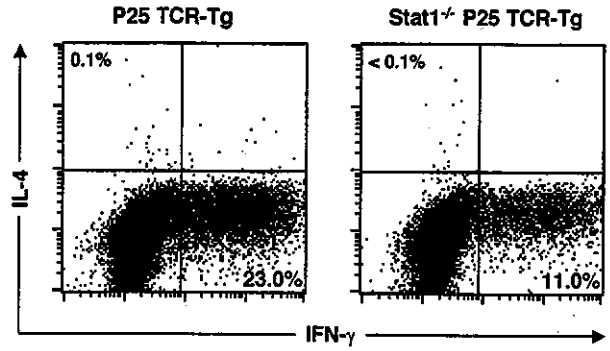

Fig. 5. Induction of Th1 differentiation of naive CD4+ T cells from STAT1 deficient P25 TCR-Tg mice upon stimulation with Peptide-25. Naive CD4+ T cells in the spleen from STAT1 deficient P25 TCR-Tg mice were stimulated with 10 μg/ml of Peptide-25 for 6 days. On day 6, the cells were washed and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h. IFN-γ- and IL-4-producing cells were assessed by intracellular staining and FACS analysis. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

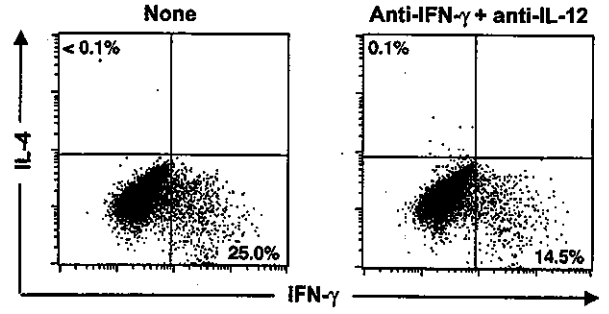

Fig. 6. Induction of Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg mice upon stimulation with Peptide-25-loaded I-A^b-CHO. Naive CD4+ T cells from P25 TCR-Tg mice were stimulated for 6 days *in vitro* with Peptide-25-loaded I-A^b-CHO in the presence or absence of anti-IFN-γ and anti-IL-12. Six days after the culture, the proliferated cells were harvested and re-stimulated with 1 μg/well of immobilized anti-CD3 for 24 h and subjected to cytoplasmic staining for IFN-γ and IL-4. The percentages of IL-4- and IFN-γ-producing cells are presented in the upper left and the lower right regions, respectively.

transfectants. CD4 expression may facilitate the interaction between TG40-BP1 and APC, resulting in augmented IL-2 production. Intriguingly, the APL could stimulate TG40-BP1/CD4 IL-2 production to a much lesser extent even at higher peptide concentrations (Fig. 1B). As APL fully preserves the I-A^b-binding amino acids of Peptide-25, the APL/I-A^b complex may have lower avidity for the TCR compared with Peptide-25.

Expression profiles of cell surface activation markers on splenic T cells from P25 TCR-Tg mice were similar to these from WT mice, and mRNA expression of neither T-bet nor IFN-γ was observed, suggesting that CD4+ T cells in P25 TCR-Tg mice are not pre-activated. Naive CD4+ T cells from P25 TCR-Tg mice could differentiate into IFN-γ- and IL-4-producing cells upon anti-CD3 stimulation (Fig. 2), indicating that they keep their potential to differentiate into either Th1- or Th2-lineage cells upon TCR ligation. Interestingly, naive CD4+ T cells differentiated solely to IFN-γ-producing cells, but not to IL-4-producing cells upon Peptide-25 stimulation (Fig. 3). This preferential Th1 differentiation induced by Peptide-25 stimulation was also dependent on APC from C57BL/6 mice. As we described, stimulation of naive CD4+ T cells from P25 TCR-Tg mice with Peptide-25 at 10 μg/ml (6.0 μM) preferentially induces Th1 development. In contrast, when we stimulated the T cells with Peptide-25 at 0.1 μg/ml (0.06 μM), we observed a Th2-dominant response (data not shown). These observations are consistent with the published data (31) addressing that IFN-γ production is preferentially induced at 1.6–6.2 μM of OVA peptide in the OVA TCR-Tg mouse model. These results further support the notion that the Peptide-25 has an intrinsically highly potential to induce Th1. Intriguingly, stimulation with APL in place of Peptide-25 induced solely IL-4-producing cells (Fig. 3). When we analyzed APC cell surface marker expression after stimulation with either Peptide-25 or APL, we did not observe an activation-dependent alteration of cell surface marker expression such as CD80, CD86, or CD40 (data not shown). The differences between Peptide-25 and APL regarding Th1 and Th2 differentiation may be due to differences in avidity between Peptide-25/I-A^b and APL/I-A^b to TCR.

Differentiation of naive CD4+ Th precursors to Th1 and Th2 is affected by the manner and environment that they encounter (2,32,33). The strength of interaction between the TCR and MHC/peptide complex affects the lineage commitment of Th cells (15,17,31,34). It is well known that Th1 cell development involves IFN-γ signaling through STAT1 and IL-12 signaling through STAT4 activation (35,36). Peptide-25-induced Th1 differentiation of naive CD4+ T cells from P25 TCR-Tg mice was observed even in the presence of anti-IFN-γ and anti-IL-12 (Fig. 4). We obtained similar results using T cells of STAT1 deficient P25 TCR-Tg mice (Fig. 5). This indicates that both IFN-γ/STAT1 and IL-12 signals are not essential for preferential induction of P25 TCR-Tg naive CD4+ T cells to Th1.

The activation and differentiation of naive CD4+ T cells appears to require at least three separate signals. The first signal is delivered through the TCR/CD3 complex after its interaction with MHC/peptide complex on APC. The second signal is provided by a number of co-stimulatory or accessory molecules on the APC that interact with their ligands on T cells such as CD28/CD80/86, CTLA-4/CD80/86, LFA-1/ICAM-1, OX40/OX40L or ICOS/B7h (37–43). The dose or antigen concentration is also important in determining the Th1-dominated immune response. Third, cytokines such as IFN-γ, IL-12 or IL-18 play a role in the expansion of the committed Th1 cells (10,11,44–46). Stimulation of naive CD4+ T cells from P25 TCR-Tg mice with Peptide-25-loaded I-A^b-CHO in primary culture lead to lower proliferation and cell recovery after culturing compared to stimulation with Peptide-25-loaded splenic APC (data not shown). Interestingly, anti-CD3 stimulation of the T cells, recovered from culture with Peptide-25-loaded I-A^b-CHO, could induce Th1 development preferentially as shown in T cells stimulated with Peptide-25 and splenic APC in primary culture (Fig. 6). As Chinese hamster ovary cells do not express detectable levels of CD80, CD86, ICAM-1, OX40L or B7h, we are in favor of the hypothesis that preferential induction of Th1 development in P25 TCR-Tg naive CD4+ T cells may be independent of these well-known co-stimulating signals from APC.

