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Cutting Edge: Deletion of Ezrin in B Cells of Lyn-Deficient Mice Downregulates Lupus Pathology

Debasis Pore, Emily Huang, Dina Dejanovic, Neetha Parameswaran, Michael B. Cheung, and Neetu Gupta

Genetic deletion of the Src family tyrosine kinase Lyn in mice recapitulates human systemic lupus erythematosus, characterized by hyperactive BCR signaling, splenomegaly, autoantibody generation, and glomerulonephritis. However, the molecular regulators of autoimmunity in Lyn-deficient mice and in human lupus remain poorly characterized. In this study, we report that conditional deletion of the membrane–cytoskeleton linker protein ezrin in B cells of Lyn-deficient mice (double knockout [DKO] mice) ameliorates B cell activation and lupus pathogenesis. B cells from DKO mice respond poorly to BCR stimulation, with severe downregulation of major signaling pathways. DKO mice exhibit reduced splenomegaly as well as significantly lower levels of autoantibodies against a variety of autoantigens, including dsDNA, histone, and chromatin. Leukocyte infiltration and deposition of IgG and complement component C3 in the kidney glomeruli of DKO mice are markedly reduced. Our data demonstrate that ezrin is a novel molecular regulator of B cell–associated lupus pathology. *The Journal of Immunology, 2018, 201: 1353–1358.*

Systemic lupus erythematosus (SLE) is an aggressive autoimmune disorder that afflicts 1.5 million Americans. B cell hyperactivity and anti-dsDNA autoantibody production are hallmarks of SLE (1–3), signifying that altered B cell function is central to the pathogenesis of SLE. Human genome-wide association studies and genetic knockouts have revealed a powerful biochemical pathway that keeps BCR signaling under check (4–6); Lyn tyrosine kinase is a key component of this pathway. Lyn phosphorylates CD22 on inhibitory tyrosines (7), which then recruits the phosphatase Shp-1, resulting in downregulation of BCR signaling (8). Genetic variations in Lyn (9) and reduced expression of Lyn (10, 11) are associated with strong susceptibility to human SLE, and mice deficient in Lyn, CD22, or Shp-1 exhibit SLE-like disease, including hyperactive BCR signaling, autoantibody production, and glomerulonephritis (12–18). As Lyn−/− mice represent a clinically relevant model of SLE, insights from characterization of proteins and processes that regulate B cell hyperactivation and autoantibody production in this model may lead to better molecular understanding of SLE.

Ezrin is a member of the ERM family of membrane–cytoskeleton cross-linking proteins. It contains an N-terminal FERM domain that binds transmembrane proteins such as CD44 (19) and Cbp/PAG (20). The C-terminal domain contains a conserved threonine residue (T567) whose phosphorylation is critical for its conformational activation and binding to F-actin (19). We have previously reported that ezrin modulates B cell function owing to its ability to regulate BCR organization, signaling (21), B cell chemotaxis (22), and IL-10 production (23). Moreover, we recently showed that ezrin supports pathogenic BCR signaling in germinal center–derived diffuse large B cell lymphoma (24). However, it is not known whether ezrin regulates abnormal BCR signaling and B cell–associated pathogenesis in Lyn−/− mice.

In this study, we employed Lyn−/− mice in which ezrin was conditionally deleted in B cells to investigate the impact on BCR signaling, autoantibody levels, and kidney pathology. Our data show that loss of ezrin in Lyn−/− B cells resolves these major hallmarks of SLE-associated autoimmune pathology.

**Materials and Methods**

**Mice**

Ezfl/flMB1cre/+ (Ez-def), MB1cre/+, and Lyn−/− mice have been previously described (12, 13, 21). Two- to eight-month-old animals were used, and all experiments were performed in compliance with the guidelines approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

**Flow cytometry**

Single-cell suspensions were prepared from spleens, blocked with anti-CD16/32 (clone 2G12), and stained with FITC- or PE-conjugated Abs to surface IgM (BD Pharmingen). Developmental and mature B cell subsets were identified by flow cytometry based on previously described gating strategies (21). Flow cytometry data were collected on BD LSRFortessa and analyzed using FlowJo software (Tree Star).

The online version of this article contains supplemental material.

Abbreviations used in this article: DKO, double knockout; Ez-def, Ezfl/flMB1cre/+; FO, follicular; SLE, systemic lupus erythematosus.

