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A CD19-Dependent Signaling Pathway Regulates Autoimmunity in Lyn-Deficient Mice

Minoru Hasegawa,* Manabu Fujimoto,* Jonathan C. Poe,* Douglas A. Steeber,* Clifford A. Lowell,† and Thomas F. Tedder‡*†

CD19 and the Src family protein tyrosine kinases (PTKs) are important regulators of intrinsic signaling thresholds in B cells. Regulation is achieved by cross-talk between Src family PTKs and CD19; Lyn is essential for CD19 phosphorylation, while CD19 establishes an Src family PTK activation loop that amplifies kinase activity. However, CD19-deficient (CD19−/−) B cells are hyporesponsive to transmembrane signals, while Lyn-deficient (Lyn−/−) B cells exhibit a hyper-responsive phenotype resulting in autoimmunity. To identify the outcome of interactions between CD19 and Src family PTKs in vivo, B cell function was examined in mice deficient for CD19 and Lyn (CD19/Lyn−/−). Remarkably, CD19 deficiency suppressed the hyper-responsive phenotype of Lyn−/− B cells and autoimmunity characterized by serum autoantibodies and immune complex-mediated glomerulonephritis in Lyn−/− mice. Consistent with Lyn and CD19 each regulating conventional B cell development, B1 cell development was markedly reduced by Lyn deficiency, with further reductions in the absence of CD19 expression. Tyrosine phosphorylation of Fyn and other cellular proteins induced following B cell Ag receptor ligation was dramatically reduced in CD19/Lyn−/− B cells relative to Lyn−/− B cells, while Syk phosphorylation was normal. In addition, the enhanced intracellular Ca2+ responses following B cell Ag receptor ligation that typify Lyn deficiency were delayed by the loss of CD19 expression. BCR-induced proliferation and humoral immune responses were also markedly inhibited by CD19/Lyn deficiency. These findings demonstrate that while the CD19/Lyn amplification loop is a major regulator of signal transduction thresholds in B lymphocytes, CD19 regulation of other Src family PTKs also influences B cell function and the development of autoimmunity. The Journal of Immunology, 2001, 167: 2469–2478.

Keywords: B lymphocytes, autoimmunity, BCR, CD19, Lyn, signal transduction, Ca2+.

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Abbreviations used in this paper: BCR, B cell Ag receptor; PTK, protein tyrosine kinase; CD19−/−, CD19-deficient; Lyn−/−, Lyn-deficient; SHP-1, Src homology 2 domain-containing phosphotyrosine phosphatase; SHIP, Src homology 2 domain-containing inositol phosphatase; CD19/Lyn−/−, CD19-deficient and Lyn-deficient; [Ca2+]i, intracellular Ca2+; ANA, anti-nuclear Ab; DNP-KLH, 2,4-dinitrophenyl-keyhole limpet hemocyanin; SLE, systemic lupus erythematosus.
general rheostat that defines signaling thresholds critical for expansion of the peripheral B cell pool (29, 41). While CD19 and CD19 interact during B cell activation, and Lyn expression is required for detectable CD19 phosphorylation (16), Lyn−/− B cells and CD19−/− B cells exhibit contrasting phenotypes. Lyn−/− B cells are hyper-responsive to transmembrane signals, since Lyn activation also influences signaling pathways that negatively regulate B cell activation (7, 13, 14, 42–44). In addition, Lyn is expressed by other leukocyte subpopulations, including monocytes (45, 46), where it also negatively regulates activation (C. A. Lowell, unpublished observation). Consequently, Lyn−/− mice develop autoimmunity characterized by serum autoantibodies and immune complex-mediated glomerulonephritis (7–9). To determine whether autoimmunity induced by Lyn deficiency proceeds through a CD19-dependent pathway, mice deficient in both CD19 and Lyn expression (CD19/ Lyn−/−) were generated. This also allowed us to determine whether CD19 expression is required for the in vivo activation of Src family PTKs in addition to Lyn. Since CD19 is not phosphorylated at detectable levels in Lyn−/− B cells, we predicted that CD19 would be functionally inactive in Lyn−/− mice and that Lyn−/− and CD19/Lyn−/− mice would share similar phenotypes. Surprisingly, CD19 deficiency inhibited activation of Src family PTK-dependent signaling pathways in Lyn−/− B cells and suppressed the hyper-responsive phenotype and development of autoimmunity in Lyn−/− mice.

Materials and Methods

Mice

CD19−/− (129 × C57BL/6) and Lyn−/− (129 × C57BL/6) mice were generated as previously described (7, 22). CD19/Lyn−/− double-deficient mice were generated through breedings of homozygous single-mutant mice and their heterozygous offspring. All mice were housed in a specific pathogen-free barrier facility. Control age-matched wild-type mice were generated in a gen-free barrier facility. Control age-matched wild-type mice were generated in a gen-free barrier facility.

