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Genetic Interdependence of Lyn and Negative Regulators of B Cell Receptor Signaling in Autoimmune Disease Development

Evelyn Tsantikos,*†‡ Mhairi J. Maxwell,*† Nicole Kountouri,† Kenneth W. Harder,†,1 David M. Tarlinton,§ and Margaret L. Hibbs*†

Ab-mediated autoimmune disease is multifaceted and may involve many susceptibility loci. The majority of autoimmune patients are thought to have polymorphisms in a number of genes that interact in different combinations to contribute to disease pathogenesis. Studies in mice and humans have implicated the Lyn protein tyrosine kinase as a regulator of Ab-mediated autoimmune disease. To examine whether haploinsufficiency of Lyn gives rise to cellular and clinical manifestations of autoimmune disease, we evaluated the phenotype of Lyn+− mice. We find that their B cell compartment is significantly perturbed, with reduced numbers of marginal zone and transitional stage 2 B cells, expansion of plasma cells, downregulation of surface IgM, and upregulation of costimulatory molecules. Biochemical studies show that Lyn+− B cells have defects in negative regulation of signaling, whereas Lyn+− mice develop IgG autoantibodies and glomerulonephritis with age. Because Lyn has a pivotal role in the activation of inhibitory phosphatases, we generated mice harboring double heterozygous loss-of-function mutations in Lyn and SHP-1 or Lyn and SHIP-1. Partial inactivation of SHP-1 or SHIP-1 amplifies the consequence of Lyn haploinsufficiency, leading to an accelerated development of autoantibodies and disease. Our data also reveal that the BALB/c background is protective against autoimmune-mediated glomerulonephritis, even in the face of high titer autoantibodies, whereas the C57BL/6 background is susceptible. This study demonstrates that Lyn is a haploinsufficient gene in autoimmune disease and importantly shows that quantitative genetic variation in Lyn-regulated pathways can mirror the complete loss of a single critical inhibitory molecule. The Journal of Immunology, 2012, 189: 1726–1736.

H uman diseases are rarely caused by a single genetic mutation. For instance, systemic lupus erythematosus (SLE) is a complex polygenic autoimmune disease that may involve up to 100 susceptibility genes, and it is likely that polymorphisms in different genes with distinct combinations contribute to SLE pathogenesis (1). In mice, several homozygous genetic mutations mimic, to varying degrees, the pathogenesis of SLE, suggesting that pathways regulated by the missing or overactive gene products in mice are similarly affected in humans. For example, augmented CD19 signaling, which triggers autoimmunity in mice (2), are seen in patients with Sjögren’s syndrome (5). Furthermore, it is known that mutations in the complement pathway in mice and humans are risk factors for SLE (reviewed in Ref. 6). However, for the most part, human patients are less likely to suffer from the overexpression or complete absence of just one regulatory factor but, rather, are anticipated to show quantitative genetic variation in several factors along a biochemical pathway.

Lyn tyrosine kinase is an important regulator of Ab-mediated autoimmune disease in mice and humans (reviewed in Ref. 7). Both Lyn-deficient and Lyn gain-of-function mutant mice develop circulating autoreactive Abs and severe glomerulonephritis caused by the deposition of immune complexes in the kidney, a pathology reminiscent of SLE (8–10). Early studies of human SLE patients showed some to have abnormal levels of Lyn (11, 12), whereas more recent data suggested that Lyn expression and localization to lipid raft-signaling domains were altered in a majority of patients with SLE (13). Moreover, genome-wide association scans and population-based studies identified Lyn as an SLE susceptibility locus, with single nucleotide polymorphisms being found in the 5′ untranslated region of Lyn (14, 15). Collectively, these studies imply that alteration of Lyn activity or expression may be associated with human disease.

Although Lyn has long been known to be an activatory component of the BCR-signaling complex, it is now clear that Lyn is also an essential inhibitory-signaling molecule downstream of the BCR (7). Lyn activity is required for the tyrosine phosphorylation of inhibitory cell surface receptors, such as FcγRIIB1, CD22, and PIR-B, generating binding sites for negative regulatory phosphatases, such as the 5′ inositol phosphatase SHIP-1 and the protein

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Abbreviations used in this article: ANA, anti-nuclear Ab; MZB, marginal zone B cell; SLE, systemic lupus erythematosus; T2, transitional stage 2.

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tyrosine phosphatase SHP-1 (16-21). Consistent with the importance of inhibitory signaling pathways for limiting cellular activation, loss-of-function mutations in these regulatory molecules invoke immune system defects. Loss of SHIP-1 leads to deregulated signaling in the PI3K pathway, which, in turn, results in myeloproliferative disease in mice (22, 23), perturbations in B cell development and function (24-26), and an SLE-like autoimmune disease that is B cell dependent (27). The failure to appropriately recruit and/or activate SHP-1 leads to catastrophic deregulation of the immune system, as evidenced by the severe phenotypes of mice carrying loss-of-function mutations in SHP-1 (Me, Me', spin), which include systemic autoimmunity (28-30). Loss of CD22, a site of membrane recruitment for SHP-1, leads to a mild phenotype, dependent on genetic background synergizing with the effects of CD22 deficiency to promote autoantibody development (31-34). Although there is some ambiguity about the importance of CD22 in autoimmune disease, it is clear that loss of CD22 function is associated with a hyperresponsive B cell phenotype. In studies exploring the CD22, Lyn, SHP-1–signaling pathway, haploinsufficiency of Lyn, CD22, and SHP-1 in triple-heterozygous CD22+/Lyn+/-Mev+/- mice led to reduced CD21 and IgM expression and increased MHC class II expression on mature B cells compared with single- and double-heterozygous mice, whereas the mice showed increased serum IgM and increased negative selection in the presence of a neo self-Ag (17). Whether these B cell changes are the extent of the consequences of this multilocus heterozygosity or, more importantly, whether they result in autoimmunity, has not been determined. In more recent studies, we found that haploinsufficiency of Lyn in mice leads to the development of autoantibodies, albeit with delayed onset compared with Lyn-/- mice; however, we did not assess disease development (35).

