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Autoimmune Disease in Lyn-Deficient Mice Is Dependent on an Inflammatory Environment Established by IL-6

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Lyn-deficient mice develop Ab-mediated autoimmune disease resembling systemic lupus erythematosus where hyperactive B cells are major contributors to pathology. In this study, we show that an inflammatory environment is established in Lyn−/− mice that perturbs several immune cell compartments and drives autoimmune disease. Lyn−/− leukocytes, notably B cells, are able to produce IL-6, which facilitates hyperactivation of B and T cells, enhanced myelopoiesis, splenomegaly, and, ultimately, generation of pathogenic autoreactive Abs. Lyn−/− dendritic cells show increased maturation, but this phenotype is independent of autoimmunity as it is reiterated in B cell-deficient Lyn−/− mice. Genetic deletion of IL-6 on a Lyn-deficient background does not alter B cell development, plasma cell accumulation, or dendritic cell hypermatur, suggesting that these characteristics are intrinsic to the loss of Lyn. However, hyperactivation of B and T cell compartments, extramedullary hematopoiesis, expansion of the myeloid lineage and autoimmune disease are all ameliorated in Lyn−/−/−IL-6−/− mice. Importantly, our studies show that although Lyn−/− B cells may be autoreactive, it is the IL-6-dependent inflammatory environment they engender that dictates their disease-causing potential. These findings improve our understanding of the mode of action of anti–IL-6 and B cell-directed therapies in autoimmune and inflammatory disease treatment. The Journal of Immunology, 2010, 184: 000–000.

Inflammation has evolved to protect the host against infectious pathogens; however, many pathological conditions, including autoimmune diseases, are triggered and sustained by persistent inflammation (1–3). There is evidence that during adaptive immune responses against pathogens, the stimulation of immune effector cells and the release of proinflammatory cytokines can lead to the establishment of autoimmune disease in susceptible individuals (reviewed in Ref. 4). Indeed, bacteria and viruses can have epitopes similar to self-Ags (5, 6). In these cases, the recognition of a foreign Ag may lead to the recognition of self by molecular mimicry, and as a consequence of these infections, a prolonged immune response can lead to the establishment of chronic inflammation and autoimmune disease (7–9). In addition, the inflammatory cascade accompanying infection may be sufficient to stimulate self-reactive bystander cells (10, 11), which would normally be suppressed. Given that conventional anti-inflammatory agents are variably effective in autoimmune diseases, an understanding of the specific role of inflammation in autoimmune pathology is imperative as we seek to improve disease management.

Autoimmune diseases are thought to arise via loss of tolerance to self-Ags, with a reduced threshold of immune cell activation being a vital predisposing factor in the initiation of autoimmunity (12, 13). Damage can be mediated by both autoreactive T cells and B cells, with much effort over the last decade having been spent in trying to identify and classify autoimmunity susceptibility loci. Studies on naturally occurring mouse mutants (lpr, gld, moth-eaten), genetically susceptible strains (NZB, NZW, BXSb), gene-targeted mice (FcγRIIIB−/−, C1q−/−, TACI−/−, PTEN+/−), and transgenic (Tg) animals (BAFF Tg) have provided important insights into autoimmune diseases, and have made valuable contributions to defining the genetics of autoimmune diseases such as systemic lupus erythematosus (SLE) (reviewed in Ref. 14). One well characterized mouse model of Ab-mediated autoimmune disease is the germline loss-of-function mutation in the Src-family protein tyrosine kinase Lyn (15–17). Lyn-deficient mice develop autoimmunity in a manner akin to SLE in humans (15–17). In this disease, the production of autoreactive Abs against cellular elements, primarily nuclear, leads to immune complex-mediated glomerulonephritis; a common manifestation of humoral autoimmune diseases. The mechanisms behind autoimmune disease development in Lyn−/− mice have been studied in detail, and include a dominant B cell hyper-responsiveness, a lowered threshold to B cell stimulation, and an inability to downmodulate signaling after stimulation (17–21). The biochemical basis for these phenotypes lies in the ability of Lyn to phosphorylate inhibitory receptors in B cells that contain immunoreceptor tyrosine-based inhibition motifs, which ordinarily recruit negative regulatory phosphatases, including SHIP-1 and Src homology region 2 domain-containing phosphatase 1 (reviewed in Ref. 22).

B cells play a major role in the development of autoimmune disease in Lyn-deficient mice. Lyn-deficient mice lacking B cells fail to develop autoimmune disease (23), as do Lyn−/− mice with null mutations in molecules essential for early B cell activation such as Btk and CD19 (24–26). Although few studies have investigated the role of other hematopoietic cells and the contribution of cytokines, a recent report has shown that disruption of TLR
signaling in Lyn−/−MyD88−/− mice prevented the production of pathogenic auto-Abs and associated glomerulonephritis, and has suggested a role for proinflammatory cytokine secretion by dendritic cells (DCs) in disease pathogenesis (27). Indeed, Lyn is suggested a role for proinflammatory cytokine secretion by den-
pathogenic auto-Abs and associated glomerulonephritis, and has inflammatory environment. We have shown that although Lyn−/− B cells have the capacity to produce class-switched, pathogenic auto-Abs, it is the inflammatory environment in which the B cells reside that determines their propensity to do so. These findings contribute to a better understanding of the mode of action of anti-
IL-6 and B cell-directed therapies, and to their future refinement in the treatment of diverse autoimmune conditions.

