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Novel Roles for Lyn in B Cell Migration and Lipopolysaccharide Responsiveness Revealed Using Anti-Double-Stranded DNA Ig Transgenic Mice

Su-jean Seo, Jodi Buckler, and Jan Erikson

Lyn-deficient mice produce Abs against dsDNA, yet exhibit exaggerated tolerance to the model Ag hen-egg lysozyme. To investigate this apparent contradiction, and to further examine the function of Lyn in Ag-engaged cells, we have used an anti-dsDNA Ig transgenic model. Previously, looking at these anti-dsDNA B cells in Lyn-sufficient BALB/c mice, we showed that they are regulated by functional inactivation (anergy). In the absence of Lyn, these anti-dsDNA B cells remain unable to secrete Ab. This suggests that functional inactivation of anti-dsDNA B cells does not depend on Lyn, and that the anti-dsDNA Abs that are produced in lyn−/− mice arise from a defect in another mechanism of B cell tolerance. Although the anti-dsDNA B cells remain anergic, Lyn deficiency does restore their ability to proliferate to LPS. This reveals a novel role for Lyn in mediating the LPS unresponsiveness that normally follows surface Ig engagement. Furthermore, Lyn deficiency leads to an altered splenic localization and EBV-induced molecule 1 ligand chemokine responsiveness of anti-dsDNA B cells, as well as an absence of marginal zone B cells, suggesting additional roles for Lyn in controlling the migration and development of specific B cell populations. The Journal of Immunology. 2001, 166: 3710–3723.

 lyn is a src family tyrosine kinase expressed in many hematopoietic cell types, including B cells; little to no expression has been found in T cells (1, 2). Expressed throughout B cell development (3), Lyn participates in signaling from multiple cell surface receptors. Upon engagement of surface Ig, Lyn associates with the B cell Ag receptor (BCR) complex and is rapidly tyrosine phosphorylated, with an associated increase in its enzymatic activity (4–6). Lyn is also activated after engagement of the CD40 receptor (7), and in the absence of Lyn, CD40-induced Fas expression was reported to be substantially reduced, as was germinal center (GC) formation (8, 9). Lyn thus plays an important positive role in B cell activation; however, BCR signaling can occur in the absence of Lyn. Indeed, knockout mice have shown that the net effect of Lyn deficiency is to render B cells hyperactive to BCR stimulation (9–11), suggesting that the most crucial role for Lyn in vivo is in the down-modulation of B cell responses. In this regard, Lyn has been implicated in inhibitory signaling from the CD22 and FcγRIIB receptors (12, 13).

Mice deficient in Lyn have been shown to produce anti-dsDNA Abs and to develop glomerulonephritis with immune complex deposition (10, 11, 14), suggesting that Lyn is crucial to the negative regulation of autoreactive B cells. There are two major sites of autoreactive B cell regulation. During B cell development in the bone marrow (BM), the process of V(D)J recombination may yield autoreactive Abs. In the BM, B cells bearing these autoreactive receptors are regulated by editing of the receptor, by deletion, or by functional inactivation (anergy). The form of the Ag appears to dictate these opposing outcomes (reviewed in Refs. 15 and 16). Autoantibodies in Lyn−/− mice could be due to perturbed regulation of B cells generated in either the BM or the periphery.

To see whether the mechanism of B cell tolerance termed anergy is maintained in the absence of Lyn, Cornall et al. (20) studied the fate of lyn−/− B cells directed against hen-egg lysozyme (HEL). B cell anergy can be defined by one fundamental property: although autoreactive B cells survive and are altered by their encounter with Ag, they do not secrete Ab. This inability to secrete Ab is exhibited by lyn−/− anti-HEL B cells that develop in the presence of soluble HEL (18); anti-dsDNA B cells have also been described that share this characteristic (19). Intriguingly, lyn−/− anti-HEL B cells undergo an exaggerated tolerogenic response to soluble HEL and, instead of being anergized, are deleted in the BM (20). This is consistent with a role for Lyn as a negative regulator of B cell signaling.