Both mouse and human studies suggest that Lyn-dependent pathways contribute to the development of a subset of autoimmune conditions that, in humans, includes SLE. However, patients are unlikely to possess single genetic changes that confer constitutive Lyn activity or inactivity; rather, it seems more probable that they have perturbations in steps along Lyn-regulated pathways that collectively have an effect similar to that observed in mice. To mimic this type of situation, we investigated the consequence of haploinsufficiency of Lyn, either alone or in combination with SHIP-1 or SHP-1. We establish that Lyn is a haploinsufficient gene in autoimmune disease and demonstrate that simultaneous haploinsufficiency of SHP-1 or SHIP-1 greatly accelerates autoimmune disease development. These types of genetic interactions more appropriately reflect the human condition, where susceptibility to, and manifestation of, autoimmune disease is more likely to be dependent on the accumulation of effects on multiple genes rather than the single complete loss of a particular gene.

Materials and Methods

Animals

Lyn-/- mice have been described (8), and mice that had been backcrossed for 20 generations to the C57BL/6 background were used. Motheaten viable (Me') mice (29, 36) were on a C57BL/6 background and were maintained by interbreeding heterozygotes and genotyping. Haploinsufficient Lyn+/mice were generated by crossing Lyn+/mice with C57BL/6 mice; double-heterozygote Lyn+/Mev+/- mice were generated by interbreeding Lyn+/- mice and Mev+/- mice and genotyping. To generate compound mutants involving Lyn and SHIP-1, both C57BL/6 and BALB/c genetic background mice were used, which were described previously (27, 35, 37, 38). Lyn-/- SHIP-1-/- mice were generated by interbreeding male SHIP-1-/- mice with female Lyn-/- mice. All experiments were performed in accordance with National Health and Medical Research Council of Australia guidelines for the care and use of animals for scientific purposes and were approved by the Animal Ethics Committees of the Ludwig Institute for Cancer Research/Department of Surgery and the Baker Heart and International Diabetes Institute, Melbourne.

Cell preparation and flow cytometry

Single-cell suspensions of spleen were prepared by extruding the cells from the capsule and then passing through a 40-μm filter. Multicolor flow cytometry was used to examine lymphoid tissue composition, as described (35).

Proliferation assay

Splenic cells were isolated from 10-wk-old mice, and red cells were removed by lysis with Tris-buffered ammonium chloride. Single-cell suspensions were incubated with biotinylated Abs to CD5, CD43, Mac-1, CD11c, Ter-119, and Gr-1 (BD Biosciences Pharmingen, San Diego, CA), and non-B cells were depleted using streptavidin microbeads and the MiniMACS columns (Milteny) Biotec, North Ryde, NSW, Australia). Purified cells were resuspended in DMEM, 5% FCS, 100 U/ml penicillin-streptomycin, and 4 mM glutamine and then cultured for 3 d with no stimulus, 10 μg/ml Flb(2) goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), 10 μg/ml intact goat anti-mouse IgM (Jackson ImmunoResearch Laboratories), or 5 μg/ml LPS (Sigma). On day 3, cells were pulsed with [3H]thymidine for 6 h prior to harvesting and counting with a β-scintillation counter (Perkin Elmer, Wellesley, MA).

SDS-PAGE and Western blot

For signaling studies, B cells were purified from spleen, as outlined for the proliferation assay. B cells were stimulated with 20 μg/ml intact goat anti-mouse IgM for 0, 5, or 30 min and then lysed at a concentration of 106 cells/ml in 1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN). Following protein determination by BCA (Pierce Chemical Company, Rockford, IL), total-cell lysates were separated by SDS-PAGE, and proteins were transferred to nitrocellulose. Blots were probed with anti-p-ERK and anti-Erk (Cell Signaling Technology, Danvers, MA) as a loading control and were detected using the Odyssey detection system (LI-COR Biosciences, Lincoln, NE).

Detection of autoantibodies by ELISA

Mice were bled from the tail vein every 2-4 wk from the age of 10 wk, and anti-nuclear Abs (ANAs) were measured, as described (35). IgG ANA values are defined as the optical density reading measured at 450 nm with correction at 595 nm.

Myeloid colony assays

Myeloid progenitor cells responsive to defined growth factors were enumerated by semisolid agar cultures, as described (39).

Histopathology

Tissues for light microscopy were fixed in Bouin’s solution for 24 h and then embedded in paraffin. For histopathology studies, 3-μm sections were cut and stained with H&E, according to standard procedures. Pathological assessment was performed by determination of glomerular area as follows. Serial photographs were taken of H&E-stained kidney sections. ImageJ software was used to determine the cross-sectional area of individual glomeruli from the images obtained using a known reference scale. Between 20 and 30 glomeruli were analyzed/mouse, and the mean glomerular area was determined, as described (40). For each genotype, the mean glomerular area of individual mice was plotted to determine the mean value for each genotype.

Statistical analysis

Statistical analysis was performed on all numerical data using GraphPad Prism software (GraphPad, San Diego, CA).

Results

Perturbation of the B cell compartment in Lyn+/mice

Previous studies by us and other investigators found severe perturbations in the B cell compartment of Lyn-/- mice, which precede autoimmune disease development (8, 9, 41). To assess whether the loss of one allele of Lyn affected this lineage, we...
examined the B cell compartment of Lyn−/− mice by flow cytometry, assessing the expression of cell surface markers indicative of developmental and activation status (Fig. 1). Unlike in Lyn−/− mice (38), the total number of splenic B cells (Fig. 1A) did not differ between control and Lyn−/− mice. However, similar to Lyn−/− mice, plasma cell numbers were significantly elevated in the spleens of Lyn−/− mice (0.82 ± 0.14 × 10^6 in C57BL/6 versus 2.53 ± 0.45 × 10^6 plasma cells in Lyn−/− spleen; Fig. 1A). Lyn−/− mice also show a total loss of the marginal zone B cell (MZB) compartment and a block in development from transitional stage 1 to transitional stage 2 (T2) B cells (38, 42). We determined the effect of Lyn heterozygosity on these populations and found that both the MZB and T2 cell compartments were reduced to approximately half of the number of cells present in control spleens (Fig. 1B). Thus, loss of one allele of Lyn is sufficient to affect the plasma cell, MZB, and T2 populations.

