A Kinase-Dead Allele of Lyn Attenuates Autoimmune Disease Normally Associated with Lyn Deficiency

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Lyn kinase, a member of the Src family of tyrosine kinases, functions as both a positive and negative regulator of B cell activation. In the absence of Lyn, BCR signaling is unregulated, leading to perturbed B cell development, hyperactive B cells, and lethal Ab-mediated autoimmune disease. We have generated a mutant mouse pedigree, termed Mld4, harboring a novel mutation in the gene encoding Lyn, which renders the protein devoid of kinase activity. Despite similarities between the phenotypes of LynMld4/Mld4 and Lyn−/− mice, the spectrum of defects in LynMld4/Mld4 mice is less severe. In particular, although defects in the B cell compartment are similar, splenomegaly, myeloid expansion, and autoimmune disease, characteristic of Lyn−/− mice, are absent or mild in LynMld4/Mld4 mice. Critically, immune complex deposition and complement activation in LynMld4/Mld4 glomeruli do not result in fulminant glomerulonephritis. Our data suggest that BCR hypersensitivity is insufficient for the development of autoimmune disease in Lyn−/− mice and implicate other cell lineages, particularly proinflammatory cells, in autoimmune disease progression. Furthermore, our results provide evidence for an additional role for Lyn kinase, distinct from its catalytic activity, in regulating intracellular signaling pathways. The Journal of Immunology, 2009, 182: 2020–2029.

The Journal of Immunology

The Src family kinases (SFKs) are important mediators of signal transduction in hematopoietic cells, including signaling through immunoreceptors as well as cytokine and growth factor receptors (1, 2). Src kinases share similar domain structures that contain SH2, SH3, and kinase domains and a regulatory domain at the carboxyl terminus. A defining feature of SFKs is an intramolecular interaction between the SH2 domain and phosphorylated Tyr527 in the C-terminal regulatory domain that holds the protein in an inactive state. Dephosphorylation of Tyr527 by CD45 permits an open, active conformation of Src. Full Src kinase activity requires autophosphorylation of Tyr416 in the activation loop of the catalytic domain (3).

Lyn is the predominant SFK expressed in B lymphocytes and acts as both a positive and negative regulator of BCR signaling pathways (4). Lyn is activated following BCR ligation and initiates signaling cascades that promote BCR-mediated signaling by phosphorylating proximal signaling molecules, including CD19 and the ITAMs of the Igα/Igβ BCR subunits. Lyn is not essential, however, for the initiation of BCR signaling, as this process still occurs in Lyn-deficient cells, suggesting that this function is shared with other SFKs such as Blk and Fyn (5).

In contrast, Lyn plays a critical and nonredundant role in the negative regulation of BCR signaling. Upon BCR ligation, Lyn phosphorylates ITIM-bearing coreceptors, including CD22 and FcγRIIB, resulting in the recruitment and activation of the phosphatases SH2 domain-containing phosphatase 1 (SHP-1) and SHIP-1. In turn, these phosphatases dephosphorylate receptors, adaptors, and other signaling intermediates to switch off the activation pathways (6). The consequence of Lyn deficiency is profound, exemplified by Lyn−/− mice which have uncontrolled BCR responses and develop lethal Ab-mediated glomerulonephritis (7, 8).

The phenotype of Lyn-deficient mice is complex, reflecting the expression of Lyn in all hematopoietic cells except T cells (9). For example, analogous to its role in B cells, Lyn phosphorylates ITIM-containing receptors in mast cells and macrophages, limiting signaling in response to FcεRI and FcγR ligation, respectively (10, 11). In addition, Lyn also regulates signaling in response to cytokines and growth factors, including stem cell factor (12), G-CSF (13), GM-CSF and M-CSF, (14), IL-4 (15), erythropoietin (EPO) (16), and thrombopoietin (17), as well as regulating cytokine production itself (18). In general, the precise role of Lyn in each of these signaling pathways has not been determined. Although it has been proposed that Lyn directly phosphorylates STAT5 in response to EPO (16), EPO-dependent signaling appeared normal in studies of Lyn-deficient erythroblasts (19). Alternatively, it has been suggested that there may be cross-talk between cytokine and growth factor receptor signaling and signal transduction through ITIM-containing receptors (20).
In this study, we describe the characterization of mice expressing a kinase-dead allele of Lyn. We show that many of the abnormalities typical of Lyn−/− mice are substantially ameliorated in mice expressing kinase-dead Lyn. In particular, despite similar defects in the B cell compartment, including the extent of B lymphopenia and strength of BCR signaling, few mutant mice develop autoantibodies and autoimmune disease development is markedly reduced compared with Lyn−/− mice. Our data indicate that B cell extrinsic factors play a critical role in the development of autoimmune disease in the absence of Lyn and also provide evidence for an additional role for Lyn kinase, distinct from its catalytic activity, in regulating intracellular signaling pathways.