A complex network of gene transcription events is likely to be involved in establishing an environment that promotes Th1 development. T-bet, a recently discovered member of T-box transcription factor is expressed selectively in thymocytes and Th1 cells, and controls the expression of the hallmark Th1 cytokine, IFN-γ (47). T-bet expression correlates with IFN-γ expression in Th1 and NK cells. Ectopic expression of T-bet both transactivates the IFN-γ gene and induces endogenous IFN-γ production (47). T-bet appears to initiate Th1 lineage development from naive Th cells both by activating Th1 genetic programs and by repressing the opposing Th2 programs (47). It has been reported that T-bet is regulated by IFN-γ signaling through STAT1 activation in the context of TCR ligation (10,11) and induces chromatin remodeling of the *ifn*-γ locus (48). As naive $CD4^+$ T cells are capable of differentiating into IFN-γ producing cells even in the presence of anti-IFN-γ, the interaction between Peptide-25/I-A^b and TCR may directly induce T-bet that leads to Th1 differentiation. We are currently investigating T-bet expression during Th1 differentiation in P25 TCR-Tg naive $CD4^+$ T cells in response to Peptide-25-loaded I-A^b-CHO.

There are several possibilities to account for the immunogenicity and adjuvant activity of Peptide-25 for Th1 development. First, Peptide-25 may activate DCs directly or indirectly through Th cells to enhance expression of co-stimulatory molecules leading to activate Th1 precursors by enhancing well-known transcription factors such as T-bet or unidentified 'master cytokine' for Th1 development. Second, the avidity of Peptide-25 to its specific TCR would be potent enough leading to Th1 development preferentially. Third, Peptide-25 might enhance activation or selection of unidentified T cell subpopulations that suppress GATA-3 leading to Th2 development.

In conclusion, we have presented data showing that naive $CD4^+$ T cells from P25 TCR-Tg mice stimulated with Peptide-25/I-A^b that polarize to Th1 differentiation preferentially in the absence of IFN-γ or IL-12. We propose the hypothesis that direct interaction of the specific antigenic peptide/MHC class II complex and TCR may primarily influence the determination of naive $CD4^+$ T cell fate in development towards the Th1 subset. Therefore, P25 TCR-Tg mice may provide us with new insights and help us understand how Th cell fate is determined.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Abbreviations

APL	altered peptide ligand of Peptide-25
I-A^b-CHO	Chinese hamster ovary cells expressing I-A^b
P25 TCR-Tg	TCR-Tg line of mice expressing TCR-Vα5-Vβ11
TCRVβ11	Vβ11 of TCR
TG40-BP1	TG40 cells expressing TCR-αβ
TG40-BP1/CD4	TG40-BP1 cell line for expression of CD4

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Abrogation of autoimmune disease in Lyn-deficient mice by the deletion of IL-5 receptor α chain gene

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Abstract

Lyn, the *src*-family protein tyrosine kinase, plays a crucial role in the regulation of B cell antigen receptor (BCR)- and IL-5-receptor (IL-5R)-mediated signaling. *Lyn*-deficient mice have been reported to exhibit an increase in B-1 cell numbers, splenomegaly and accumulation of lymphoblast-like cells in the spleen with age, resulting in hyperimmunoglobulinemia and glomerulonephritis caused by the deposition of autoantibody complexes. To elucidate the role of IL-5 in B-1 cell activation, autoantibody production and autoimmune diseases, *Lyn*-deficient mice were crossed with IL-5Rα chain (IL-5Rα)-deficient mice and generated *Lyn*- and IL-5Rα-deficient (DKO) mice. In contrast to *Lyn*-deficient mice, DKO mice showed significantly reduced splenomegaly and lymphoadenopathy and reduced B-1 cell number in the peritoneal cavity. DKO mice also secreted low levels of IgM and IgG autoantibodies. Biochemical and histological analyses revealed that DKO mice showed milder pathogenesis of autoimmune-like disorders than *Lyn*-deficient mice. These results suggest involvement of IL-5 in enhanced B-1 cell activation, autoantibody production, and development of autoimmune disease in *Lyn*-deficient mice.

Keywords: B-1 cell; Autoimmune disease; Cytokine; Autoantibody; Interleukin-5; Lyn

1. Introduction

Lyn, one of the Src-family protein kinases, is expressed preferentially in hematopoietic cells and physically associates with receptors such as the B cell antigen receptor [1–3], CD40 [4], CD14 [5], the high affinity FcεRI complex [6], GM-CSF receptor [7], and IL-5[1] receptor [8–10]. *Lyn*-deficient ($Lyn^{-/-}$) mice exhibit splenomegaly and accumulation of lymphoblast-like cells and plasma cells in the spleen with age, resulting in hyperimmunoglobulinemia and glomerulonephritis caused by the deposition of immune complexes containing autoreactive antibodies [11]. These unusual lymphoblast-like cells were thought to belong to the B-1 lineage, which can be distinguished from conventional B-2 cells on the basis of surface phenotypes, cytokine secretion, anatomical location, and self-renewing activity [12,13].

B-1 cells have different surface markers from B-2 cells, such as $CD45^{lo}$, IgM^{hi}, $CD23^{-}$, $CD43^{+}$, and IgD^{lo}. B-1 cells in the peritoneal cavity constitutively express IL-5 receptor α chain (IL-5Rα), while only a small proportion (2–4%) of naive B-2 cells express IL-5Rα [8,14]. B-1 cells are rarely seen in the spleen and lymph nodes of healthy adult mice, while greatly expanded B-1 cell populations can be detected in the spleen of IL-5 transgenic mice [15]. Clonally expanded B-1 cells appear to invade in all lymphoid tissues [16]. B-1 cells have been implicated in strong association with autoimmune disease in the

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[1] *Abbreviations used:* IL, interleukin; IL-5R, interleukin-5 receptor; dsDNA, double strand DNA; Abs, antibodies; DKO, Lyn- and IL-5Rα-double knockout.

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murine experimental model [17]. The antibodies produced by B-1 cells have frequent reactivity with the autoantigens, such as bromelain-treated erythrocytes, immunoglobulins, and double-stranded (ds) DNA. These findings raise the possibility that autoantibody is produced mainly by B-1 cells. Actually, several strains of autoimmune-prone mice, such as motheaten, New Zealand Black (NZB) mice and their related strains of mice, exhibit increased frequencies of B-1 cells and autoantibodies to various autoantigens that are secreted by B-1 cells [18].

IL-5 binds to the IL-5R complex that consists of two distinct membrane proteins, α and βc, each of which is a member of the cytokine receptor superfamily. The binding of IL-5 occurs through the IL-5Rα, and the βc forms a high-affinity IL-5R in combination with the IL-5Rα and transduces signals [19–22]. IL-5 acts on B-1 cells and induces proliferation and differentiation into IgM-producing cells [23,24]. Transgenic mice expressing the *IL-5* gene exhibit elevated levels of IgM, IgA, and IgE in serum, an increase in the number of B-1 cells in the spleen and production of poly-reactive autoantibody in IgM class [15,25]. In contrast, IL-5Rα$^{-/-}$ and IL-5$^{-/-}$ mice show a decrease in B-1 cells in the peritoneal cavity and B-1 cell-derived surface (s) IgA^{+} cells in the lamina propria [26–29].

