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Requirement for MyD88 Signaling in B Cells and Dendritic Cells for Germinal Center Anti-Nuclear Antibody Production in Lyn-Deficient Mice

Zhaolin Hua,* Andrew J. Gross,‡† Chrystelle Lamagna,§ Natalia Ramos-Hernández,‡ Patrizia Scapini,§,‡ Ming Ji, † Haitao Shao,* Clifford A. Lowell,§ Baidong Hou,*‡† and Anthony L. DeFranco‡

The intracellular tyrosine kinase Lyn mediates inhibitory receptor function in B cells and myeloid cells, and Lyn−/− mice spontaneously develop an autoimmune and inflammatory disease that closely resembles human systemic lupus erythematosus. TLR-signaling pathways have been implicated in the production of anti-nuclear Abs in systemic lupus erythematosus and mouse models of it. We used a conditional allele of Myd88 to determine whether the autoimmunity of Lyn−/− mice is dependent on TLR/MyD88 signaling in B cells and/or in dendritic cells (DCs). The production of IgG anti-nuclear Abs, as well as the deposition of these Abs in the glomeruli of the kidneys, leading to glomerulonephritis in Lyn−/− mice, were completely abolished by selective deletion of Myd88 in B cells, and autoantibody production and glomerulonephritis were delayed or decreased by deletion of Myd88 in DCs. The reduced autoantibody production in mice lacking Myd88 in B cells or DCs was accompanied by a dramatic decrease in the spontaneous germinal center (GC) response, suggesting that autoantibodies in Lyn−/− mice may depend on GC responses. Consistent with this view, IgG anti-nuclear Abs were absent if T cells were deleted (TCRβ−/− TCRβ−/− mice) or if T cells were unable to contribute to GC responses as the result of mutation of the adaptor molecule SAP. Thus, the autoimmunity of Lyn−/− mice was dependent on T cells and on TLR/MyD88 signaling in B cells and in DCs, supporting a model in which DC hyperactivity combines with defects in tolerance in B cells to lead to a T cell–dependent systemic autoimmunity in Lyn−/− mice. The Journal of Immunology, 2014, 192: 875–885.

The human autoimmune disease systemic lupus erythematosus (SLE) is characterized by production of autoantibodies against multiple self-Ags, of which nuclear autoantigens, such as dsDNA and ribonucleoproteins (RNPs), are predominant (1). A similar spontaneously developing autoimmunity characterized by anti-nuclear Ab production is seen in a variety of genetically determined mouse models, some of which are multigenic and others of which result from spontaneous or targeted mutations of known genes (2). One of the better studied of the latter category is the Lyn−/− mouse, which develops a highly penetrant autoimmune and inflammatory disease characterized by anti-dsDNA IgG Abs and glomerulonephritis (3–5). Lyn is a Src family protein tyrosine kinase that is required for the function of a number of inhibitory receptors on B cells and myeloid cells. In B cells, the functions of both the sialic acid–binding Ig superfamily member CD22 and of the inhibitory FcγRIIB depend on the ability of Lyn to phosphorylate tyrosines in their cytoplasmic tails, catalyzing the recruitment to the membrane of the inhibitory phosphatases SHP-1 and SHIP-1 (4, 6, 7). Autoimmunity of Lyn-deficient mice likely involves a combination of compromised tolerance of B cells, which is due to the loss of these inhibitory pathways, and hyperactivity of myeloid cells, which drive activation of T cells and inflammatory disease (8–11).

Like most human autoimmune diseases, lupus has a strong genetic susceptibility component that is multigenic in the great majority of patients (1, 12). Among the genes that contribute to lupus susceptibility in humans are those encoding components of Lyn-inhibitory pathways. For example, some individuals of European descent have a single nucleotide polymorphism in the 5′ untranslated region of the Lyn gene that is mildly protective for the development of lupus (odds ratio, 0.80) (12). More impressively, loss-of-function alleles of SIAE, which encodes a sialic acid acetyl esterase that is necessary to create the ligand for CD22, contributes a large increase in the susceptibility to lupus and several other autoimmune diseases (odds ratio, 8) in a small, but significant, fraction of individuals (13). Given that Lyn−/− mice exhibit a mild lupus phenotype (14), it is possible that less frequent alleles of Lyn, other than those examined in genome-wide association analysis, and/or alleles of genes

*Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China; ‡Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94143; §Department of Medicine, University of California, San Francisco, San Francisco, CA 94143; †Department of Laboratory Medicine, University of California, San Francisco, San Francisco, CA 94143; ‡Division of Pathology, Department of Pathology and Diagnostics, University of Verona, Verona 37134, Italy

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Address correspondence and reprint requests to Dr. Anthony L. DeFranco or Dr. Baidong Hou, Department of Microbiology and Immunology, University of California, San Francisco, 513 Parnassus Avenue, Box 0414, HSE-1001F, San Francisco, CA 94143 (A.L.D.) or Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, China (B.H.). E-mail addresses: anthony.defranco@ucsf.edu (A.L.D.) or baidong_hou@sun5.ibp.ac.cn (B.H.)