Materials and Methods

Animals

Lyn−/− mice (15) were crossed to the C57BL/6 background for 20 gen-
erations. IL-6−/− mice (30) on the C57BL/6 background were obtained from the Walter and Eliza Hall Institute of Medical Research. Lyn−/− IL-
6−/− mice were derived by intercrossing. Lyn−/− mice lacking B cells (Lyn−/−MjM−/− mice) were generated by crossing C57BL/6 Lyn−/− mice with C57BL/6 mice carrying a mutation in the membrane exon of the μ-chain gene (31). C57BL/6 mice were used as controls, and mice were either young (8–12 wk) or aged (30–wk). Experiments were performed in accordance with guidelines for the care and use of animals for scientific purposes established by the National Health and Medical Research Council of Australia, and approved by the Ludwig Institute for Cancer Research/ Department of Surgery Animal Ethics Committee.

Flow cytometry

Single-cell preparations of the spleen were obtained by extruding cells, followed by passage through 40-μm filters. Three-color flow cytometry was used to assess cell populations, cellular activation, and IL-6 production. The mAbs conjugated to FITC, PE, and APC were from BD Pharmingen. For intracellular cytokine staining, spleno-
cytes were incubated with brefeldin-A (Sigma-Aldrich, Castle Hill, Aus-
tralia) overnight, surface stained, fixed with MTPBS/2% paraformaldehyde/0.2% Tween-20, washed, and then stained with PE-conjugated anti–IL-6 (BD Pharmingen). Cells were analyzed on a FACS-
Calibur system (BD Biosciences, San Jose, CA) after exclusion of dead cells with propidium iodide. Data were analyzed using FlowJo software (Stanford University, Stanford, CA) and presented in the form of two-
color dot plots or histograms after gating of indicated cell populations.

ELISAs

Mice were bled fortnightly to monthly from the tail vein. To detect anti-
nuclear Abs (ANAs), ELISA plates precoated with purified nuclear Ags (The Binding Site, Birmingham, U.K.) were incubated with serum and bound Abs detected with HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Serum Ig titters were determined by ELISA using goat anti-mouse Ig as a capture agent and a polyclonal goat anti-mouse HRP conjugate to the relevant specific Ig as the detecting agent (Southern Biotechnology Associates). Serum IL-6 levels were determined using an OptEIA kit from BD Biosciences.

Proliferation assay

Splenocytes were isolated from 10-wk-old mice and red cells removed by Tris-buffered ammonium chloride lysis. Single-cell suspensions were incu-
bated with biotinylated Abs to CD5, CD43, Mac-1, CD11c, Ter-119, and Gr-1 (BD Pharmingen) and non-B cells were depleted using streptavidin microbeads (Miltenyi Biotec, North Ryde, Australia). Purified cells were resuspended in B cell proliferation media (DMEM, 5% FCS, 100 U/ml penicillin-streptomycin, and 4 mM glutamine) and cultured for 3 d with either no stimulus, 10 μg/ml F(ab′)2 goat anti-mouse IgM (Jackson Im-
munoresearch Laboratories, West Grove, PA), or 5 μg/ml LPS. On day 3, cells were pulsed with [3H]-thymidine for 6 h prior to harvesting and β-scintillation counting (PerkinElmer, Wellesley, MA).

Colony assays

Myeloid progenitors were quantitated using semisolid agar cultures (32).

Histology

Kidneys were fixed in Bouin’s reagent for 24 h, washed in alcohol and embedded in paraffin. For light microscopy, sections were cut and stained with H&E.

Immunofluorescent staining

Kidneys were harvested from 42- to 46-wk-old mice in optimal cutting temperature medium and snap-frozen in liquid nitrogen. Cryostat sections (5 μm) were cut onto glass slides and acetone-fixed. Sections were rehy-
drated in MT-PBS for 30 min, blocked in 5% BSA/M-T-PBS for 30 min, and then stained with the indicated Abs for 60 min. Sections were then washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Anti-mouse IgM-biotin (BD Pharmingen) was used at 5 μg/ml, followed by streptavidin-FITC (BD Pharmingen) at 5 μg/ml. Sheep anti-
nouse IgG-FITC (Jackson ImmunoResearch Laboratories) was used at 15 μg/ml, and FITC-conjugated F(ab′)2 goat anti-mouse C3 fragment (Cupp Laboratories, Cochrannie, PA) was used at 10 μg/ml.