Although this result clearly exposes distortions in lyn−/− B cell tolerance, it does not address whether anergy of anti-dsDNA B cells is likewise disrupted in lyn−/− mice. This is particularly relevant given that lyn−/− mice spontaneously produce anti-dsDNA Abs. Furthermore, considering that anergic anti-HEL B cells and anergic anti-DNA B cells have been shown to exhibit differences in cell surface phenotype and response to B cell mitogens (18, 19, 21, 22), it is quite conceivable that anti-dsDNA B cells would respond differently to the absence of Lyn. To examine this issue,
we used the VH3H9 Ig transgene (Tg) to follow a population of BM-generated anti-dsDNA B cells. Previously, we have described a regulated phenotype for these anti-dsDNA B cells in BALB/c mice: in vitro, they do not proliferate in response to LPS stimulation; in vivo, they are developmentally arrested, halted at the T/B interface in the splenic white pulp, and fail to secrete Ab (19, 22). Strikingly, in Fas-deficient mice, a murine model of systemic lupus erythematosus, anti-dsDNA B cell anergy is not maintained; the anti-dsDNA B cells exhibit phenotypic differences and spontaneously secrete autoantibody (23). In lyn−/− mice, we find that anti-dsDNA B cells exhibit the same anergic cell surface phenotype seen in the presence of Lyn. Additionally, they are unable to secrete Ab, indicating that Lyn is not required for the induction or maintenance of B cell anergy. However, this study does reveal that Lyn significantly impacts several other B cell responses to autoantigen encounter. In particular, we document previously undescribed roles for Lyn in controlling the LPS responsiveness and migration of Ag-engaged B cells.

Materials and Methods

Mice

Lyn−/− mice (backcross 4 onto the C57BL/6 background from a mixed C3H/C57BL6 background) were obtained from E. Pure (The Wistar Institute, Philadelphia, PA), who received them originally from C. Lowell (University of California, San Francisco, CA). VH3H9 BALB/c mice were crossed with lyn−/− mice, and the resulting progeny were intercrossed to create Tg−/+ lyn+/+, Tg−/+ lyn−/−, VH3H9 lyn+/+, and VH3H9 lyn−/− mice. Some of these Tg−/+ lyn−/− and VH3H9 lyn−/− mice were also mated to generate experimental mice; similar results were obtained from both generations of lyn−/− mice. In this work, the designation lyn−/− refers to Lyn-sufficient mice of this genetic background, distinguishing them from BALB/c mice, which are also Lyn sufficient. The mice were bred and maintained in a specific pathogen-free room at The Wistar Institute animal facility, and they were sacrificed for experiments at 6–10 wk of age. All animal work was conducted in accordance with institutional guidelines.

PCR amplification of tail DNA was used to determine genotypes of mice. VH3H9 PCR was conducted as described (24). The presence of a wild-type lyn locus was determined as described (10). The presence of a neomycin insert (indicating a mutant lyn locus) was detected by PCR using primers derived from sequences in the 5′ and 3′ portions of the neomycin insert. The sequences used were: 5′-TCAAGCCAGGGGGCCGGGTTCTTC-3′ and 5′-ATCGA CAAGACGGGTTCATCCGGA-3′. PCR conditions were 94°C for 5 min; 70°C for 1 min, 72°C for 1 min, and 94°C for 1 min for 40 cycles; 70°C for 1 min; and 72°C for 5 min.

Cell preparations

One femur and one tibia were removed from each mouse for BM analysis, with the cells from both bones being pooled. Single cell suspensions from spleen and BM were prepared by disrupting the organ of interest and passing the resulting solution through a 70-μm cell strainer (Fisher Scientific, Pittsburgh, PA). RBC were then removed with RBC lysing buffer (Sigma, St. Louis, MO), and total live cell numbers were determined by trypan blue exclusion.