We next characterized the activation status of B cells from Lyn−/− mice and found the amount of IgM on the cell surface to be significantly downregulated compared with controls. Curiously, the fold reduction in IgM on Lyn−/− B cells was greater than observed for Lyn−/− B cells (Fig. 1A, 1C). This could be a reflection of the unusual B cell populations present in Lyn−/− mice (38). Although the amounts of MHC class II and CD86 on Lyn−/− B cells were significantly elevated over those on control B cells, their expression was not as high as on Lyn−/− B cells (Fig. 1C).

To determine whether the activated phenotype of Lyn−/− B cells was associated with enhanced B cell responses, we first measured proliferation of Lyn−/− B cells after exposure to a variety of agonists, including BCR-specific and -independent stimuli (Fig. 1D). Although Lyn−/− B cells responded like control B cells to LPS, they were mildly hyperresponsive to BCR cross-linking. Interestingly, they displayed marked hyperresponsiveness to stimulation with intact IgM Ab, which normally provides an inhibitory signal to B cells through coligation of the BCR and inhibitory FcγR. This suggests that this Lyn-dependent inhibitory pathway in B cells is faulty when Lyn expression is reduced. To examine whether this response had a biochemical corollary, B cells were stimulated with intact IgM Ab, and phosphorylation of Erk was assessed by probing with anti–p-Erk Ab, followed by anti-Erk as a loading control (Fig. 1E). In control and Lyn−/− B cells, phosphorylation of Erk was maximal at 5 min poststimulation; however, the signal was more intense in Lyn−/− B cells, as determined by densitometry (Fig. 1E). At 30 min poststimulation, when the intensity of p-Erk in control B cells had largely diminished to baseline levels, the signal was sustained in Lyn−/− B cells.

T cell activation occurs in the absence of early myeloid cell defects in Lyn−/− mice

We previously showed that although Lyn is not expressed in T cells, T cell perturbations occur in aged Lyn−/− mice (35, 38). We concluded that this was due to the effects of the inflammatory environment and other cellular compartments in Lyn−/− mice on T cells. Therefore, we were interested to determine whether ab-
errant T cell activation occurred in Lyn<sup>+/−</sup> mice, in which changes in the B cell compartment are not as extreme as in Lyn<sup>−/−</sup> mice. 

The splenic CD4 T cell compartment of young mice was assessed for the activation markers CD62L and CD69, which are shed and upregulated, respectively, upon cellular activation (Fig. 2A). We found that CD62L levels on the cell surface were unchanged, whereas CD69 was slightly upregulated, indicating a minor increase in activation. However, assessment of aged Lyn<sup>+/−</sup> mice revealed the T cell compartment to be significantly activated compared with younger Lyn<sup>−/−</sup> mice and aged control mice (Fig. 2B). These proportional changes were mirrored by increases in the absolute number of activated CD4 T cells (Fig. 2B).

We found that T cell activation in aged Lyn<sup>−/−</sup> mice correlates with expansion of the myeloid compartment (35, 38). Therefore, we assessed the myeloid compartment in Lyn<sup>+/−</sup> mice to determine whether there were changes present that may be occurring prior to marked T cell activation. We did not observe any significant expansion of macrophages and granulocytes in young Lyn<sup>−/−</sup> mice (Fig. 2C); however, in aged mice, myeloid expansion was evident, and differences from control mice were statistically significant (Fig. 2D). Thus, although myeloid expansion is a major and early feature of Lyn<sup>−/−</sup> mice, it is not a hallmark of young Lyn<sup>+/−</sup> mice.

**ANA development and kidney disease occur in Lyn<sup>+/−</sup> mice, albeit with delayed onset and severity**

Although several changes in Lyn<sup>−/−</sup> mice resemble homozygous deficiency of Lyn, the apparently healthy appearance of Lyn<sup>+/−</sup> mice and their longer lifespan (data not shown) brought into question whether they were susceptible to the development of autoimmune disease or whether they were protected as a result of a reduced inflammatory environment, as exemplified by Lyn<sup>+/−</sup> IL-6<sup>−/−</sup> mice (38, 43). In previous studies, we observed that BALB/c background Lyn<sup>−/−</sup> mice develop ANAs with age, but we did not assess disease development (35). To determine whether C57BL/6 background Lyn<sup>+/−</sup> mice were prone to autoimmune disease, we examined ANA levels in serum at regular time intervals (Fig. 3A). We found that Lyn<sup>+/−</sup> mice accumulated ANAs in serum, albeit at a markedly slower rate compared with Lyn<sup>−/−</sup> mice (cf. Lyn<sup>−/−</sup> maximal titers at 30 wk (38)). We also assessed the extent to which aged Lyn<sup>−/−</sup> mice develop glomerulonephritis and found that, at 1 y of age, glomeruli in the kidneys of Lyn<sup>−/−</sup> mice showed mild signs of disease (Fig. 3B), although the disease was not as severe as that in matched Lyn<sup>−/−</sup> mice (Fig. 3B). Quantification of glomerular size as a disease indicator (40) revealed that Lyn<sup>+/−</sup> glomeruli were not significantly enlarged compared with control mice at 40–50 wk; an age at which Lyn<sup>−/−</sup> mice have extensive glomerulonephritis. However, significant pathology, as indicated by glomerular enlargement, was evident in Lyn<sup>+/−</sup> mice at >60 wk of age (Fig. 3C).