**Materials and Methods**

**Generation and screening of mutant mice**

Male Mpl−/− C57BL/6 mice (21) were treated with N-ethyl-N-nitrosourea (ENU) as previously described (22). ENU-treated mice were mated with isogenic females to yield first generation (G1) progeny. G1 mice were intercrossed to yield G2 progeny, which were brother/sister mated to produce third generation (G3) mice. G3 mice were bred at 7 wk of age and peripheral blood cell values were determined using an Advia 120 automated hematological analyzer (Bayer). The percentage of B and T lymphocytes in peripheral blood was determined using flow cytometry. Upon isolation in a G3 pedigree, multilineage defect 4 (Mld4) was bred to Mpl−/+ C57BL/6 mice. All animals used in the studies described were on a Mpl−/+ C57BL/6 genetic background, unless otherwise noted. Lyn−/− mice, backcrossed onto the C57BL/6 background for 20 generations, were as described previously (7). Mice were routinely housed in clean, conventional facilities and were used for experiments at 7–10 wk of age, unless otherwise described. Mice were housed side-by-side in identical conditions, with the exception of mice used for the colony assays (described in Table I and see Fig. 3, A and B) and the B cell analyses (see Fig. 2, A–D and F–H). The B cell phenotyping data were confirmed subsequently in mice housed in identical conditions (data not shown). All experiments using mice were approved by St. Vincent’s Hospital Animal Ethics Committee.

**Genetic mapping**

Male C57BL/6 Mld4/Mld4 (m/m) Mpl−/− mice were mated to +/+ Mpl−/+ 129/SV females. Random pairs of F1 mice were mated, a quarter of which produced affected m/m F2 mice, which were identified using flow cytometry of peripheral blood samples. DNA from 16 affected F2 mice and 15 unaffected siblings was genotyped for 64 simple sequence length polymorphisms spaced evenly genome-wide (23), upon which Mld4 was assigned to the proximal region of chromosome 4. The candidate interval was refined via analysis of additional MIT and in-house CA repeat markers in the region.

**Sequencing and genotyping**

DNA was prepared from tail biopsy and the coding exons and splice junctions of candidate genes were PCR amplified and products were treated with ExoSap-IT (GE Healthcare). Sequencing was conducted using Big-Dye Terminator chemistry (Applied Biosystems) and reaction mixtures were resolved and analyzed on an Applied Biosystems 3730S Genetic Analyzer at the Micromon DNA Sequencing Facility (Monash University, Clayton, Victoria, Australia). Residue numbering corresponding to chicken c-src is used. Genotyping of the Mld4 mutation was performed by PCR amplification of exon 12 of Lyn, followed by digestion with Hpy8I (Fermentas). The Mld4 mutation destroys an Hpy8I site, resulting in a 350-bp band instead of two bands of 180 bp.

**Abs and flow cytometry**

Splenocyte suspensions were prepared by dissociating total spleens and erythrocytes were lysed by incubation in 5 ml of warm 156 mM ammonium chloride, pH 7.3 (red cell removal buffer) at 37°C for 3 min. Leukocytes were enriched in blood samples by lysing erythrocytes twice in red cell removal buffer. Cells were pretreated with a blocking Ab against FcγR, 2.4G2, then stained with Abs specific for CD45R/B220 (clone RA-6-8B2), CD90.2/Thy-1.2 (53-2.1), CD71 (C2), Ly-76 (Ter-119), Ly-6G/Gr-1 (RB6-8C5), CD11b/Mac-1 (M170), CD21/CD53 (7G6), CD23 (B34), IgM (B9/41), CD40 (3/23), CD19 (clone D3), MHC class II (AF6-202.1), CD5 (73-3.7), and CD43 (57; BD Biosciences) and analyzed on a FACS Calibur flow cytometer (BD Biosciences). Dead cells were excluded based on propidium iodide staining.

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**Table I. Colony formation by cells from Lyn+/+, LynMld4Mld4, and Lyn−/− mice**