Flow cytometry

Cells (0.5–1 × 10^6) were surface stained according to standard protocols (25). The following Abs were used: RA3-6B2 biotin (anti-B220), 1D3 biotin (anti-CD19), 7G6 FITC (anti-CD21/35), Cy3.1 FITC (anti-CD22), and B3B4 FITC (anti-CD23). 187.1 Rhodamin Red670 was purchased from Life Technologies (Gaithersburg, MD). All samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software. Forty thousand events, gated for live lymphocytes based on forward and side scatter, were collected for each sample.

Calculation of spleen cell numbers

Spleen cells were stained with anti-B220 biotin/streptavidin Red670 and anti-IgA PE. Gates were drawn as shown in Fig. 1, with the B220+ IgA+ gate being used to calculate the number of IgA cells/spleen. The percentage of B220+IgA+ cells of live cells (based on forward and side scatter) was multiplied by the total number of live cells per spleen (quantified by trypan blue exclusion using a hemacytometer and light microscope). The number of IgA cells/spleen was calculated in a similar fashion. Because staining with anti-IgA FITC shows that the B220+ IgA+ population consists of IgA cells (data not shown), the B220+ IgA+ gate was used to calculate the number of IgA cells/spleen.

In vitro proliferation assay

Spleen cells were labeled with CFSE (Molecular Probes, Eugene, OR), as described (26). CFSE-labeled spleen cells (2 × 10^6/ml) were then cultured in either medium alone (RPMI 1640/10% FCS/5 × 10^-5 M 2-ME) or LPS (10 μg/ml, Sigma). Cells were removed from culture after 4 days to determine viability, as assessed by trypan blue exclusion under a light microscope, and proliferation. Proliferation was measured by flow cytometry as a decrease in CFSE intensity relative to unstimulated cells. Each cell division is accomplished by a decrease in CFSE intensity by one-half. Percentage of recovery was determined by dividing the number of cells recovered on day 4 by the number of cells initially placed in culture.

B cell hybridoma production

Spleen cells from an 8-wk-old VH3H9 lyn−/− mouse were cultured for 2 days with 10 μg/ml LPS (Sigma). Subsequently, hybridomas were generated, and Ab isotype and specificity were determined as described (27).

Immunohistochemistry

Spleens were suspended in OCT (Fisher Scientific), frozen in 2-methylbutane cooled with liquid nitrogen, sectioned, and fixed with acetone. The 5- to 8-μm spleen sections were stored at -20°C and then stained according to the protocol described (28). Briefly, the sections were blocked using PBS/5% normal goat serum (Sigma)/0.1% Tween 20, and then stained with RA3-6B2 biotin (anti-B220; PharMingen), GK1.5 biotin (anti-CD4; grown as supernatant and conjugated), MOMA-1 (antimarginal zone (MZ) metallophilic macrophages; Bachem, Torrance, CA), Cy3.1 FITC (anti-CD22; PharMingen), or anti-IgA alkaline phosphatase (AP; Southern Biotechnology Associates, Birmingham, AL). Streptavidin HRP (Southern Biotechnology Associates), polyclonal anti-rat HRP (Jackson ImmunoResearch, West Grove, PA), and anti-FITC AP (Sigma) were used as secondary Abs. HRP and AP were developed using the substrates 3-aminio-9-ethyl-carbazole and Fast Blue BB base (Sigma), respectively.

Chemotaxis assays

Chemotaxis assays were performed in 5-μm Transwell plates (Corning Costar, Cambridge, MA). Single cell suspensions were prepared in endotoxin-free RPMI 1640 (Life Technologies) containing 10% FCS and 5 × 10^-5 M 2-ME. EBV-induced molecule 1 ligand chemokine (ELC) and B lymphocyte chemotactic attractant (BLC) (R&D Systems, Minneapolis, MN) were diluted in the same medium. Transwells were preincubated with medium for 10 min at 37°C. Subsequently, this medium was removed, and chemokine solution (600 μl) was added to the bottom chamber of the Transwell. A total of 1 × 10^6 spleen cells (in 100 μl) was added to the upper well, and the cells were allowed to migrate for 4 h at 37°C. Migrated cells were harvested, quantified by trypan blue exclusion, and identified by flow cytometry.