**Genetic association of Lyn and SHP-1 in autoimmunity**

Lyn was also shown to interact with SHP-1 in inhibitory signaling in B cells (16, 17), so we next examined whether there was a genetic interaction between Lyn and SHP-1 in autoimmunity. Mice carrying heterozygote loss-of-function mutations in Lyn and SHP-1 were generated previously, with B cells from Lyn<sup>+/−</sup>Mev<sup>−/−</sup> mice showing downregulated expression of IgM; however, the effect of this B cell phenotype on autoimmune disease susceptibility or the effect of these mutations on other cellular compartments was not assessed (17). Thus, we produced double-heterozygote Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice and sampled their serum regularly to test for the presence of IgG ANAs, comparing their levels with those of control and single-heterozygote mice (Fig. 4A). Although previous studies showed that heterozygote Mev<sup>−/−</sup> mice are healthy and show only very subtle immune system defects (44, 45), we found that Mev<sup>−/−</sup> mice developed ANAs in their serum in a manner similar to Lyn<sup>−/−</sup> mice (Figs. 3A, 4A). Strikingly, when Lyn<sup>−/−</sup> and Mev<sup>−/−</sup> mutations were compounded, ANA titers were drastically increased (Fig. 4A). In some mice, the titers were near the maximal detectable limit at only 20 wk of age, a feature characteristic of Lyn<sup>−/−</sup> mice, which develop severe disease (38).

**FIGURE 2.** Aged Lyn<sup>+/−</sup> mice show expanded representation of activated T cells and myeloid cells. Representative flow cytometry of spleen cells from young (10-wk-old) (A, C) and aged (30-wk-old) (B, D) control or Lyn<sup>+/−</sup> mice stained with the indicated Abs. (A, B) CD4<sup>+</sup> spleen cells were gated and assessed for expression of activation markers CD62L (upper panels) and CD69 (lower panels). (C, D) Staining of spleen cells with Abs to myeloid cell markers. For data in (B) and (D), absolute cell numbers of the indicated populations were calculated from individual mice based on total cell counts and flow cytometry; horizontal bars denote the mean value of the data points in the plot. Data are representative of three independent experiments and a minimum of n = 8 mice. *p < 0.05, **p < 0.01, ***p < 0.001.
Because haploinsufficiency of Lyn and SHP-1 strongly influenced exaggerated B cell perturbations. Mice with heterozygous deficiencies in Lyn and SHP-1 show some minor changes but were not significantly enlarged. To determine whether the exaggerated B cell population changes occurring in compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> heterozygote mice were accompanied by increased B cell activation, additional cell surface markers were assessed by flow cytometry. These studies showed that both Lyn<sup>−/−</sup> and Mev<sup>−/−</sup> B cells had reduced surface IgM expression, as observed previously (17). Interestingly, the amount of IgM on Mev<sup>−/−</sup> B cells was significantly lower than on Lyn<sup>−/−</sup> B cells and almost identical to that on B cells from compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice (Fig. 5A). To further assess the level of B cell activation, we measured the expression of MHC class II and CD86, which were already increased on Lyn<sup>−/−</sup> B cells (Fig. 1C). Levels were further increased on the surface of compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> heterozygote mice B cells, indicating significant activation (Fig. 5C); however, there was no significant difference from control B cells in the expression of MHC class II and CD86 on Mev<sup>−/−</sup> B cells.

To determine whether the changes in the B cell compartment of Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice had a biochemical basis, we stimulated purified B cells from control, Lyn<sup>−/−</sup>, and Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice with intact IgM Ab, and phosphorylation of Erk was assessed (Fig. 5D). Enhanced and more sustained p-Erk was observed in compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> B cells compared with Lyn<sup>−/−</sup> B cells, which had enhanced and more sustained p-Erk compared with control and Mev<sup>−/−</sup> B cells. These data suggest that compound mutant B cells showed enhanced signaling (Fig. 5D).

The T cell compartment is highly activated in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice

We next examined the CD4 T cell compartment of young Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice, finding minimal differences (Fig. 6A). However, as previously observed in Lyn<sup>−/−</sup> mice (35, 38) and Lyn<sup>−/−</sup> Mev<sup>−/−</sup> mice (Fig. 2B), T cell activation became apparent in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice: interestingly, these T cells showed almost complete loss of CD62L expression, indicating substantial activation (Fig. 6B). The T cells of aged Mev<sup>−/−</sup> mice showed some evidence of activation compared with those from control mice; however, this was minor compared with the T cell activation observed in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice or Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice (Fig. 6B).

Myeloid expansion occurs in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice

The compound heterozygous loss-of-function mutations in Lyn and SHP-1 had far greater effects on B and T cell activation than did either mutation alone. We previously showed that, in C57BL/6 Lyn<sup>−/−</sup> mice, expansion of the myeloid compartment precedes perturbations in the T cell compartment (38). However, in Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice, expansion of the myeloid compartment was marked (Fig. 4B, 4C). Glomeruli from matched Lyn<sup>−/−</sup> and Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice at the indicated ages by ELISA. Values refer to the absorbance reading obtained at 450 nm with correction at 595 nm; horizontal bars denote the mean value of the data points at each time point. For each genotype, one point represents the mean glomerular area of a single mouse taken from an average of 20–30 glomeruli/mouse. Horizontal bars represent the mean glomerular area for that genotype where n = number of points on the graph (n ≥ 4). **p < 0.01, ***p < 0.001.

To assess pathological signs of disease, we studied kidney sections of aged control, Lyn<sup>−/−</sup>, Mev<sup>−/−</sup>, and Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice and determined glomerular cross-sectional area (Fig. 4B, 4C). Glomeruli from 40–50-wk-old Lyn<sup>−/−</sup> mice and Mev<sup>−/−</sup> mice showed some minor changes but were not significantly enlarged. In contrast, glomeruli from matched Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice were globular and significantly enlarged (Fig. 4B, 4C), indicating that when heterozygous mutations in Lyn and SHP-1 were compounded, the effect on kidney pathology was pronounced. In 60–90-wk-old mice, the glomeruli of both Lyn<sup>−/−</sup> and Mev<sup>−/−</sup> mice were significantly enlarged compared with controls, whereas the glomeruli of Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice showed further enlargement and remained significantly larger than did those of Lyn<sup>−/−</sup> mice (Fig. 4C).