| Cells Cultured | Stimulus | BL | G | GM | M | Eo | Meg |
|---------------|----------|----|---|----|---|----|-----|
| Adult bone marrow (25,000) | G-CSF | 0 ± 0 | 21 ± 3 | 4 ± 1 | 32 ± 11 | 3 ± 1 | 0 ± 0 |
| Lyn+/+ | G-CSF | 0 ± 0 | 10 ± 1 | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| M-CSF | 0 ± 0 | 2 ± 1 | 4 ± 1 | 48 ± 13 | 0 ± 0 | 0 ± 0 |
| IL-3 | 5 ± 3 | 22 ± 3 | 11 ± 4 | 16 ± 8 | 3 ± 2 | 4 ± 3 |
| LynMld4/Mld4 | SCF + IL-3 + EPO | 15 ± 6 | 23 ± 2 | 10 ± 1 | 22 ± 3 | 3 ± 2 | 19 ± 4 |
| Lyn+/+ | SCF + IL-3 + EPO | 0 ± 0 | 18 ± 5 | 11 ± 4* | 34 ± 10 | 3 ± 1 | 0 ± 0 |
| M-CSF | 0 ± 0 | 11 ± 3 | 5 ± 1 | 61 ± 8 | 0 ± 0 | 0 ± 0 |
| IL-3 | 0 ± 0 | 17 ± 4 | 10 ± 5 | 24 ± 5 | 3 ± 2 | 4 ± 3 |
| Lyn+/+ | SCF + IL-3 + EPO | 13 ± 2 | 28 ± 9 | 12 ± 2 | 26 ± 8 | 3 ± 2 | 23 ± 3 |
| Lyn+/+ | GM-CSF | 1 ± 1 | 28 ± 8 | 11 ± 6 | 46 ± 16 | 3 ± 1 | 0 ± 0 |
| Lyn+/+ | G-CSF | 0 ± 0 | 16 ± 5* | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Lyn+/+ | M-CSF | 0 ± 0 | 3 ± 1 | 8 ± 3* | 81 ± 20* | 0 ± 0 | 0 ± 0 |
| IL-3 | 11 ± 3 | 21 ± 3 | 11 ± 3 | 30 ± 3* | 2 ± 2 | 6 ± 1 |
| SCF + IL-3 + EPO | 17 ± 8 | 27 ± 3* | 13 ± 3 | 34 ± 8* | 2 ± 2 | 22 ± 8 |
| Adult spleen (50,000) | GM-CSF | 0 ± 0 | 1 ± 1 | 0 ± 1 | 0 ± 1 | 0 ± 1 | 0 ± 0 |
| Lyn+/+ | G-CSF | 0 ± 0 | 0 ± 0 | 2 ± 2 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Lyn+/+ | M-CSF | 0 ± 0 | 0 ± 0 | 8 ± 2** | 0 ± 0 | 0 ± 0 |
| Lyn+/+ | SCF + IL-3 + EPO | 4 ± 2* | 2 ± 1* | 2 ± 2 | 2 ± 2 | 1 ± 0 | 0 ± 0 |
| Lyn+/+ | GM-CSF | 1 ± 1 | 6 ± 3* | 2 ± 1* | 15 ± 7** | 0 ± 0 | 0 ± 0 |
| Lyn+/+ | M-CSF | 0 ± 0 | 0 ± 0 | 1 ± 1 | 16 ± 12 | 0 ± 0 | 0 ± 0 |
| Lyn+/+ | SCF + IL-3 + EPO | 8 ± 8 | 6 ± 4 | 3 ± 3 | 7 ± 4* | 1 ± 1 | 20 ± 14 |

Cultures were scored after 7 days of incubation. Data are mean colony numbers in the entire culture ± SDs counted in cultures stained with acetyl cholinesterase, Luxol Fast Blue, and hematoxylin. BL, blast; G, granulocytic; GM, granulocyte-macrophage; M, macrophage; Eo, eosinophil; Meg, megakaryocyte colonies; SCF, stem cell factor.

* p < 0.05 and **, p < 0.01, relative to Lyn+/+ data.
ELISA

Anti-dsDNA Abs (IgG) were quantitated in mouse serum using an ELISA kit (Alpha Diagnostic International) according to the manufacturer’s instructions. IgE and IgM were quantitated in mouse serum using a BD Biosciences OptEIA IgE ELISA set or anti-mouse IgM Abs (capture: clone II/41; detection: clone R6-60.2; BD Biosciences).

Histology and immunohistochemistry

Kidneys were fixed in 10% buffered formalin, sectioned, and stained with H&E. Frozen kidney sections were fixed in acetone for 5 min at room temperature, followed by staining with FITC-conjugated goat anti-mouse IgG (Millipore) or rabbit anti-mouse C3c complement (Abcam).

Immunoblotting

B cells were purified from mouse spleen by negative selection using MACS beads. Briefly, following BRC lysis of splenocyte suspensions, non-B cells were stained with biotinylated Abs specific for Mac-1, Gr-1, Ly-76 (TER-119), CDS (clone 53-7.3), and CD43 (ST7), washed, incubated with streptavidin microbeads (Miltenyi Biotec) and depleted using a MACS column (Miltenyi Biotec). The purity of the B cell preparation was typically >90%. Purified B cells were stimulated with 30 μg/ml goat anti-mouse IgM F(ab')2 (Jackson ImmunoResearch Laboratories) at 37°C and lysed for 1 h in Triton X-100 lysis buffer (1% Triton X-100, 1% glycerol, 150 mM Tris (pH 7.5), and 2 mM EDTA) supplemented with protease and phosphatase inhibitors (Complete protease inhibitor and PhosSTOP, Roche). The nuclear and cellular debris were removed by centrifugation. SDS-PAGE of total cell lysates and immunoblots using Abs specific for ERK1/2 (K-23/ sc-15; Santa Cruz Biotechnology), Lyn (Lyn-01; Abcam), phosphorylated forms of CD19 (Tyr^295; catalog no. 3571), Akt (Ser^473; catalog no. 9272), Stress-activated protein kinase/ERK1/2 (Thr^202/ Tyr^204; catalog no. 9801), and c-Src Tyr^416 (catalog no. 3265), all from Cell Signaling Technology, phosphotyrosine (4G10; Millipore), or phospho-Akt (Ser 473; catalog no. 9271), phospho-SHP-1 (Tyr536; catalog no. SP1571), phospho-SAPK/JNK (Thr^183/Tyr^185; catalog no. 9251), phospho-p38 MAPK, (Thr^180/Tyr^182; catalog no. 9211), Akt (Ser^473; catalog no. 9272), Stress-activated protein kinase/ERK1/2 (Thr^202/ Tyr^204; catalog no. 9801), and c-Src Tyr^416 (catalog no. 2101), all from Cell Signaling Technology, phosphotyrosine (4G10; Millipore), or phospho-

Calcium flux

Calcium flux was performed essentially as previously described (25). Briefly, splenocytes (1 × 10^7) were loaded with Indo-1 AM (Molecular Probes) and stained with Abs specific for IgM and CD23. The ratio of blue:green fluorescence (FL5/FL3) was measured on an LSR flow cytometer (BD Biosciences) before and after stimulation of cells with 4 μg/ml goat anti-mouse IgM F(ab')2. Flux was measured for at least 5 min.