Cribitida luciliae assay

The presence of anti-dsDNA Abs in serum was detected using fixed, permeabilized C. luciliae (29) as the substrate (Antibodies, Davis, CA). Serum samples were tested at serial 10-fold dilutions from 1/100 to 1/1,000,000. Anti-dsDNA Ab binding (defined as staining of the kinetoplast) was detected either with a combination of FITC-conjugated goat anti-mouse IgG and anti-mouse IgM reagents, or with a FITC-conjugated goat anti-mouse IgA reagent (Southern Biotechnology Associates). The samples were then visualized under a fluorescent microscope and scored without knowledge of age or genotype of the mice. The serum titer was defined as the reciprocal of the last dilution at which kinetoplast staining was seen.

Statistical analysis

Statistical significance was determined using an unpaired, two-sample Student’s t test and Instat software.

Results and Discussion

The anti-dsDNA Ig Tg model

The VH3H9 Tg is an Ig heavy chain Tg that, in combination with the endogenous α1-light chain, forms an anti-dsDNA Ab (27, 30).
By using anti–λ reagents, we can specifically identify anti-dsDNA B cells within VH3H9 Tg mice. Endogenous κ-light chains can also combine with the VH3H9 Tg, yielding both anti-DNA Abs and nonautoreactive specificities (24, 27, 30); therefore, the Igλ anti-dsDNA B cells can be studied within a diverse B cell repertoire. Using this approach, we have examined anti-dsDNA B cells in a variety of contexts, including in this study in lyn−/− mice.

Anergic anti-dsDNA B cells are not deleted in lyn−/− mice

We have previously reported that Igλ anti-dsDNA B cells from VH3H9 BALB/c mice are present in both the BM and spleen and, consistent with Ag encounter, express a decreased level of surface Ig compared with Tg−/− Igλ B cells (19). This remains true for the Igλ anti-dsDNA B cells in the lyn−/+ genetic background investigated in this study (Fig. 1A). Igλ cells are also present in the BM and spleens of VH3H9 lyn−/− mice with decreased levels of surface Ig, suggesting that whether or not Lyn is present, the anti-dsDNA B cells see their Ag at the same early stage of development. Additionally, their absolute number is the same in VH3H9 lyn−/− spleens as in VH3H9 lyn−/+ (Fig. 1B).

The ability of Igλ anti-dsDNA B cells to persist in VH3H9 lyn−/− mice is particularly striking when compared with the dramatic reduction in the number of VH3H9/Igκ cells. Consistent with published data, we found that the number of B cells in Tg−/− lyn−/− mice was decreased 54–89% (10, 11, 14), and both Igλ and Igκ cell numbers were equally reduced. Although similar reductions in overall B cell number were found in VH3H9 lyn−/− mice, in this case the decrease occurred only in the Igκ population. Thus, compared with the majority of B cells in VH3H9 lyn−/− mice, Igλ anti-dsDNA B cells appear better able to survive in the absence of Lyn. One explanation for this is that anti-dsDNA B cells are rescued by encounter with Ag. In this regard, anti-Ig treatment of lyn−/− B cells was shown to result in hyperactivation of Akt, a B cell survival factor (31, 32).

Interestingly, lyn−/− anti-dsDNA B cells also have a survival advantage over lyn−/− anti-HEL B cells that have encountered Ag (20). One obvious difference between lyn−/− anti-HEL B cells (which are deleted) and lyn−/− anti-dsDNA B cells (which survive) is the Ag specificity. This suggests that the survival of lyn−/− B cells (and possibly the extent of Akt activation) is not only

**FIGURE 1.** Igλ anti-dsDNA B cells are present with decreased surface Ig. A, BM (left) and spleen (right) cells from Tg−/− (top) and VH3H9 (bottom) mice were stained with Abs against B220 and Igλ. The mean fluorescence intensity is given for the B220+ Igλ+ cells in the boxed region to the right. B, Using the gates shown in A, the total number of splenic Igλ (left) and Igκ (right) cells was calculated as described in Materials and Methods. Brackets indicate significant differences with p values as indicated. There is no significant difference between the number of Igλ anti-dsDNA B cells in VH3H9 lyn+/+ and VH3H9 lyn−/− mice (p = 0.1855). These are representative plots of n ≥ 6 mice of each genotype.
linked to Ag encounter, but is also determined by the strength of signaling through surface Ig. Given that the nature of the in vivo Ag regulating anti-dsDNA B cells has not been defined, this is difficult to directly evaluate.