There are two possibilities to account for autoimmune diseases in Lyn$^{-/-}$ mice. The role of Lyn in the maintenance of immunological tolerance may be broken in Lyn$^{-/-}$ mice. Another possibility is that Lyn negatively regulates B-1 cell activation resulting in autoantibody production. We tried to elucidate whether the IL-5/IL-5R system plays an important role in the autoantibody production and autoimmune disease, which were seen in Lyn$^{-/-}$ mice, using *Lyn*- and *IL-5Rα*-deficient (DKO) mice. We show here that IL-5 is involved in autoantibody production and autoimmune disease in *Lyn*-deficient mice.

2. Materials and methods

2.1. Mice

Wild type (WT) C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). *Lyn*-deficient (Lyn$^{-/-}$) mice were prepared and maintained according to procedures previously described [11]. Lyn$^{-/-}$ and the IL-5Rα null mutant (IL-5Rα$^{-/-}$) mice [26] used in this study were backcrossed with C57BL/6J mice for more than 10 generations. Lyn$^{-/-}$, IL-5Rα$^{-/-}$, and WT mice were bred and maintained in our animal facilities under specific pathogen-free conditions. Lyn$^{-/-}$ IL-5Rα$^{-/-}$ (DKO) mice were obtained by mating Lyn$^{-/-}$ and IL-5Rα$^{-/-}$ mice. From 5- to 6-month-old mice were used in this study. All experiments were conducted according to our institution's guidelines for the care and treatment of experimental animals.

2.2. Cell preparation

Single cell suspensions were prepared from the lymphoid organs of 6-month-old mice. A standard procedure was used to prepare single cell suspensions from the peritoneal cavity, lymph nodes, and spleen. Briefly, peritoneal exudate cells were obtained by washing the peritoneal cavity with Hank's balanced salt solution (Gibco-BRL, Grand Island, NY) containing 3% FCS. Mononuclear cells from the lymph nodes or spleen were isolated by a mechanical method using a stainless steel screen.

2.3. Flow cytometry

Cells ($1–10 \times 10^5$) were stained with predetermined optimal concentrations of the respective antibodies together with antibodies together with 2.4G2 (10 μg/ml). After washing, the cells were analyzed on a FACSCalibur instrument (BD Bioscience, San Jose, CA) with CELLQuest software. The following mAbs were used: biotinylated anti-IL-5Rα (T21) [14]; PE-labeled or biotinylated anti-CD5 (53–7.3) and biotinylated anti-CD3 (145-2C11) (all purchased from BD PharMingen, San Diego, CA); FITC-labeled B220, PE-labeled B220, biotinylated anti-B220 (RA3-6B2), PE-labeled anti-IgM F(ab')$_2$, and FITC-labeled or biotinylated anti-Mac-1 (M1/70) (all obtained from Caltag Laboratories, Burlingame, CA). PE-labeled streptavidin (Ancell, Bayport, MN) or allophycocyanin-conjugated streptavidine (BD Pharmingen) were used for biotin-coupled antibody staining. In some staining, 2 μg/ml 7-amino-actinomycin D (Sigma–Aldrich, St. Louis, MO) were used to gate out dead cells.

2.4. ELISA

Amounts of each Ig isotype in sera were measured by sandwich enzyme-linked immunosorbent assay (ELISA) with antibodies specific for each mIg isotype. In brief, 96-well trays (Greiner, Frickenhausen, Germany) were coated with 10 μg/ml of isotype-specific goat anti-mIg polyclonal Abs for total Igs or 10 μg/ml of pBluescript II (Stratagene, La Jolla, CA) plasmid DNA for anti-dsDNA Abs. Samples were added to the wells and the trays were incubated at room temperature for 2 h. After washing with PBS containing 0.05% Tween 20 (washing buffer), biotinylated isotype-specific goat anti-mIg polyclonal Abs were added to each well and the trays were incubated for another 2 h. After washing with washing buffer, HRP–streptavidine was added to each well and the incubation was continued for 1 h at room temperature. Finally, trays were washed with the buffer, and 100 μl aliquots of substrate, *o*-phenylene-diamine (final

0.4 mg/ml), and hydrogen peroxide (final 0.015%), dissolved in 0.1 M citrate buffer (pH 5.0), were added to each well. The enzyme reaction was terminated by adding 50 μl of 2 M sulfuric acid and optical density at 495 nm was measured with a V-max kinetic Micro Plate Reader (Molecular Devices, Sunnyvale, CA). Using myeloma proteins (BD PharMingen), standard curves were generated for each isotype and the concentration of mIg was determined. To measure amounts of IL-5 in sera, we used Quantikine Immunoassay kit (R&D Systems, Minneapolis, MN) for mouse IL-5 and followed the manufacturer's instructions.

2.5. Histological examination

For histological examination, the spleen, kidney, and liver were fixed in 1% formaldehyde solution for 1 day and embedded in paraffin. Sections (3 μm thick) were prepared and stained with haematoxylin–eosin according to standard procedures.

2.6. Urine examination

Urine was collected from 5- to 6-month-old mice. The contents of urobilinogen, protein, glucose, and occult blood in the urine were measured by URO paper (Eiken Chemical, Tokyo, Japan). Results were expressed according to the manufacturer's recommendation. The pH of the urine was also examined by URO paper.

3. Results

3.1. Autoantibody levels in $Lyn^{-/-}$ mice and $Lyn^{-/-}$ IL-$5R\alpha^{-/-}$ mice

Lyn-deficient ($Lyn^{-/-}$) mice exhibit splenomegaly and accumulation of lymphoblast-like cells and plasma cells in the spleen with age, resulting in hyperimmunoglobulinemia and glomerulonephritis caused by the deposition of immune complexes containing autoreactive antibodies [11]. To examine the involvement of IL-5 in autoantibody production in $Lyn^{-/-}$ mice, we generated $Lyn^{-/-}$ IL-$5R\alpha^{-/-}$ (DKO) mice by crossing $Lyn^{-/-}$ and IL-$5R\alpha^{-/-}$ mice. ELISA for serum levels of immunoglobulin (Ig) subclasses in 5- to 6-month-old was carried out. The results revealed that $Lyn^{-/-}$ mice exhibited approximately 8- and 3-fold increase in total IgM and IgA levels in serum, respectively, compared with WT mice (Fig. 1A), while the IgG1 and IgG3 levels were comparable each other (data not shown). Although DKO mice showed higher IgM and IgA in serum than WT mice, the levels were 50% lower than those in $Lyn^{-/-}$ mice (Fig. 1A). We then measured serum anti-dsDNA autoantibodies in IgM and IgG classes and found that significantly elevated levels of anti-dsDNA Abs were detected in serum of both $Lyn^{-/-}$ and DKO mice. Interestingly, the levels of anti-dsDNA Abs in DKO mice were less than 50% of those in $Lyn^{-/-}$ mice (Fig. 1B). Anti-dsDNA autoantibody in serum of age-matched WT mice was undetectable.

