-/-} mice are substantially decreased, as is also true of mature naive follicular B cells. It is possible that at least some fraction of the pathogenic IgGs in these animals is derived from extrafollicular B cells, under the influence of TLR signaling, as has been suggested

in other autoimmune strains (42). However, the observation that crossing the *lyn*^{-/-} mice with signaling lymphocyte activation molecule-associated protein-deficient animals, which lack T follicular helper cells and GC B cells, causes a large decrease in IgG antinuclear Ab formation (52), suggests a GC origin for most pathogenic Abs in this model. Therefore, the developmental history

Table I. Histological scoring

	Nephritis	C3	IgG
Control	0.00 ± 0.00	0.00 ± 0.00	0.0 ± 0.0
<i>lyn</i> ^{-/-}	1.40 ± 0.20	1.40 ± 0.89	1.40 ± 0.40
<i>B-lyn</i> ^{-/-}	<i>p</i> ≤ 0.01 versus control 1.50 ± 0.19	<i>p</i> ≤ 0.05 versus control 0.63 ± 0.26	1.12 ± 0.35
<i>B-lyn</i> ^{-/-} <i>myd88</i> ^{-/-}	<i>p</i> ≤ 0.001 versus control 0.40 ± 0.24	0.17 ± 0.17	0.33 ± 0.21
	<i>p</i> ≤ 0.05 versus <i>lyn</i> ^{-/-} <i>p</i> ≤ 0.01 versus <i>B-lyn</i> ^{-/-}	<i>p</i> ≤ 0.05 versus <i>lyn</i> ^{-/-}	

Kidney inflammation was scored in a blinded fashion as described in *Materials and Methods*. Briefly, the presence and severities of the disease in the glomerular and interstitial compartments were arbitrarily graded as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Data are averaged from at least five microscopic fields per specimen, with five to seven mice (8 mo old) per group. The *p* values were determined by a one-way ANOVA.

of autoreactive B cells in this model, and other lupus-like mouse models, remains an open and important question.

We have also examined the effects of deletion of *Lyn* selectively in DCs. Surprisingly, when the *lyn*^{fl/fl} mice were crossed with *Cd11c-cre* animals, to generate *Lyn* deficiency in DCs specifically (DC-*lyn*^{-/-} mice hereafter), the resulting mice developed a much more severe autoimmune/inflammatory disease than is present in either *lyn*^{-/-} or *B-lyn*^{-/-} animals (18). Whereas the DC-*lyn*^{-/-} mice developed similar titers of IgG anti-sm/RNP and anti-dsDNA as seen in the *lyn*^{-/-} animals, they had much higher levels of T cell activation, myeloexpansion, and much more severe glomerulonephritis. The DC-*lyn*^{-/-} animals developed diffuse lymphadenopathy as well as a number of tissue inflammatory infiltrates, including hepatitis, pneumonitis, and dermatitis, which are rarely observed in *lyn*^{-/-} animals (or in *B-lyn*^{-/-} mice). As a result, the DC-*lyn*^{-/-} mice showed decreased survival. In contrast, DC-*lyn*^{-/-} mice showed none of the B cell homeostatic defects seen in *lyn*^{-/-} or *B-lyn*^{-/-} strains; in fact, the DC-*lyn*^{-/-} mice developed ele-

vated levels of splenic and lymph node mature B cells, as well as increased plasma cell numbers. Importantly, deletion of *myd88* in DCs alone in the DC-*lyn*^{-/-} strain reversed the disease process completely, indicating that TLR signaling in DCs is necessary to sustain the inflammatory/autoimmune phenotype in this strain.

Taken together, these studies indicate that there are multiple pathways by which *Lyn* deficiency can initiate autoimmunity, leading to some similar and some distinct phenotypes. In the *B-lyn*^{-/-} model, we propose that loss of inhibitory signaling in B cells leads to alterations in B cell homeostasis, which is likely caused by reduced survival of mature B cells with elevated tonic BCR signaling. In these mice, selection of self-reactive B cell clones by a mechanism requiring TLR signaling in the B cells leads to autoantibody production, probably in a T cell-dependent manner. This in turn leads to immune complex formation with kidney deposition, which we hypothesize drives the myeloexpansion and mild systemic inflammatory response (i.e., elevated cytokines) seen in the *B-lyn*^{-/-} mice.