Antibodies

The Abs used in this study included purified rabbit polyclonal Abs specific for Fyn and Syk (Santa Cruz Biotechnology, Santa Cruz, CA) and SHP-1 (Upstate Biotechnology, Lake Placid, NY); mouse IgA anti-mouse CD19 (MB19-1) (27); goat F(ab')2 specific for mouse IgM (IC Biomedicals (Cappel), Irvine, CA); biotinylated or FITC-conjugated goat anti-mouse IgM isotype-specific Abs (Southern Biotechnology Associates, Birmingham, AL); PE-conjugated anti-IgD (Southern Biotechnology Associates); biotinylated or FITC-conjugated anti-B220 (RA3-6B2); biotinylated anti-CD11b (Mac1, Pharmingen, San Diego, CA); biotinylated anti-CD23 (Pharmingen); and PE-conjugated anti-CD3 (53-7.3, Pharmingen). PE-conjugated streptavidin (Pharmingen) was used to reveal biotin-coupled Ab staining.

Anti-nuclear Ab (ANA) analysis

ANA levels in the serum were assessed by indirect immunofluorescence staining of HeLa cells cultured on tissue culture slides (Lab-Tek Products, Naperville, IL) for 48 h. Cells were washed and fixed in 4% formaldehyde in PBS for 20 min and permeabilized with 0.15% Triton X-100 (Bio-Rad, Richmond, CA) in PBS. Cells were washed and blocked with 10% FCS (Sigma, St. Louis, MO) in PBS for 1 h and then washed and treated with serum appropriately diluted with 1% FCS in PBS for 1 h. The slides were washed and incubated with FITC-conjugated goat F(ab')2 specific for mouse IgG (γ-chain specific; Caltag Laboratories, Burlingame, CA) or mouse IgM (μ-chain specific; Southern Biotechnology Associates).

Immunofluorescence analysis

Single-cell suspensions of lymphocytes from spleen, bone marrow, peripheral lymph nodes were isolated before two- or three-color immunofluorescence analysis. Lymphocytes (0.5 × 10^7) were stained at 4°C using predetermined optimal concentrations of Abs for 20 min. Blood erythrocytes were lysed after staining using FACS lysing solution (Becton Dickinson Biosciences, San Jose, CA). Cells with the forward and side light scatter properties of lymphocytes were analyzed on a FACScan flow cytometer (BD Biosciences) with fluorescence intensity shown on a four-decade log scale. Fluorescence contours are shown as 50% log density plots. Positive and negative populations of cells were determined using unreactive isotype-matched mAbs (Caltag Laboratories) as controls for background staining. Background levels of staining were delineated using gates positioned to include 98% of the control cells.

Histopathology and immunohistochemistry

For light microscopy, tissues were fixated in 10% formaldehyde solution for 24 h and embedded in paraffin. Sections were stained with hematoxylin and eosin. Frozen sections of kidneys in OCT compound (Sakura Finetek USA, Torrance, CA) were sectioned with a cryostat and fixed in cold acetone for 5 min. The sections were blocked with 10% normal goat serum in PBS for 20 min, washed, and then stained with FITC-conjugated goat anti-mouse IgG (γ-chain specific, Sigma) directly conjugated to detect immune complexes.

Immunization of mice

Six-week-old mice were immunized i.p. with 100 μg of the T cell-dependent Ag, 2,4-dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH; Calbiochem-Novabiochem, La Jolla, CA), in CFA and were boosted 21 days later. Serum was obtained before and after immunization.

Anti-dsDNA and isotype-specific ELISAs

To determine serum Ig concentrations, ELISAs were conducted as previously described (22), using affinity-purified mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA (Southern Biotechnology Associates) to generate standard curves. The relative Ig concentration in individual samples was calculated by comparing the mean OD obtained for duplicate wells to a semilog standard curve of titrated standard Ab using linear regression analysis. DNP-specific Ab titers of sera were measured as previously described (22), using ELISA plates coated with DNP-BSA (5 μg/ml; Calbiochem-Novabiochem). ELISA color development was allowed to progress until the wells containing the highest Ab levels reached OD levels of about 2.0. These OD values were determined to be within the linear range of the ELISA using sera over multiple dilutions. Serum IgM and IgG anti-dsDNA levels were determined by ELISAs using 96-well microtiter plates (Costar, Cambridge, MA) coated with 5 μg/ml calf thymus dsDNA (Sigma) as previously described (27). Relative levels of dsDNA-specific IgM and IgG were also determined for each group of mice using pooled serum samples. Sera were diluted at log intervals (1/10 to 1/10^4) and assessed for relative Ig levels as described above, except the results were plotted as OD vs dilution.

Measurement of [Ca^{2+}]_{i}

Splenocytes were isolated at room temperature, washed, resuspended at 1 × 10^7/ml in RPMI 1640 medium (Sigma) containing 5% FCS and 10 mM HEPES (Life Technologies, Grand Island, NY), and loaded with 1 μM Indo-1/AM ester (Molecular Probes, Eugene, OR) at 37°C for 30 min. The cells were stained with FITC-conjugated anti-B220 Abs for 15 min at room temperature, washed, and resuspended at 2 × 10^6 cells/ml. Baseline fluorescence ratios were collected for 1 min before the addition of 10 μg/ml F(ab')2 anti-mouse IgM or 40 μg/ml anti-mouse CD19 Abs. Fluorescence ratios were obtained at 20-s intervals with the background subtracted. An increase in the fluorescence ratio indicates an increase in [Ca^{2+}]_{i}.