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Abbreviations used in this article: ANA, anti-nuclear Ab; C3, complement 3; DC, dendritic cell; GC, germinal center; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SmRNP, Smith Ag ribonucleoprotein; TIB, follicular helper T.

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encoding the other components of Lyn-dependent inhibitory pathways contribute significantly to lupus susceptibility in humans.

Recent studies (15) in several mouse models of lupus implicated TLR9 and TLR7 in the spontaneous production of anti-dsDNA and anti-RNP IgG, respectively. For example, MRL/lpr mice are protected from the development of glomerulonephritis when combined with loss-of-function mutation of TLR7, either alone or in combination with mutation of TLR9 (16). Similarly, deletion of the TLR-signaling component MyD88 prevents spontaneous lupus-like disease in Lyn-deficient mice (17). Conversely, the Yaa autoimmune accelerator locus of mice turns out to be a duplication onto the Y chromosome of a small region of the X chromosome that includes TLR7, resulting in increased expression of TLR7 (18–20). The possible relevance of TLR7 and TLR9 to lupus-like autoimmunity was initially suggested by in vitro studies of Marshak-Rothstein and coworkers (15), demonstrating a marked synergy for B cell activation in vitro between BCR engagement and TLR9 or TLR7 engagement. This synergy was shown to operate in vivo as well (21). Although those studies strongly suggest that the contribution of TLR7 and TLR9 to lupus-like autoimmunity is by their action in nucleic acid–recognizing B cells, TLR7 and TLR9 are also potent activators of dendritic cells (DCs) and, moreover, induce type 1 IFN production by plasmacytoid DCs (22). A number of studies implicated type 1 IFNs in the pathogenesis of human lupus (23), so it is also possible that the nucleic acid–recognizing TLRs play key roles in DCs for the development or propagation of lupus-like autoimmunity.

Recently, we used cell type–specific deletion of the key TLR-signaling component MyD88 to dissect the cellular basis for TLR9 stimulation of Ab responses to immunization. We found that in vivo IgG responses to soluble protein–CpG oligonucleotide conjugates were dependent primarily on TLR9/MyD88 signaling in DCs, whereas IgG responses to virus-like particles or inactivated influenza virus particles were primarily dependent on TLR7/MyD88 signaling in B cells (24). In this study, we addressed the role of MyD88 signaling in B cells and in DCs for the spontaneous development of lupus-like autoimmunity in Lyn−/− mice. We found that anti-dsDNA and anti-RNP IgG in this model were likely produced by germinal center (GC) responses that were highly dependent on TLR7/MyD88 signaling in B cells. TLR7/MyD88 signaling in DCs also played a major, although less essential, role in autoantibody production. In contrast, TLR7/MyD88 signaling in DCs was essential for accumulation of activated T cells and splenomegaly, reinforcing the notion that Lyn-deficient autoimmune disease has both autoimmune and inflammatory components and that TLR/MyD88 signaling contributes to both disease manifestations.

Materials and Methods

Mice

B6 (000664; C57BL/6J) mice were from The Jackson Laboratory. Lyn−/− MyD88−/− mice (B6.129P2-MyD88tm1(Tg)Y, MyD88−/− Cd11c-Cre mice, and MyD88−/− Cd79a-Cre mice were described previously (25–27) and were backcrossed onto the C57BL/6 background for ≥10 generations. These mice were bred to generate Lyn−/− MyD88−/−, Lyn−/− MyD88−/− Cd11c-Cre, or Lyn−/− MyD88−/− Cd79a-Cre mice used in this study. Sex-matched littersmates were used in all experiments, and data were pooled and analyzed according to the age group. Sar+ mice (28) were obtained from Dr. Pamela Schwartzberg (National Institutes of Health, Bethesda, MD). tcR+ mice (29, 30) were from The Jackson Laboratory. Animals were housed in specific pathogen–free animal facilities at the University of California, San Francisco or the Institute of Biophysics under conditions that met the respective institutional and national guidelines. Animal use in each facility was approved by the respective Institutional Animal Care and Use Committee.

Anti-nuclear Ab immunofluorescence

Serum was diluted at 1:40 or 1:160 in PBS containing 1% FBS and applied to fixed and permeabilized HEp-2 anti-nuclear Ab (ANA) slides (Bio-Rad).

After overnight incubation at 4˚C, ANAs were detected by Alexa Fluor 488–conjugated goat anti-mouse IgG (Fcy fragment specific; Jackson ImmunoResearch). DAPI was included in the last wash of slides. Slides were visualized with a regular fluorescence microscope under a 40× objective lens and imaged with an Olympus digital camera. Nuclear regions were defined with DAPI staining, and the average Alexa 488 fluorescence intensity of nuclear regions was quantified with ImageJ (National Institutes of Health).

