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Perturbation of the CD4 T Cell Compartment and Expansion of Regulatory T Cells in Autoimmune-Prone Lyn-Deficient Mice

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Regulatory T cells (Tregs) are a subset of T lymphocytes that are responsible for suppressing the function of other immune cells, and preventing potentially harmful autoimmune responses. Studies in autoimmune-prone mice and human autoimmune diseases have shown reduced Treg number or function as a causative factor for the apparent loss of tolerance that contributes to disease. We have found that Lyn-deficient mice, which develop high titers of autoantibodies with age, have a perturbed Treg compartment. Contrary to what has been observed in some strains of autoimmune-prone mice, aged Lyn-deficient mice have increased numbers of Tregs. This expansion occurs in the presence of elevated serum IL-2 and diminished TGF-β. Despite expansion of the Treg compartment, Lyn-deficient mice succumb at ~1 year of age due to immune complex-mediated glomerulonephritis. We have shown that Lyn is not expressed in Tregs or indeed in any T cell subset, suggesting that the expansion and apparent functional deficiency in Tregs in Lyn-deficient mice is due to extrinsic factors rather than an intrinsic Treg defect. Indeed, using an in vivo colitis model, we have shown that Lyn-deficient Tregs can suppress inflammation. These results suggest that Tregs are expanding in Lyn-deficient mice in an effort to control the autoimmune disease but are simply overwhelmed by the disease process. This study highlights the role of the inflammatory setting in autoimmune disease and its consideration when contemplating the use of Tregs as an autoimmune therapy. The Journal of Immunology, 2009, 183: 2484–2494.

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utoimmune disease develops as a result of self-reactive CD4⁺ or CD8⁺ effector T cell activation or the generation of autoreactive B cells to produce autoantibodies (1). For many human autoimmune diseases, the target proteins and mechanisms of induction are unknown; however, they all involve the breakdown of tolerance mechanisms, where self-tissues that are normally ignored are attacked as foreign.

The Src family tyrosine kinase Lyn is renowned for its ability to negatively regulate signaling pathways (2, 3). In B cells, Lyn can participate in transmission of positive signals (2); however, Lyn’s key role is in the tyrosine phosphorylation of B cell-specific inhibitory receptors such as CD22 and FcγRIIB1 (4–7), negative regulatory enzymes such as Src homology region 2 domain-containing phosphatase 1 (SHP-1) (8, 9), and adaptor proteins such as Dok-3 that negatively regulate calcium signaling (10). Lyn-deficient B cells show an impairment in signal termination, the biochemical defects of which manifest as hyperresponsive signaling upon BCR engagement. Lyn−/− mice develop an autoimmune disease that resembles systemic lupus erythematosus (SLE), which is characterized by high titer autoantibodies against nuclear Ags and immune complex-mediated glomerulonephritis (11–13). Many studies have focused on the role of Lyn in B cells to fully elucidate the mechanisms behind autoimmune disease development in Lyn−/− mice, fittingly so as B cell-deficient Lyn−/− mice do not develop autoimmunity (14), demonstrating a dependency on B cells. However, although Lyn is not expressed in T cells (15, 16), T cells are clearly implicated in the disease through their ability to induce B cell class-switching, leading to the production of pathogenic IgG autoantibodies, and yet no studies have described the role of T cells in this process or defined how T cells are recruited into the disease. Indeed, a recent study (17) has observed no change in the T cell compartment of Lyn-deficient mice.

Regulatory T cells (Tregs) have an important role in autoimmune disease by suppressing the activation and effector function of self-reactive cells. Sakaguchi et al. (18) showed that CD4⁺ CD25⁺ T cells were essential for maintaining immune tolerance by transferring congenic CD25-depleted CD4⁺ T cells into athymic nude mice, which rapidly developed a milieu of autoimmune diseases, including gastritis, thyroiditis, and insulinitis (18). Others (Hori et al. (19) and Fontenot et al. (20)) independently established the link between Foxp3 and the function of Tregs by showing that Foxp3 is specifically expressed in CD4⁺ CD25⁺ cells and is required for their development. Numerous studies have shown that function-affecting mutations in Foxp3 confer a complete loss of Treg function and result in a wide spectrum of autoimmune diseases (reviewed by Ziegler (21)). Autoimmune-prone strains of mice, including the NZB × NZW F₁ and SWR × NZB F₁ strains, have

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Abbreviations used in this paper: SHP-1, Src homology region 2 domain-containing phosphatase 1; ANA, anti-nuclear Abs; IBD, inflammatory bowel disease; MFI, mean fluorescence intensity; SLE, systemic lupus erythematosus; Treg, regulatory T cell; WT, wild type.