B cell proliferation

Splenic B cells were purified by removing T cells with anti-Thy 1.2 Ab-coated magnetic beads (Dynal, Lake Success, NY). Purified B220+ (wild type, 95%; CD19−/− mice, 92%; Lyn−/− mice, 88%; CD19/Lyn−/− mice, 87%) splenic B cells (2 × 10^7/well) were cultured in 0.2 ml RPMI 1640 medium containing 10% FCS in 96-well flat-bottom tissue culture plates with LPS (Escherichia coli serotype 0111:B4, Sigma) or F(ab')2 anti-mouse IgM Abs as indicated for 64 h. Proliferation was assessed by the incorporation of [H]-labeled thymidine (1 μCi/well) added during the last 16 h of culture, followed by scintillation counting. All treatments were conducted in triplicate wells for each group.
Immunoprecipitation and Western blotting

Splenic B cells were purified as described above, stimulated with Fab', anti-mouse IgM Abs (40 µg/ml), and lysed in buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 50 mM NaF, 2 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitors. The lysates were either analyzed directly by SDS-PAGE or were subjected to immunoprecipitation with Abs against proteins of interest. For immunoprecipitation, the cell lysates were precleared twice with rabbit IgG plus protein G-Sepharose beads (Amersham Pharmacia, Uppsala, Sweden), followed by incubation with protein G beads plus specific Abs or rabbit IgG control Abs for 3 h at 4°C. After washing with lysis buffer four times, immunoprecipitates were subjected to SDS-PAGE, with subsequent electrophoretic transfer to nitrocellulose membranes. Membranes were incubated with HRP-conjugated anti-phosphotyrosine Abs (4G10, Upstate Biotechnology) and were developed using an ECL kit (Pierce, Rockford, IL). To verify equivalent amounts of protein in each lane, the same blots were stripped and reprobed with Abs reactive with the appropriate proteins. Relative band intensities of immunoblot bands were determined using National Institutes of Health Image software (version 1.60).

Statistical analysis

ANOVA was used to analyze the data, and Student’s t test was used to determine the level of significance for differences between sample means.

Results

CD19 loss inhibits the development of autoimmunity in Lyn−/− mice

Autoimmunity reminiscent of systemic lupus erythematous (SLE) is a remarkable feature of Lyn−/− mice (7–9). Therefore, the effect of CD19 loss on ANA titers was determined by indirect immunofluorescence staining using sera from 5-mo-old mice. ANAs in Lyn−/− mice were predominantly of the IgG isotype and showed a homogeneous chromosomal staining pattern (Fig. 1A). Mean IgM and IgG ANA titers in sera from Lyn−/− mice were about 20-fold higher than those in sera from CD19/Lyn−/− mice (Fig. 1B). IgM ANAs were present in 100% of Lyn−/− mice, but in only 38% of CD19/Lyn−/− mice (Fig. 1B). IgG ANAs were present in 100% of sera from Lyn−/− mice, but in only 25% of sera from CD19/Lyn−/− mice. IgM and IgG ANAs were not detected in wild-type or CD19−/− mouse sera.

Aged Lyn−/− mice develop glomerulonephritis that is caused by IgG immune complex deposition in the kidneys, a pathological process reminiscent of SLE in humans (7–9). Immunohistochemical staining of frozen kidney sections revealed the deposition of IgG immune complexes within the glomeruli of 5-mo-old Lyn−/− mice (Fig. 1C). Modest deposits of IgG immune complexes were observed in the glomeruli of some CD19/Lyn−/− mice, but were not seen in wild-type or CD19−/− mice. Similarly, kidneys from 5-mo-old Lyn−/− mice showed evidence of chronic glomerulonephritis consisting of hypercellularity and hyperlobularity (Fig. 1D). By contrast, glomeruli of CD19/Lyn−/− mice were similar to those of wild-type and CD19−/− mice.

The homogeneous chromosomal staining pattern of sera from Lyn−/− mice is probably due to the existence of anti-dsDNA Abs, which are characteristic of SLE (7–9). Therefore, anti-dsDNA Ab