Statistical analysis

Statistical analysis was performed with the Mann-Whitney nonparametric U test using GraphPad Prism software (GraphPad, San Diego, CA). Sta-
tistically significant differences between groups of data are indicated by asterisk(s), whereas nonsignificant differences are unmarked.

Results

Perturbed B cell development and plasmacytosis in Lyn−/− mice

The B cell defects and development of Ab-mediated autoimmune disease in Lyn−/− mice have been documented by three in-
dependent groups (15–17). The nature of the disease and the production of pathogenic IgG Abs suggested a dependence on B cells and a role for T cells (15–17). We undertook studies to reevaluate the role of the B cell compartment and to determine whether B cells, in addition to their role as terminal effectors of the disease, are able to drive disease progression, and to define the contributions of the T cell and DC compartments.

We analyzed the splenic B cell compartment by flow cytometry, specifically assessing cell surface receptors that reflect de-
velopmental and activation status. As reported (33), we found a complete deficit of marginal zone B cells (MZBs), reductions in the follicular B cell (FOB) compartment, and a loss of transitional 2 (T2) B cells (Fig. 1A). IgM and CD21 levels on FOBS were downregulated (Fig. 1A) suggesting the B cells were activated. Consistent with previous histology and ELISPOT studies (15–17, 19), we found a significant increase in the number of plasma cells, defined as B220+CD138+ (Fig. 1B). As previously recognized (19), B lymphocytes from Lyn−/− mice had increased expression of MHC class II (MHC-II), with a 2-fold higher mean fluorescence intensity (MFI) versus control B cells (Fig. 1B). Furthermore, there was increased expression of CD80 and CD86 on Lyn−/− B cells (Fig. 1B), supporting an activated phenotype.

To evaluate whether the B cell phenotype varied with autoim-

une disease development, we examined mice >27 wk of age, by which time most Lyn−/− mice had maximal titers of circulating
ANAs (Fig. 1C) and kidney disease (data not shown), similar to what we and others have reported previously (15, 16). With age, there was a greater deficit in conventional B cells, the appearance of B cells with unusual characteristics (B220loIgM2; B2202IgM+), and a further expansion of B220loCD138hi plasma cells (population highlighted with an ellipse), and B220 versus IgM to determine proportions of B cells and levels of surface IgM. Assessment of costimulation and activation markers (MHC-II, CD80, and CD86) on splenic B220+ cells. Lyn−/− B cells have increased expression measured by MFI as well as proportion of cells with positive staining (bars). C, Quantitation of IgG ANAs in the serum of Lyn−/− mice of the indicated ages by ELISA, and of C57BL/6 mice of 30 wk of age (WT). D, Representative three-color flow cytometric analysis of spleen cells from aged (30 wk) control (WT) and Lyn−/− mice; staining as for (B). All studies are representative of at least six mice of each genotype in three independent experiments.

Alteration of the T cell and DC phenotype in Lyn−/− mice

We focused also on the CD4 T cell and DC compartments because of their importance in the initiation and progression of autoimmune disease. Although Lyn is not expressed in T cells (28, 34), we occasionally observed mild CD4+ T cell activation in young Lyn−/− mice; however, this was not a common feature (data not shown). With age, expression of the activation marker CD69 became significantly elevated on CD4+ T cells from Lyn−/− mice, with concomitant severe downregulation of CD62L, the homing marker that is lost on activation (Fig. 2A). The exaggerated T cell changes in aged Lyn−/− mice suggested that increased activation occurred simultaneously with disease progression. Because Lyn is not expressed in T cells, the increase in T cell activation in aged Lyn−/− mice is a secondary event and most likely driven by another cell population, possibly activated B cells or DCs.

In the absence of infection or inflammation, most DCs in peripheral tissues and lymphoid organs have a resting, immature phenotype characterized by low surface expression of MHC-II and costimulatory molecules. However, interaction with microbial ligands, proinflammatory cytokines, or CD40-ligand induces DCs to acquire an activated phenotype and become efficient at T cell priming. We examined the DC compartment in the spleens of Lyn−/− mice using CD11c to detect myeloid DCs. In a manner akin to B cells, DCs from young Lyn−/− mice had increased expression of MHC-II and CD86 on their surface compared with controls (Fig. 2B), suggesting enhanced maturation and Ag-presenting capability, although this phenotype was less striking in aged Lyn−/− mice, and in fact, DCs from both young and aged Lyn−/− mice had reduced CD80 expression (Fig. 2C and data not shown). Collectively, these results indicate that the autoimmune disease in Lyn−/− mice may be dependent on B cells, with the disease having an effect on, or being driven by, additional populations, including T cells and DCs.
The B cell compartment is central to the T cell phenotype in aged Lyn−/− mice, whereas the DC trait is independent of B cells

To define the contribution of the B cell compartment to the T cell and DC activation observed in Lyn−/− mice, Lyn−/− mice lacking B cells (Lyn−/− μMT−/− mice) were analyzed. We have previously shown that these mice do not develop autoimmune disease (23). Consistent with the results in Fig. 2A, CD4+ T cells from 20-wk-old Lyn−/− mice had an activated phenotype, whereas CD4+ T cells from Lyn−/− μMT−/− mice looked similar, or even less activated, than CD4+ T cells from control mice (Fig. 3A). However, CD11c+ DCs from Lyn−/− μMT−/− mice resembled those from Lyn−/− mice having increased MHC-II, reduced CD80 and elevated CD86 expression (Fig. 3B). These results indicate that Lyn−/− B cells are required for the exacerbated myelopoiesis in aged Lyn−/− mice, and furthermore, support a role for B cell-driven disease in mediating this process.