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Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

ORCID: 0000-0002-7393-1282 (E.H.); 0000-0002-0021-3802 (M.B.C.)

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Address correspondence and reprint requests to Dr. Neetu Gupta, Department of Immunology, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue, NE40, Cleveland, OH 44195. E-mail address: guptan@ccf.org

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BCR stimulation, immunoblotting, and calcium flux

Spleenic B and T cells were purified by negative selection using CD43 beads or mouse Pan T Cell Isolation Kit II (Miltenyi Biotec), respectively. Purified B cells were stimulated with 10 μg/ml F(ab')2 fragment of anti-mouse IgM (Jackson ImmunoResearch Laboratories) for the indicated times. B and T cell lysates were prepared and immunoblotting was performed as described (21). All immunoblotting Abs were from Cell Signaling Technology, except for actin (Santa Cruz Biotechnology), Igα (Abcam), phosphoryrosynine, and ezrin (EMD Millipore). To measure intracellular-free calcium levels, purified splenic B cells were loaded with Fluo-3 AM (Molecular Probes) at 37°C for 20 min. Cells were washed, resuspended in DMEM supplemented with 1% BSA (Sigma-Aldrich) and 20 mM HEPES (Sigma-Aldrich), warmed to 37°C for 20 min. Cells were washed, resuspended in DMEM supplemented with 1% BSA (Sigma-Aldrich) and 20 mM HEPES (Sigma-Aldrich), warmed to 37°C for 5 min, and analyzed by flow cytometry. After the baseline was established for 30–40 s, cells were stimulated with 10 μg/ml F(ab')2 fragment of anti-mouse IgM for the indicated duration.

Autoantigen array and autoantibody ELISA

The profiling of IgM and IgG autoantibodies in sera was done using 98-plex autoantigen arrays. Arrays were hybridized with mouse sera, detected by Cy5-labeled anti-mouse IgM and Cy3-labeled anti-mouse IgG Abs, and scanned with GenePix 4400A Microarray Scanner to generate TIFF images. The images were analyzed using GenePix Pro 6.0 software to generate GPR files. The averaged net fluorescent intensity of each autoantigen was normalized to internal controls (IgM or IgG). ELISA plates coated with purified dsDNA (Invitrogen) were used to quantify serum anti-dsDNA Abs, and those coated with Abs to IgM or IgG, respectively, were used to quantify total IgM and IgG levels.

Histopathology and immunofluorescent staining

Kidneys were fixed in 10% formalin (Sigma-Aldrich) for 24 h, washed in alcohol, and embedded in paraffin. For light microscopy, sections were cut and stained with H&E. IgG immune complex and complement C3 deposits in kidney were detected by direct immunofluorescence of OCT-embedded frozen kidney sections (5 μm thick) using Texas Red-conjugated goat anti-mouse IgG (Molecular Probes) or FITC-conjugated anti-C3 complement (Thermo Fisher Scientific), respectively. The slides were mounted in VECTASHIELD (Vector Laboratories) and observed under the microscope.

Statistical analysis

All analyses were performed with Prism 4 software (GraphPad Software, La Jolla, CA). Statistical differences between any two genotypes were calculated using an unpaired t test. A p value <0.05 was considered significant.

Results and Discussion

Reciprocal regulation of ezrin and Lyn

Both ezrin and Lyn act at very proximal steps during B cell activation by Ag, the former through regulation of lipid raft and BCR dynamics and the latter by initiating BCR phosphorylation as well as terminating B cell activation through recruitment of tyrosine and lipid phosphatases. To test if ezrin and Lyn regulate each other, we employed Ez-def and Lyn<−/−>B cells and examined the BCR-dependent phosphorylation of Lyn and ezrin, respectively. Purified splenic B cells from 2- to 3-mo-old C57BL/6 and Lyn<−/−>mice (Supplemental Fig. 1A) or MB1<cre/+> and Ez-def mice (Supplemental Fig. 1B) were stimulated with F(ab')2 fragment of anti-IgM for 1 and 10 min, and the phosphorylation of ezrin and Lyn was assessed in cell lysates. Lyn<−/−>B cells showed constitutively higher ezrin phosphorylation in unstimulated cells and a resistance to dephosphorylation upon BCR stimulation (Supplemental Fig. 1A). Similarly, phosphorylation of Lyn (pS53, pS56) and Fyn (pS59) was increased in Ez-def B cells as compared with MB1<cre/+> B cells (Supplemental Fig. 1B), indicating a reciprocal regulatory relationship between ezrin and Lyn.