![FIGURE 1. Autoimmunity in Lyn/CD19−/− mice. A, Nuclear staining by sera from wild-type, CD19−/−, Lyn−/−, and CD19/Lyn−/− mice. ANAs were assessed using sera (diluted 1/50) from 5-mo-old mice and FITC-labeled IgG-specific secondary Abs in indirect immunofluorescence staining assays with HeLa cells as substrate (magnification, ×400). These results represent those obtained from eight mice of each genotype. B, IgG and IgM ANA titers in 5-mo-old mice. Each symbol represents the maximal dilution of sera from individual mice that generated higher than background staining. Titers greater than 1/50 were considered significant. The small horizontal bars indicate mean serum titers. C, Frozen kidney tissue sections from 5-mo-old mice stained with FITC-conjugated anti-mouse IgG (γ-specific) Abs to detect immune complex deposition (magnification, ×150). These results represent those obtained with at least three mice of each genotype. For A and C, all cell samples and tissue sections were assayed simultaneously to prevent variability between samples. Moreover, all slides were developed, photographed, and reproduced with identical times, microscope settings, and exposures. Thereby, the levels of background staining were equivalent in all cases and are represented by the data shown. D, Histopathology of kidneys from 5-mo-old wild-type, CD19−/−, Lyn−/−, and CD19/Lyn−/− mice. Tissue sections were stained with hematoxylin and eosin (magnification, ×200). These results represent those obtained from at least three mice of each genotype.](http://www2.jimmunol.org/doi/abs/10.4049/jimmunol.1100270)
production was assessed in 2-mo-old Lyn−/− and CD19/Lyn−/− littersmates. CD19/Lyn−/− mice had mean IgM anti-dsDNA Ab levels similar to those of wild-type and CD19−/− littersmates (Fig. 2A). By contrast, Lyn−/− mice had mean anti-dsDNA IgM Ab levels that were 90% higher than those found in wild-type littersmates, although significantly lower than those of age-matched, SLE-prone, MRL+/−/− mice (Fig. 2A). IgG anti-dsDNA Ab levels were low in all four genotypes at 2 mo of age. At 5 mo of age, CD19/Lyn−/− mice had IgM and IgG anti-dsDNA Ab levels that remained comparable with those of wild-type and CD19−/− mice. However, mean IgM anti-dsDNA Ab levels were significantly elevated in Lyn−/− mice compared with wild-type mice (540% increase; Fig. 2B) and were comparable with levels in MRL+/−/− mice. Mean IgG anti-dsDNA Ab levels were also significantly elevated in Lyn−/− mice relative to wild-type littersmates (240% increase; Fig. 2B). Similar results were obtained when dsDNA-specific IgM and IgG levels were determined by ELISA using serially diluted pooled samples of sera (Fig. 2B). These data collectively demonstrate that CD19 loss inhibits the development of autoimmunity in Lyn−/− mice.

CD19 deficiency does not affect B cell development in Lyn−/− littersmates

Whether CD19 deficiency inhibits autoantibody production by inhibiting B cell development in CD19/Lyn−/− mice was assessed. Deletion of Lyn and/or CD19 had modest effects on the frequency of IgM+ B220low pro/pre-B cells or IgM+ B220low immature B cells in the bone marrow (Fig. 3A and Table I). However, the frequency of IgM+ B220high mature B cells in the bone marrow of Lyn−/− and CD19/Lyn−/− mice was significantly decreased relative to that in wild-type (73 and 53% reduction, respectively) and CD19−/− littersmates (Fig. 3A and Table I). Similarly, circulating B cell numbers were significantly reduced in both Lyn−/− and CD19/Lyn−/− littersmates compared with wild-type mice (84 and 68% reduction, respectively), while circulating B cell numbers were less significantly reduced in CD19−/− mice (Table I). Lyn−/− and CD19/Lyn−/− littersmates had reduced numbers of
Table I. Frequency, number, and cell surface IgM density of B lymphocytes in CD19–/–, Lyn–/–, and CD19/Lyn–/– littermates

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CD19–/–</th>
<th>Lyn–/–</th>
<th>CD19/Lyn–/–</th>
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<tr>
<td></td>
<td>No. (%)</td>
<td>IgM+ (%)</td>
<td>IgM%</td>
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<tr>
<td>Bone marrow</td>
<td>40 ± 1</td>
<td>35 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>25 ± 1</td>
<td>32 ± 1</td>
<td>36 ± 5</td>
</tr>
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Values represent mean ± SEM results obtained from 5 to 11 of 8- to 16-mo-old mice of each genotype. Numbers represent the percentage of lymphocytes (based on side and forward light scatter properties) expressing the indicated surface markers as determined in Fig. 3 using 2-color immunofluorescence staining. The percentage or number was significantly different from wild-type littermates (Table I). Relative cell surface receptor densities were determined by comparing the channel numbers of the mean linear fluorescence intensity between B cells from wild-type and CD19–/–, Lyn–/–, or CD19/Lyn–/– littermates. The percentage or number was significantly different from wild-type littermates (Table I).

Effect of CD19 deficiency on B1 cell development in Lyn–/– littermates

At 2 mo of age, peritoneal IgM+ B220+ B cell numbers were significantly lower in Lyn–/– and CD19/Lyn–/– mice (77 and 69%, respectively) compared with wild-type littermates (Table I). This resulted from significant reductions in both CD5+ B220low and Mac1+ IgM+ or CD23– IgMhigh IgDlow (Fig. 3E and Table I). CD19–/– littermates also had lower numbers of peritoneal B cells, including a reduction in CD5+ B220low conventional B cells and a more significant (79–85%) reduction in B1 cells (Fig. 3E and Table I). In fact, B1 cell numbers were similar in CD19–/– and CD19/Lyn–/– littermates. Similar results were obtained for peritoneal and spleen CD5+ B220low, Mac1+ IgM+ or CD23– IgMhigh IgDlow B1 cells in 5-mo-old littermates (Fig. 3D and data not shown). Thus, CD19 expression had a dominant influence on B1 cell development in Lyn–/– mice.