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been shown to have decreased frequencies of Tregs compared with nonautoimmune strains (22). There is also evidence suggesting Tregs from these mice have reduced suppressive capabilities; however, this is in concert with activated B and T cells, which appear resistant to suppression by Tregs (22–24). Parallels to human SLE have also been found, suggesting that either reduced Treg numbers or reduced suppressive function is correlated with disease severity (25–28). However, the distinction between active disease in the form of SLE flares and inactive disease is becoming important in the assessment of the phenotype of Tregs from these patients. This is illustrated by studies that show reductions in either number (25) or suppressive function (26, 28) are only seen when disease is active, whereas inactive disease in patients renders the phenotypic characteristics of their Tregs comparable to healthy controls. Therefore, active disease in both mouse models and SLE patients is positively associated with a reduction in Treg number or function (reviewed in Ref. 29). There is also evidence that defects in Treg number or function are implicated in other human autoimmune diseases (30–32).

In this study, we have reassessed the development of autoimmune disease in fixed genetic background Lyn-deficient mice, paying particular attention to the role of T cells. We have shown that perturbations in the B lymphocyte compartment precede a skewing of the CD4 T cell compartment. Although young Lyn-deficient mice show no obvious T cell defects, aged mice have increased numbers of activated effector CD4+ T cells and dramatically expanded numbers of Tregs. These phenotypes are extrinsic to the T cell compartment and driven by the Lyn-deficient environment as we have demonstrated that Lyn is not expressed in any T cell compartment.

**Materials and Methods**

**Animals**

Lyn−/− mice have been described previously (11). The mice used in this study were backcrossed to the BALB/c genetic background by microsatellite screening of each generation of backcrossed mice (16). Young mice were classified as 8–12 wk of age, whereas aged mice were ≥30 wk old. BALB/c wild-type (WT) mice were used as controls in all experiments. SCID mice were purchased from the Animal Resources Centre. All experiments were performed in accordance with National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes and were approved by the Ludwig Institute for Cancer Research/Department of Surgery Animal Ethics Committee.

**Detection of autoantibodies by ELISA**

Mice were bled fortnightly to monthly from the tail vein. To detect antinuclear Abs (ANA), ELISA plates precoated with purified nuclear Ags (The Binding Site) were incubated with serum, and bound Abs were detected with HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates).

**Cell preparation and flow cytometry**

Single-cell suspensions were prepared from spleen by extruding the cells from under the capsule and from lymph node using a wire sieve. In experiments involving lymph node cells, the axillary, mesenteric, and inguinal lymph nodes were pooled and used. All cell suspensions were passed through a 40-μm filter before use. B cells were purified from the spleen of WT mice or Lyn−/− mice by negative selection using MACS chromatography (Miltenyi Biotec).

Four-color flow cytometry was used to determine the composition of lymphoid tissue using the following Abs: GK1.5 (CD4), 53-6.7 (CD8), PC61 (CD25), DTA-1 (GITR), RA3-6B2 (B220), CD45RB (16A), syndecan-1 (CD138), IM7 (CD44), MEL-14 (CD62L), H1.2F3 (CD69), GL1 (CD86), AMS-32.1 (I-Ak), 11/41 (IgM), RB6-8C5 (Gr-1), M1/70 (Mac-1), TER-119 (Ter-119), and C2 (CD71) (all from BD Pharmingen). Tregs as defined by Foxp3 expression were detected using a kit (eBioscience). Cells were analyzed on a FACS Calibur (BD Biosciences), and data were analyzed with FloJo software (Stanford University). Dead cells were excluded on the basis of propidium iodide uptake, and at least 10,000 events were acquired.