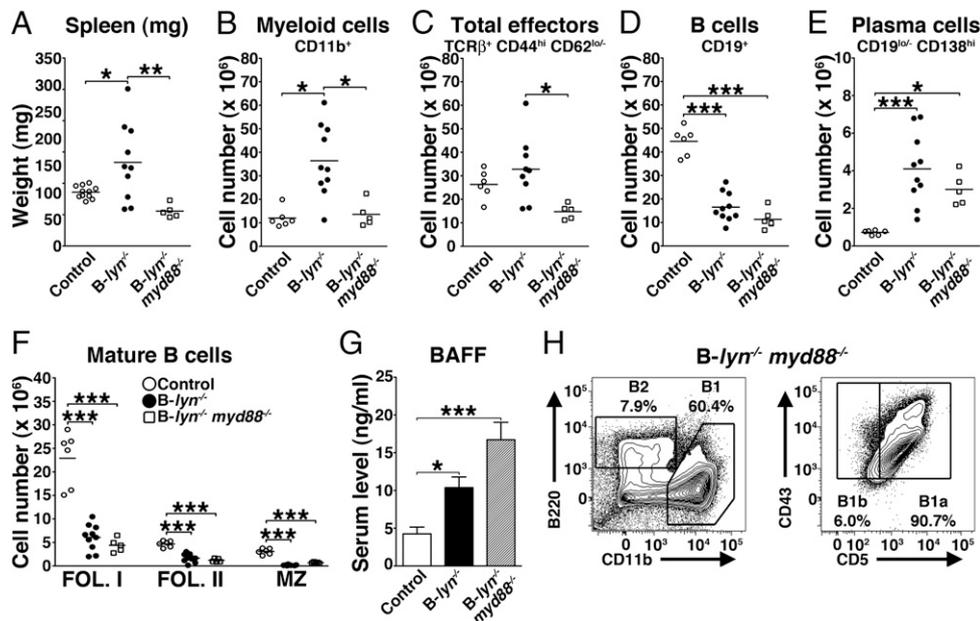


FIGURE 8. Deletion of MyD88 in *Lyn*-deficient B cells rescues the myeloproliferative phenotype but not the defect in B cell homeostasis observed in *Lyn*-deficient B cells. (A) Weights of spleens harvested from 8-mo-old control, *B-lyn*^{-/-}, and *B-lyn*^{-/-}*myd88*^{-/-} mice. (B–F) Absolute numbers of myeloid (B), total effector (C), B (D), plasma (E), and mature B cells (F) in the spleens of 8-mo-old control, *B-lyn*^{-/-}, and *B-lyn*^{-/-}*myd88*^{-/-} mice. (A–F) Data from control and *B-lyn*^{-/-} groups are the same as represented in Figs. 1, 2, 6, and 7 to perform comparisons to the *B-lyn*^{-/-}*myd88*^{-/-} mice. Data represent means of independent experiments. Each dot represents an individual mouse. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001 (one-way ANOVA). (G) BAFF levels in the serum of control, *B-lyn*^{-/-}, and *B-lyn*^{-/-}*myd88*^{-/-} mice (8 mo old) were determined by ELISA. Bars represent means ± SEM from 6 to 10 mice per group. (H) Representative FACS contours showing the percentages of B1 and B2 cells in the peritoneal cavity of 8-mo-old *B-lyn*^{-/-}*myd88*^{-/-} mice. B1 cells were determined as CD19⁺CD11b⁺B220^{lo}CD43⁺ cells and further defined as B1a (CD5⁺) or B1b (CD5⁻) subsets. B2 cells were determined as CD19⁺B220^{hi}CD11b⁻ cells.