Mice with heterozygous deficiencies in Lyn and SHP-1 show exaggerated B cell perturbations

Because haploinsufficiency of Lyn and SHP-1 strongly influenced predisposition to autoimmune disease, we examined various immune cell compartments in Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice to provide a possible cellular basis for this mechanism. We first assessed B cell subpopulations in the spleen by flow cytometry (Fig. 5); although there was no difference in spleen weight among any groups (data not shown), the numbers of splenic B cells in Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice were significantly reduced, although B cell numbers in single heterozygote animals were unchanged (Fig. 5A). Interestingly, the expansion of plasma cells observed in Lyn<sup>−/−</sup> mice was not exaggerated in compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> heterozygote mice, despite mice with a heterozygous loss-of-function mutation in SHP-1 (Mev<sup>−/−</sup>) showing a minor, yet significant, expansion of the plasma cell compartment (Fig. 5A). The MZB and T2 populations were significantly diminished in Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice compared with control mice, approaching the low numbers observed in Lyn<sup>−/−</sup> mice (compare Fig. 1B versus Fig. 5B). Intriguingly, Mev<sup>−/−</sup> mice showed considerable variation in their MZB and T2 B cell compartments, with some mice showing normal numbers and others showing severe diminution, making the differences from control mice not significant (an extreme example is shown in Fig. 5B).

To determine whether the exaggerated B cell population changes occurring in compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> heterozygote mice were accompanied by increased B cell activation, additional cell surface markers were assessed by flow cytometry. These studies showed that both Lyn<sup>−/−</sup> and Mev<sup>−/−</sup> B cells had reduced surface IgM expression, as observed previously (17). Interestingly, the amount of IgM on Mev<sup>−/−</sup> B cells was significantly lower than on Lyn<sup>−/−</sup> B cells and almost identical to that on B cells from compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice (Fig. 5A, 5C). To further assess the level of B cell activation, we measured the expression of MHC class II and CD86, which were already increased on Lyn<sup>−/−</sup> B cells (Fig. 1C). Levels were further increased on the surface of compound Lyn<sup>−/−</sup>Mev<sup>−/−</sup> heterozygote mice B cells, indicating significant activation (Fig. 5C); however, there was no significant difference from control B cells in the expression of MHC class II and CD86 on Mev<sup>−/−</sup> B cells.

The T cell compartment is highly activated in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice

We next examined the CD4 T cell compartment of young Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice, finding minimal differences (Fig. 6A). However, as previously observed in Lyn<sup>−/−</sup> mice (35, 38) and Lyn<sup>−/−</sup> Mev<sup>−/−</sup> mice (Fig. 2B), T cell activation became apparent in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice; interestingly, these T cells showed almost complete loss of CD62L expression, indicating substantial activation (Fig. 6B). The T cells of aged Mev<sup>−/−</sup> mice showed some evidence of activation compared with those from control mice; however, this was minor compared with the T cell activation observed in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice or Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice (Fig. 6B).

Myeloid expansion occurs in aged Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice

The compound heterozygous loss-of-function mutations in Lyn and SHP-1 had far greater effects on B and T cell activation than did either mutation alone. We previously showed that, in C57BL/6 Lyn<sup>−/−</sup> mice, expansion of the myeloid compartment precedes perturbations in the T cell compartment (38). However, in Lyn<sup>−/−</sup>Mev<sup>−/−</sup> mice.
myeloid cell expansion similar to aged Lyn−/− mice at the indicated ages. Values refer to the absorbance reading obtained at 450 nm with correction at 595 nm; horizontal bars denote the mean value of the data points in each group. (B) H&E-stained sections through the cortex of kidneys of 48–52-wk-old C57BL/6, Lyn−/−, Mev−/−, and Lyn−/−Mev−/− mice. Shown are representative examples of each genotype. Scale bar, 50 μm. (C) Quantification of glomerular area from 40–50- and 60–90-wk-old C57BL/6, Lyn−/−, Mev−/−, and Lyn−/−Mev−/− mice. For each genotype, one point represents the mean glomerular area of a single mouse taken from an average of 20–30 glomeruli. Horizontal bars represent the mean glomerular area for that genotype where n = number of points on the graph (n ≥ 4). *p < 0.05, **p < 0.01, ***p < 0.001.

Minor myeloid progenitor expansion occurs in Lyn−/−Mev−/− mice

Myeloid progenitor expansion is another component of autoimmune disease in Lyn−/− mice (36, 38, 39). Young Lyn−/− mice have augmented numbers of progenitors responsive to myeloid cytokines, which increase dramatically as the mice age and autoimmune disease becomes severe. Because Lyn−/−Mev−/− mice only show myeloid cell expansion with age, we next determined whether myeloid progenitor cells were present at an increased frequency in Lyn−/−Mev−/− mice. In young Lyn−/−Mev−/− mice, splenic myeloid progenitors were increased, but this increase was minor and not statistically significant compared with the large numbers of progenitors observed in the spleens of young Lyn−/− mice (Fig. 7). Lyn−/− and Mev−/− mice were also included for comparison and showed no statistically significant differences in myeloid progenitor numbers. Interestingly, aged Lyn−/−Mev−/− mice showed a statistically significant increase in splenic myeloid progenitors (Fig. 7), but this was considerably less than the expansion observed in aged Lyn−/− mice (36, 38, 39). Thus, although Lyn−/−Mev−/− mice show myeloid cell expansion similar to aged Lyn−/− mice (Fig. 6D) (38), this occurs without the dramatic enhancement in myeloid progenitor number.