Absolute numbers of cells were calculated from viable cell counts and proportions, as determined by flow cytometry. Cell populations were defined as the following: Tregs, CD4+ CD25+ Foxp3+; activated T cells, CD4+ CD25− CD69+; conventional B cells, B220−/highCD138−/low/; plasma cells, B220−/lowCD138−/high; myeloid cells, Mac-1+ Gr-1+; and erythroblasts, Ter119+CD71+.

**Histology, immunohistochemistry, and immunofluorescence**

Tissues for histopathology and immunohistochemistry were fixed in Bouin’s solution for 24 h and then embedded in paraffin. Immunohistochemical analysis was performed after dehydrating and rehydrating sections. Slides were microwaved for 10 min in target retrieval solution (Dako-Cytomation) to expose antigenic sites. To block nonspecific binding, sections were treated with CAS block (Zymed Laboratories), followed by primary and secondary Abs. Sections were stained with mAbs to B220 and IgM as markers of B cells and plasma cells, respectively. Under the Ag retrieval conditions used, the mAb to IgM is largely specific for cytoplasmic IgM and does not significantly recognize membrane-bound IgM. For immunofluorescence, lymph node from aged WT and Lyn−/− mice were imbedded in optimal cutting temperature compound (Sakura Finetek) and snap-frozen in liquid nitrogen. Acetone-fixed sections were rehydrated in PBS and blocked in 5% RSA/PBS. Sections were stained with anti-FOXp3-biotin (eBioscience) and streptavidin-FITC (BD Biosciences), and immunofluorescence was detected on a Nikon 90i inverted microscope fitted with a SPOT RT3 SLIDER camera.

**Assessment of Lyn expression in Tregs and evaluation of Treg function**

CD4+ CD25− CD45RB+ T cells and CD4+ CD25+ CD45RB− T cells were purified from the lymph node by FACS sorting using a FACSaria (BD Biosciences). For expression studies, CD4+ CD25− CD45RB+ T cells were lysed at a concentration of 105 cells/ml in 1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and complete protease inhibitor mixture (Roche Diagnostics). After protein determination by bicinchoninic acid (Pierce Chemical), total cell lysates were separated by SDS-PAGE, and proteins were transferred to nitrocellulose. Blots were probed with anti-Lyn (Santa Cruz Biotechnology) and anti-SHP-1 as a protein loading control.

Treg function was assessed by adoptive transfer using a model of inflammatory bowel disease (IBD) developed by Powrie et al. (33). Briefly, 5 × 106 WT CD4+ CD25− CD45RB+ T cells were injected i.p. into SCID recipients to induce colitis. Treg function was assessed when 105 WT or Lyn−/− CD4+ CD25+ CD45RB− T cells were cotransferred along with CD4+ CD25− CD45RB− WT T cells. Mice were weighed twice a week to monitor disease development and sacrificed after losing >10% of their body weight. All mice were sacrificed ~12 wk postinjection, and their colons removed for subsequent histopathology. Results are expressed as percentage of mice disease-free using Kaplan-Meier plots.

**Measurement of TGF-β levels in serum**

To determine the levels of active TGF-β in serum, a luciferase reporter cell line assay was used (34). Briefly, NIH-3T3 cells were stably transfected with a TGF-β3-smad3 luciferase reporter p(CAGA)12-Luc. The resultant reporter cell line has a sensitivity of 2-fold increase to ~1 pg/ml TGF-β stimulation (overnight) and a dynamic range of >300-fold at ~1 ng/ml as measured by luciferase assay (Promega) in a 96-well format. The TGF-β activity in mouse serum (5–10 μl) was measured (in triplicate) as the readout of luciferase activity upon substrate addition to cells, and the concentrations were calculated on a standard titration curve. The SE for each concentration is <10%.