CD19 deficiency up-regulates surface IgM expression on Lyn–/– B cells

Circulating B cells from Lyn–/– mice had an approximately 50% decrease in surface IgM expression relative to wild-type littermates (Fig. 3B and Table I), perhaps as a consequence of augmented transmembrane signaling. IgM expression by circulating B cells in CD19/Lyn–/– and wild-type littermates was more comparable (Fig. 3B and Table I). By contrast, circulating B cells from CD19–/– mice had 2-fold higher surface IgM expression (Fig. 3B and Table I), consistent with decreased transmembrane signaling.
(26, 27). Splenic B cells from CD19/Lyn−/− and CD19−/− mice had elevated IgM expression levels (Fig. 3C and Table I). Consistent with a previous report (14), surface IgM levels on splenic B cells from Lyn−/− and wild-type littermates were similar. IgM expression levels on B cells from peripheral lymph nodes were significantly elevated in both CD19/Lyn−/− and CD19−/− mice compared with wild-type mice (147 and 60%, respectively; Table I). Thus, IgM expression by circulating CD19/Lyn−/− B cells was intermediate between the decreased IgM levels found on Lyn−/− B cells and the elevated levels found on CD19−/− B cells, while CD19 deficiency resulted in increased IgM expression by CD19−/− and CD19/Lyn−/− B cells in the periphery. These results suggest that IgM expression levels are influenced by both Lyn and CD19 expression and that CD19 expression may play a more prominent role than Lyn in regulating basal signaling thresholds.

**CD19 deficiency inhibits Fyn phosphorylation in Lyn−/− B cells**

The consequences of Lyn and CD19 loss on BCR-induced signal transduction were first evaluated by assessing total cellular protein tyrosine phosphorylation. Although tyrosine phosphorylation of some proteins was increased in Lyn−/− B cells compared with phosphorylation in wild-type B cells, tyrosine phosphorylation of most cellular proteins was significantly decreased in Lyn−/− B cells both before and after BCR ligation (Fig. 4A). Resting B cells from both CD19/Lyn−/− and CD19−/− mice had significantly lower levels of endogenous protein tyrosine phosphorylation than B cells from wild-type littermates (Fig. 4A) (15). After BCR ligation, protein phosphorylation was delayed and modest in CD19/Lyn−/− B cells compared with that in Lyn−/− or CD19−/− B cells. Protein phosphorylation increased modestly in CD19−/− B cells relative to wild-type B cells, as described previously (15). Protein phosphorylation was delayed in Lyn−/− B cells compared with wild-type B cells, although the intensity of tyrosine phosphorylation for some proteins was normal after 10 min of BCR ligation as previously reported (7). Therefore, CD19 loss reduced signal transduction in Lyn−/− B cells.

**CD19 deficiency delays BCR-induced \([\text{Ca}^{2+}]\), responses in Lyn−/− B cells**

The combined loss of CD19 and Lyn in B cells resulted in a marked delay in initial IgM-induced \([\text{Ca}^{2+}]\), responses beyond the effect of either CD19 or Lyn deficiency alone (Fig. 5). CD19 loss resulted in a modest delay during the acute phase \([\text{Ca}^{2+}]\), response as previously reported (39). By contrast, \([\text{Ca}^{2+}]\), responses were dramatically augmented in Lyn−/− B cells, with a delayed peak during the acute phase and a prolonged late phase response, as previously reported (13). CD19 ligation alone generated a \([\text{Ca}^{2+}]\), response in Lyn−/− B cells that was delayed, but comparable to that in wild-type B cells (Fig. 5). Thus, CD19-induced \([\text{Ca}^{2+}]\), responses were generated in the absence of Lyn expression, although the lack of Lyn expression delayed the initiation of the response.

**FIGURE 4.** BCR signal transduction in CD19/Lyn−/− B cells. A, Total protein tyrosine phosphorylation in B cells from wild-type, CD19−/−, Lyn−/−, and CD19/Lyn−/− mice following BCR cross-linking. Purified splenic B cells (1 × 10^7/lane) were incubated with either medium alone (time zero) or with F(ab’)2 anti-IgM Abs for the indicated times and detergent solubilized. Cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose with anti-SHPI Ab to verify equivalent amounts of proteins loaded in each lane. These results are representative of those obtained in five independent experiments. Fyn (B) and Syk (C) phosphorylation were similarly assessed following IgM ligation. Proteins were immunoprecipitated from cell lysates (1 × 10^7 cells/lane) with specific Abs, fractionated by SDS-PAGE, and transferred onto nitrocellulose for subsequent anti-phosphotyrosine immunoblotting. Blots were subsequently stripped of Ab and reprobed with anti-SHIP-1 Ab to verify equivalent amounts of immunoprecipitated protein. Results obtained in four independent experiments are shown as histograms. Values are the relative mean OD (±SEM) of band intensities determined by scanning densitometry from independent experiments. Values obtained for wild-type control B cells incubated with medium alone (time zero) were adjusted to 100%, with all other density values shown relative to this. Asterisks indicate where differences in mean values were significantly different from values for wild-type control B cells (p < 0.05).
**CD19 deficiency impairs Lyn−/− B cell proliferation**