In vitro kinase assays

Cell lysates from purified splenic B cells (10^7 cells/100 μl) were precleared with protein G-Sepharose (Fast Flow 4; GE Healthcare) for 1 h at 4°C. Lyn was immunoprecipitated from precleared lysates (100 μl/immunoprecipitation) for 2 h at 4°C using 1 μg of anti-Lyn Ab (sc-15; Santa Cruz Biotechnology) and 20 μl of protein G-Sepharose. Control immunoprecipitations were performed with protein G-Sepharose alone. Immunoprecipitates were washed three times in Triton X-100 lysis buffer and twice in kinase buffer (50 mM Tris (pH 6.8), 50 mM NaCl, 0.1% Triton X-100, 10 mM MgCl2, 5 mM MnCl2, and 0.1 mM sodium vanadate) and resuspended in 20 μl of kinase buffer supplemented with 0.5 mM DTT, 5 μM Src optimal peptide (60222-1; AnaSpec), and 0.5 μM [γ-32P]ATP (3000 Ci/mmol; PerkinElmer) at 30°C. At regular intervals, 4-μl aliquots of supernatant were transferred onto 2 × 2-cm squares of Whatman P81 chromatography paper, which were washed, shaking, for 1 h in 0.4% orthophosphoric acid, rinsed briefly in 100% ethanol, air dried, immersed in scintillation fluid (Ultima Gold; PerkinElmer), and counted on a beta counter (Tri-Carb 2500 TR; PerkinElmer). Kinase reactions were also performed using lysates (100 μg of precleared extract/immunoprecipitation) from macrophages derived from the bone marrow of mice by culture in L cell-conditioned medium for 6–7 days. Equivalent Lyn protein input for B cell and macrophage in vitro kinase reactions was established by immunoblotting lysates with anti-Lyn Ab (Lyn-01; Abcam).

Colony assays

Clonal cultures were prepared in 35-mm petri dishes containing 1 ml of DMEM with 20% FCS and 0.3% agar. Cultures contained 25,000 bone marrow or 50,000 spleen cells and were stimulated by final concentrations of one or more of the following purified recombinant mouse proteins produced in-house (The Walter and Eliza Hall Institute) or purchased from PeproTech: 10 ng/ml GM-CSF, 10 ng/ml M-CSF, 10 ng/ml IL-3, 100 ng/ml stem cell factor, 2 IU/ml EPO, and 10 ng/ml human G-CSF. Culture conditions and analysis were as described previously (26).

Statistical analyses

Significant differences were determined by Student’s two-tailed t tests for independent events.

Results

Generation of the Mld4 pedigree

To identify genes important for immune regulation, we conducted a forward genetic screen for recessive mutations using the chemical mutagen ENU. The mutant pedigrees used in this study had originally been generated in a genetic screen for mutations that attenuate thrombocytopenia using Mpl^−/− mice, which lack the receptor for thrombopoietin (27). We isolated a pedigree in which multiple animals showed reduced numbers of white blood cells, RBC, and platelets compared with control Mpl^−/− mice in their peripheral blood (Fig. 1A). Flow cytometric analysis of the blood from mice of this pedigree, named multilineage-deficient 4 (Mld4), showed that the lymphopenic phenotype was due predominantly to a loss of B, rather than T, lymphocytes (Fig. 1B).

The Mld4 mutation was mapped to a 6.48-Mb region of chromosome 4 using a standard positional cloning approach (Fig. 1C). The gene encoding Lyn kinase, located within this region, was considered a strong candidate because Lyn^−/− mice are profoundly B lymphopenic (7, 8). Sequence analysis of the exons and intron/exon boundaries of Lyn revealed a C-to-A conversion at base 25 in exon 12 which was not found in either parental Mpl^−/− nor C57BL/6 wild-type (wt) mice (Fig. 1D). This mutation is predicted to cause the substitution of a lysine for a threonine codon at residue 429 in the highly conserved Src activation loop (Fig. 1E).

B cell lymphopenia is similar in Lyn^Mld4/Mld4 and Lyn^−/− mice

B lymphopenia is a hallmark of Lyn^−/− mice (5, 7, 8). To determine the effect of the Mld4 mutation on B cell development, we analyzed splenic B cell subsets by flow cytometry. Similar to Lyn^−/− mice, there was a profound reduction in the proportion of transitional stage 2, marginal zone, and CD23^+ (mainly follicular) B cells in Lyn^Mld4/Mld4 spleen (Fig. 2A). This B lymphopenia was reflected in reduced total cellularity in both Lyn^Mld4/Mld4 and Lyn^−/− spleens and a significant decrease in follicular and marginal zone B cell number (Fig. 2, B and C).