**Measurement of IL-2 levels in serum**

IL-2 levels in serum were determined using an IL-2 ELISA kit following the manufacturer’s instructions (eBioscience). Briefly, 96-well immunosorb plates (Nunc) were coated overnight at 4°C with anti-IL-2 capture Ab. The next day, plates were washed in PBS containing 0.05% Tween 20, and samples and standards were added. After overnight incubation, plates were washed, and detection Ab with avidin-HRP was added for 1 h. Plates were then washed, and substrate was added. Color reaction was stopped with concentrated H2SO4, and plates were analyzed on a FACS Calibur (BD Biosciences), and data were analyzed with FloJo software (Stanford University). Dead cells were excluded on the basis of propidium iodide uptake, and at least 10,000 events were acquired.
were read at 450 nm, with correction at 595 nm. The sensitivity of the assay was determined to be 3.2 pg/ml.

Statistics

Unless otherwise stated, statistical comparisons were performed using an unpaired t test. Where indicated, the Mann-Whitney U test was used when the variance of the samples was large.

Results

Age-dependent development of autoantibodies in fixed background Lyn-deficient mice

Previous studies have shown that older Lyn−/− mice develop autoimmune disease. These studies were conducted on both mixed (129 × C57BL/6) mice (11–13) or C57BL/6 mice (17, 35). We generated Lyn−/− mice on a BALB/c background by rapid backcrossing using microsatellite screening (16). Older BALB/c background Lyn−/− mice (30 wk) developed severe lymphadenopathy (Fig. 1A), a feature not characteristic of C57BL/6 Lyn−/− mice. This was also reflected by increased cellularity in these nodes compared with aged WT mice (Fig. 1A), and the proportion of nonviable cells in the lymph nodes of aged Lyn−/− mice was high (data not shown). In addition, aged Lyn−/− BALB/c mice also developed splenomegaly (WT: 0.146 ± 0.010 g, n = 5 vs Lyn−/−: 0.827 ± 0.173 g, n = 6), a trait of both mixed (129 × C57BL/6) (8, 12, 13) and C57BL/6 Lyn−/− mice (17, 35). Similar to mixed (129 × C57BL/6) and C57BL/6 Lyn−/− mice, BALB/c Lyn−/− mice had a reduced median survival (360 days; Fig. 1B). To determine whether the lymphadenopathy phenotype and decreased survival tracked with autoimmune disease development, we tested sera from a cohort of mice for the presence of IgG ANA (Fig. 1C). When assayed fortnightly, Lyn−/− mice showed incremental increases in serum ANA titers, whereas WT mice had negligible titers. By 30 wk of age, when the lymphadenopathy phenotype was pronounced, most Lyn−/− mice had maximal titers. Aged Lyn−/− mice also displayed macroscopic evidence of kidney disease (Fig. 1D), and H&E-stained sections of kidney from aged Lyn−/− mice showed severe glomerular damage (Fig. 1D).

To determine whether Lyn is a haploinsufficiency gene for autoantibody development, BALB/c mice carrying one copy of the Lyn null mutation were tested for the presence of serum ANA (Fig. 1E). Although ANA development was delayed in Lyn+/− mice compared with Lyn−/− mice, Lyn+/− mice showed an incremental increase in serum ANA and had modest titers by 30 wk; most mice had high titers by ~1 year of age. These results demonstrate that ANA development in Lyn−/− mice is genetic background independent, and moreover, they show that Lyn is a haploinsufficient gene for autoantibody development.

Plasma cells are expanded in Lyn-deficient mice

To understand the nature of the severe lymphadenopathy in BALB/c Lyn−/− mice, we performed flow cytometry studies on lymph node and spleen cells from young (8–12 wk) and old (30

9-mo-old WT and Lyn−/− mice, and live cell counts of single-cell suspensions of pooled lymph nodes (axillary, mesenteric, and inguinal) from young and aged WT and Lyn−/− mice; ***, p < 0.0001 by Mann-Whitney U test. B, Kaplan-Meier survival curve of BALB/c mice (■) and BALB/c background Lyn−/− mice (○). C, Serum ANA titers of BALB/c background Lyn−/− mice of the indicated ages. On the right of the graph are ANA titers of 30-wk-old WT mice. D, Photographs of the peritoneal cavity of 9-mo-old WT and Lyn−/− mice. Note the pale kidney (K) in the Lyn−/− photograph and the severe lymphadenopathy (arrows). H&E sections through the cortex of kidneys from WT and Lyn−/− mice. E, Serum ANA titers of BALB/c background Lyn−/− mice at the indicated time points.