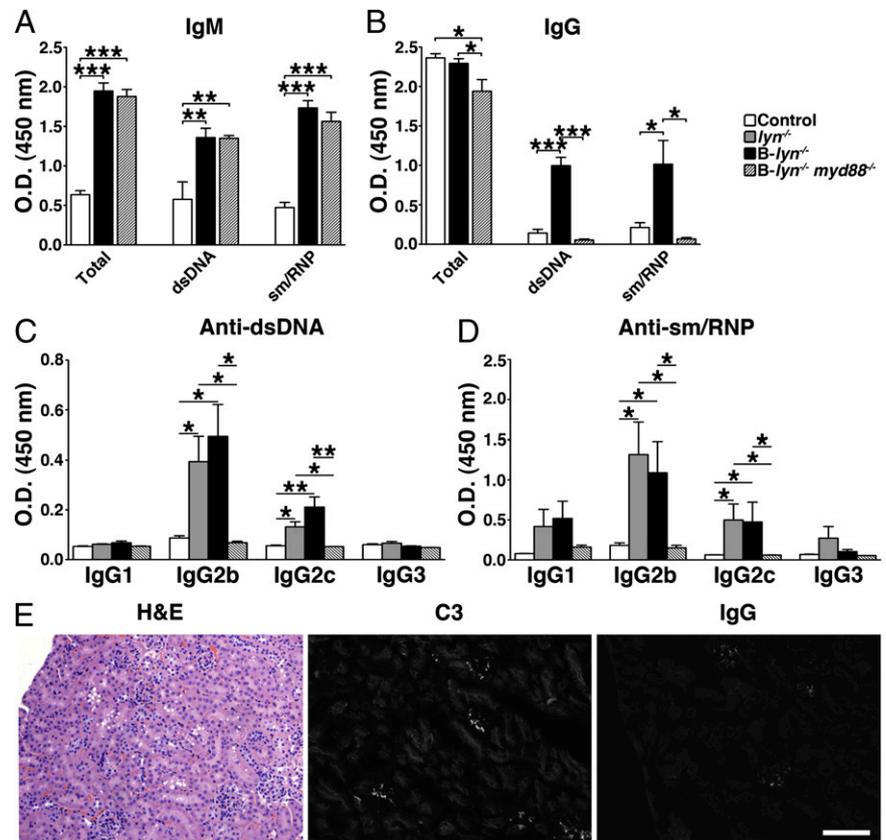


FIGURE 9. Specific deletion of Lyn and MyD88 in B cells prevents the development of pathogenic class-switched autoantibodies and autoimmunity. (**A–D**) Detection of autoreactive IgM isotype (**A**), IgG isotype (**B**), and pathogenic class-switched IgG anti-dsDNA (**C**) or anti-sm/RNP (**D**) Abs in the serum of 8-mo-old control, *B-lyn*^{-/-}, and *B-lyn*^{-/-} *myd88*^{-/-} mice. Bars represent means \pm SEM from six mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (one-way ANOVA). (**E**) H&E, C3, and IgG staining of kidney sections from 8-mo-old *B-lyn*^{-/-} *myd88*^{-/-} mice. Representative images are shown. Scale bars, 50 μ m.

In contrast, in the DC-*lyn*^{-/-} model the primary initiating response is dysregulated MyD88-mediated signaling in DCs probably in response to commensal organisms. This leads to exaggerated proinflammatory cytokine production, which drives T cell activation and production of IFN- γ (among other inflammatory cytokines) that in turn feeds back on the DCs to further activate them, leading to a self-amplifying loop of inflammation (17). This proinflammatory state, which includes high BAFF levels even in the presence of elevated mature B cell numbers, leads to the selection of self-reactive B cell clones producing autoantibodies. The autoantibodies deposit in tissues, which we hypothesize further exacerbates the inflammatory disease. The very proinflammatory nature of the process in the DC-*lyn*^{-/-} mice, with the lymphoproliferation/myeloproliferation, the T cell activation, and the high systemic cytokine production, likely accounts for why the disease is lethal in these mice compared with the *B-lyn*^{-/-} animals. The MyD88 requirement for both the DC-initiated pathway of autoimmune and inflammatory disease and the B cell-initiated lupus-like autoimmunity is consistent with the earlier observation that the autoimmunity of the global *lyn*^{-/-} mice is ablated by crossing to *Myd88*^{-/-} animals (43).

Similar comparisons between B cell- versus DC-specific knockouts of SHP-1 and A20 have recently been reported and reinforce the conclusion that autoimmunity can be initiated by defective negative regulation of receptor signaling in either of these cell types. Deficiency of the tyrosine phosphatase SHP-1 selectively in B cells results in a mild autoimmunity with mild renal immune complex deposition (44). Lyn phosphorylation of the ITIM of CD22 leads to recruitment of SHP-1, and this is likely a key pathway affected by Lyn deficiency of B cells because the phenotypes resulting from deletion of Lyn or SHP-1 in B cells or deletion of CD22 are all similar (45). Compared to deletion of SHP-1 in B cells, DC-specific deletion of SHP-1 produces a much more profound inflammatory and autoimmune process, which similar to the DC-

lyn^{-/-} mice is dependent on expression of MyD88 in DCs (46). Similarly, mice lacking the *tnfrsf25* (A20) gene specifically in B cells develop a mild autoimmunity similar to the *B-lyn*^{-/-} animals (47). A20 is a negative regulator of NF- κ B pathways downstream of the BCR, TLRs, and a number of other cell types (48). DC-specific A20-deficient mice, as with DC-specific deletion of Lyn or SHP-1, have a stronger systemic inflammatory and autoimmune disease with rapid end-organ damage (49, 50). Thus, in all three cases, dysregulation of B cells or DCs can lead to autoimmune and inflammatory disease. Understanding the mechanisms of the cellular interactions in genetic models of autoimmunity is critical for future studies. The approach of cell type-specific deletion of autoimmune susceptibility genes is clearly informative in this regard.

These studies illustrate that complex regulatory and counter-regulatory networks are operating in the autoimmune Lyn-deficient model. It is very likely that similar processes are present in other autoimmune mice as well as in SLE patients. Indeed, because genome-wide-associated studies have demonstrated that SLE is multigenic and likely genetically diverse among different groups of patients (51), it is likely that dysregulation of multiple cellular and molecular pathways can predispose to autoimmunity in different individuals, as is the case in mice defective in key negative regulators of receptor signaling such as Lyn, SHP-1, and A20. Therefore, defining which pathways are dysregulated in any given SLE patient will be required to develop appropriate therapy to that patient's disease process. The variable results seen with many current SLE therapies may simply reflect the fact that one cannot treat a heterogeneous disease process with a single agent that affects a single pathway. Thus, disentangling the pathogenic mechanisms in mouse models such as the Lyn-deficient animal may lead to practical improvements in our approach to treating complex autoimmune diseases in humans.

Disclosures

The authors have no financial conflicts of interest.

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