Previous studies have characterized differences in the expression of cell surface molecules on B cells between wt and Lyn^−/− mice, which are thought to reflect compensatory adjustments in response to uncontrolled BCR signaling (28). Surprisingly, receptor density on Lyn^Mld4/Mld4 follicular B cells differed from that of Lyn^−/− cells. As reported previously (28), Lyn^−/− B cells expressed lower levels of CD21 compared with wt, but this difference was not seen in Lyn^Mld4/Mld4 B cells (Fig. 2D). In contrast, surface IgM and CD19 expression were invariably increased in Lyn^Mld4/Mld4 B cells, as was CD23 expression (Fig. 2, E–G). A small but significant increase in CD19 and CD23 expression was also seen in Lyn^−/− cells compared with wt, but this difference was less pronounced than for Lyn^Mld4/Mld4 cells (Fig. 2, F and G). No difference in surface IgM expression on Lyn^−/− cells was observed (Fig. 2E). B cells were activated in both Lyn^−/− and Lyn^Mld4/Mld4 mice, with significant increases in MHC class II expression (Fig. 2H). Elevated levels of circulating IgE and IgM, characteristic of Lyn^−/− mice (7, 8, 15), were also seen in Lyn^Mld4/Mld4 mice (Fig. 2I).
The B lymphopenia was mainly restricted to mature B cell populations. No defect in early B cell development in the bone marrow was seen, while the proportion of recirculating mature B cells in the bone marrow was reduced in both Lyn<sup>Mld4/Mld4</sup> and Lyn<sup>−/−</sup> mice as expected (Fig. 2, J and K). The effect of Lyn deficiency in the B1 cell compartment has been contentious, with some studies indicating an increase in B1 B cell number in Lyn<sup>−/−</sup> mice (5, 8) and others reporting little or no change (7, 29–31). We found no consistent change in the number of B1a or B1b cells in the peritoneal cavity of either Lyn<sup>Mld4/Mld4</sup> or Lyn<sup>−/−</sup> mice (Fig. 2, L and M). The proportion and number of peritoneal T cells, however, was increased in both Lyn<sup>Mld4/Mld4</sup> and Lyn<sup>−/−</sup> mice, which may be a reflection of an ongoing immune response in these mice (Fig. 2, L and M).

Intermediate enhancement of myelopoiesis and erythropoiesis in Lyn<sup>Mld4/Mld4</sup> mice

Hypersensitivity to myeloid growth factors in the absence of Lyn has been shown to result in an age-dependent increase in myeloid, erythroid, and primitive hematopoietic progenitor numbers and the development of splenomegaly and macrophage tumors (14, 19). To assess the effect of the Mld4 mutation on myelopoiesis, we determined the myeloid progenitor number in 8-wk-old Lyn<sup>+/+</sup>, Lyn<sup>Mld4/Mld4</sup>, and Lyn<sup>−/−</sup> mice using colony assays. As reported previously, there was a significant increase in the number of colonies generated in response to GM-CSF, M-CSF, or IL-3 in both the bone marrow and spleen of Lyn<sup>−/−</sup> mice (Table I and Fig. 3, A and B) (14, 19). The number of Lyn<sup>Mld4/Mld4</sup> myeloid progenitors was also enhanced relative to Lyn<sup>+/+</sup> cells, but in general was intermediate between that of Lyn<sup>−/−</sup> and Lyn<sup>+/+</sup> cells (Table I and Fig. 3, A and B). Unlike Lyn<sup>−/−</sup> mice, which develop a myeloproliferative syndrome, the increase in myelopoiesis in Lyn<sup>Mld4/Mld4</sup> mice resulted in only minor increases in the proportion of neutrophils and macrophages in peripheral lymphoid tissues, even in 1-year-old mice (Fig. 3C) (14). The expanded splenic myeloid compartment contributes to the development of splenomegaly in Lyn<sup>−/−</sup> mice (average spleen weight of 0.61 g in 1-year-old mice) (14). In contrast, no difference was observed between the spleen weight of wt and Lyn<sup>Mld4/Mld4</sup> mice (Fig. 3D). Similar to Lyn<sup>−/−</sup> mice, erythropoiesis was enhanced in Lyn<sup>Mld4/Mld4</sup> mice, with a large increase in the proportion of CD71<sup>+</sup>Ter-119<sup>+</sup> erythroblasts in the spleens of Lyn<sup>Mld4/Mld4</sup> mice (Fig. 3E).

Lyn<sup>Mld4</sup> is a kinase-dead allele of Lyn

To address how the Mld4 mutation affects Lyn function, we measured the catalytic activity of Lyn using an in vitro trans kinase assay. Lyn immunoprecipitated from purified wt splenic B cell lysates effectively phosphorylated Src optimal peptide over a time course (Fig. 4A). In contrast, despite normal levels of Lyn in Lyn<sup>Mld4/Mld4</sup> B cells, mutant Lyn immunoprecipitated from these cells completely lacked kinase activity. Similar results were seen using macrophage lysates (Fig. 4B). In addition, Lyn immunoprecipitated from Lyn<sup>Mld4/Mld4</sup> macrophage lysates was completely devoid of activity in an autokinase assay (data not shown). Although our results strongly suggest that the Lyn<sup>Mld4/Mld4</sup> mutant protein is catalytically inactive, we cannot completely exclude the possibility that some activity remains that is below the threshold for detection, but given the sensitivity of these assays, any residual activity would be expected to be minimal. Surprisingly, mutant Lyn expressed in Lyn<sup>Mld4/Mld4</sup> B cells was phosphorylated on Tyr<sup>416</sup> in the catalytic domain in response to BCR ligation, albeit to a lesser extent than in wt samples (Fig. 4C). Although this critical tyrosine residue is generally considered to be an autophosphorylation site, these results indicate that it can be phosphorylated in trans by other kinases as well.