Serum Ig and autoantibody measurement

Levels of serum IgM and IgG were measured using ELISA–based quantification kits (Bethyl Laboratories), according to the manufacturer’s instructions. For anti-dsDNA Ig ELISA, 96-well flat-bottom plates (BD Falcon) were coated with 20 ng/well linearized pUC19 plasmid in 100 mM Tris-HCl (pH 7.3). For anti-Smith Ag ribonucleoprotein (SmRNP) Ig ELISA, plates were coated with 2 μM Smith Ag RNP complex (ImmuNvision) in carbonate buffer (pH 9.6). After overnight incubation at 4˚C, plates were blocked with PBS containing 1% BSA and 0.05% Tween 20 for 1 h. Serially diluted sera were added to the plates and incubated for 2 h at room temperature. After washing with PBS containing 0.05% Tween 20, 200-μl anti-IgM or anti–PD-1 (RMP1-30), anti-TCR chain (RPMI 1640, 25 mM HEPES, 50 μg/ml Liberase [Roche] and 100 μg/ml DNase I [Worthington]), Bone marrow cells were obtained by flushing tibia and femur with medium. Kidney cells were prepared as previously described (8). Briefly, kidneys were pressed through a 70-μm cell strainer (BD Falcon). After washing, the cells were resuspended in 33% Percoll solution and centrifuged at 2000 rpm for 20 min at room temperature. Kidney-infiltrating cells were obtained from cell pellets. For staining for flow cytometry, single-cell suspensions were blocked with anti-CD16/CD32 Ab and then stained with fluorescent Abs in ice-cold flow cytometry buffer (PBS supplemented with 2 mM EDTA, 1% heat-inactivated FBS, and 0.02% sodium azide). The Abs included FITC-labeled anti-Ly6G (1A8), anti-Ly6C (HK1.4), anti-CD44 (IM7), anti–GL-7, and anti–CD43 (57) Abs; PE-labeled anti-CD86 (GL1), anti–PD-1 (RMP1-30), anti–TCRβ (H57-597), and anti–CD23 (B3B4) Abs;
PerCP-Cy5.5-labeled anti-F4/80 (BM8), anti-ICOS (7E.17G9), and anti-IgM (RM-M-1) Abs; PE-Cy7-labeled anti-CD11b (N1/170), anti-CD95 (Jo2), and anti-CD62L (MEL-14) Abs; allotrophocytin- or Alexa Fluor 647-labeled anti-CD11c (HL3), anti-CD8 (53-6.7), and anti-CD38 (28-1-2), and anti-CD26L (MEL-14) Abs; Alexa Fluor 700-labeled anti-I-A<sup>ß</sup> (M5/114.15.2), anti-CD11b (M1/70), and anti-CD45.2 (104) Abs; allotrophocytin-Cy6 (6D6), and anti-CD3 (AA4.1) Abs were detected with streptavidin-conjugated Pacific Orange (Invitrogen). After the final wash, the cells were resuspended in flow cytometry buffer containing 0.4 μM DAPI (Invitrogen). All data were collected on an LSR II flow cytometer (Becton Dickinson) and analyzed with FlowJo software (TreeStar).

Immature, transitional, and mature B cell populations were identified in the spleen and bone marrow of mice using flow cytometry. B cells against surface B220, CD23 (BD Biosciences), CD93 (clone AA4.1; eBioscience), and IgM F(ab’<sup>2</sup>) antigen (Jackson ImmunoResearch). In the spleen, surface CD3<sup>+</sup> was used to distinguish immature (B220<sup>+</sup>, CD93<sup>+</sup>) and mature (B220<sup>+</sup>, CD93<sup>+</sup>) B cells. Within the immature B cell population, surface expression of CD23, CD21, IgM, and CD43 were used to identify immature-transitional T1 (IgM<sup>+</sup>, CD23<sup>lo-neg</sup>), T2 (IgM<sup>+</sup>, CD23<sup>lo-neg</sup>), and T3 (IgM<sup>+</sup>, CD23<sup>lo-neg</sup>) subsets. Within the mature B cell population, surface expression of CD23, CD21, IgM, and CD43 were used to distinguish follicular B cells (CD23<sup>+</sup>, CD21<sup>+</sup>, IgM<sup>+</sup>, CD43<sup>+</sup>), and B1 B cells (CD23<sup>+</sup>, CD21<sup>+</sup>, IgM<sup>+</sup>, CD43<sup>+</sup>). These markers were also used to distinguish immature and pro/pre-B cells (B220<sup>+</sup>, CD93<sup>+</sup>) and mature recirculating B cells (B220<sup>+</sup>, CD93<sup>+</sup>) in the bone marrow. Surface IgM and CD23 levels within the CD3<sup>+</sup> population distinguish newly formed immature B cells (IgM<sup>+</sup>, CD23<sup>lo-neg</sup>), BM-T1 cells (IgM<sup>+</sup>, CD23<sup>lo-neg</sup>), BM-T2 cells (IgM<sup>+</sup>, CD23<sup>+</sup>), and pro/pre-B cells (IgM<sup>+</sup>, CD23<sup>-</sup>). Absolute cell numbers within each population were back-calculated from total splenocytes or total cells.