Serum IL-6 levels in Lyn−/− mice, and IL-6 production by resting Lyn−/− B cells

The nature of the autoimmune disease in Lyn−/− mice, namely, production of ANAs and immune complex-mediated glomerulonephritis, bears a striking resemblance to human SLE (15, 16), and indeed some SLE patients may have decreased Lyn expression (35). Studies have shown that human SLE B cells produce IL-6 in culture, and patients with SLE and other inflammatory diseases have elevated serum IL-6 (36–39). The association of these diseases with IL-6 and the presence of large numbers of plasma cells in Lyn−/− mice, which are normally dependent on IL-6, prompted us to assess the contribution of IL-6 to disease development in
Lyn−/− mice. We measured serum IL-6 levels in young and aged Lyn−/− mice and found that although young mice had low amounts similar to those observed in control mice, some aged Lyn−/− mice had higher concentrations of IL-6, although there was variation between individual mice. Furthermore, many aged Lyn−/− mice had serum IL-6 levels below the detection limit of the assay, and the differences over control mice were not statistically significant (Fig. 4A).

As serum cytokine levels may not always indicate local inflammatory niches, we assessed production of IL-6 by immune cells. Resting splenocytes were cultured in brefeldin-A to prevent cytokine secretion and then analyzed by flow cytometry. Although little IL-6 was present in control splenocytes, a significant proportion of Lyn−/− splenocytes were IL-6+. The IL-6–expressing cells were mostly B cells as they coexpressed B220, whereas the B220− cells, T cells, and macrophages, had low levels (Fig. 4B, 4C). Nonetheless, because others have shown that in vivo-derived Lyn−/− DCs are able to produce increased IL-6 in culture (27), we examined IL-6 production by other leukocytes, and quantitated absolute numbers of IL-6–expressing cells (Fig. 4C). These studies showed that significantly more Lyn−/− B cells than control B cells were IL-6+. Furthermore, leukocytes other than B cells were also IL-6+, although the numbers were much lower than Lyn−/− B cells, and with the exception of CD4+ T cells, they were not statistically significantly different from control cells (Fig. 4C). To determine whether B cells were a major source of IL-6 in Lyn−/− mice, we also measured IL-6
levels in the serum of aged Lyn−/− μMT−/− mice and found that most mice had undetectable IL-6 levels (Fig. 4A). However, the differences between Lyn−/− mice and Lyn−/− μMT−/− mice were not significant; IL-6 was detected in the serum of two Lyn−/− μMT−/− mice indicating that cells other than Lyn−/− B cells also contribute to serum IL-6 levels.

Effect of crossing Lyn-deficient mice with IL-6-deficient mice
Finding increased production of IL-6 by Lyn−/− leukocytes, notably B cells, and the association between IL-6 and SLE, made it of interest to determine the contribution of this cytokine to disease in Lyn−/− mice. Thus, C57BL/6 Lyn−/− mice were crossed with C57BL/6 mice deficient in IL-6 to generate double knockout (DKO) mice. We first assessed the B cell compartment to see if loss of IL-6 affected the B cell phenotype in Lyn−/− mice. Mice lacking both Lyn and IL-6 had a B cell phenotype similar to that of mice lacking Lyn alone; including a severe B cell deficit with the loss of MZBs and T2 cells (Fig. 5A). DKO B cells also had slightly restored IgM and CD21 expression compared with Lyn−/− B cells, but not to wild-type (WT) levels. Interestingly, DKO mice, even though they lacked IL-6, showed an expansion of plasma cells, although this was not as marked as that in Lyn−/− mice (Fig. 5B, 5C). Examination of cell surface activation/costimulatory markers on B cells from aged mice revealed DKO B cells had MHC-II expression similar to control B cells, and well below that expressed by Lyn−/− B cells (Fig. 5D). CD86 and CD80 expression was also close to control B cell levels (Fig. 5D). In addition, with age the plasma cell population increased in DKO mice in the same manner as in Lyn−/− mice (Fig. 6A). This suggests that in Lyn−/− mice, the B cell developmental defects are intrinsic to loss of Lyn, whereas the activation phenotype is dependent on IL-6.