3.2. Reduction of B-1 cells in the peritoneal cavity of $Lyn^{-/-}$ IL-$5R\alpha^{-/-}$ mice

The lymphoblast-like cells expanded in $Lyn^{-/-}$ mice were thought to belong to the B-1 lineage and to be the main cell source of autoantibody producer [30,31]. To elucidate association of B-1 cells with autoantibody production in $Lyn^{-/-}$ mice, we compared proportions of IgM^+ $CD5^+$ B-1 cells in DKO mice with those in $Lyn^{-/-}$ mice. As shown in Fig. 2A, $Lyn^{-/-}$ mice showed an increased proportion of B-1 cells in the peritoneal cavity compared to WT and DKO mice. DKO mice displayed a similar extent of B-1 cells to WT mice (Fig. 2A, upper panel). Although no significant differences in total number of IgM^+ $CD5^+$ B-1 cells were observed in the peritoneal cavity between $Lyn^{-/-}$ and WT mice, these B-1 cell numbers in DKO mice were significantly reduced, about 50 and 35% compared with that in $Lyn^{-/-}$ and WT mice,

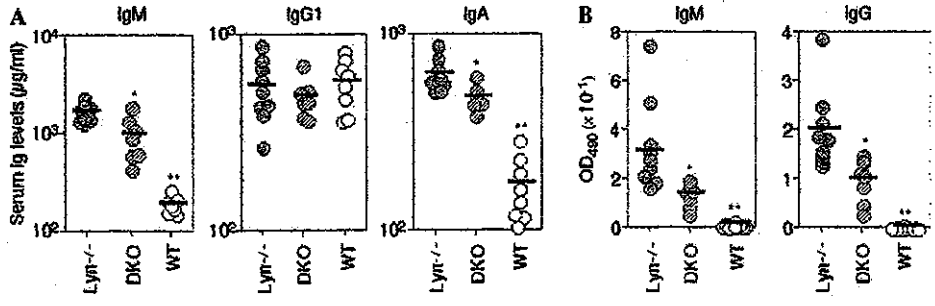

Fig. 1. Decrease of elevated immunoglobulin levels in $Lyn^{-/-}$ IL-$5R\alpha^{-/-}$ mice. Concentrations of immunoglobulin subclasses in serum of $Lyn^{-/-}$, $Lyn^{-/-}$ IL-$5R\alpha^{-/-}$ (DKO), and $Lyn^{+/+}$ IL-$5R\alpha^{+/+}$ (WT) mice were determined by isotype-specific ELISA. Anti-dsDNA antibody in serum was determined according to procedures described in the Materials and methods. The levels of immunoglobulins (A) and anti-dsDNA antibodies (B) are shown. Horizontal bar indicates mean values for each group. The mean values of indicated groups of mice are represented as a bar. $^*P < 0.05$, $^{**}P < 0.01$ by the Student's t test, compared with $Lyn^{-/-}$ mice.

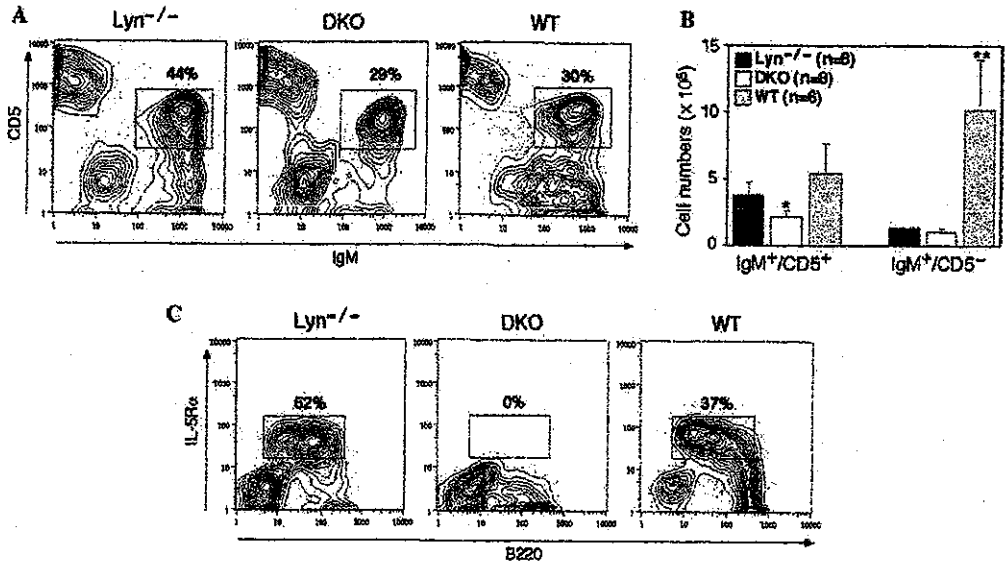

Fig. 2. Reduction of B-1 cells in $Lyn^{-/-}$ IL-5R$\alpha^{-/-}$ mice. (A) Representative two-color contour plots show the expression of IgM and CD5 on peritoneal exudate cells from the indicated group of mice. (B) Cellularity of IgM^+ $CD5^+$ cells and IgM^+ $CD5^-$ cells in the peritoneal exudate cells. $*P < 0.05$, $**P < 0.01$ by the Student's t test, compared with $Lyn^{-/-}$ mice. (C) Representative two-color contour plots show the expression of IL-5Rα and B220 on peritoneal exudates cells from each group of mice. Percentages represent the fractions of the lymphocyte-gated live cells that fall into the indicated boxes (A,C). The representative results of three independent experiments are shown (A,C).

respectively (Fig. 2B). The cell number of IgM^+ $CD5^-$ B cells in the peritoneal cavity was comparable between $Lyn^{-/-}$ and DKO mice, whose levels were significantly lower than that of WT mice (Fig. 2B). More than 50% of B cells in the peritoneal cavity of $Lyn^{-/-}$ mice expressed IL-5Rα that was approximately 40% higher than WT mice (Fig. 2C). As expected, DKO mice showed that IL-5Rα^+ B cells in the peritoneal cavity were absent. We did not observe the increase in proportion or number of IL-5Rα^+ B cells in the spleen and lymph nodes of $Lyn^{-/-}$ mice (data not shown).

3.3. Reduction of splenomegaly and lymphoadenopathy in $Lyn^{-/-}$ IL-5R$\alpha^{-/-}$ mice

$Lyn^{-/-}$ mice exhibited splenomegaly and lymphoadenopathy besides weight increase of the spleen and lymph nodes (Figs. 3A and C), as previously described [10]. Deletion of IL-5Rα in $Lyn^{-/-}$ mice resulted in splenomegaly and lymphoadenopathy that were much milder than those observed in $Lyn^{-/-}$ mice. We calculated the number of total cells, lymphocytes, and myeloid cells in the spleen and lymph nodes. As shown in Table 1, there were 50% more spleen and lymph node cells in $Lyn^{-/-}$ mice than those in DKO and WT mice. The total number of lymphocytes in the spleen was also increased in $Lyn^{-/-}$ mice. In $Lyn^{-/-}$ mice, $CD3^+$ T cells were comparable to WT mice, while myeloid cells were increased about 3-fold.