**Maintenance of the anergic phenotype in lyn−/− anti-dsDNA B cells**

Previously, using flow cytometric analysis, we established that anti-dsDNA B cells in VH3H9 BALB/c mice display an altered cell surface phenotype that correlates with their functional inactivation (19). This phenotype, which includes decreased levels of B220, CD21/35, CD22, and CD23, is also found in the VH3H9 mice investigated in this work (Fig. 2). Before examining cell surface marker expression in VH3H9 lyn−/− mice, we established the phenotype of B cells from Tg ( lyn−/−) mice, expressing lower levels of CD21/35, CD22, and CD23 (Fig. 2). As the VH3H9 Tg can combine with lyn−/− light chains to form autoreactive specificities, this altered phenotype may reflect an increased frequency of anergic B cells in the Igκ population of VH3H9 lyn−/− mice.

To verify the functional inactivation of Igκ anti-dsDNA B cells, serum samples from 40-wk-old Tg ( lyn−/−) mice were tested for anti-dsDNA Abs by the C. luciliae assay. Confirming previous reports (10, 11), high titers of autoantibodies were detected in Tg ( lyn−/−) mice (mean titer = 1.50 × 10^5; n = 17). This contrasts with Lyn-sufficient mice, which have undetectable levels of anti-dsDNA Abs (titers <10; n = 10). Both Igκ and Igλ Abs contributed to serum titers in 15 of 17 Tg ( lyn−/−) mice; in the remaining two mice, only Igκ anti-dsDNA Abs were detected. We have previously reported that for the MRL-1pr/lpr mouse model of lupus, autoantibody production is not affected by the presence of the VH3H9 Tg, and Igλ autoantibodies arise at the same time as Igκ autoantibodies (23). However, in VH3H9 lyn−/− mice, anti-dsDNA Ab titers are reduced (mean titer = 1 × 10^3; n = 13), and, importantly, Igλ anti-dsDNA Abs are not detected.

Given that anti-dsDNA B cell anergy remains intact in the absence of Lyn, the mechanism for anti-dsDNA Ab production in lyn−/− mice remains unknown. In terms of BM-induced tolerance, anti-dsDNA B cells are a heterogeneous group that are regulated not only by anergy, but also by deletion. For example, anti-dsDNA B cells that use the VH3H9 Tg in combination with the Vκ4-light chain are deleted in the BM (33). One possibility, then, is that deletion of anti-dsDNA B cells in the BM is defective. This could be tested by tracking the fate of VH3H9/Vκ4 anti-dsDNA B cells in the absence of Lyn.

**FIGURE 2.** Cell surface phenotype of splenic lyn−/− B cells. Histograms (gated as in Fig. 1) show representative staining of Igκ cells (bold histogram) overlaying staining of Igλ cells (thin histogram). Bold histograms have been scaled 10^3 to allow for comparison of the smaller pool of Igλ cells with Igκ cells. Except for lacking a CD21/35^{high} peak, Tg ( lyn−/−) B cells closely resemble Tg ( lyn−/−) B cells. Regardless of Lyn expression, Igκ anti-dsDNA B cells in VH3H9 mice look strikingly different from Tg ( lyn−/−) cells, expressing lower levels of CD21/35, CD22, and CD23, as well as slightly lower levels of B220 (15–35% lower, p = 0.0371). In VH3H9 lyn−/− mice, the majority of Igκ cells also express low levels of CD21/35 and CD23. These are representative plots of n ≥ 6 mice of each genotype.
A second possibility is that although BM-induced tolerance remains intact, there is a failure to regulate anti-dsDNA B cells produced in GCs. We have previously suggested that this is the case for bcl-2 Tg mice (34). Strikingly, in both lyn−/− and bcl-2 Tg mice, autoimmune antibody production is reduced by the presence of the VH3H9 Tg. One explanation for this is that the VH3H9 Ig Tg increases production of autoreactive cells in the BM, where they are appropriately regulated, and by doing so limits the pool of nontolerized B cells available for recruitment into a GC. This would predict that the B cell repertoire in VH3H9 lyn−/− mice contains a much larger fraction of anergic B cells than Tg−/− lyn−/− mice. Consistent with this, a large proportion of the Igκ population in VH3H9, but not Tg−/− lyn−/− mice appears altered, expressing low levels of CD21/35 and CD23 (Fig. 2).