Hyper-IgM and B cell hyper-responsiveness is maintained in DKO mice
One of the features of Lyn−/− mice is an increased serum IgM titer (15, 16). Given that deficiency of IL-6 on a Lyn−/− background did not normalize either B cell development (Fig. 5A) or the number of B cells and plasma cells (Figs. 5C, 6A), we were interested to see if serum IgM levels in DKO mice were altered. As reported previously (15, 16), Lyn−/− mice had a 10-fold increase in serum IgM (Fig. 6B). DKO mice had significantly higher levels of IgM compared with control mice; however, their levels were 2-fold decreased compared with Lyn−/− mice (Fig. 6B), possibly a reflection of the lower absolute numbers of plasma cells in DKO mice (Fig. 6A). Interestingly, although serum IgM titers increased with age, the same fold-differences between the three genotypes were maintained.

To determine whether loss of IL-6 altered the hyper-responsiveness of Lyn−/− B cells, proliferation assays were performed on B cells purified from the spleen. As found previously, Lyn−/− B cells were hyper-responsive to BCR cross-linking (17) and had reduced responses to LPS (15, 16) (Fig. 6C). B cells lacking both Lyn and IL-6 behaved similarly to Lyn−/− B cells (Fig. 6C), demonstrating this phenotype to be intrinsic to loss of Lyn.

T cell activation characteristic of disease-bearing Lyn−/− mice is absent in DKO mice
CD4 T cell activation is a feature of aged, disease-bearing Lyn−/− mice (Fig. 2A). Given that loss of IL-6 on a Lyn-deficient background affected B cell activation, we speculated that it may have similarly affected T cell activation. Flow cytometry of CD4+ T cells from young mice showed identical staining regardless of genotype, with the cells expressing similar amounts of both CD69 and CD21 (Fig. 6D). However, as expected, young DKO mice had a substantial decrease in CD69 expression on T cells compared with both Lyn−/− and Lyn−/− mice, indicating an intrinsic activation defect.

FIGURE 5. The B cell developmental defects in Lyn−/− mice are reiterated in Lyn−/− IL-6−/− mice. Splenic B cell compartment of young (8–10 wk) control (WT), Lyn−/− and Lyn−/− IL-6−/− (DKO) mice. A, Representative three color flow cytometry of spleen cells to delineate the various B cell subsets as follows: FOB, T2, transitional 1, and MZBs delineated by Abs to CD21, IgM, and CD23. Staining was performed on various B cell subsets as follows: FOB, T2, transitional 1, and MZBs delineated by Abs to CD21, IgM, and CD23. Staining was performed on whole spleen, and gates set on CD23+ cells to examine CD21 versus IgM, and on CD23− cells to examine IgM versus CD21. B, Spleen cells stained with Abs to B220 and CD138 to detect plasma cells, and with Abs to B220 and IgM to assess cell surface levels of BCR. C, Absolute numbers of plasma cells in the spleen of young C57BL/6 (WT), Lyn−/−, and DKO mice. Numbers were calculated based on the percentage of plasma cells and total numbers of cells in spleen. Statistical significance is shown by asterisk; *p < 0.05; **p < 0.01. D, Assessment of the expression levels of B cell activation and costimulatory markers on B220+ spleen cells from aged mice. MFI is indicated. In A–D, studies are representative of at least six mice of each genotype in three independent experiments.
and CD62L (data not shown). With age, control mice and IL-6-/- mice showed evidence of some T cell activation with increased expression of CD69 and diminished CD62L expression (Fig. 7A). CD4+ T cells from DKO mice showed the same trend as control mice and IL-6-/- mice, but this was less pronounced and significantly different to the highly activated CD4+ T cell phenotype in aged Lyn-/- mice (Fig. 7A). This indicates that IL-6 is required for the T cell activation phenotype in Lyn-/- mice. The increased proportion of activated CD4 T cells in Lyn-/- mice is also reflected by an increase in absolute number of CD69+ and CD62L- T cells, despite similar CD4 T cell numbers (Fig. 7B). However, in the absence of IL-6, CD4 T cell skewing in Lyn-/- mice does not occur, and the numbers of activated CD4 T cells resemble those of control mice (Fig. 7B).

The DC compartment in young and aged DKO mice was also examined. Interestingly, MHC-II and CD86 levels were increased on DCs from young DKO mice, significantly higher than control and similar to Lyn-/- DCs (Fig. 8A), and as with aged Lyn-/- mice, this phenotype was less pronounced in aged DKO mice (Fig. 8B). This result indicates that the DC phenotype in Lyn-/- mice is independent of IL-6.