Splenomegaly seen in aged-$Lyn^{-/-}$ mice is mainly due to the massive increase of Mac-1^+ $B220^-$ and cytoplasmic IgM^+ lymphoblast-like cells [11]. We confirmed severely increased proportion of Mac-1^+ $B220^-$ cells in the spleen and lymph nodes in $Lyn^{-/-}$ mice compared with WT mice (Figs. 3B and D). As shown in Table 1, absolute cell number of Mac-1^+ $B220^-$ cells (Mac-1^+ cells) and Mac-1^- $B220^+$ cells ($B220^+$ cells) was also changed significantly in $Lyn^{-/-}$ mice compared to that in WT mice. The total number of Mac-1^+ cells in the spleen of DKO mice was reduced to a half of those in $Lyn^{-/-}$ mice, even the proportion of splenic Mac-1^+ cells was comparable (Fig. 3B), and was 6-fold more cells than WT mice. Splenic $B220^+$ B cells in DKO mice were reduced to 75 and 30% compared with $Lyn^{-/-}$ and WT mice (Fig. 3B and Table 1). $B220^+$ cells in the lymph nodes of DKO mice were greatly reduced compared to those of WT or $Lyn^{-/-}$ mice (Fig. 3D and Table 1). Mac-1^+ cells were also reduced to 3-fold in the lymph nodes of DKO mice compared to those of $Lyn^{-/-}$ mice, though the number of the cells was 160% increased compared to those of WT mice (Fig. 3D and Table 1). These data indicate that splenomegaly and lymphoadenopathy observed in $Lyn^{-/-}$ mice can be rescued partially by the blocking of IL-5/IL-5R interaction.

3.4. Splenic architecture and glomerulonephritis in $Lyn^{-/-}$ IL-5R$\alpha^{-/-}$ mice

Histological sections of the spleen, kidney, and liver from each group of $Lyn^{-/-}$, DKO and WT mice were prepared and stained with haematoxylin–eosin. Pathological examinations revealed that mature lymphocytes in lymphoid follicles were disappeared and large lymphoblast-like cells and reticular cells were accumulated in white

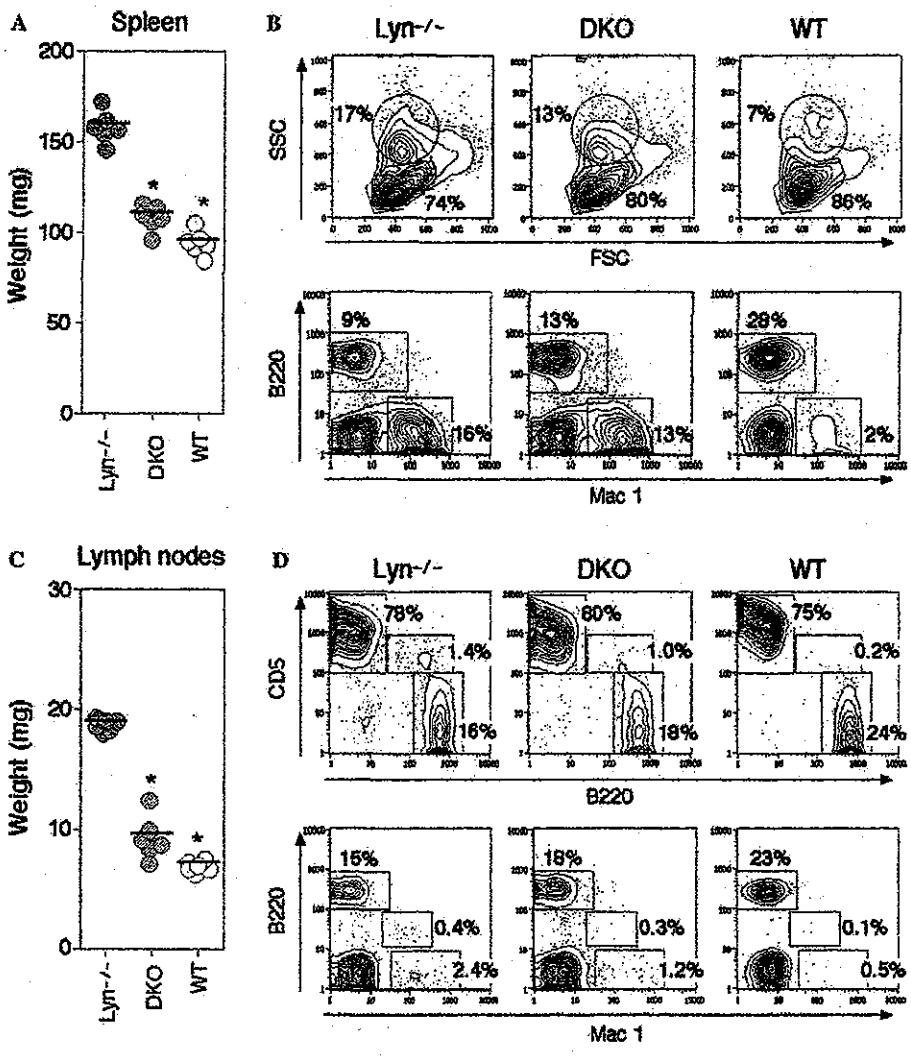

Fig. 3. Reduced splenomegaly and lymphoadenopathy in Lyn$^{-/-}$ IL-5Rα$^{-/-}$ mice. Weights of the spleen (A) or lymph nodes (C) from 5- to 6-month-old each group of mice were measured. The mean values of indicated group of mice are represented as a bar. *$P < 0.01$ by the Student's t test, compared with Lyn$^{-/-}$ mice (A,C). Representative two-color contour plots showing expression of FSC, SSC, B220, and Mac-1 on cells from the spleen (B) or CD5, B220, and Mac-1 on cells from lymph nodes (D). Percentages represent the fractions of the total live cells that fall into the indicated regions. Representative result of at least three independent experiments is shown.

pulp of the spleen from 6-month-old Lyn$^{-/-}$ mice (Fig. 4A, left panel). Moreover, Lyn$^{-/-}$ mice showed severe hyperplasia of fibrous tissue in the spleen. DKO mice showed above abnormalities in the spleen that were much milder than those seen in Lyn$^{-/-}$ mice (Fig. 4A, middle panel). We saw no abnormalities in the spleen from 6-month-old WT mice (Fig. 4A, right panel). Histological examinations of the kidney sections showed that both Lyn$^{-/-}$ and DKO mice developed glomerulonephritis with age but glomerulonephritis in DKO mice was also much milder than that observed in Lyn$^{-/-}$ mice (Fig. 4B, left panel vs. Fig. 4B, middle panel). We saw no abnormality in the liver from Lyn$^{-/-}$, DKO or WT mice (Fig. 4C).

When we examined the biochemical data in urine of these mice at 20–25-week old of age, DKO mice showed significantly lower levels of protein in their urine (0–10 mg/dL) than Lyn$^{-/-}$ mice (30–100 mg/dL) (data not shown). Thus, milder development of disruptions in splenic architecture and glomerulonephritis in DKO mice than in Lyn$^{-/-}$ mice appears to be related with the blocking of the IL-5/IL-5R signal.