Deletion of ezrin in Lyn<−/−> mice reduces splenomegaly

We hypothesized that ezrin may regulate BCR signaling in Lyn<−/−> B cells and impact downstream autoimmune pathology. To test this, we bred the Lyn<−/−> mice with Ez-def mice to generate Lyn<−/−> Ez-def, MB1<cre/+>, double knockout (DKO) mice that lack the expression of Lyn systemically and ezrin exclusively in B cells. Deletion of both ezrin and Lyn expression in B cells from DKO mice was confirmed by immunoblotting of cell lysates (Fig. 1A, left panel). The expression of ezrin was intact in purified MB1<cre/+>, Ez-def, Lyn<−/−>, and DKO T cells (Fig. 1A, right panel), indicating exclusive targeting in B cells. Conditional deletion of ezrin in the B cells of Lyn<−/−> mice (DKO) led to reduction in spleen size (Fig. 1B), weight (Fig. 1C), and the number of splenocytes (Fig. 1D). Thus, splenomegaly, which is a characteristic feature of Lyn<−/−> mice, was ameliorated in DKO mice. The splenic tissue in DKO mice also appeared less fibrous during mechanical disruption as compared with Lyn<−/−> mice. We next analyzed the B cell compartment in 2- to 3-mo-old MB1<cre/+>, Ez-def, Lyn<−/−>, and DKO mice to examine if loss of ezrin in Lyn<−/−> mice affected bone marrow B cell development and peripheral B cell subset composition. The total number CD19<+> B cells in the spleen, lymph nodes, and bone marrow was not altered in DKO mice as compared with Lyn<−/−> mice (Fig. 1E). Bone marrow B cell development was also not affected in DKO mice, as indicated by comparable numbers of pro/pre-B cells and immature B cells (Fig. 1F), and long-lived plasma cells in the bone marrow were similar in number between the Lyn<−/−> and DKO mice (Fig. 1F). Lyn<−/−> mice were reported to show reduced mature follicular (FO) B cells and T1 and T2 developmental stages of B cells in the spleen (13, 25). We observed that FO B cells were not affected by deletion of ezrin in Lyn<−/−> mice, whereas T1 and T2 B cells were further decreased in DKO mice (Fig. 1G). Splenic B1 B cell numbers were also reduced in DKO mice compared with Lyn<−/−> mice, whereas marginal zone, germinatal center, and splenic plasma cell numbers remained unaltered (Fig. 1G).

Deletion of ezrin reduces activation of Lyn<−/−> B cells

Lyn<−/−> B cells are known to be hyperresponsive to BCR stimulation and display hyperactive proximal and distal signaling with delayed but greatly increased magnitude of calcium flux (12). Therefore, we examined whether the loss of ezrin in Lyn<−/−> B cells would affect their response to BCR stimulation. Purified splenic B cells from 2- to 3-mo-old MB1<cre/+>, Ez-def, Lyn<−/−>, and DKO mice were stimulated with F(ab')2 fragment of anti-IgM for 5, 15, and 30 min to mimic ex vivo response to Ag stimulation, and the activation of major proximal and distal BCR signaling pathways was analyzed in cell lysates by immunoblotting with specific Abs. The data were quantified, and statistical significance of differences is shown in Fig. 2A, right panels. Ez-def B cells exhibited stronger induced phosphorylation of Igα, PLCγ2, and ERK, but not Akt, as compared with MB1<cre/+> B cells, consistent with our previous findings (21) (Fig. 2A, left panels). All of these signaling pathways were also more robustly activated in Lyn<−/−> B cells compared with MB1<cre/+> B cells, as was Akt phosphorylation (Fig. 2A, left panels), as previously reported (12, 13). However, the DKO B cells were remarkably hyporesponsive to BCR stimulation, with signification reduction in activation of Igα and Akt and a complete loss of induction of PLCγ2 phosphorylation (Fig. 2A, left and right panels). Interestingly, DKO B cells showed a significant increase in basal ERK phosphorylation compared with MB1<cre/+>.
and Ez-def B cells but significantly reduced induction of ERK phosphorylation upon anti-IgM cross-linking (Fig. 2A, left and right panels). DKO B cells also failed to mobilize intracellular calcium upon anti-IgM stimulation, whereas MB1cre/+ and Ez-def B cells showed similar induction of calcium flux, and Lyn−/− B cells were characteristically hyperresponsive (Fig. 2B). We tested if the reduced activation of DKO B cells was due to altered surface IgM expression. However, flow cytometry analysis revealed that the level of surface IgM was similar in Lyn−/−, and Ez-def B cells but significantly reduced in DKO mice (Fig. 3B). Because human SLE patients also develop Abs to a variety of other nuclear and nonnuclear Ags, we employed a commercial autoantigen microarray to examine the effect of B cell–specific ezrin deletion in Lyn−/− mice on other autoantibody levels. Sera from four randomly selected Lyn−/− and five randomly selected DKO mice were compared. Lyn−/− mouse sera contained higher than average levels of autoantibodies to most autoantigens; in contrast, most of the DKO mouse sera tested had markedly reduced IgM (Supplemental Fig. 2A) and IgG (Supplemental Fig. 2B) autoantibodies to antibodies to most autoantigens. These data indicate that the loss of ezrin impacts not only B cell activation but also downstream autoantibody production.