B cell proliferation in response to BCR engagement or LPS stimulation was assessed in young CD19/Lyn−/− mice to determine how Lyn and CD19 interact to regulate B cell proliferation. Augmented BCR-induced proliferation and normal LPS-induced proliferation are characteristic for Lyn−/− B cells from young mice (7), while proliferative responses to BCR cross-linking and LPS are severely impaired in aged Lyn−/− mice (8, 9). In this study the proliferation of CD19/Lyn−/− B cells in response to BCR ligation was significantly reduced compared with that of wild-type B cells (53% inhibition at 20 μg/ml; p < 0.05) and Lyn−/− B cells (Fig. 6). BCR-induced proliferation of Lyn−/− B cells was significantly augmented compared with that of wild-type littermates (46% increase at 20 μg/ml; p < 0.05). Proliferation of CD19−/− B cells was even lower following BCR ligation (77% inhibition at 20 μg/ml; p < 0.05), consistent with previous results (22). In response to LPS stimulation, proliferation of CD19/Lyn−/− and CD19−/− B cells was similarly low (81% inhibition at 10 μg/ml; p < 0.01), while Lyn−/− and wild-type B cell proliferation responses were comparable (Fig. 6). Thus, proliferative responses of Lyn−/− B cells were significantly inhibited when combined with CD19 loss.

**Humoral immunity in CD19/Lyn−/− mice**

The effects of combined Lyn and CD19 loss on B cell differentiation were assessed by determining serum Ig levels in CD19/Lyn−/− mice. Strikingly, CD19 deficiency suppressed Ab production in Lyn−/− mice and reduced serum IgM, IgG1, IgG2b, and IgA concentrations to levels comparable with those found in wild-type littermates (Fig. 7A). These decreases corresponded to the significantly reduced levels of these Ig isotypes found in CD19−/− mice (22). Serum Ig levels were dramatically higher in Lyn−/− mice than in wild-type littermates (IgM, 1300% increase; IgG1, 350% increase; IgG2b, 590% increase; and IgA, 75% increase) as previously described (7–9).

The influence of Lyn and CD19 loss on humoral immune responses was determined by immunizing mice with the T cell-dependent Ag, DNP-KLH. Endogenous anti-DNP IgM levels were comparable in CD19/Lyn−/−, CD19−/−, and wild-type littermates (Fig. 7B). Lyn−/− mice exhibited remarkably higher serum anti-DNP IgM levels before immunization, probably due to high levels of cross-reactive Abs, as previously reported (8). CD19/Lyn−/− mice generated delayed IgM anti-DNP Abs after immunization that were significantly lower on day 7 compared with those in wild-type mice, but were equivalent after day 14 and after rechallenge. IgM responses in Lyn−/− mice were modest on day 7 after immunization, with a significant increase found after day 14, as previously reported (8, 9). CD19−/− mice had markedly impaired primary and secondary IgM responses. IgG1 responses in CD19/Lyn−/− mice were delayed and modest (Fig. 7B). CD19−/− mice also showed modest IgG1 anti-DNP responses even after secondary immunization. In Lyn−/− mice, anti-DNP IgG1 responses were modest on day 7 after the first immunization, while the titers were comparable with those of wild-type littermates by day 14 as previously reported (9). Thus, B cell differentiation and humoral immune responses in CD19/Lyn−/− littermates were significantly down-regulated by CD19 deficiency, but were influenced to a small extent by Lyn- and CD19-independent pathways.

**Discussion**

The current study demonstrates that CD19 expression is required for the development of autoimmunity in Lyn−/− mice (Fig. 1). Aged Lyn−/− mice produced autoantibodies, including ANAs and anti-dsDNA Abs (Figs. 1 and 2), that correlated with immune complex-related glomerulonephritis (Fig. 1, C and D) as previously described (7–9). However, CD19 deficiency reduced mean IgM and IgG ANA titers in aged Lyn−/− mice by about 20-fold to near normal levels (Fig. 1). Glomerulonephritis, IgG immune complex deposition, and serum IgM and IgG anti-dsDNA Ab production were also dramatically reduced in Lyn−/− mice when CD19 was not expressed (Fig. 1, C and D, and Fig. 2). Although serum Ig levels are elevated in Lyn−/− mice, this alone does not account for autoantibody production (8, 9). Similarly, Lyn−/− mice still produced significant autoantibodies relative to CD19/Lyn−/− mice, even when Ab concentrations were equilibrated in the sera tested.
Thus, CD19 expression specifically influences signaling pathways that contribute to autoimmunity.