4. Discussion

Lyn is expressed in various hematopoietic cells, including B lineage cell but little to no expression has been found in T cells [2]. In B cells, Lyn expression is maintained throughout B cell development and plays an important role in signaling through BCR and CD22 [32]. We have shown that Lyn is activated in IL-5-stimulated B cells [22]. Extensive analysis of Lyn$^{-/-}$ mice revealed

Table 1
Comparison of total cell number, lymphocytes, and myeloid cells in the spleen and lymph nodes from $Lyn^{-/-}$, $Lyn^{-/-}$ IL-5R$\alpha^{-/-}$ (DKO), or WT mice

	$Lyn^{-/-}$ ($n=10$)	DKO ($n=10$)	WT ($n=6$)
Spleen ($\times 10^7$)			
Total cells	18.5 ± 2.1	$13.3 \pm 1.8^{**}$	$12.3 \pm 2.4^{**}$
Lymphocytes	14.7 ± 1.7	$10.6 \pm 1.6^{**}$	$10.5 \pm 1.8^{**}$
$B220^+$ cells	1.62 ± 0.09	$1.16 \pm 0.12^{**}$	$4.12 \pm 0.14^{**}$
Mac-1^+ cells	3.25 ± 0.12	$1.63 \pm 0.13^{**}$	$0.26 \pm 0.03^{**}$
$CD3^+$ cells	5.71 ± 0.54	5.12 ± 0.41	5.14 ± 0.44
Myeloids	3.15 ± 0.21	$1.73 \pm 0.14^{**}$	$1.05 \pm 0.08^{**}$
Lymph nodes ($\times 10^5$)			
Total cells	19.9 ± 2.8	$12.9 \pm 1.6^{**}$	$11.6 \pm 1.9^{**}$
Lymphocytes	18.8 ± 2.1	$11.8 \pm 1.2^{**}$	$10.9 \pm 1.7^{**}$
$B220^+$ cells	2.51 ± 0.23	$1.65 \pm 0.15^{**}$	$2.81 \pm 0.13^{*}$
Mac-1^+ cells	8.31 ± 0.21	$2.98 \pm 0.15^{**}$	$1.81 \pm 0.09^{**}$
$CD3^+$ cells	6.74 ± 0.59	6.12 ± 0.38	6.22 ± 0.41
Myeloids	1.12 ± 0.12	1.14 ± 0.15	1.05 ± 0.14

$^*P < 0.05$, $^{**}P < 0.01$ by the Student's t test, compared with $Lyn^{-/-}$ mice.

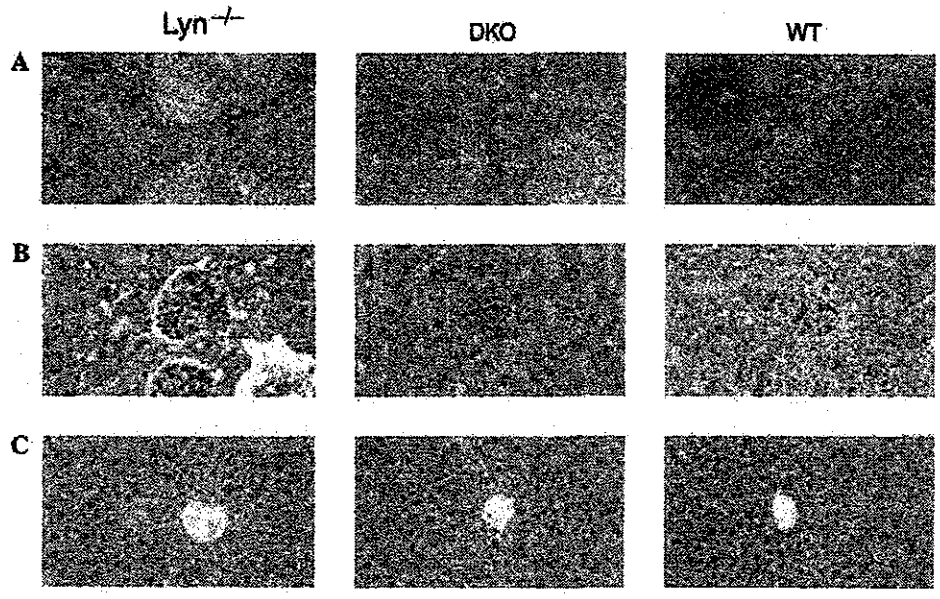

Fig. 4. Histological analyses of the spleen (A), kidney (B), and liver (C) from $Lyn^{-/-}$, $Lyn^{-/-}$ IL-5R$\alpha^{-/-}$, and IL-5R$\alpha^{+/+}$ mice. The tissues from 6-month-old mice were fixed in formalin and embedded in paraffin. The histological sections were stained with haematoxylin–eosin.

various abnormalities including reduction of mature B cells, reduction of recirculating B cells, hyper IgM in serum, production of dsDNA autoantibodies, splenomegaly, lymphoadenopathy, the dramatically increase of Mac-1^+ cells in the white pulp of spleen and development of renal defects probably associated with autoimmune disease [11]. $Lyn^{-/-}$ B-2 cells also showed the impaired proliferation and Ig production in response to LPS, CD38, and TI-2 antigen [33,34]. Furthermore, $Lyn^{-/-}$ B-2 cells in spleen showed enhanced expression of IL-5Rα within 2 days of culture in the absence of exogenous stimuli and responded to IL-5 resulting in significantly augmented IgM production [33]. In contrast, the IL-5Rα expression was tightly regulated in wild type B-2 cells in spleen and did not produce significant amounts of IgM for 5-day culture in the presence of IL-5. These results suggest that Lyn regulates the IL-5Rα expression on B-2 cells resulting in IL-5-dependent IgM production. Taking results together, Lyn is involved in the regulation of B-2 cell activation to various stimuli including IL-5 though IL-5R.

B-1 cells, a subpopulation of B cells, are suggested to be associated with autoimmune diseases because the number of B-1 cells is increased in certain autoimmune mouse strains. B-1 cells are generated from B cell precursors early in life and maintained by self-renewal. Mutation of genes encoding signal transduction molecules, such as Syk, Btk, CD19, and Vav, affects the development of B-1 cells and leads to a loss of this subpopulation. Abnormal expansion and activation of B-1 cells

associate with a B cell hyperplasia, particularly of the B-1 cells, hyper-IgM, and autoantibody production [35]. For example, viable motheaten mice, which have mutations of gene encoding the SH2-containing phosphotyrosine phosphatase (SHP1), contain only B-1 cell and are affected by a severe autoimmune condition early in life [36]. In contrast to $Lyn^{-/-}$ mice showing expansion of B-1 cells in the peritoneal cavity and autoimmune disease, $Lyn^{-/-}$ mice having the *xid* gene exhibit disappearance of B-1 cells, markedly reduced serum IgM and IgG3 revels, repaired splenomegaly and no evidence for autoimmune disease [31]. These support the notion that Lyn and Btk regulate B-1 cell development and activation. $Lyn^{-/-}$ B-1 cells showed higher proliferation and IgM production in vitro in response to IL-5 than these of wild type B-1 cells (data not shown), suggesting that Lyn negatively regulates IL-5-induced B-1 cell triggering. It remains unclear at this moment, however, whether B-2 cells or B-1 cells in $Lyn^{-/-}$ mice play crucial roles for the development of autoantibody production and autoimmune diseases.