Perturbations in the erythroid compartment occur in Lyn−/−Mev−/− mice

Finally, we analyzed the erythroid cell compartment in double-heterozygous Lyn−/−Mev−/− mice, because our previous studies suggested a close relationship between Lyn and SHIP-1 in regulating erythropoiesis (36). Although we observed slight changes in this compartment in young Lyn−/−, Mev−/−, and Lyn−/−Mev−/− mice, the differences from control mice were not significant (Fig. 8A). However, in aged mice, a significant expansion in Ter119+CD71+ erythroblasts occurred in Lyn−/−Mev−/− mice. Although there was little change in aged Mev−/− mice, aged Lyn−/− mice showed considerable variation in the numbers of erythroblasts, making the differences from control mice nonsignificant (Fig. 8B).

Genetic interaction between Lyn and SHIP-1 in autoimmunity

Previous studies showed that Lyn and the inositol phosphatase SHIP-1 are in the same biochemical pathway that negatively regulates B cell signaling; membrane recruitment of SHIP-1 is severely impaired in Lyn-deficient B cells through loss of Lyn-dependent FcγRIIB1 phosphorylation (18, 19). Because inhibitory signaling through FcγRIIB1 is also impaired in Lyn−/−B cells (Fig. 1D, 1E), we next asked whether a genetic interaction existed between Lyn and SHIP-1 in autoimmunity. For these studies, we generated double-heterozygote Lyn−/−SHIP-1−/− mice on both the C57BL/6 and BALB/c genetic backgrounds. When measuring ANA titers, we had to use BALB/c genetic background mice, because ANAs in C57BL/6 background SHIP-1 mutant mice are not detectable using a commercial ELISA kit (27). We found that, although BALB/c Lyn−/− mice developed high ANAs in their serum, SHIP-1−/− mice did not, even when close to a year in age (Fig. 9A). Nonetheless, when the SHIP-1−/− mutation was compounded with the Lyn−/− mutation, the double-heterozygote mice developed ANAs at a significantly faster rate, demonstrating a clear genetic interaction between Lyn and SHIP-1 in the development of autoimmunity. To assess whether the production of
ANAs correlated with SLE-like disease in the form of glomerulonephritis, kidney sections from age-matched BALB/c background mice were examined. Surprisingly, despite developing high titers of ANAs, BALB/c background Lyn
\(^{+/-}\) did not develop significant glomerulonephritis, even when >1 y of age, with kidney disease confined to slight, but not significant, glomerular enlargement (Fig. 9B, 9D; 70-wk-old mice shown). However, BALB/c Lyn
\(^{+/-}\)SHIP-1
\(^{+/-}\) mice displayed signs of mild glomerulonephritis, with glomeruli being significantly enlarged compared with controls and single-heterozygote mice (Fig. 9B, 9D). To determine whether this effect was replicated on the C57BL/6 background, we also assessed the kidneys of aged C57BL/6 Lyn
\(^{+/-}\), SHIP-1
\(^{+/-}\), and Lyn
\(^{+/-}\)SHIP-1
\(^{+/-}\) mice (Fig. 9C, 9D). Unlike on the BALB/c background, we observed mild, yet significant, kidney pathology in aged C57BL/6 Lyn
\(^{+/-}\) and SHIP-1
\(^{+/-}\) mice, with glomerular size being significantly increased compared with controls. Enhanced kidney pathology was observed in Lyn
\(^{+/-}\)SHIP-1
\(^{+/-}\) mice, in which glomeruli showed extensive leukocytic infiltrates, globularity, and enlargement (Fig. 9C). In several mice, extensive kidney damage had occurred, as reflected by severely distended glomeruli or empty spaces in the cortex where glomeruli had been destroyed (one example shown in Fig. 9C). When glomerular size was measured, glomeruli were significantly larger than were those of C57BL/6 Lyn
\(^{+/-}\) and SHIP-1
\(^{+/-}\) mice (Fig. 9D).

**Discussion**

The importance of Lyn tyrosine kinase as a regulator of autoimmune disease was first revealed in analyses of Lyn-deficient mice in the mid-1990s (8, 9), and it is becoming increasingly clear in more recent human disease studies (14, 15). We have examined the consequences of haploinsufficiency of Lyn, alone or in combination with heterozygous loss-of-function mutations in inhibitory-signaling molecules known to be activated by Lyn, on the development of inflammation and autoimmune disease. We show that Lyn
\(^{+/-}\) mice develop many features of their homozygous counterparts, albeit in a milder form. These include alterations in B cell subsets and plasma cells; increased cellular activation, as indicated by downregulation of cell-surface IgM and increased MHC class II and CD86 levels; and heightened B cell responsiveness to external stimuli. Furthermore, Lyn
\(^{+/-}\) mice display age-dependent dysregulation of both T cell and myeloid compartments, features reminiscent of Lyn
\(^{-/-}\) mice. Finally, Lyn
\(^{+/-}\) mice on a C57BL/6 background develop an autoimmune disease that is clinically similar to that of Lyn
\(^{-/-}\) mice. Collectively, these results show that Lyn is a key inhibitor of autoimmune disease development and one that is extremely sensitive to gene dose, clearly demonstrating that Lyn is a haploinsufficient gene for autoimmune disease.

We also show that partial inactivation of key inhibitory molecules downstream of Lyn, specifically SHIP-1 and SHP-1, amplifies the consequences of Lyn insufficiency and leads to an acceleration of B cell responsiveness to external stimuli. Furthermore, Lyn
\(^{+/-}\) and Lyn
\(^{+/-}\) mice display age-dependent dysregulation of both T cell and myeloid compartments, features reminiscent of Lyn
\(^{-/-}\) mice. Finally, Lyn
\(^{+/-}\) mice on a C57BL/6 background develop an autoimmune disease that is clinically similar to that of Lyn
\(^{-/-}\) mice. Collectively, these results show that Lyn is a key inhibitor of autoimmune disease development and one that is extremely sensitive to gene dose, clearly demonstrating that Lyn is a haploinsufficient gene for autoimmune disease.