RNA extraction and quantitative RT-PCR

For quantifying cytokine expression, mouse spleens were harvested and snap-frozen in liquid nitrogen. Total RNA was extracted with the RNAeasy kit (QIAGEN) with on-column DNase digestion. cDNA was transcribed from total RNA with the iScript cDNA Synthesis Kit (Bio-Rad). The primer pairs for IL-12p40, IL-6, and BAFF were described previously (8). Transcripts were quantified by PCR with iTaq SYBR Green SuperMix with ROX (Bio-Rad), and the levels of cytokine transcripts were normalized to the levels of HPRT mRNA.

Statistical analysis

Statistical significance for data with multiple groups was calculated with a one-way ANOVA; if a significant difference was observed among the groups, the Bonferroni posttest was used to assess the statistical difference between specific groups and either the wild-type (WT) group or the Lyn<sup>−/−</sup> group. In cases in which there was a large difference between the WT group and the three groups with Lyn deficiency (B cell number data, Fig. 5E, Fig. 6B, Supplemental Fig. 1B), this analysis was inadequate to detect differences between the Lyn-deficiency groups, because all were greatly different from the WT group. In these cases, a secondary statistical analysis was performed with just the Lyn-deficiency groups. In the figures, this secondary statistical analysis is denoted by different symbols. When there were three such groups, this secondary analysis again used one-way ANOVA; however, in cases in which only two groups remained, the Mann–Whitney U test was used. All <i>p</i> values ≤ 0.05 were considered significant and are indicated in the figures.

Results

Ablation of MyD88 signaling in either B cells or DCs attenuates autoimmunity in Lyn<sup>−/−</sup> mice

To examine the contribution of TLR signaling in B cells and DCs to the spontaneous autoimmunity caused by Lyn deficiency, mice with a conditional allele of Myd88 (Myd88<sup>lo</sup>) were crossed to Lyn<sup>−/−</sup> mice, and transgenic Cre alleles that express selectively in B cells (Cd79a-Cre) or in DCs (Cd11c-Cre) were also introduced. Progeny of genotypes Lyn<sup>−/−</sup> Myd88<sup>lo</sup>, Lyn<sup>−/−</sup> Myd88<sup>lo</sup>Cd79a-Cre, or Lyn<sup>−/−</sup> Myd88<sup>lo</sup>Cd11c-Cre (hereafter referred to as Lyn<sup>−/−</sup>, Lyn<sup>−/−</sup>B-Myd88<sup>lo</sup>, and Lyn<sup>−/−</sup>DC-Myd88<sup>lo</sup>, respectively) were generated, housed under specific pathogen–free conditions, and examined for autoimmune phenotypes at different ages.

The development of autoantibodies by these mice was initially screened by ANA immunofluorescence, with mouse sera collected at 5–7 mo of age. As previously described (4, 5), most Lyn<sup>−/−</sup> mice exhibited elevated titers of ANAs by this age (Fig. 1A, 1B). The development of IgG ANAs was almost completely ablated in Lyn<sup>−/−</sup>B-Myd88<sup>−/−</sup> mice, and it was substantially attenuated in mice lacking MyD88 selectively in DCs (Fig. 1A, 1B).

Next, we used ELISA to examine the development of Abs to dsDNA and smRNP, two major types of ANAs seen in SLE patients (1), as well as in animal models of SLE. All Lyn<sup>−/−</sup> mice developed IgM autoantibodies against both of these nuclear Ags, typically by 4 mo of age, and a substantial majority also developed IgG anti-dsDNA and IgG anti-smRNP. Deletion of MyD88 in DCs delayed production of these autoantibodies, but 8–10-mo-old mice still had substantial titers of IgG and IgM anti-dsDNA and IgM anti-smRNP. In contrast, deletion of MyD88 in B cells prevented the production of IgG autoantibodies in Lyn<sup>−/−</sup>B-Myd88<sup>−/−</sup> mice at all time points tested and attenuated the production of IgM autoantibodies (Fig. 1C–F). Total serum IgM and IgG levels were somewhat reduced in these mice compared with Lyn<sup>−/−</sup> mice (Fig. 1G, 1H), but the differences were generally ≤2-fold, indicating that there was a selective defect in the production of autoantibodies.

Anti-dsDNA IgG Abs are often deposited in the glomeruli of the kidneys and can lead to glomerulonephritis, as observed in Lyn-deficient mice (8, 31). Indeed, immunofluorescent staining clearly revealed a large amount of C3 deposition in the glomeruli of most of the 5–7-mo-old Lyn<sup>−/−</sup> mice (Fig. 2A, 2B). In agreement with the almost complete abolishment of IgG autoantibodies in Lyn<sup>−/−</sup>B-Myd88<sup>−/−</sup> mice, C3 deposition was not evident in the glomeruli of these mice at a corresponding age. Moreover, the nephritis typically seen in old Lyn<sup>−/−</sup> mice by H&E staining of kidney tissue sections was substantially decreased, but not completely absent, in Lyn-deficient mice deleted for MyD88 in either B cells or DCs (Fig. 2C, 2D); the numbers of infiltrating inflammatory cells (CD4<sup>+</sup>), mainly CD11b<sup>+</sup> neutrophils and macrophages, were correspondingly reduced (Fig. 2E). The splenomegaly and myeloproliferation that are characteristic of aged Lyn<sup>−/−</sup> mice (≥6 mo old) were corrected by deletion of Myd88 in DCs but apparently not by deletion of MyD88 in B cells (Fig. 3A, 3B, Suplemental Fig. 1A). Together, these results are consistent with the observation that ablation MyD88 signaling in B cells or DCs reduces IgG autoantibody production in Lyn<sup>−/−</sup> mice but also indicates that some of the inflammatory phenotypes are independent of autoantibody production.