Other researchers and we have reported that IL-5 is an important cytokine for development, survival, and activation of B-1 cells. $IL\text{-}5^{-/-}$ and $IL\text{-}5R\alpha^{-/-}$ mice or anti-IL-5-treated mice show reduced B-1 cell number, impaired homeostatic proliferation of B-1 cells and suppressed B-1 cell activation in response to CD40 or LPS [26,27,37]. $IL\text{-}5R\alpha^{-/-}$ mice having enforced expression of IL-5Rα by crossing with IL-5Rα transgenic mice show fully restored B-1 cells in number and in function [29]. IL-5 administration activates autoreactive B-1 cells to secrete autoantibody in the anti-red blood cell (RBC) Ig-transgenic (HL) mouse model [38]. In HL mice, which B-2 cells are deleted by spontaneous exposure to RBC but B-1 cells escape from clonal deletion, autoimmune hemolytic anemia is induced in conventional conditions, for which B-1 cells are responsible because deletion of B-1 cells by RBC injection cures autoimmune anemia in HL mice [39]. Furthermore, these anti-RBC Ig-producing B-1 cells are undetectable in the peritoneal cavity of HL mice, which *IL-5Rα* gene is deleted ($IL\text{-}5R\alpha^{-/-} \times$ HL mice), although they contain 30% of the peritoneal B-1 cells as compared with HL mice [40]. These results indicate that IL-5 plays a critical role in differentiation of autoreactive B-1 cells. Actually, most peritoneal B-1 cells express IL-5Rα and respond to IL-5 in high frequency for IgM production (data not shown) [14].

Molecular mechanisms of autoimmune disease observed in $Lyn^{-/-}$ mice have not been fully elucidated. There are at least two possibilities. First, Lyn is involved in maintenance of self-tolerance and clonal deletion in B cells. In this case, IL-5Rα deficiency may not affect significantly on autoimmune disease observed in $Lyn^{-/-}$ mice, because DKO mice still exhibit significant amounts of autoantibody production. Another possibility is that Lyn solely regulates expansion and activation of B-1 cells negatively. If this is the case, IL-5R deficiency may decrease B-1 cell number and activation leading to prevent from autoimmune disease observed in $Lyn^{-/-}$ mice. To address this issue, we examined the extent to which IL-5 is involved in autoantibody production and autoimmune disease observed in Lyn-deficient conditions. In the present study, we directly demonstrated, using $Lyn^{-/-}$ $IL\text{-}5R\alpha^{-/-}$ (DKO) mice, that IL-5Rα deficiency partially reduced the accelerated expansion and activation of B-1 cells and rescued from the production of pathogenic autoantibody observed in Lyn kinase deficiency.

There are several explanations for abrogation of autoantibody production and autoimmune diseases in DKO mice compared with $Lyn^{-/-}$ mice. First, B-1 cells in the peritoneal cavity of DKO mice may be reduced leading to abrogate autoimmune diseases in DKO mice. As shown in Fig. 2, DKO mice showed reduced proportion and absolute cell number of B-1 cells in the peritoneal cavity. Second, abnormal expansion of $IL\text{-}5R\alpha^{+}$ B-1 cells other than in the peritoneal cavity such as in the spleen of $Lyn^{-/-}$ mice may be occurred that might be involved in onset of autoimmune diseases and DKO mice might show reduced B-1 cells in the organ. Our extensive analysis of $IL\text{-}5R\alpha^{+}$ B-1 cells in the spleen and lymph nodes revealed that the proportion of $IL\text{-}5R\alpha^{+}$ cells at least in the spleen and lymph nodes of both $Lyn^{-/-}$ and DKO mice was nearly equivalent (data not shown). Third, splenic $Mac\text{-}1^{+}$ cells that were reported to produce IgM in $Lyn^{-/-}$ mice without any exogenous stimulation [11] may be reduced in DKO mice. Those splenic $Mac\text{-}1^{+}$ cells may be plasmablast generated though unusual maturation of B-1 cells upon encounter with self-antigens. We did not see significant differences in proportions of splenic $Mac\text{-}1^{+}$ cells between $Lyn^{-/-}$ and DKO mice (Fig. 3B), even DKO mice showed decreased $Mac\text{-}1^{+}$ cell number in spleen and splenomegaly compared to $Lyn^{-/-}$ mice (Table 1). Fourth, IL-5 may be produced in $Lyn^{-/-}$ mice in response to autoantigens by unknown mechanisms in the absence of exogenous stimulation that is reduced in DKO mice. We titrated serum IL-5 by ELISA and found that significant levels of IL-5 were detected in serum of three out of eight $Lyn^{-/-}$ mice (8–16 pg/ml) and four out of eight DKO mice (8–13 pg/ml) (data not shown). In WT mice, we did not detect IL-5 in serum (data not shown). No significant differences were observed in serum IL-5 levels between $Lyn^{-/-}$ and DKO mice. Fifth, IL-5 might synergize with other cytokines such as IL-9 or IL-10 to induce expansion and activation of B-1 cells to produce autoantibody.

In conclusion, the present study provides evidence that inhibition of IL-5 signaling rescues to a certain extent from autoimmune diseases induced by deletion of Lyn. The results imply that IL-5 plays a role to certain extent in regulating B-1 cell activation and autoantibody production in Lyn-deficient background. We are in favor

of hypothesis that Lyn deficiency causes expansion of B-1 cell compartment and induces B-1 cell proliferation and activation leading to autoantibody production and onset of autoimmune diseases that may be associated with IL-5 signaling. However, results do not exclude possibilities that factors or microenvironment other than IL-5 signaling also play important roles in the breakdown of self-tolerance in Lyn-deficient conditions.

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Increased Numbers of B-1 Cells and Enhanced Responses against TI-2 Antigen in Mice Lacking APS, an Adaptor Molecule Containing PH and SH2 Domains

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APS (adaptor molecule containing PH and SH2 domains) is an intracellular adaptor protein that forms an adaptor family along with Lnk and SH2-B. While experiments using cultured cell lines have demonstrated that APS is phosphorylated in response to various stimuli, its in vivo functions remain unclear. We attempted to determine the physiological roles of APS by generating *APS*-deficient (*APS*$^{-/-}$) mice. *APS*$^{-/-}$ mice were viable and fertile and showed no abnormalities or growth retardation. Immunologically, *APS*$^{-/-}$ mice showed normal development and distribution of lymphocytes and myeloid cells, except for increased numbers of B-1 cells in the peritoneal cavity. *APS*$^{-/-}$ mice exhibited an enhanced humoral immune response against trinitrophenol-Ficoll, a thymus-independent type 2 antigen, while *APS*$^{-/-}$ B-2 cells exhibited normal proliferative responses and tyrosine phosphorylation of intracellular proteins upon B-cell receptor (BCR) cross-linking. APS colocalized with filamentous actin (F-actin) accumulated during the capping of BCRs in *APS*-transgenic B cells. After BCR stimulation, F-actin contents were lower in *APS*$^{-/-}$ B-1 cells than in wild-type B-1 cells. Our results indicate that APS might have a novel regulatory role in actin reorganization and control of B-1 cell compartment size.