BCR signaling defects are similar in Lyn<sup>−/−</sup> and Lyn<sup>Mld4/Mld4</sup> B cells

Following BCR ligation, Lyn is rapidly activated and phosphorylates substrates involved in both the positive and negative regulation of BCR signaling (5, 32, 33). We compared the strength of
FIGURE 2. B lymphopenia in LynMld4/Mld4 mice is similar to that of Lyn−/− mice. Splenocytes from 8-wk-old Lyn+/+ (+/+), LynMld4/Mld4 (m/m), and Lyn−/− (−/−) mice were stained with the indicated Abs and analyzed by flow cytometry. A, Dot plots gated on CD23+ and CD23− populations show the proportions of marginal zone (MZ) and transitional stage 1 (T1) (middle), and transitional stage 2 (T2) and follicular (Fo) (lower) B cells. Total splenocytes (B) and numbers of follicular and marginal zone/B-1 B cells (C) per spleen are graphed at the right. Splenocytes were stained with Abs specific for CD21 (D), surface IgM (sIgM; E), CD19 (F), CD23 (G), or MHC class II (H) and analyzed by flow cytometry. The median fluorescence intensity (MFI) ± SEM of data gated on CD23+ (follicular, T2) B cells is shown. I, Levels of IgE and IgM in the serum of resting 27- to 30-wk-old Lyn+/+ and LynMld4/Mld4 mice (n = 6–12). Horizontal bar indicates mean level. J, Bone marrow from Lyn+/+, LynMld4/Mld4, and Lyn−/− mice was stained with the indicated Abs and...
BCR signaling in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} B cells to determine whether expression of a kinase-dead Lyn allele could lead to partial regulation of these pathways. Although BCR-induced tyrosine phosphorylation of cellular proteins was evident for all three genotypes, phosphorylation of several proteins was absent or substantially reduced in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} lysates relative to Lyn\textsuperscript{+/+} (Fig. 5A). The phosphorylation of these proteins was equally reduced in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} lysates. A 130-kDa protein, inducibly phosphorylated in Lyn\textsuperscript{+/+} but to a lesser extent in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} cells, was subsequently confirmed to be CD22, an inhibitory coreceptor that is directly phosphorylated by Lyn (Fig. 5A and data not shown) (28, 32, 34). Other proteins of low molecular mass were more highly phosphorylated in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} lysates than in wt, suggesting increased activity of another tyrosine kinase or decreased activity of a tyrosine phosphatase in cells lacking catalytically active Lyn (Fig. 5A).

Further detailed biochemical analyses of cellular lysates were undertaken with a panel of Abs that detect phosphorylated proteins involved in B cell signaling. BCR ligation results in Lyn-induced phosphorylation of ITIMs on the inhibitory coreceptors FcyRIIB and CD22, enabling recruitment, phosphorylation, and activation of the inositol phosphatase SHIP-1 and tyrosine phosphatase SHP-1 (25, 28, 32–34). BCR ligation failed to induce phosphorylation of SHIP-1 in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} lysates, whereas ligand-induced phosphorylation of SHP-1 was reduced (Fig. 5B)

The tyrosine kinase Syk also becomes phosphorylated on several sites following BCR ligation, resulting in Ca\textsuperscript{2+} mobilization. One of these sites, Tyr\textsuperscript{323}, has been shown to be a negative regulatory site that is phosphorylated by Lyn, resulting in suppression of the Ca\textsuperscript{2+} signal (35). Consistent with this, phosphorylation of Tyr\textsuperscript{323} on Syk was substantially reduced in the absence of active Lyn (Fig. 5B). Thus, negative regulatory actions attributable to Lyn appeared to be perturbed to a similar extent in Lyn\textsuperscript{Mld4/Mld4} and Lyn\textsuperscript{−/−} cells.

Lyn also has a key role in the initiation of pathways that promote B cell activation, although this role is likely to be shared with other SFKs (5). The requirement for Lyn in CD19 phosphorylation is controversial, with reports in the literature either supporting or refuting an essential role for Lyn in this process (36, 37). In our experiments, CD19 phosphorylation did not appear to depend on Lyn activity, as CD19 phosphorylation was induced with similar...
kinetics and intensity in wt, Lyn<sup>Mld4/Mld4</sup>, and Lyn<sup>-/-</sup> splenic B cells (Fig. 5C). Conversely, BCR ligand-induced activation of ERK1/2, JNK, and Akt was grossly elevated in both Lyn<sup>Mld4/Mld4</sup> and Lyn<sup>-/-</sup>/H11002 lysates relative to wt (Fig. 5C). Further, calcium flux was amplified to a similar extent in Lyn<sup>Mld4/Mld4</sup> and Lyn<sup>-/-</sup>/H11002 cells (Fig. 5D).