Autoantibody production in Lyn<sup>−/−</sup> mice requires SAP expression and T cell help

Abs against dsDNA obtained from MRL/lpr mice exhibit somatic mutations that increase reactivity to DNA (32), which is consistent with a GC origin for these autoantibody responses. However, Ig-transgenic B cells with a rheumatoid factor specificity in these mice make an extracellular Ab response with substantial numbers of somatic mutations, even in the absence of Ag-specific T cell help (21, 33). Moreover, mice overexpressing BAFF also spontaneously produce ANAs in a T cell–independent fashion (34). Thus, there are precedents for extracellular production of ANAs in some mouse models.

To address this issue, we first examined Lyn<sup>−/−</sup> mice for the frequency of GC phenotype B cells. The frequency of CD95<sup>−/−</sup>G-7<sup>−</sup> GC B cells relative to the number of splenic B cells already was significantly increased in Lyn<sup>−/−</sup> mice compared with WT mice.
by 2–4 mo, and this increase became more dramatic at 5–7 mo (Fig. 3C, 3D), correlating with the time course of the development of IgG ANAs. However, it should be noted that Lyn−/− mice have substantially fewer splenic B cells than do WT mice (Supplemental Fig. 1B), so the absolute numbers of GC B cells were similar in Lyn−/− mice and WT mice (Fig. 3E). In agreement with flow cytometry analysis, staining of spleen sections also clearly revealed the existence of IgDlowGL-7+ GC structures in multiple follicles of Lyn−/− mice (Fig. 3F) at 5–7 mo of age. In contrast, GCs were not common in WT mice housed in the same mouse room (data not shown). Interestingly, the spontaneous GC response seen in Lyn−/− mice was greatly reduced upon deletion of Myd88 in B cells (Fig. 3D, 3E). In contrast, the mesenteric lymph nodes of Lyn−/− and Lyn−/− B-Myd88−/− mice contained similar numbers of GC B cells (data not shown), suggesting that the latter mice did not have a general defect in mounting GC reactions.

To examine whether Th cells and the GC reaction contribute to the generation of IgG ANAs, we crossed Lyn−/− mice to mice deleted for TCR genes (tcrβ−/− tcrδ−/−) or to mice lacking the SAP signaling adaptor protein, which is critical for follicular helper T (Tfh) cells to interact with GC B cells and promote GC Ab responses (35, 36). Lyn-deficient mice lacking T cells or lacking the SAP adaptor molecule made greatly attenuated levels of anti-dsDNA IgG Abs, as measured by ELISA (Fig. 3G). These genetic results strongly support the hypothesis that there is a GC-origin of lupus-like autoantibodies in Lyn-deficient mice.

Activation and expansion of self-reactive T cells in Lyn−/− mice require MyD88 signaling in DCs and B cells

Lyn−/− mice exhibit a dramatic expansion of activated T cells as autoimmune disease progresses (8, 37). Therefore, we next investigated the role of MyD88 in DCs and B cells in the hyper-reactivity of T cells in Lyn−/− mice. As previously reported (8), Lyn−/− mice accumulate increasing numbers of CD4+ and CD8+ T cells with an activated or effector memory phenotype (CD44+ CD62L−) as they age. As Lyn-deficient mice lacking MyD88 in

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**FIGURE 1.** Roles of MyD88 signaling in B cells and in DCs for development of autoantibodies in Lyn−/− mice. (A and B) Anti-nuclear IgG Abs of individual mice were screened using sera of 5–7-mo-old mice. Representative ANA fluorescence images (original magnification ×40) of 1:40 diluted sera of the indicated genotype (A), as well as summarized relative fluorescence intensity of sera from individual mice (C) and means of each mouse group (bars) (B). Serum IgM and IgG Abs to dsDNA (C, D) and to anti-smRNP (E, F) of individual mice of the four genotypes indicated and of different age groups were measured by ELISA. The relative amount of autoantibody in each mouse (C) and the mean of each mouse group (bars) shown. (G and H) Total serum IgM and IgG levels of mice of the different genotypes and age groups were quantified by ELISA. Data are presented as mean ± SE of WT (n = 6, 6, 6 mice, respectively, in each age group), Lyn−/− (n = 6, 13, 11), Lyn−/− B-MyD88−/− (n = 6, 10, 7), and Lyn−/− DC-MyD88−/− (n = 7, 8, 7) mice. Differences between WT or Lyn−/− and other groups are indicated by brackets. *p < 0.05, **p < 0.01.
FIGURE 2. Effect of ablation of MyD88 signaling in B cells and in DCs for complement deposition and inflammation in the kidneys of Lyn−/− mice. Deposition of C3 was detected by immunofluorescent staining of frozen kidney sections of 5–7-mo-old mice of the indicated genotype; representative images (original magnification ×20) (A) and summarized data (B) are shown. (C) Kidney sections of the different genotypes at 8–10 mo of age were stained with H&E to assess pathological changes associated with glomerulonephritis (original magnification ×40). (D) Sections from individual mice were graded, in a blinded fashion, on a scale of 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe) for the degree of histological abnormality in the glomeruli and for the degree of inflammation in the interstitial regions. (E) Sections from individual mice were graded, in a blinded fashion, on a scale of 0 to 3 (0 = absent, 1 = mild, 2 = moderate, 3 = severe) for the degree of histological abnormality in the glomeruli and for the degree of inflammation in the interstitial regions. (F) Numbers of inflammatory cells in the kidneys were measured by flow cytometry in six mice of each genotype at 8–10 mo of age. Data are mean ± SE and are representative of two separate experiments.