Loss of IL-6 prevents myeloid expansion in central and peripheral lymphoid tissues

To determine whether IL-6 plays a role in the exacerbated extramedullary hematopoiesis (EMH) and myeloid cell expansion in Lyn-deficient mice, colony assays and flow cytometry studies were performed on DKO mice. In 8-wk-old mice, numbers of myeloid progenitors in the spleen were significantly lower in DKO mice when compared with Lyn-/- mice, but were higher than in control mice (Fig. 9A). In 30-wk-old mice, spleenic hematopoiesis was largely unchanged in control mice, whereas Lyn-/- mice had large numbers of myeloid progenitors (Fig. 9A); ~10-fold greater than in 8-wk-old mice and 3- to 4-fold greater than in 20-wk-old mice (Fig. 3C). However, in DKO mice, numbers of spleen progenitors did not increase with age and were only slightly increased.
over those found in control mice (Fig. 9A). Similarly, splenomegaly was observed in aged Lyn−/− mice as described previously (23), but this was not a feature of aged DKO mice and in fact, the spleens of DKO mice were significantly smaller than those of control mice (Fig. 9B). Furthermore, macrophages (Mac-1+c-fms+) and granulocytes (Mac-1+Gr-1+) were reduced in proportion and number in aged DKO mice compared with Lyn−/− mice (Fig. 9C, 9D), indicating that myeloid cell expansion in Lyn-deficient mice is dependent on IL-6. These results indicate that IL-6 is dispensable for normal hematopoiesis but plays a role in the exacerbated hematopoiesis, splenomegaly and myeloid cell accumulation in aged Lyn−/− mice.

FIGURE 8. The DC compartment is not restored in mice lacking both Lyn and IL-6. Representative three-color flow cytometric analysis of spleen cells from (A) young (8–10 wk) and (B) old (30 wk) control (WT), Lyn−/−, and Lyn−/−IL-6−/− (DKO) mice. A and B, Staining of spleen cells with Abs to B220 and CD11c to determine proportions of B cells and myeloid DCs. CD11c+ cells were gated and assessed for expression of markers of costimulation and activation; MFI of the CD11c+ cells is indicated. Studies are representative of at least six mice of each genotype in three independent experiments.

Lyn−/−IL-6−/− mice do not develop pathogenic ANAs or autoimmune disease

To determine whether loss of IL-6 on a Lyn-deficient background affected autoimmune disease development, we determined ANA titers over time in DKO mice. When assayed for pathogenic IgG ANAs, DKO mice did not show titers above controls, even at the age when Lyn−/− mice had maximum titers (Fig. 10A). Examination of IgM ANAs showed that DKO mice had detectable titers of serum IgM ANAs, which were higher than control mice, but not as high as those observed in Lyn−/− mice (Fig. 10B). This result indicates that DKO mice still have autoreactive B cells but in the absence of IL-6 are unable to produce class-switched, pathogenic auto-Ab. Because IL-6–deficient mice are thought to have generalized defects in class switching during T-dependent immune responses to certain pathogens (40, 41), we measured total serum IgG titers in resting DKO mice to determine whether the failure of DKO mice to produce pathogenic IgG ANAs was due to an overall reduction of IgG. IL-6 deficiency did not affect switched Ab levels as there was no statistically significant differences between IgG titers in Lyn−/−, DKO, and IL-6−/− mice (Fig. 10C).

To assess development of glomerulonephritis, kidneys from aged mice were also examined. Although 30-wk-old Lyn−/− mice had severe glomerulonephritis, the kidneys of DKO mice showed no evidence of glomerular destruction and resembled those of control mice (Fig. 10D). Kidneys from aged mice were also examined for the presence of Ig and complement deposition by immunofluorescence (Fig. 10E). Abs detected the presence of IgM in the glomeruli of all aged mice, with more extensive staining in both Lyn−/− and DKO kidneys compared with control kidneys. However, only the glomeruli in Lyn−/− kidneys showed the presence of IgG, with no staining apparent in control or DKO kidneys. Abs to the complement fragment C3 revealed intense staining in Lyn−/− glomeruli, with only diffuse weak staining in control and DKO glomeruli along with some background staining of tubules. This analysis shows that loss of a single cytokine, IL-6, from Lyn-deficient mice ameliorates IgG auto-Ab production and associated severe kidney pathology brought about by pathogenic IgG immune complex deposition.
Discussion

In this study, we have shown that inflammation, and in particular the proinflammatory cytokine IL-6, plays a critical role in autoimmune disease development in Lyn-deficient mice. We have demonstrated that Lyn-deficient leukocytes, notably B cells, overproduce IL-6, and this establishes an inflammatory environment leading to the activation of Lyn−/− B cells, and a plethora of changes in other cellular compartments that ultimately give rise to severe autoimmune pathology. Activation of Lyn−/− B cells precedes an alteration in the CD4 T cell compartment, which becomes highly activated in aged, disease-bearing mice. The perturbation of the CD4 T cell compartment is a secondary effect, as Lyn is not ordinarily expressed in T cells (28, 34, 42). However, T cells are major contributors to autoimmune disease in Lyn−/− mice through their ability to induce B cell class switching that leads to the production of pathogenic IgG auto-Abs (15–17). It is possible that both B cells and DCs may be responsible for driving T cell activation and the autoimmune phenotype in Lyn−/− mice, and it is clear that both cell types ordinarily express Lyn at high levels (15, 28, 29). In this study, we have found that Lyn−/− DCs have a mature/activated phenotype, which does not change with the age or disease-state of the mice, suggesting that DCs may play a role in triggering autoimmunity in Lyn−/− mice. However, the fact that we found the same DC characteristics in B cell-deficient Lyn−/− mice and Lyn−/− IL-6−/− mice, both of which are autoimmune disease-free (Fig. 10 and Ref. 23), strongly suggests that this DC phenotype is intrinsic to Lyn deficiency and independent of B cells, IL-6, and autoimmune disease.