CD19 expression by Lyn−/− B cells was required for optimal tyrosine phosphorylation of cellular proteins before and after BCR stimulation (Fig. 4A). In addition, CD19 expression was required for optimal Fyn phosphorylation in wild-type and Lyn−/− B cells (Fig. 4B). These results suggest that CD19 augments both Fyn and Lyn activation through processive amplification. This is consistent with earlier in vitro observations that the CD19 cytoplasmic domain amplified both Lyn and Fyn kinase activities (15). CD19 is likely to also regulate the activity of other Src family PTKs, since Lyn, Fyn, Blk, and Lck phosphorylation are severely impaired in CD19-deficient B cells (15). Lyn and Lck coimmunoprecipitate with CD19 in cell lines (31, 47). Thus, CD19 may be a general regulator of Src family PTK activity in B cells. That CD19 was functionally active in Lyn−/− B cells was surprising, since CD19 is not phosphorylated at detectable levels in Lyn−/− B cells (16). In addition, CD19/Fyn interactions were not detectable in B cells from Lyn−/− mice. Although it remains possible that CD19 regulates Src family PTK activity through heretofore unknown phosphorylation-independent pathways, it is possible that a small pool of phosphorylated CD19 is sufficient for Src family PTK amplification. CD19 may also regulate the spatial distribution of the Src family PTKs at the membrane/cytoplasm interface. Since tyrosine-phosphorylated CD19 and the Src family PTKs preferentially localize within detergent-insoluble lipid raft microsignaling domains (48, 49), CD19 expression may help recruit or retain Src family PTKs within lipid rafts and thereby regulate downstream signaling pathways. Alternatively, Src family PTKs may recruit CD19 into lipid rafts, where the adapter function of CD19 amplifies kinase activity. Nonetheless, CD19 expression was not required for Syk phosphorylation in Lyn−/− B cells (Fig. 4C) as previously shown (15). Since Syk phosphorylation and kinase activity can be upregulated through an efficient autophosphorylation loop (50), BCR-induced Fyn activation may be sufficient to activate Syk in the absence of CD19-mediated Src family PTK amplification. Thus, CD19 is likely to contribute to signaling and the hyperresponsive phenotype of Lyn−/− B cells by amplifying Src family PTK activity.

Altered CD19 phosphorylation and signaling may also contribute indirectly to the hyper-responsive phenotype of Lyn−/− B cells by dysregulating CD22 function. Despite its positive regulatory roles, Lyn activation initiates inhibitory pathways through CD22 phosphorylation, which recruits the SHP-1 and SHIP phosphatases (13, 42–44, 51, 52). Lyn phosphorylation of CD22 is primarily dependent on Lyn expression and Lyn amplification of Lyn kinase activity (35, 39). The CD22/SHP-1 inhibitory pathway, in turn, regulates CD19 phosphorylation (39, 40). It is likely that

**FIGURE 7.** A, Serum Ig levels in CD19/Lyn−/− mice. Values are the mean ± SEM Ig levels for at least nine wild-type, CD19−/−, Lyn−/−, and CD19/Lyn−/− mice as determined by isotype-specific ELISAs. B, Humoral immune responses of CD19/Lyn−/− mice. Wild-type, CD19−/−, Lyn−/−, and CD19/Lyn−/− mice were injected i.p. with 100 μg DNP-KLH in CFA on days 0 and 21 (arrows) and bled at the indicated times. Serum levels of anti-DNP Abs for five or six mice of each genotype were determined by isotype-specific ELISAs. Mean values significantly different from wild-type levels are indicated: *p < 0.05; **p < 0.01.
activated SHP-1 down-regulates CD19 phosphorylation by dephosphorylating Lyn (40) or by dephosphorylating CD19 directly. Thus, the loss of Lyn expression may effectively block/reduce CD19 and CD22 phosphorylation and thereby disarm the negative regulatory role of CD22. Consistent with CD22 involvement, Lyn−/− B cells generate dramatically higher and prolonged [Ca2+]i increases following BCR engagement that are similar to the exaggerated [Ca2+]i responses (Fig. 5) observed in CD22- and SHP-1-defective B cells (13, 43, 53–55). However, the role of CD19 during [Ca2+]i responses is more complex, since CD19 loss delays the initial peak [Ca2+]i response in both Lyn−/− and wild-type B cells following BCR engagement (Fig. 5). Therefore, CD19 may influence early phase [Ca2+]i responses by amplifying Src family kinase activity, which expedites efficient and rapid CD79a/b and Syk phosphorylation following BCR ligation (39).

Thus, altering Lyn and CD19 expression affects multiple downstream signaling pathways, which is likely to include CD22 regulatory function.

CD19 loss down-regulated the hyper-responsive phenotype of Lyn−/− B cells, but did not dramatically affect peripheral B cell development in young Lyn−/− mice (Fig. 3 and Table I). Specifically, CD19 deficiency up-regulated surface IgM expression levels on Lyn−/− B cells in the blood, lymph node, and spleen (Fig. 3 and Table I), presumably reflecting a less responsive phenotype to transmembrane signals (19, 26, 27, 55). These findings suggest that CD19 and Lyn interact to modulate IgM expression and signal transduction thresholds in resting B cells. Although BCR- and LPS-induced proliferation were reported to be abrogated by Lyn deficiency (8, 9), enhanced BCR-induced proliferation and normal LPS-induced proliferation are observed for B cells from young Lyn−/− mice (7). CD19 loss dramatically inhibited the hyperproliferative response of Lyn−/− B cells following BCR engagement (Fig. 6). Since B cell proliferation was slightly higher in CD19/ Lyn−/− B cells than in CD19−/− B cells, Lyn deficiency may also contribute to B cell proliferation in part through CD19-independent pathways. By contrast, LPS-induced proliferation was modest for both CD19/Lyn−/− and CD19−/− B cells (Fig. 6). This suggests that LPS-induced proliferation signals proceed through a CD19-dependent pathway that is predominantly Lyn independent. Nonetheless, CD19 expression was essential for the hyper-responsive phenotype and hyperproliferation of Lyn−/− B cells in response to BCR engagement.