Development of autoimmune disease is attenuated in Lyn<sup>Mld4/Mld4</sup> mice

Previous studies have shown that Lyn<sup>-/-</sup> mice develop Ab-mediated autoimmune disease as they age, with autoantibodies detectable in virtually all Lyn<sup>-/-</sup> mice by 4 mo of age (5, 7, 8). We
LynMld4/Mld4 mice assayed (LynMld4/Mld4, n = 7; Lyn−/−, n = 6), along with the deposition of complement C3 (86% of LynMld4/Mld4 (n = 7) and 83% Lyn−/− (n = 6) kidneys positive for C3; Fig. 6B, lower panels and data not shown). Neither IgG nor C3 aggregates were seen in kidney sections from wt mice of similar ages (n = 4; Fig. 6B).

These results suggest that despite being undetectable in many mice by ELISA, autoantibodies are present in young LynMld4/Mld4 and Lyn−/− mice at sufficient concentrations to aggregate in glomeruli. Alternatively, autoantibodies specific for Ags other than dsDNA or nuclear Ags may be present that would not be detected by this assay.

More than 90% of Lyn−/− mice aged 6 wk or older show histological signs of autoimmune disease (7). Despite the appearance of autoantibodies and IC deposition in the glomeruli, there was no evidence of either hematopoietic cellular infiltration in the kidney, pancreas, or thyroid of LynMld4/Mld4 mice or major glomerular damage by histological staining (n = 6 mice, 6–12 mo of age; Fig. 6C and data not shown). Histological changes in LynMld4/Mld4 kidneys were minimal and were limited to mild hypercellularity apparent in one of the mice analyzed (12 mo of age; data not shown). This is in contrast to the severe glomerulonephritic changes characteristic of Lyn−/− mice, including global sclerosis, crescent formation and lobularity (7, 8). At 1 yr of age, 90% of LynMld4/Mld4 mice were healthy (n = 10; data not shown), whereas 40% of Lyn−/− mice had died by this age (25). These results suggest that the presence of kinase-dead Lyn affords protection against the fatal glomerulonephritis characteristic of Lyn-deficient mice.

Discussion
We describe here the characterization of a mutant mouse strain that expresses a kinase-dead allele of Lyn kinase. Despite displaying a similar spectrum of defects to Lyn−/− mice, the overall phenotypic severity of LynMld4/Mld4 mice is substantially reduced. In particular, the extent of splenomegaly, myeloid expansion, and autoantibody production in LynMld4/Mld4 mice is reduced compared with Lyn−/− mice, and they do not develop the fulminant glomerulonephritis characteristic of Lyn−/− mice.

The Mld4 mutation is predicted to cause the substitution of a lysine for a threonine codon in the Src activation loop. This nonconservative substitution, resulting in a change of charge, is predicted to affect Lyn structure in this region, but it is unclear how catalytic activity is affected by this change. The tyrosine residue in the activation loop critical for catalytic activity, Tyr416, is still accessible to kinases, but phosphorylation of this residue is clearly insufficient for kinase activity in the presence of the Mld4 mutation. Although there is indisputable evidence that Tyr416 is an autophosphorylation site in all SFK members (3), our data indicate that other tyrosine kinases, most likely other SFKs, can also phosphorylate this site. Phosphorylation of Tyr416, located between the two lobes of the kinase domain, is thought to trigger a conformational reorganization of the activation loop, allowing displacement of the C-terminal helix into the active site (38). It is conceivable that this displacement may be affected by the Mld4 mutation.

Given that there are multiple mutations present in the genome of LynMld4/Mld4 mice, there is a small possibility that linked mutations segregating in these mice may affect the autoimmune phenotype. We consider this possibility to be unlikely, however, since linked mutations would be segregating randomly and would not be present in all mice with the LynMld4/Mld4 genotype, leading to an inconsistency in the phenotype of the LynMld4/Mld4 mice that is contrary to our observation. The most likely explanation is that the mutant Lyn protein retains some function in regulating signaling, at least in some lineages. Although catalytically inactive, intact interaction motifs such as the SH2 and SH3 domains, as well as quantitated serum autoantibodies (IgG isotype) recognizing dsDNA from mice at various ages by ELISA. In young mice (7–9 wk), autoantibodies were detectable in only 10% of LynMld4/Mld4 sera (mean anti-dsDNA Abs = 29.0 μg/ml, n = 10; Fig. 6A). Serum levels of these Abs increased moderately in older mice (mean, 111 μg/ml at 42–54 wk, n = 9; Fig. 6A). Slightly higher titers of anti-dsDNA Abs were measured in the sera of young Lyn−/− mice (mean, 352 μg/ml at 7–9 wk, n = 18; Fig. 6A), but dramatic increases in autoantibody concentration were evident as Lyn−/− mice aged (mean, 5.6 mg/ml at 27–30 wk, n = 2; Fig. 6A). The titer of anti-dsDNA Abs in the sera of compound heterozygotes (Lyn−/Mld4, −/m) was intermediate between that of LynMld4/Mld4 and Lyn−/− mice. Similar results were obtained by measuring Abs to nuclear Ags (IgA, IgG, and IgM isotypes; data not shown).