There is some controversy about the role of Lyn in DC maturation and activation. Previous studies, including our own, have reported that Lyn−/− DCs have a maturation defect expressing lower levels of MHC-II and costimulatory molecules. Earlier studies, however, used DCs that were derived from the bone marrow in the presence of GM-CSF (28, 29). In the current study, we have found that splenic CD11c+ DCs have an activated/mature phenotype, expressing elevated levels of costimulatory molecules on their surface when analyzed ex vivo from Lyn−/− mice, consistent with the recent findings of Silver et al. (27). An explanation for these apparent contradictions may lie in the fact that Lyn−/− cells are hyper-responsive to GM-CSF (32).

IL-6 is a pleiotropic and proinflammatory cytokine that has been studied for >20 y (43). Its role in immunity and disease has been highlighted by the generation of IL-6 null and Tg mice (44, 45). IL-6 null mice have impaired survival and self-renewal of hematopoietic progenitor cells, and an impairment of T cell function in immune responses to certain T-dependent Ags (44). IL-6 Tg mice, which overexpress the human form of IL-6, display a spectrum of immune hyperactivation, including polyclonal hyper-IgG syndrome, plasmacytosis in central and peripheral lymphoid organs, progressive kidney pathology, and death between 12–20 wk of age.

![Figure 9](http://www.jimmunol.org/DownloadedFrom/332.jpg)
because of kidney failure (45). These studies emphasize the importance of steady-state levels of IL-6 for responses to infection, as well as the ability of IL-6 to cause severe immunopathology when expressed at high levels. Given the long association between IL-6 and SLE (36, 37, 46–49), it is surprising that there are very few studies that have targeted the IL-6 axis in SLE, and no significant clinical trials using anti–IL-6 (tocilizumab), even though targeting of IL-6 has been shown to be successful in the treatment of autoimmune-prone animals (50–52). This is particularly surprising given that therapies targeting IL-6 have been shown to be very effective in the treatment of other autoimmune diseases such as rheumatoid arthritis (53–55). Interestingly, treatment of rheumatoid arthritis patients with the anti-B cell mAb rituximab provides significant benefits (56–58), and although the mechanism of action is still largely unclear, ablation of B cell-derived IL-6 may be a contributing factor. The treatment of SLE with anti-B cell-directed therapies has not been as successful. Early small scale prospective studies and phase I/II trials using rituximab yielded encouraging results (59–61), although it has recently been announced that a large phase II/III randomized, double-blind, placebo-controlled, multicenter trial has failed to reach critical clinical end points (62). This may lie in the difference in disease pathogenesis between rheumatoid arthritis and SLE, and the absolute dependence on B cells in these diseases.

FIGURE 10. Autoimmune disease and associated kidney pathology are ameliorated in compound Lyn−/−-IL-6−/− mice. A, IgG ANAs in the serum of four C57BL/6, four Lyn−/− and seven Lyn−/−-IL-6−/− (DKO) mice at the indicated ages by ELISA. B, IgM ANAs in the serum of C57BL/6, Lyn−/− and DKO mice at the indicated ages by ELISA; n = 4 mice of each genotype. C, Titers of IgG isotypes in the serum of 8- to 10-wk-old C57BL/6, Lyn−/−, Lyn−/−-IL-6−/−, and IL-6−/− mice; n = 6 mice of each genotype. Statistical significance: *p < 0.05; **p < 0.01. D, H&E stained sections through the cortex of kidneys from 30-wk-old control (WT), Lyn−/−, and Lyn−/−-IL-6−/− (DKO) mice. E, Immunofluorescence images of kidneys from 42- to 46-wk-old mice stained with Abs to IgM (upper panels), IgG (middle panels), and complement fragment C3 (lower panels). The data in D and E are representative of at least four mice of each genotype in two independent experiments (original magnification ×20).
In both SLE patients and autoimmune-prone mutant mice, it is not known whether there is an intrinsic loss of tolerance to self-Ags in B cells, or whether there are inactivating mutations in tolerance checkpoints that, in a cumulative manner, establish an inflammatory environment and thereby promote cultivation of a pathogenic phenotype leading to increased immunoreactivity and disease. This is especially of interest given the ability of SLE B cells to produce large amounts of IL-6 (37). It is unknown whether the inflammatory environment begets hyper-responsive B cells or if they are intrinsically misprogrammed. By generating Lyn−/− mice deficient in IL-6 (DKO) we have been able to assess those characteristics intrinsic to B cells and those due to an inflammatory environment. We found that DKO B cells remained unchanged in their development and for the most part were identical to Lyn−/− B cells, indicating that the severe B cell deficit, hyper-responsive phenotype, expansion of plasma cells, loss of MZB and block in transition to T2 are intrinsic to Lyn deficiency. The only differences observed were in the levels of activation and costimulatory markers, which were high on Lyn−/− B cells but reduced to control levels on DKO B cells. In parallel with a reduction in B cell activation, T cell activation was significantly reduced in DKO mice. The importance of B cell and T cell activation in autoimmune disease development in Lyn−/− mice is highlighted by the lack of pathogenic ANAs, kidney pathology, myeloid expansion, and splenomegaly in DKO mice. This clearly demonstrates that IL-6 is an important mediator of autoimmune disease in Lyn−/− mice. Although IL-6 can be produced by many different cell types (43), our studies suggest that B cells may be a significant source of IL-6 in Lyn−/− mice, although it is clear that other Lyn-deficient cells can also produce IL-6. It remains to determine why deficiency of Lyn results in hyperproduction of IL-6, but this finding suggests that Lyn is a negative regulator of the IL-6 pathway. The pathway leading to IL-6 production is likely to be related to TLR signaling, and this is supported by a recent study that has shown that loss of the important TLR signaling intermediate, MyD88, on a Lyn-deficient background prevents autoimmune disease development (27). Interestingly, this study showed that CD11c+ DCs from Lyn−/− mice treated with TLR agonists were a source of IL-6; however, purified splenic DCs were used and these cultures, which were reported to be ∼83% pure (27), may have been contaminated with B cells, which we show are a potent source of IL-6.