**Anti-dsDNA B cells are LPS responsive in the absence of Lyn**

In addition to the induction of tolerance, there are many other ways in which B cells can be altered by Ag encounter. For example, B cells that have received an initial signal through the BCR appear hyporesponsive to B cell mitogens such as LPS (35). To see whether Lyn is involved in this process, we tested the response of lyn−/− anti-dsDNA B cells to LPS stimulation. We have previously noted that Igκ anti-dsDNA B cells in several genetic backgrounds (BALB/c, MRL−/−, and MRL-lpr/lpr) proliferate in response to a combination of CD40L + IL-4, but are markedly deficient in their response to LPS. Not only do the anti-dsDNA B cells fail to proliferate, but they also do not survive (22). The ability of lyn−/− B cells to proliferate in response to LPS has been disputed, with two groups reporting impaired proliferation in the absence of Lyn (11, 14) and one group finding no change (10). In this study, looking at Tg(lyn−/−) mice, we show that although lyn−/− B cells proliferate to LPS, they do not divide as many times as lyn+/+ B cells, as measured by decreases in CFSE intensity (Fig. 3, left panels). This could be explained by recent studies demonstrating that Lyn is essential to signaling from the Toll-like receptor protein RP105 (36), which in turn is necessary for optimal B cell responses to LPS (37).

Given that signals through Ig are reported to impair proliferation to LPS (35), as does the absence of Lyn, lyn−/− anti-dsDNA B cells might be expected to exhibit profound deficits in their response to LPS. However, remarkably, Lyn deficiency actually restores their ability to proliferate in the presence of LPS (Fig. 3, right panels). This suggests that Lyn is crucial to the pathways by which BCR signaling inhibits the LPS response. In support of this idea, it has been shown that coligation of the FcγRIIb (from which Lyn mediates signaling) enhances the inhibitory effect of surface Ig engagement on RP105-induced proliferation (38). Thus, Lyn...
transduces signals from at least one receptor that can affect Ig-mediated inhibition of the LPS response.

The altered LPS responsiveness of lyn−/− anti-dsDNA B cells was also reflected in hybridomas generated from VH3H9 lyn−/− B cells. In previous experiments, anti-dsDNA B cells were not captured in hybridoma panels generated after LPS stimulation (24). However, after LPS stimulation of VH3H9 lyn−/− B cells, 6 of 102 Ig− hybridomas secreted IgA autoantibody (data not shown).

**Altered localization and ELC responsiveness of anti-dsDNA B cells in lyn−/− mice**

We have previously reported that anti-dsDNA B cells in VH3H9 BALB/c mice are restricted from entering the B cell follicles of the spleen white pulp, localizing instead at the interface between the periarteriolar lymphoid sheath (PALS) and the B cell follicle (19). Anti-dsDNA B cells also line up along the T/B interface in VH3H9 lyn−/− mice (Fig. 4A). To determine whether Lyn affects the migration of Ag-experienced B cells, spleen sections from lyn−/− mice were stained with anti-CD22 and anti-IgA, or anti-CD4 and IgA. In Tg− lyn−/− and lyn+/+ mice, IgA cells disperse throughout the B cell follicles. In VH3H9 lyn−/− mice, as in VH3H9 lyn+/+ mice, IgA anti-dsDNA B cells are excluded from the B cell follicles; intriguingly, however, instead of lining up at the T/B interface, the IgA cells are spread throughout the PALS.