FIGURE 1. Development of lymphadenopathy, impaired survival, and Ab-mediated autoimmune disease development in BALB/c background Lyn−/− mice. A, Photographs of the axillary lymph nodes (arrows) of
wk) mice. Flow cytometric analysis of lymph node cells from young BALB/c background mice showed that Lyn−/− mice had a diminution of mature B220+ B cells, as reported previously (Refs. 11–13; Fig. 2A). Interestingly, Lyn−/− B cells showed an increased expression of CD138, a marker of developing B cells and plasma cells (mean fluorescence intensity (MFI) of 42 on Lyn+/+ B cells vs 108 on Lyn−/− B cells). No plasma cells (B220 low CD138 high) were obvious in the lymph node of young mice of either genotype; however, analysis of 30-wk-old mice showed a substantial increase in B220 low CD138 high plasma cells in Lyn−/− lymph node, whereas this population was negligible in WT lymph node (Fig. 2B). In the spleen of young Lyn−/− mice, as well as the diminution of B cells and increased expression of CD138 on B cells seen in lymph node, there was an increase in B220 low CD138 high plasma cells (Fig. 2C). With age, there was a significant expansion of this population, which mirrored the expansion occurring in the lymph node (Fig. 2D). Given the lymphadenopathy and splenomegaly exhibited by older Lyn−/− mice, this represents a substantial increase in absolute numbers of plasma cells in aged mice (Fig. 2E). In addition, B cells from young Lyn−/− mice had significantly elevated levels of the activation and costimulation markers CD86 and class II on their surface (Fig. 2F), supporting a hyperactivation phenotype and potentially a prelude to plasma cell expansion.

The myeloid and erythroid compartments are also altered in BALB/c background Lyn-deficient mice

In previous studies, we have found that Lyn−/− mice have enhanced hemopoiesis, leading to an age-dependent increase in numbers of myeloid and erythroid cells (8, 14). To determine whether BALB/c Lyn−/− mice also showed similar phenotypes, we used flow cytometry to assess numbers of myeloid and erythroid cells in secondary lymphoid tissue. Almost no myeloid cells were present in the lymph nodes of WT mice regardless of age, and this was also true of young Lyn−/− mice; however, aged Lyn−/− mice showed an expansion of neutrophils and macrophages with myeloid cells comprising ~10% of the lymph node cell population (Fig. 3A). This represents a 100-fold increase in the absolute number of Mac-1+ Gr-1+ myeloid cells in the lymph node of aged Lyn−/−
mice (Fig. 3A). Similar numbers of myeloid cells were present in the spleen of young WT and Lyn−/− mice; however, aged Lyn−/− mice showed a significant expansion of myeloid cells, particularly neutrophils (Fig. 3B). When we assessed the erythroid compartment, we found that both young and aged Lyn−/− mice had a significantly increased proportion as well as absolute number of erythroblasts in spleen compared with WT mice (Fig. 3C). These studies show that BALB/c Lyn−/− mice, such as mixed genetic
background and C57BL/6 background mice (8, 14), have severely perturbed myeloid and erythroid compartments.

Age-dependent CD4 T cell perturbations in Lyn-deficient mice

Previous studies have shown that Lyn is not normally expressed in T cells (15, 16). Nonetheless, the T cell compartment is clearly implicated in the development of autoimmune disease in Lyn \(^{-/-}\) mice, as the mice develop class-switched pathogenic IgG autoantibodies. Using flow cytometry, we analyzed CD4 T cell populations, including effector/pathogenic T cells and immunosuppressive Tregs in young and disease-bearing aged mice (Figs. 4 and 5). Young Lyn-deficient mice showed no obvious differences in their effector or regulatory T cell compartments in either the lymph node or spleen (Figs. 4A and 5A; data not shown). Similar numbers of activated effector (CD4\(^+\)CD25\(^-\)CD44\(^-\)CD69\(^-\)) and CD4\(^+\)CD25\(^-\)CD62L\(^-\)) T cells were present in young WT and Lyn \(^{-/-}\) mice, and no differences in the Tregs, defined as either CD4\(^+\)CD25\(^+\), CD25\(^-\)CD45RB\(^-\)CD25\(^-\), CD4\(^+\)CD25\(^-\)CD62L\(^-\)), were apparent (Figs. 4A and 5A).
The CD4^+CD25^+ T cell compartment in the lymph node and spleen of Lyn-deficient mice showed evidence of activation displaying increased expression of CD69 and CD44 and down-regulated CD62L (Fig. 4. A and B). When we calculated absolute numbers of activated T cells in lymph node, defined as CD4^+CD25^+CD69^+Tregs, there was a statistically significant increase (Fig. 4D). In spleen, however, although the differences in absolute number were not statistically significant (Fig. 4E), it is clear that the CD4^+CD25^+ T cell population is skewed toward activation (Fig. 4C).