IL-6 is known to play a role in regulating hematopoietic progenitor number and myelopoiesis. Injection of IL-6 into mice can lead to elevated myeloepoiesis and myeloproliferative disease (63), and IL-6 can synergize with other cytokines such as IL-3 and stem cell factor to enhance the production of immature hematopoietic progenitors (64, 65). Furthermore, IL-6 is able to support emergency granulopoiesis in animals that simultaneously lack G-CSF and GM-CSF (66). Conversely, deletion of IL-6 in mice leads to abnormal numbers of committed progenitors and an altered recovery after hematopoietic ablation (67). We have found that Lyn-deficient mice have increased numbers of myeloid progenitors and EMH that progressively increases with the age and autoimmune disease state of the mice. This phenotype is abrogated in DKO mice suggesting that IL-6 is an important component of this process. However, B cell-deficient Lyn-deficient mice, like DKO mice, do not develop autoimmune disease and do not exhibit EMH, yet they have the ability to produce IL-6. Thus, this suggests that the inflammatory milieu that accompanies autoimmune disease in Lyn-deficient mice, which is perhaps established by IL-6, is responsible for the EMH.

Perhaps surprisingly, the plasmacytosis associated with Lyn deficiency is not markedly altered by loss of IL-6, even though IL-6 is a known growth and survival factor of plasma cells in both steady-state and disease settings (68–70). Lyn-deficient mice lacking IL-6 nonetheless exhibited a profound plasmacytosis, and IgM levels in DKO mice were significantly higher than in control mice, yet clearly no pathogenic IgG Abs were produced. An important area of further investigation will be to assess germinal center formation and maintenance following T-dependent immune responses, as IL-6 has been previously reported to be important for germinal center progression (71), as well for the development of T cell subsets, two of which, Th follicular and Th17 cells, have been shown to be involved in germinal center development in a normal and an autoimmune disease setting, respectively (72, 73). However, it is important to note that IL-6 deficiency on a Lyn-null background does not affect switched Ab levels as there are no significant differences between IgG titers in Lyn−/− and DKO mice. This finding bodes well for the targeting of IL-6 in autoimmune disease settings, as it suggests that, under such conditions, conventional immune responses may be unaffected. Clearly, IL-6 may act at a multitude of points in the development and progression of autoimmune disease and refining our understanding of its mode of action will be important in optimizing the use of anti−IL-6 agents for the treatment of disease.

In summary, in this study, we have been able to separate in part the defects that are intrinsic to Lyn deficiency in B cells from those that are induced in response to an inflammatory environment. Our data support a scheme in which the overproduction of IL-6 in Lyn-deficient mice, notably by Lyn−/− B cells, establishes an inflammatory milieu that supports the development of autoimmune disease. Although mature DCs are key orchestrators of immune responses and thought to be central to autoimmune disease, our data show that DC maturation is not sufficient for disease development, whereas B cell and ultimately CD4 T cell activation are limiting factors in allowing disease to establish. Inflammation is not the cause of the severe B cell phenotypes in Lyn-deficient mice, as developmental and population differences are maintained in Lyn−/−IL-6−/− mice. Notably, inflammation determines the pathogenic potential of B cells that have broken tolerance to self-Ags. Importantly, these results support the use of inhibitors targeting B cells and IL-6 in the therapeutic arsenal against autoimmune disease.

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Disclosures

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References

AUTOIMMUNITY IN LYN-/- MICE IS DEPENDENT ON IL-6