*p < 0.05, **p < 0.01.

B cells or in DCs aged, these populations did not increase at all (CD4+ T cells) or nearly as much (CD8+ T cells) (Fig. 4A–C, Supplemental Fig. 1C, 1D). Thus, increased spontaneous activation of T cells in Lyn−/− mice was largely dependent on MyD88 signaling in both DCs and B cells.

Many of the activated CD4+ T cells also expressed ICOS and PD-1, two markers that have been used to identify Tfh cells. The percentage and absolute number of those cells already were slightly elevated in Lyn−/− mice at 2–4 mo of age compared with WT mice, and they especially increased in older mice (Fig. 4E, 4F). In another recent study (38), the number of Tfh cells at 4–5 mo of age was roughly normal in WT mice, and they especially increased in older mice (Fig. 4E, 4F). Further, deletion of MyD88 in either DCs or B cells substantially reduced the expression of these cells in Lyn−/− mice (Fig. 4E, 4F).

Splenic B cells and DCs in Lyn−/− mice have elevated expression of MHC class II and CD86 that is not dependent on MyD88 signaling

TLR agonists have well-established adjuvant properties for promoting T cell responses (39). To investigate the possibility that TLRs of DCs or B cells promote their ability to present self-Ags to T cells, we examined the expression of MHC class II and of the costimulatory molecule CD86 on splenic B cells and on DCs of Lyn−/− mice with or without DC or B cell MyD88. In Lyn−/− mice, an increased proportion of B cells exhibited elevated levels of MHC class II and CD86 by 2–4 mo of age (Fig. 5A, 5B). The number of such B cells was not dependent on the expression of MyD88 in B cells (Fig. 5A, 5B), suggesting that the loss of Lyn-based attenuation of BCR signaling in B cells may be sufficient for this phenotypic alteration. Similarly, DCs in young Lyn−/− mice had spontaneously elevated MHC class II expression and CD86 expression. Deletion of MyD88 in DCs had little effect on their expression of MHC class II and CD86 (Fig. 5C, 5D). Together, these data indicate that the requirement for MyD88 in B cells and in DCs for development of autoimmunity in Lyn−/− mice was not caused by changes in the expression of MHC class II and CD86.

Next, we examined the role of MyD88 signaling in DCs and B cells in Lyn−/− mice for the induction of cytokines that have been implicated in the pathogenesis of autoimmunity and inflammation in this model (8, 40). In total splenocytes from Lyn−/− mice at 2 mo of age, increased mRNA expression of the cytokines IL-12p40 and BAFF was readily detected (Fig. 5E–G), demonstrating that enhanced inflammatory responses arise even before the development of overt autoimmunity in these mice. Interestingly, deletion of Myd88 in DCs substantially reduced the expression of IL-12p40 and IL-6 in the spleens of young Lyn−/− mice (Fig. 5E, 5F), suggesting that Lyn-deficient DCs are sensitized to have enhanced responses to endogenous or ubiquitous TLR ligands and are a major producer of proinflammatory cytokines in these mice. This is in agreement with other recent reports (10, 11) describing a proinflammatory role for Lyn-deficient DCs. As mentioned above, the incidence of splenomegaly was greatly reduced in aged (8–10 mo) Lyn−/− DC-MyD88−/− mice compared with Lyn−/− mice (Fig. 3A, 3B). In comparison, deletion of...
B cell Myd88 had little or no effect on the expression of IL-12p40 or BAFF (Fig. 5E–G).