**B cell-specific ezrin deletion in Lyn−/− mice lowers autoantibodies**

Because generation of autoantibodies to dsDNA is a hallmark clinical manifestation of autoimmune disease in human SLE as well as in aging Lyn−/− mice and is a direct consequence of hyperactive B cells, we examined whether loss of ezrin in B cells of Lyn−/− mice would affect autoimmune disease development. ELISA was used to determine the levels of total IgM and total IgG Abs in the sera of 6- to 8-mo-old MB1cre/+ Ez-def, Lyn−/−, and DKO mice. Total IgM levels were significantly lower in DKO mice compared with Lyn−/− mice (Fig. 3A), whereas total IgG levels were similar between all four groups (Supplemental Fig. 1E). As reported previously (13, 14), Lyn−/− mice had detectable anti-dsDNA IgM and IgG autoantibodies within 2–3 mo (Fig. 3A), which were further increased in 6- to 8-mo-old mice (Fig. 3B). Both serum IgM and IgG levels of Abs against dsDNA were significantly reduced in the DKO mice as compared with Lyn−/− mice at 2–3 mo (Fig. 3A) as well as at 6–8 mo of age (Fig. 3B). Interestingly, low levels of IgM Abs to dsDNA were detectable in 6- to 8-mo-old MB1cre/+ mice, but they were decreased in Ez-def mice (Fig. 3B). Because human SLE patients also develop Abs to a variety of other nuclear and nonnuclear Ags, we employed a commercial autoantigen microarray to examine the effect of B cell–specific ezrin deletion in Lyn−/− mice on other autoantibody levels. Sera from four randomly selected Lyn−/− and five randomly selected DKO mice were compared. Lyn−/− mouse sera contained higher than average levels of autoantibodies to most autoantigens; in contrast, most of the DKO mouse sera tested had markedly reduced IgM (Supplemental Fig. 2A) and IgG (Supplemental Fig. 2B) autoantibodies against a majority of nuclear and nonnuclear autoantigens, including histones, chromatin, ssDNA, ssRNA, small nuclear ribonucleoproteins, collagen, cytochrome C, and others. These data indicate that the loss of ezrin impacts not only B cell activation but also downstream autoantibody production.

**B cell-specific ezrin deletion in Lyn−/− mice inhibits kidney pathology**

The kidneys of Lyn−/− mice have been reported to contain infiltrating inflammatory leukocytes resulting in glomerulonephritis, a clinical feature that is conserved in a proportion of human SLE patients, as well as deposition of IgG autoantibodies and fixation of complement component C3 (26). Because the DKO B cells appear to be hypoactive and do not sustain autoantibody production in DKO mice, we examined the kidney pathology in 8-mo-old MB1cre/+ Ez-def, Lyn−/−, and DKO mice. The kidneys of Lyn−/− mice showed enlarged glomeruli filled with leukocytes (see insets.
marked by white boxes in Fig. 4A), whereas DKO mice displayed little or no evidence of glomerulonephritis (Fig. 4A). The kidneys of MB1cre/+ and Ez-def mice showed no IgG staining and only interstitial complement C3 staining, whereas in Lyn2/2 kidneys, both IgG deposition and C3 fixation was observed in the glomeruli. The deposition of IgG and complement component C3 was strikingly reduced in DKO mice (Fig. 4B). These data demonstrate that deletion of ezrin in the B cells of Lyn2/2 mice ameliorates autoantibody production and associated severe kidney pathology, suggesting that ezrin regulates the development and progression of autoimmune disease in Lyn2/2 mice.