![FIGURE 5.](http://www.jimmunol.org/)

**FIGURE 5.** B cell population perturbations in Lyn
\(^{+/-}\) mice are exacerbated in Lyn
\(^{-/-}\)Mev
\(^{+/-}\) mice. Representative flow cytometry of spleen cells from young (10-wk-old) control, Lyn
\(^{+/-}\), Mev
\(^{+/-}\), and Lyn
\(^{-/-}\)Mev
\(^{+/-}\) mice. (A) Staining with Abs to B220 and CD138 to detect B220
\(^{+}\)CD138
\(^{hi}\) plasma cells (population highlighted with an ellipse) and Abs to B220 and IgM to determine proportions of B cells and levels of surface IgM (MFI indicated). (B) Staining with Abs to CD21, CD23, and IgM to distinguish follicular zone B and MZB cell subsets (upper panels). Gates were set on CD23
\(^{+}\) cells to examine the T2 cell population (lower panels). For data in (A) and (B), absolute cell numbers of the indicated populations were calculated from individual mice based on total cell counts and flow cytometry; horizontal bars denote the mean value of the data points in each group. (C) B220
\(^{+}\) spleen cells were gated to examine activation marker expression. MFI is presented in graphical form as a ratio of the control. For all flow cytometry data, plots shown are representative of three independent experiments and a minimum of n = 7 mice. (D) Western blot analysis of purified splenic B cells from control, Lyn
\(^{-/-}\), Mev
\(^{+/-}\), and Lyn
\(^{-/-}\)Mev
\(^{+/-}\) mice stimulated for 0, 5, or 30 min with 20 μg/ml anti-IgM and probed with anti-p-Erk or anti-Erk as a loading control. Densitometry ratios of p-Erk/Erk are shown, and the Western blot is representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
of disease development. However, within these pathways, Lyn appears to be the definitive regulatory component, because the reduction in active Lyn is required for disease development, whereas haploinsufficiency of SHIP-1 or SHP-1 alone is of limited consequence. These studies clearly show that Lyn expression needs to be tightly regulated to achieve immune homeostasis.

Recently, it was proposed that the myeloid compartment plays a crucial role in the development of autoimmunity in Lyn\(^{-/-}\) mice (46). The investigators suggested that deregulated BAFF production by Lyn-deficient myeloid cells promoted T cell activation and the induction of IFN-\(\gamma\) secretion, which is then able to sustain myeloid cell activation and BAFF production. This does not appear to be a complete explanation of disease development because the B cell compartment of Lyn\(^{-/-}\) mice does not resemble that of BAFF transgenic mice, which show expanded follicular B2 and MZB cells (4), two populations that are absent in Lyn\(^{-/-}\) mice and compromised in Lyn\(^{+/+}\) mice. Previous studies showed that Lyn\(^{-/-}\) mice on either mixed or C57BL/6 backgrounds show early-onset myeloid hyperplasia (36, 38, 39). These myeloid compartment changes correlate with early-onset ANAs in serum; however, at this early time point, their T cell compartment is ostensibly normal and only becomes perturbed as the mice age and develop disease (38). Intriguingly, young C57BL/6 Lyn\(^{+/+}\) mice have normal numbers of myeloid cells and only develop an expanded myeloid compartment as they age. These observations suggest that, in the Lyn\(^{+/+}\) setting, age-dependent perturbations in the T cell compartment occur in the absence of early myeloid cell defects, implying that these effects may be driven by B cell defects initially, in addition to eventual perturbations in myeloid cells.

**FIGURE 6.** T cell and myeloid changes are exaggerated in aged Lyn\(^{-/-}\)-Mev\(^{-/-}\) mice. Representative flow cytometry of spleen cells from young (10-wk-old) (A, C) and aged (30-wk-old) (B, D) control, Lyn\(^{-/-}\), Mev\(^{-/-}\), and Lyn\(^{-/-}\)-Mev\(^{-/-}\) mice. (A, B) CD4\(^+\) spleen cells were gated and assessed for expression of CD62L. (C, D) Staining of spleen cells with Abs to myeloid cell markers. For data in (C) and (D), absolute cell numbers of the indicated populations were calculated based on total cell counts and flow cytometry; horizontal bars denote the mean value of the data points in each group. For all flow cytometry data, plots shown are representative of three independent experiments and a minimum of \(n = 6\) mice. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).

**FIGURE 7.** Moderate myeloid progenitor expansion occurs in aged Lyn\(^{-/-}\)-Mev\(^{-/-}\) mice. Quantitation of myeloid progenitors in the indicated young (10-wk-old) (A) and aged (30-wk-old) (B) mice. In young mice, Lyn\(^{-/-}\) mice were included for comparison. The data shown are representative of \(n = 4\) mice in two independent experiments and are presented as mean ± SEM. **\(p < 0.01\) versus control.
Interestingly, early expansion of plasma cells in Lyn−/− mice did not result in production of IgG ANAs in young mice, in contrast to Lyn−/− mice, a feature also observed by other investigators (43). It appears that plasma cells do not produce ANAs in the absence of an inflammatory environment (early myeloid expansion). This is reminiscent of Lyn−/− IL-6−/− mice, in which significant numbers of plasma cells are present; however, because of the absence of IL-6–mediated inflammatory components, they lack IgG ANAs and remain disease-free throughout life (38, 43). In contrast, in aged Lyn−/− mice, myeloid expansion and significant T cell activation eventually come to the fore, concomitant with the production of pathogenic IgG ANAs, leading to nephritis. We previously found that Lyn−/− mice on the BALB/c genetic background develop IgG autoantibodies (35). In this study, we recapitulated these findings on the C57BL/6 background while also demonstrating that these mice develop autoimmune disease, albeit with a longer latency and a reduced severity relative to Lyn−/− mice. Another study failed to detect IgG autoantibodies in Lyn−/− mice (43); however, they only examined mice up to 18 wk of age, a time point at which IgG autoantibodies in C57BL/6 background Lyn−/− mice are low (Fig. 3A). This milder, yet significant, disease phenotype observed in mice with a heterozygous mutation in Lyn is reminiscent of human diseases that also result from heterozygous, rather than null, mutations in critical genes. For instance, heterozygous loss-of-function mutations in the sialic acid esterase gene have been identified in rheumatoid arthritis and type 1 diabetes, indicating that these mutations act in a dominant fashion to influence disease (47). In addition, a heterozygous mutation in TNFRSF1A was associated with SLE in Japanese patients (48), whereas heterozygous mutations in AIRE were shown to be associated with multiple autoimmune syndromes, including hepatitis (49) and hypoparathyroidism (50). To extrapolate from our studies in mice, we suggest that there may be a subset of SLE patients that shows loss of one allele of Lyn, or at least single nucleotide polymorphisms in regulatory elements that affect Lyn gene expression. Indeed, this is supported, in part, by studies that show reduced expression of Lyn in lupus patients (11–13).