**Effect of cell-intrinsic MyD88 signaling on peripheral numbers of B cells in Lyn−/− mice**

Lyn deficiency has substantial B cell–intrinsic effects on the numbers of B cells in the spleen (8), decreasing their overall numbers (Supplemental Fig. 1B), apparently as a result of enhanced cell death at the T1 or T2 immature stages in the spleen and decreased maturation to the follicular mature state and/or decreased survival of these cells (41–43). Interestingly, although deletion of Myd88 in Lyn−/− B cells did not affect bone marrow populations of B cell precursors or immature B cells compared with Lyn−/− mice (Fig. 6A), the decreased number of B cells in the spleens of young Lyn−/− mice was further exacerbated by deletion of Myd88 in B cells (Fig. 6B). The reduction in B cells in Lyn−/− B-Myd88−/− mice began at the T1 stage in the spleen and persisted in subsequent developmental stages until maturation (Fig. 6B, 6C). Despite these decreases in mature B cell number, the numbers of plasma cells in the spleen were elevated in Lyn−/− and Lyn−/− B-Myd88−/− mice compared with WT mice.
These results suggest that MyD88 signaling in B cells promotes their survival at the immature T1 stage in the spleen, but it does not greatly affect maturation or survival at subsequent stages of development in the spleen and is not required for differentiation into plasma cells, in agreement with the circulating levels of total IgM and IgG in these mice (Fig. 1G, 1H). We observed a modest increase in the percentage of CD93+CD23+IgMlow T3 B cells and a small decrease in the percentage of follicular B cells in the spleens of Lyn−/−B-Myd88−/− mice, such that the ratio of T3/follicular B cells was slightly, but significantly, increased by the loss of MyD88 in B cells (Fig. 6D). The T3 B cell population previously was shown to include many anergic autoreactive B cells (44). Our data suggest that intrinsic MyD88 signaling may keep some self-reactive B cells from becoming anergic, although other explanations are possible.

Discussion

Lyn is an intracellular protein tyrosine kinase that is critical for inhibitory receptor function in B cells and in DCs, and Lyn−/− mice spontaneously develop a lupus-like autoimmune and inflammatory disease (4, 5). To address the mechanism by which nucleic acid–recognizing TLRs contribute to spontaneous production of ANAs in the Lyn−/− mouse model of SLE, we used cell type–specific deletion of the gene encoding MyD88, a key adaptor molecule required for intracellular signaling by most TLRs, including TLR7 and TLR9. Deletion of Myd88 selectively in B cells completely blocked production of the anti-dsDNA and anti-RNP IgG Abs, which are among the most characteristic autoantibodies seen in human SLE and are rarely produced in other human diseases (1), and ameliorated glomerulonephritis. In addition, deletion of Myd88 selectively in DCs delayed production of IgG anti-dsDNA, blocked production of IgG anti-smRNP, largely abrogated accumulation of activated phenotype T cells in the spleen, and ameliorated glomerulonephritis. These results demonstrate that TLR/MyD88 signaling is required in both B cells and DCs for the development of autoimmune disease in these mice.

The demonstration that MyD88/TLR signaling in B cells is required for anti-nuclear IgG production in Lyn−/− mice adds to the accumulating evidence indicating that dual stimulation of DNA- and RNP-specific B cells by the BCR and by TLR9 or TLR7, respectively, is a key underlying mechanism in the breakdown of tolerance to nuclear self-Ags in mouse models of lupus (15, 45, 46). Although TLR signaling can boost Ab responses in multiple ways (47), we recently showed that it can boost GC responses dramatically in response to virus-like particles and inactivated virions (24). It is likely that an analogous mechanism, perhaps in response to fragments from apoptotic cells, participates in the production of anti-dsDNA and anti-RNP IgG Abs in Lyn−/− mice, because it was largely dependent on Myd88 expression in B cells, on the presence of T cells, and on the expression of SAP, which is required for GC responses (Fig. 3G). SAP is an adaptor for SLAM family adhesion molecules, and its function in T cells is important at early stages of the GC response by acting to stabilize interactions between activated B cells and cognate Th cells (36). Moreover, a recent report from another group (38) found that Lyn−/−IL-21−/− mice failed to produce anti-dsDNA IgG Abs, which is also consistent with an
important role for the GC response in the production of these Abs, given the known role of IL-21 in the GC response. Curiously, in that study, glomerulonephritis was not ameliorated despite a substantial decrease in most IgG autoantibodies. Thus, a number of results strongly implicate the GC response in the production of anti-nuclear IgG Abs in Lyn-deficient mice.

Our finding of a requirement for T cells in the autoimmunity of Lyn\(^{-/-}\) mice is consistent with previous data demonstrating that the T cell production of IFN-\(\gamma\) plays a critical role in the autoimmune phenomena of Lyn-deficient mice (8) and that IL-10 from B cells decreases the inflammatory reaction generated by T cells in these mice (9). Interestingly, a previous study (48) found that CTLA4-Ig blockade of T cell costimulation via CD28 prevented IgG ANA production but there was now production of IgA. The mechanism of IgA ANA production in these treated mice is unclear at this time.

The GC response has been implicated in lupus-like autoantibody production in several other mouse models of SLE, including the NZB \(\times\) NZW F1 mouse (46, 49, 50). However, it should be noted that autoantibody production occurs independently of T cells in BAFF-overexpressing transgenic mice (34), or in MRL/lpr mice expressing a rheumatoid factor Ig transgene (21). Therefore, the nature of the genetic alterations causing susceptibility to SLE apparently can affect, to some degree, the underlying immunological mechanism of autoantibody production. These results suggest that human SLE may also be mechanistically diverse; therefore, it will be of interest to develop methods for analyzing immune cells from SLE patients to assess such possible heterogeneity because this may be relevant to their treatment.