The CD4^+CD25^+ T cell compartment in Lyn^-/- mice also showed age-dependent perturbations. When expressed as a proportion of CD4 cells, the CD25^+ population comprised ~10% in lymph node and 20% in the spleen of aged WT mice, whereas this was increased to 35% in lymph node and ~50% in the spleen of aged Lyn^-/- mice (Fig. 4, B and C). Given the size of the lymph nodes and spleen in aged Lyn^-/- mice, this represents a significant expansion in absolute number (Fig. 4, D and E). To confirm that the CD4^+CD25^+ cells in the lymph nodes of young and aged Lyn^-/- mice expressed Foxp3 and were in fact Tregs, cells were stained with Abs to CD25 and CD45RB, fixed, permeabilized, and then stained with Abs to Foxp3 (Fig. 5, A and B). Approximately 80% of CD25^+CD45RB^low cells from the lymph nodes of aged WT mice were Foxp3^+ whereas this was ~50% in Lyn^-/- samples (Fig. 5B). At present, it is unclear why cells with many characteristics of Tregs (CD4^+CD25^+CD45RB^low) are Foxp3^-, although this population may also include activated effector T cells. Interestingly, the MFI of Foxp3 expression was slightly reduced on Tregs from both young and aged Lyn^-/- mice, which may have some bearing on Treg function. To prove conclusively that Tregs were expanded in aged Lyn^-/- mice, we calculated absolute numbers of CD4^+CD25^+Foxp3^+ cells in lymph node and found that they were significantly elevated (Fig. 5C).

**Tregs do not express Lyn**

Previous studies have shown that Lyn is not expressed in T cells (15, 16), except under specific circumstances (36). Because aged Lyn^-/- mice display alterations in the T cell compartment favoring Treg skewing, we questioned whether Lyn was expressed in Tregs and had a role in the function of these cells. Because Tregs comprise only a small proportion of total T cells in WT mice, Lyn expression was analyzed specifically in this subset. To obtain a highly purified population of cells, WT Tregs were FACS sorted from lymph node on the basis of CD4, CD25, and CD45RB^low expression, lysed, and analyzed by Western blotting (Fig. 6A). The two isoforms of Lyn were clearly observed in lysates of WT B cells, even when diluted 20-fold; however, Lyn was not observed in lysates of WT Tregs. These data show that Lyn is not expressed in Tregs and therefore indicates that expansion of Tregs in Lyn-deficient mice is not due to an intrinsic loss of Lyn in these cells.

**TGF-β levels are diminished while IL-2 levels are elevated in aged Lyn-deficient mice**

TGF-β is a cytokine that is able to induce Treg generation from naive CD4 T cells in the periphery (37, 38). To determine whether TGF-β was contributing to the Treg expansion in aged Lyn^-/- mice, serum was analyzed for levels of active TGF-β by a reporter cell line assay (34). When comparing young WT and Lyn^-/- mice, there was no difference in the levels of active TGF-β (data not shown); however, in aged Lyn^-/- mice, the levels of TGF-β were significantly reduced (Fig. 6B). It is possible that this reduction is associated with the increased T and B cell activation in aged Lyn^-/- mice.

Since IL-2 is an important factor for Treg expansion and survival (39), its levels were investigated in the serum of aged WT
and Lyn−/− mice. Although the serum of all WT mice contained levels of IL-2 undetectable by the limits of the assay, IL-2 was detected in the serum of most aged Lyn−/− mice (Fig. 6C), which may provide Lyn−/− Tregs a survival advantage.