One mechanism to account for the altered localization of lyn−/− anti-dsDNA B cells is a change in their chemokine responsiveness. To examine this possibility, we studied migration in response to BLC, which promotes trafficking to the B cell follicle (39), and ELC, which can mediate homing to the T cell area (40). No consistent differences in baseline migration (migration at 0 ng/ml of chemokine) were seen between lyn+/+ and lyn−/− B cells (Fig. 5). Additionally, no consistent differences in BLC responsiveness were seen between lyn+/+ and lyn−/− B cells with or without the VH3H9 Tg (data not shown). In response to ELC, B cells and T cells from Tg− lyn+/+ and Tg− lyn−/− mice migrated to a similar extent (Fig. 5A and data not shown). However, anti-dsDNA B cells from VH3H9 lyn−/− mice exhibited a significantly greater chemotactic response than did those from VH3H9 lyn+/+ mice (Fig. 5B). Thus, Lyn does not seem to affect ELC responsiveness in resting B cells; however, it does appear to play a regulatory role after BCR engagement. This increased ELC responsiveness could explain why lyn−/− anti-dsDNA B cells localize to the T cell area, where ELC is produced.

**Absence of MZ B cells in lyn−/− mice**

A second alteration in splenic architecture was also noted in lyn−/− mice. Staining with MOMA-1 (to mark MZ macrophages (41)) and anti-CD22 shows that B cells fail to fully populate the MZ in lyn−/− mice (Fig. 4B). Compared with lyn+/+ mice, there is a clear decrease in the B cell population that usually extends beyond the ring of MOMA-1. Additionally, by flow cytometry, it is evident that lyn−/− mice lack the CD21/35high population of B cells (Fig. 2). Given that CD21/35highCD23low B cells are enriched in the MZ of the spleen (reviewed in Ref. 42), this is consistent with the absence of MZ B cells seen by immunohistochemistry. Thus, in addition to influencing the migration of autoreactive B cells, Lyn signaling appears crucial to the development or localization of MZ B cells. Because CD19 is required for the induction or maintenance of anti-dsDNA B cell anergy (43) and Lyn appears necessary for CD19 function (44), it may be that defects in this pathway are responsible for the lack of MZ B cells in lyn−/− mice. It is also possible that alterations in chemokine responsiveness contribute to the loss of MZ B cells. This idea was proposed to explain the lack of MZ B cells in Pyk-2-deficient mice. In support of this, the authors demonstrated that MZ B cells are highly motile and disappear from wild-type animals upon treatment with pertussis toxin, which blocks migration to chemokines (45).

A final possibility is that the loss of MZ B cells, as well as the other B cell alterations that we have documented in this study, are secondary to abnormalities in non-B cells in lyn−/− mice. In this regard, Lyn deficiency has been shown to result in an accumulation of myeloid cells in the spleens of older (>3-mo old (14) or >23-wk-old (10)) lyn−/− mice. The studies here used 6- to 10-wk-old mice that had no obvious alterations in myeloid cells (data not shown). However, we cannot rule out the possibility that non-B cells may be primarily responsible for the alterations we detect in the lyn−/− B cells. Future experiments using mixed BM chimeras could be used to definitively answer this question.

In summary, anti-dsDNA B cells that are anergized in the BM remain functionally inactive in lyn−/− mice. Thus, Lyn is not required for the induction or maintenance of anti-dsDNA B cell anergy. In addition to this finding, lyn−/− anti-dsDNA B cells reveal...
several novel aspects of Lyn function after Ig engagement in vivo. Lyn appears to regulate B cell migration after Ag encounter; in the absence of Lyn, anti-dsDNA B cells exhibit alterations in splenic localization and responsiveness to ELC. Additionally, the absence of Lyn restores the ability of anti-dsDNA B cells to respond to LPS, implicating Lyn in the suppression of LPS responses after Ig engagement. Finally, MZ B cells are absent in lyn−/− mice, demonstrating that Lyn is crucial to the development and/or localization of this B cell population.

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