In previous studies (24) of the role of MyD88 signaling in B cells versus DCs for Ab responses to Ag–CpG oligonucleotide conjugates, we found that the cellular requirement for an optimal IgG response to the Ag depended on the physical form of the Ag. When soluble protein Ags, such as OVA or the ragweed pollen Ag Amb a1, were used, MyD88 in DCs was required, and MyD88 in B cells did not contribute to the response. In contrast, when virus-like particles containing TLR9 or TLR7 ligands or chemically inactivated influenza virus particles were used as immunogen, the IgG response to viral coat proteins was greatly augmented by MyD88 signaling in B cells but not by MyD88 signaling in DCs. Moreover, for virus-like particles, the magnitude of the TLR effect was dependent on the epitope density of the Ag on the particle, indicating that stronger BCR signaling enabled TLR7 or TLR9 in B cells to enhance the response (24). This enhancement occurred by promotion of the GC component of the response (24). Conversely, weak BCR signaling, as likely occurs with the soluble protein Ags, did not enable B cell TLR9 to boost the IgG response. Based on the results obtained in this study and the results obtained previously, we propose that the true autoantigen in SLE is a particulate form of chromatin and/or RNP, such as occurs on
apoptotic fragments released from dying cells. This proposal is consistent with the fact that many autoantibodies obtained from mouse SLE-prone strains bind to apoptotic blebs (51).

The observation mentioned above—that strong BCR signaling is required to enable TLR7 or TLR9 signaling in B cells to enhance their GC response—suggests why Lyn deficiency of B cells creates a strong susceptibility for the development of anti-dsDNA and anti-RNP IgG Abs. Low-affinity DNA- or RNP-reactive mature or anergic follicular B cells in WT mice presumably have weak BCR signaling that is attenuated by the Lyn/CD22/SHP-1 feedback-inhibitory pathway; therefore, even if they acutely encounter apoptotic blebs, their low level of BCR signaling does not synergize with TLR7 or TLR9 signaling to promote a GC response. In contrast, Lyn-deficient B cells of the same specificity have exaggerated BCR signaling; therefore, we hypothesize that they can enter into and participate in GC responses, leading to the production of class-switched and affinity-matured pathogenic autoantibodies.

Several recent studies (10, 11) implicated dysregulation of DCs by loss of Lyn as also being an important contributor to the autoimmune phenomena in Lyn<sup>−/−</sup> mice. Consistent with this view is our demonstration that MyD88 signaling in DCs contributes importantly to the autoimmune phenotypes of Lyn<sup>−/−</sup> mice. The characteristic expansion of activated phenotype T cells that is seen in Lyn<sup>−/−</sup> mice as they get older was abrogated by deletion of MyD88 in DCs (Fig. 4), indicating that TLR/MyD88 signaling in DCs provides a necessary activation signal that synergizes with the effects of loss of Lyn-dependent inhibitory signaling in these cells. This interpretation is supported by the recent report (11) that mice in which Lyn is deleted only in DCs also develop a severe lupus-like autoimmunity. Thus, we propose that Lyn<sup>−/−</sup> mice develop a severe lupus-like autoimmunity and inflammatory disease as a result of the combination of at least two defects: the deficiency of Lyn in B cells compromises their cell-intrinsic tolerance mechanisms by allowing TLR7 and TLR9 to promote activation of self DNA- and RNP-reactive B cells, and the dysregulation of Lyn-deficient DCs leads to excessive activation of T cells, which, in turn, can promote increased affinity IgG responses of the DNA- and RNP-specific B cells. In addition, Lyn-deficient DCs and the T cells activated by them provide a self-reinforcing inflammatory response that can also contribute to inflammatory disease (8, 11). This latter component of the disease of Lyn-deficient mice is especially evident if Lyn is selectively deleted in DCs (11) or if the B cells are unable to produce IL-10 to inhibit it (9).

Studies in mouse models indicate that the presence of genetic susceptibility loci is required for spontaneous breakdown of tolerance to nuclear autoantigens. Although most natural susceptibility loci in human and mouse remain poorly understood, a subset of identified loci alters regulation of BCR signaling, including ablation of Lyn (4, 31), B cell–specific deletion of SHP-1 (52), deletion of CD22 (53), and a point mutation of CD45 that increases the activity of some Src family tyrosine kinases while decreasing the activity of Lyn (54, 55). Strikingly, Lyn, CD22, and SHP-1 work together in a feedback-inhibitory pathway to limit BCR signaling, especially in mature B cells (4, 56). Genetic analysis in human SLE patients suggests that this pathway is likely compromised in some SLE patients (12, 13). Thus, the Lyn<sup>−/−</sup> mouse model of lupus is highly relevant to a subset of human SLE patients. Our results indicate that TLR/MyD88 signaling is likely to be necessary for ANA production in this subset of patients.

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Disclosures

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References