Tregs in lymph nodes of Lyn-deficient mice do not have a defined localization, whereas plasma cells are localized near the subcapsular region

Because Tregs are increased in proportion and number in Lyn−/− mice and yet do not effectively suppress autoimmune disease development, we investigated their localization by immunohistochemistry using Abs to CD25 (Fig. 6D). CD25+ cells were rare in WT lymph node, with immunohistochemical staining localizing them to the peri-follicular space of T cell areas. In contrast, the Lyn−/− lymph nodes were disorganized, and the CD25+ cells were spread abundantly throughout, showing no distinct pattern or evidence of clustering (Fig. 6D, upper panels). To confirm that the CD25+ cells were Tregs, we also used an Ab to Foxp3 and immunofluorescence to examine Treg localization. Whereas Foxp3+ cells were scarce in WT lymph nodes, they were clearly abundant in Lyn−/− nodes (Fig. 6D, lower panels).

To determine whether the Lyn−/− Tregs colocalized with the expanded population of plasma cells in the lymph nodes of aged Lyn-deficient mice, we performed histopathology analysis and further immunohistochemistry studies. When examined histologically (Fig. 6E), the lymph nodes of aged Lyn−/− mice showed severely disrupted architecture, in which distinct lymphoid follicles were not obvious, and there were no well defined B/T cell zones but instead a plane of cells without any distinctive organization. Immunohistochemical staining of lymph node sections from old WT mice showed well defined B cell follicles and the occasional plasma cell in the extra follicular region (Fig. 6E). In contrast, staining of lymph node sections from aged Lyn−/− mice revealed
poorly stained, disorganized B cells, and large numbers of plasma cells localized near the subcapsular region, suggesting that the cells may be long-lived plasma cells (Fig. 6E).

These data show that although plasma cells are localized to distinct areas in the lymph nodes of aged Lyn−/− mice, the Tregs are scattered loosely and do not colocalize with plasma cells or indeed any particular cell subset.

Tregs from Lyn-deficient mice are functional in an in vivo model of disease

Our analyses have shown that Lyn−/− mice have a perturbed B cell compartment and with age accumulate large numbers of myeloid cells, erythroblasts, plasma cells, and activated CD4+CD25− pathogenic T cells. Concomitant with these changes is a substantial expansion in numbers of Tregs, and yet the mice develop high titers of autoreactive Abs and succumb to autoimmune disease. Since development of disease questioned whether the Lyn-deficient Tregs were functioning appropriately, a colitis model, which is an established model of IBD (33), was used to assess Treg function in vivo. This model uses pathogenic CD4+CD25−CD45RBlow T cells to induce intestinal inflammation in immunocompromised mice, and the CD4+CD25−CD45RBhigh fraction was enriched in Tregs to protect against disease (33). To test the functionality of Lyn-deficient Tregs, SCID mice were injected with either FACS-sorted WT or Lyn-deficient Tregs in conjunction with WT pathogenic T cells from young donors, and the health of the mice was monitored over a period of 12 wk. As expected, all mice receiving pathogenic T cells developed IBD, and mice injected with pathogenic T cells and WT Tregs were protected against IBD development (Fig. 7, A and B). Mice receiving pathogenic T cells and Lyn-deficient Tregs showed no clinical signs of IBD, and the colons from mice euthanized 12 wk postinjection showed no lymphocyte infiltration (Fig. 7, A and B). These data demonstrate that Lyn-deficient Tregs are functional in suppressing inflammation elicited by CD4+CD25−CD45RBhigh cells and suggest that their expansion in disease-bearing Lyn−/− mice occurs as a consequence of disease. To test the function of Tregs derived from old mice, aged donors were also used, and the results obtained were identical to those using young donors (data not shown), reiterating that the Tregs from Lyn−/− mice are functional when removed from the Lyn−/− environment.