Consistent with the observation that CD19 and Lyn interact to modulate signal transduction thresholds in conventional B cells, CD19 and Lyn interactions are critical for the development of peritoneal CD5+ B1 cells (Fig. 3E). The frequency of CD5+ B1 cells in Lyn−/− mice has previously been reported to be normal (8, 9, 46). Although the emergence of a Mac1− subset of cells with increasing age makes this assessment difficult (discussed below), the frequency and total number of peritoneal CD5+ B1 cells was reduced by about 70% in the absence of Lyn expression (Fig. 3E and Table I). This reduction was comparable with the 60–80% reduction in numbers of conventional peripheral B cells in Lyn−/− mice (Table I). Thus, Lyn−/− mice generate significant levels of autoantibodies despite a dramatic reduction in conventional and CD5+ B cell numbers. The number of peritoneal CD5+ B1 cells in CD19−/− and CD19/Lyn−/− mice was reduced by 80% (Table I). Therefore, both conventional and B1 B cells are dependent on Lyn and CD19 expression for normal development, presumably reflecting a requirement for CD19 amplification of Lyn kinase activity.

As Lyn−/− mice age, lymph node and spleen size increase dramatically (7–9). However, splenomegaly in 5-mo-old Lyn−/− mice was significantly reduced (~65%) by CD19 deficiency. Lymphadenopathy and splenomegaly in aged Lyn−/− mice have been proposed to correlate with or result from the accumulation of a unique lymphoblast-like Mac-1+ cell population (7, 9). In one study of 7- to 8-wk-old and 3-mo-old mice, an unusual population of Mac1+, Thy1−, B220+, IgM−, cytoplasmic IgM− cells with the size characteristics of lymphoblasts were identified in spleen and peripheral blood (9). These cells spontaneously secreted large amounts of IgM in vitro (9). A subsequent study of 6-mo-old mice with splenomegaly reported the presence of an IgM+, Mac1+, CD5+, B220low, and IgD− blast-like population within enlarged lymph nodes that was not found in wild-type or young Lyn−/− mice (7). These cells were proposed to resemble a subtype of B1 cells (7). In the current study the above subpopulations were not apparent in spleens or lymph nodes of Lyn−/− mice by 5 mo of age, although there was a significant increase in the frequency and number of Mac1+ F4/80+ IgM− IgD− CD19− CD5− B220− Thy1− cells with the size characteristics of lymphoblasts (Fig. 3D and data not shown). These cells may represent a subset of myeloid lineage cells, since Lyn is expressed by monocytes (45, 46), and the myelomonocytic cell population is expanded in Lyn−/− mice (C. A. Lowell, unpublished observations). While autoimmune disease in Lyn−/− mice may correlate with the expansion of these abnormal cell populations, serum Ig levels and anti-DNA Abs are elevated in Lyn−/− mice at early ages before these unique cells accumulate (Figs. 2A and 7A) (44). In support of this, autoantibody production is significantly elevated in mice that overexpress CD19 due to a breakdown in peripheral B cell tolerance rather than to increased numbers of B1 cells (27, 56, 57). That CD19 deficiency suppressed autoantibody production suggests that CD19 regulates B cell signaling pathways that contribute to the genesis of autoimmunity in Lyn−/− mice regardless of the cellular source of autoantibodies.

Increased signaling thresholds and the impairment of B cell proliferation and clonal expansion in response to self-Ags are likely to explain the decreased autoimmunity in CD19/Lyn−/− mice. That autoimmunity and immune responses are regulated or fine-tuned by CD19 explains why subtle increases in CD19 expression leads to autoantibody production (19, 27, 57, 58). In addition, it is likely that CD19 establishes an Src family kinase activation loop that amplifies Fyn and Blk kinase activity in vivo when Lyn is not expressed (15, 29). Although it remains unknown whether these kinases benefit from CD19 expression when Lyn is expressed normally, Fyn phosphorylation is decreased in CD19−/− B cells relative to wild-type B cells (Fig. 4), and CD19 amplifies the activation of other Src family PTKs in vitro in addition to Lyn (15, 29). Thus, while the CD19/Lyn amplification loop is a major regulator of signal transduction thresholds in B cells, CD19 interactions with other Src family PTKs are also likely to regulate and influence B cell function. A further understanding of the molecular aspects of CD19/Src family PTK interactions may identify target molecules for therapeutic intervention during autoimmunity.

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References