Despite low or undetectable serum autoantibody levels in younger mice, glomerular immune complex (IC) deposition was evident in kidney sections from both LynMld4/Mld4 and Lyn−/− mice (Fig. 6B, upper panels). These ICs, which contained both IgG and IgM aggregates, were evident in the kidneys of all 8-wk-old
multiple potential tyrosine phosphorylation sites, may influence downstream signaling events by competing for interaction sites that might otherwise be occupied by other signaling molecules. For instance, it is conceivable that mutant Lyn could limit positive signaling events in both B and myeloid cells, emanating from the surface receptors BCR/CD19 and FcγRI/II respectively, by blocking the recruitment and activation of other Src kinases. Although we did not observe signaling differences between LynMld4/Mld4 and Lyn+/− B cells, subtle differences may occur in vivo when cells are exposed to physiological levels of stimuli or in other signaling pathways not examined in this study. Indeed, significant differences in autoantibody titers and B cell surface receptor expression suggest that LynMld4/Mld4 and Lyn−/− B cells are not functionally equivalent. The most dramatic differences we observed, however, occurred in the myeloid compartment where myeloid expansion is observed in Lyn−/− but not LynMld4/Mld4 mice, strongly suggesting a role for proinflammatory cell types in the development of autoimmunity.

Several mouse models have been generated which rescue the autoimmune phenotype of Lyn−/− mice while retaining other aspects, including B lymphopения. Mice deficient in both Lyn and Btk, as well as Lyn+/−Cd19+/− mice, do not develop autoimmunity, most likely due to reduced BCR signaling in the absence of Btk or CD19 (29, 30, 39). Introduction of a transgene expressing a low level of Btk into Lyn−/−Btk−/− mice restored B cell numbers and BCR signaling hypersensitivity but failed to restore autoimmunity, suggesting that BCR hyperresponsiveness is insufficient for the development of autoimmune disease, consistent with our data (31).

Autoimmune disease also failed to develop in mice lacking MyD88 and Lyn, indicating that TLR signaling contributes to the autoimmune phenotype and that inappropriate activation of other cell lineages collaborates in autoimmune disease development (40). We have shown that the sensitivity of LynMld4/Mld4 cells to colony-stimulating factors is intermediate between that of Lyn+/+ and Lyn−/− cells, suggesting that differences in myeloid behavior may contribute to the differences in autoimmune and inflammatory disease between the mouse models. This tempered signaling most likely also limits the development of splenomegaly in LynMld4/Mld4 mice. Colony-stimulating factors not only induce myeloid differentiation, but also increase myeloid activity, including inflammatory cytokine secretion and Ag-presenting activity, both of which may promote the progression of autoimmune disease. Indeed, renal pathology was suppressed in autoimmune-prone MRL-Fas+/− mice when generated on a M-CSF-deficient background (41).

Deposition of glomerular ICs results in complement activation and recruitment of phagocytic cells, initiating inflammation and renal injury. Phagocytosis of ICs is largely mediated by FcγR. Innate immune effector cells such as macrophages and neutrophils express combinations of FcγR, comprising both activating (FcγRI, FcγRIII) and inhibitory (FcγRIBB) receptors, thereby ensuring adequate regulation of the effector responses (42). Interestingly, disruption of the gene encoding the γ-chain of FcγR uncouples IC formation and complement activation from renal injury in lupus-prone mice, an analogous phenotype to LynMld4/Mld4 mice (43). Fcγ−/− mice do not express activating receptors but maintain expression of the inhibitory receptor FcγRIBB, which is activated by Lyn. Furthermore, expression of intact FcγR in myeloid cells was shown to be sufficient for the development of fulminant glomerulonephritis in lupus-prone mice (44). Given that Lyn regulates FcγRIBB activation, it is likely that the net effect of FcγR signaling in Lyn−/− myeloid cells is inflammatory, which may contribute to renal injury. In contrast, minimal renal injury, observed in LynMld4/Mld4 mice, may reflect the capacity of cat-

ally inactive Lyn to limit the activation of the proinflammatory response to some degree.

Collectively, our data indicate that autoimmune and inflammatory disease associated with Lyn deficiency is attenuated in mice expressing a kinase-dead allele of Lyn. Future studies will address whether signaling cascades differ in intensity in LynMld4/Mld4 phagocytes and will characterize the effect of the Mld4 mutation on Lyn function in dendritic cells and other myeloid cells.

Acknowledgments
We thank J. Corbin, L. DiRago, and S. Mifsud for first-class technical assistance; Drs. S. Nutt and P. Lock for the generous provision of flow cytometry and Western blot Abs; Dr. D. Tarlinton for the provision of mice and advice; Drs. K. Graham, M. Fuchsberger, and K. Greig for assistance with immunohistochemistry and calcium flux experiments; and M. Rowe, R. Branch, C. Van Puyenbroek, K. Hibbins, S. Ross, and S. Guzzardi for expert animal husbandry.

Disclosures
We disclose that this work was supported in part by Murigen Pty Ltd for whom W.S.A., B.T.K., and D.J.H. consult and/or have a financial interest. The remaining authors have no financial conflict of interest.

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