Haploinsufficiency of both Lyn and SHP-1 was reported previously (17); however, the extent to which the mice developed autoimmunity and associated disease was not assessed. We showed a clear genetic interaction between Lyn and SHP-1 in the development of autoimmune disease. Lyn−/− Mev−/− mice develop autoantibodies and glomerulonephritis with age, showing signs of disease that are exaggerated over Lyn−/− mice. The strength of this genetic interaction is also mirrored by IgG ANA titers, because those of 18–22-wk-old Lyn−/− Mev−/− mice resemble those of Lyn−/− mice (38). In addition, T cell activation is a major feature of aged Lyn−/− Mev−/− mice, resembling the T cell compartment of aged Lyn−/− mice. However, certain aspects of disease in Lyn−/− Mev−/− mice differ from those in Lyn−/− mice. Myeloid cell expansion, which is observed in young Lyn−/− mice, is only significant in aged Lyn−/− Mev−/− mice; interestingly, this is not reflected by a dramatic increase in splenic myeloid progenitors, a feature of Lyn−/− mice (38). A possible explanation is that expansion of the mature myeloid cell compartment and the expansion of myeloid progenitor cells require distinct forms of an inflammatory environment, which may differ between Lyn−/− and Lyn−/− Mev−/− mice. Assessment of indices of inflammation, including myeloid effector cell function and cytokine environment, may be useful in delineating the key differences between Lyn−/− Mev−/− and Lyn−/− mice, as would a comparative analysis of disease onset and degree in Lyn−/− Mev−/− mice versus Lyn−/− mice.

SHIP-1 is a key regulatory molecule known to be downstream of Lyn in inhibitory signaling (10, 18, 19). As a further example of combining heterozygous loss-of-function mutations on the Lyn-signaling pathway, we studied autoimmune disease development in Lyn−/−SHIP-1−/− mice. ANA titers were increased considerably in BALB/c background Lyn−/−SHIP-1−/− mice compared with single-heterozygote mice, with this interaction being mirrored in the development of kidney pathology. Neither Lyn−/− nor SHIP-1−/− BALB/c background mice developed significant glomerular pathology, even when >1 yr old, whereas BALB/c background Lyn−/−SHIP-1−/− compound heterozygote mice displayed mild, but significant, signs of glomerulonephritis, demonstrating a clear genetic interaction between Lyn and SHIP-1 in autoimmune disease development. The mild pathology is in stark contrast to C57BL/6 background Lyn−/−SHIP-1−/− mice, which developed severe kidney pathology consisting of extensive leukocytic infiltrates and glomerular destruction. Intriguingly, when comparing kidney disease in Lyn−/− mice on the two different genetic backgrounds, the extent of kidney pathology showed little relationship to ANA titers; BALB/c Lyn−/− mice developed high ANA titers without significant disease, whereas C57BL/6 background Lyn−/− mice developed low-moderate ANA titers but exhibited marked pathology. Therefore, ANA levels may provide an indication of autoimmunity but are not a true measure of autoimmune-mediated kidney pathology. The apparent resistance of the BALB/c background to severe glomerulonephritis, despite the presence of high-titer IgG ANAs, is supported by mild kidney disease in BALB/c Lyn−/− mice and BALB/c SHIP-1−/− mice, both of which develop high-titer IgG ANAs by 30–40 wk of age (27, 35). These studies confirm that the BALB/c genetic background itself carries lupus-suppressor loci, as was suggested previously (27, 51, 52).
Collectively, these studies highlight the importance of the Lyn tyrosine kinase as an autoimmune-susceptibility gene, showing that reducing Lyn expression by 50% has profound effects on the B cell compartment and is sufficient to drive myeloid expansion, T cell activation, and, ultimately, autoimmune disease on a susceptible genetic background. Although autoimmune disease is slower to develop under conditions of Lyn haploinsufficiency, multiple genetic mutations in Lyn-regulated pathways can accelerate and exacerbate autoantibody development and the clinical manifestations of autoimmune disease, although interactions with genetic background also contribute to disease susceptibility. Closer examination of Lyn and Lyn-regulated pathways in human disease is clearly warranted.

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Disclosures
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References

FIGURE 9. Genetic interaction between Lyn and SHIP-1 in autoimmune disease development is influenced by genetic background. (A) IgG ANAs in the serum of BALB/c background control, Lyn−/−, SHIP-1−/−, and Lyn−/− SHIP-1−/− mice at the indicated ages were quantified by ELISA. Values refer to the absorbance reading obtained at 450 nm with correction at 595 nm; horizontal bars denote the mean value of the data points in each group. H&E-stained sections through the cortex of kidneys of 56–70-wk-old BALB/c background (B) and C57BL/6 background (C) control, Lyn−/−, SHIP-1−/−, and Lyn−/− SHIP-1−/− mice. Shown are representative examples of control, Lyn−/−, and SHIP-1−/− mice and two examples of Lyn−/− SHIP-1−/− mice. Scale bars, 50 μm. (D) Quantification of glomerular area from the indicated mice in (B) and (C) on BALB/c versus C57BL/6 genetic backgrounds. For each genotype, one point represents the mean glomerular area of a single mouse taken from an average of 20–30 glomeruli. The horizontal bar represents the mean glomerular area for that genotype, where n = number of points on the graph (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001.