Discussion

Lyn-deficient mice develop an Ab-mediated autoimmune disease (11–13) that is dependent on B cells (14) and thought to be due in part to B cell hyperresponsiveness, resulting from defects in BCR signal termination (4–7, 13). We show here that autoimmune disease development is genetic background independent, because disease occurs when the Lyn null mutation is expressed on the C57BL/6 background animals (8, 12, 13) and does not occur in C57BL/6 mice (our unpublished observations). In one study on C57BL/6 Lyn−/− mice, a 2-fold increase in lymph node weight was observed in 6-mo-old mice (35), but this is substantially less than what occurs in aged BALB/c Lyn−/− mice. Flow cytometry has shown that the lymph nodes of 8- to 12-wk-old BALB/c Lyn−/− mice have diminished numbers of B cells but no overt defects in other cell compartments, whereas the enlarged lymph nodes of aged, disease-bearing mice contain large numbers of plasma cells and myeloid cells, in addition to activated B and T lymphocytes and Tregs. Although the autoimmune disease process is likely to be contributing to the lymphadenopathy phenotype in BALB/c Lyn−/− mice, it is unlikely to be the sole basis for the phenotype, because it is not characteristic of C57BL/6 Lyn−/− mice, which develop autoimmunity at a similar rate. In our unpublished studies, we have shown that aged, disease-bearing C57BL/6 Lyn−/− mice do not accumulate large numbers of plasma cells in lymph node, although they contain.
activated B and T lymphocytes, Tregs, and myeloid cells. This suggests that there must be genetic modifiers on the BALB/c background that when coupled with Lyn deficiency creates survival niches for plasma cells in lymph node in addition to an environment in the lymph node that supports leukocyte expansion leading to lymphadenopathy.

Because Lyn is not expressed in T cells (15, 16), T cells are unlikely to be the primary mediators of the autoimmune disease that aged animals develop; however, as pathogenic IgG immune complexes have been observed in the kidneys of Lyn-deficient mice and ANA are of the IgG subclass (11–13), T cells are obviously recruited into the disease. Thus, although the primary defect appears to lie with B cells that have lost the ability to maintain tolerance to self, T cells are clearly implicated in disease pathogenesis. When we examined the T cell compartment of young Lyn-deficient mice, which lack ANA but B cell show defects, their CD4 T cell compartment was normal. However, in aged Lyn-deficient mice, which have high titers of ANA and large numbers of plasma cells, the effector CD4 compartment showed evidence of activation (increased levels of the activation markers CD69 and CD44, with down-regulation of CD62L), and there was a dramatic increase in numbers of Tregs, particularly in lymph nodes. When taking into account that aged Lyn-deficient mice have severe lymphadenopathy and splenomegaly, the increase in absolute numbers of Tregs is substantial. Although previous studies, including our own, have shown that Lyn is not expressed in T cells (15, 16), we felt it necessary to confirm that Lyn is not expressed in Tregs, because they represent only a small subset of T cells. A lack of Lyn expression in Tregs indicates that the alteration in this compartment is not intrinsic to loss of Lyn but extrinsic and due to the Lyn-deficient environment.

Because Tregs are responsible for the suppression of autoimmunity and inflammation (33, 40, 41), one may expect that their expansion would be associated with marked general immunosuppression; however, this is not the case in Lyn-deficient mice as they develop severe, lethal glomerulonephritis. Because Foxp3 is essential for Treg function (20), we investigated Foxp3 expression in young and aged WT and Lyn−/−mice. Tregs were significantly expanded in number in aged Lyn−/−mice, although showed slight reductions in the expression level of Foxp3. Reductions in Foxp3 expression may contribute to reduced function; however, the level of Foxp3 is similarly reduced on Tregs from both young and aged mice, indicating that this feature is not associated with expansion. Despite this, we have shown that Lyn-deficient Tregs were functional in suppressing colitis in the IBD model developed by Powrie et al. (33), leading us to conclude that this population is not expanded in these mice (our unpublished observations), indicating a dependency on B cells and possibly disease itself. Future studies are aimed at understanding the mechanism behind the Treg expansion in Lyn-deficient mice and the role of the Lyn-deficient inflammatory environment in regulating their function. These studies will provide important insights into the role of chronic inflammation in disease settings on the potential efficacy of Tregs as therapeutics.

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Disclosures
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