Taken together, our results indicate a novel role for the membrane–cytoskeleton linker protein ezrin in controlling B cell tolerance and the development of autoimmune disease in Lyn2/2 mice. Increased basal ezrin phosphorylation observed in Lyn2/2 B cells may be due to higher protein kinase C expression and/or activity because ezrin is its known substrate. The absence of anti-IgM–induced dephosphorylation indicates inability of a Ser/Thr phosphatase to gain access to or dephosphorylate ezrin. It is well known that the tyrosine and lipid phosphatase pathways (Shp-1 and SHIP, respectively) are inactive in Lyn2/2 B cells. Our data suggest that the function of a Ser/Thr phosphatase such as PP1 or PP2 may be similarly compromised. The ezrin network supports the organization and integrity of BCR microclusters through its dynamic phosphorylation/dephosphorylation, and thus regulates BCR signaling (21, 27). Hyperphosphorylation of ezrin in Lyn2/2 mice may regulate BCR dynamics and early activation processes that are required for appropriate initiation and termination of BCR signaling. Interestingly, hyperphosphorylation of ERM proteins in peripheral T cells of lupus patients is responsible for enhanced T cell adhesion, migration, and polarization and contributes to pathogenesis (28). Thus, altered ezrin phosphorylation in Lyn2/2 mice may additionally impact B cell migration and contact with other cells within secondary lymphoid organs. A detailed analysis of Tyr, Ser/Thr, and lipid phosphatase function in conjunction with microscopic analysis of BCR clustering and recruitment of different phosphatases to BCR clusters will clarify the spatial mechanisms at play.

We have previously reported that Ez-def B cells signal more strongly through the BCR (21), whereas in this study, we demonstrate that DKO B cells signal poorly. There are at least two potential models to explain this. Higher constitutive ezrin phosphorylation observed in Lyn2/2 B cells may represent an attempt by the cells to reign in BCR activation and thus serve as a negative regulator. Upon dual loss of Lyn and ezrin in DKO mice, this control is lost, and autoreactive B cells may be subject to sustained overstimulation by autoantigens in vivo. Over time, this may result in death of immature B cells and exhaustion-induced anergy in mature B cells, and prevent...
the “exhausted” B cells from supporting anti-IgM–induced BCR signaling and autoantibody generation. The reduction of T1, T2, and B1 B cell numbers in the spleens of DKO mice compared with those of Lyn$^{-/-}$ mice supports this model. Significantly higher basal ERK activation in DKO B cells, but failure to activate PLCγ2 and mobilize calcium...
upon BCR stimulation, is also reminiscent of autoreactive anergic B cells (29), lending further support to this model. Alternately, higher ezrin phosphorylation may mediate hyperactivation of Lyn−/− B cells and act as a positive regulator. In the absence of ezrin, this positive regulatory effect may be lost, leading to reduced activation of B cells. Detailed future analyses of these possibilities will elucidate the molecular pathway(s) involved in ezrin-mediated regulation of Lyn−/− B cells.

The clinical ramifications of SLE in patients are highly complex and diverse, and observed to varying degrees within the hematological, dermatological, renal, cardiac, vascular, pulmonary, ocular, gastrointestinal, musculoskeletal, and neuropsychiatric systems (30). Specifically, the Lyn−/− mice develop hyperactivation of B cells, autoantibodies to dsDNA and other self-antigens, and leukocyte infiltration and Ig deposition in the kidney glomeruli. All of these features of clinical disease were ameliorated in DKO mice. Lyn−/− mice also exhibit splenomegaly (13, 18, 25), which was ameliorated in DKO mice. Given that B1 B cells are responsible for natural serum IgM, their reduction in the DKO mice may explain the reduction in total IgM levels observed in the DKO mice compared with Lyn−/− mice. Further, the remarkable lack of response of DKO B cells to polyclonal stimulation by anti-IgM ex vivo suggests that the DKO mice may mount poorer T cell–independent and/or T cell–dependent Ab responses to pathogens in vivo.

In summary, our findings indicate a novel context-dependent function of ezrin in B cell activation and pathogenesis in the autoimmune environment of Lyn−/− mice and suggest that ezrin may be a new target of therapeutic intervention in lupus.

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Disclosures

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