B cells differentiate from progenitor cells to form a diverse repertoire reactive to almost all potential pathogens. During this differentiation, a series of developmental programs and checkpoints regulate the production of functionally mature B cells. The pre-B-cell receptor (pre-BCR) shares many signaling components with the BCR and transmits critical signals that allow the selection of precursors that express productive immunoglobulin heavy chains (29). Self-antigens presented on stromal cells trigger BCRs, which then provide signals that help determine the fate of the lymphoid precursors (13). Various growth factors and cytokines, such as stem cell factor, Flt3-L, and interleukin 7 (IL-7), assist in the regulation of lymphoid precursor expansion by binding to c-Kit, Flk2, and IL-7 receptors, respectively (3).

Developing B-cell precursor populations, as well as peripheral B-cell subpopulations, can be characterized according to their expression of BCRs and various other surface markers (9). Immature B cells generated in the bone marrow (BM) emigrate to the periphery and give rise to a heterogeneous peripheral B-cell population, consisting of recirculating cells located in spleen and lymph node follicles and nonrecirculating cells mainly localized to the splenic marginal zone (MZ). The majority of splenic B cells in the adult mouse are follicular (FO) B cells, with MZ B cells representing only 5 to 10% (26, 34). B-1 cells are another self-renewing B-cell subset, but they do not develop in the BM. B-1 cells predominate in the peritoneal and pleural cavities (21). While MZ B cells and B-1 cells produce natural antibodies and provide a first line of defense against antigens, FO B cells are involved in thymus-dependent (TD) antibody responses, from which memory and plasma cells are generated (26).

The binding of extracellular ligands to cell surface polypeptide receptors, such as antigen receptors and growth factor receptors, initiates a cascade of events through the activation of intracellular protein kinases (2, 40). The phosphorylation events catalyzed by these kinases both modulate the catalytic activity of effector enzymes and mediate the protein-protein interactions that juxtapose critical signal transduction elements. While the details of how signaling molecules are activated or recruited to receptors have yet to be completely elucidated, recent studies have defined an array of adaptor proteins that integrate and regulate multiple signaling events (22, 25, 37). Adaptor proteins lack kinase, phosphatase, and transcriptional domains and instead contain multiple binding sites, such as SH2, SH3, or PH domains, that mediate protein-protein or protein-lipid interactions. The importance of adaptor proteins has been demonstrated in various signaling pathways. For example, mice lacking the adaptor protein SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) or LAT (linker for activation of T cells) show severe defects in T-cell development due to impaired pre-T-cell receptor signaling during T lymphopoiesis (4, 38, 52). Similarly, mice lacking BLNK/SLP-65/BASH manifest severe defects in the maturation of pro-B cells to pre-B cells (11, 20, 36).

Together with Lnk and SH2-B, APS forms an adaptor protein family that shares a homologous N-terminal region with proline-rich stretches, PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site (19, 35, 43, 50). It has previously been demonstrated that Lnk plays a critical role in

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the regulation of B-cell precursor and hematopoietic progenitor cell production. Mutant mice lacking Lnk show enhanced B-cell production due to the hypersensitivity of B-cell precursors to stem cell factor, a ligand for c-Kit (43). In addition, $lnk^{-/-}$ mice exhibit increased numbers of hematopoietic progenitors in the BM, and the ability of hematopoietic progenitors to repopulate irradiated host animals was greatly enhanced by the absence of Lnk (42). It has also been reported that SH2-B is an important signaling molecule in the insulin-like growth factor I-mediated reproductive pathway. $SH2\text{-}B^{-/-}$ mice displayed slight growth retardation and impaired fertility due to insufficient gonadal tissue maturation (33). A third member of the Lnk protein family was also isolated by using a homologous region conserved between Lnk and SH2-B as a probe (19). This protein was the murine homologue of APS that had already been cloned as a possible candidate substrate for the c-Kit tyrosine kinase receptor. Other studies have since shown that APS is phosphorylated upon stimulation with various growth factors, including insulin, nerve growth factor (NGF), and BCR cross-linking (19, 31, 39, 50). Nonetheless, the physiological role of APS has not been properly evaluated in vivo.

To investigate the in vivo function of APS in the immune system, we generated mutant mice that lacked APS ($APS^{-/-}$ mice). $APS^{-/-}$ mice were viable, fertile, and showed no abnormalities or growth retardation. Although lymphoid- and myeloid-cell developments were not severely affected, B-1 cell numbers in the peritoneal cavity were increased and humoral immune responses to thymus-independent type 2 (TI-2) antigen were significantly enhanced in the absence of APS. APS colocalized with filamentous actin (F-actin) during the capping of receptors of stimulated B cells overexpressing APS, which showed increased F-actin contents upon BCR cross-linking. In contrast, F-actin content was lower in stimulated $APS^{-/-}$ B-1 cells than in wild-type B-1 cells. These results indicated that APS might regulate the function and maintenance of B-1 cells and modulate the response against TI-2 antigen via controlling actin reorganization.

MATERIALS AND METHODS

Mice. Genomic fragments containing coding exons of the *APS* gene were isolated as described previously (19). The G418 resistance cartridge from pMC1*neo* (Stratagene, La Jolla, Calif.) was inserted between the 3.6-kb *Eco*RI fragment 5′ of the first coding exon and the 2.4-kb PCR-amplified fragment containing the seventh coding exon, and the herpes simplex virus *tk* gene was ligated to the 3′ end for selection against nonhomologous recombination. Transfection into AK7 embryonic stem (ES) cells (8) and the screening of ES clones carrying the targeted allele were performed as described previously (43). The generation of chimeric mice from $APS^{+/-}$ ES cells and germ line transmission of the mutant allele were confirmed by Southern blot analysis of tail DNA obtained from progeny mice. Experiments were performed with 129/Sv-C57BL/6 mixed-background mice. All mice were housed under specific-pathogen-free conditions and were analyzed between 6 and 12 weeks of age.

The generation of lymphocyte-specific *APS*-transgenic (*APS*-Tg) mice will be described elsewhere (M. Iseki and S. Takaki, unpublished data). Briefly, mouse APS cDNA (19) was inserted into the p1026x vector that contained the murine *lck* proximal promoter, immunoglobulin intronic heavy-chain enhancer Eμ, and the human growth hormone (GH) gene cassette (18, 44), and fragments containing the transgene were injected into mouse zygote pronuclei.

Flow cytometry. Single-cell suspensions of lymphocytes from BM, spleen, or the peritoneal cavity were prepared, stained, and analyzed as previously described (43). The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 (M1/70), phycoerythrin (PE)-conjugated and biotin-conjugated anti-CD3ε (145-2C11), PE- and biotin-anti-CD43 (S7), PE-anti-Gr-1 (RB6-8C5), FITC-anti-CD21/CD35 (7G6), PE- and biotin-anti-CD23 (B3B4), PE- and biotin-anti-CD5 (53-7.3) (all purchased from BD PharMingen, San Diego, Calif.), FITC- and PE-anti-B220 (RA3-6B2) (Caltag Laboratories, Burlingame, Calif.), biotin-anti-IL-5 receptor alpha (IL-5Rα) (T21) (15), and biotin-anti-immunoglobulin D (IgD) (CS/15; a gift from K. Miyake, University of Tokyo). FITC- and PE-conjugated $F(ab')_2$ polyclonal anti-IgM fragments were purchased from Caltag Laboratories, and PE- and TRI-COLOR-conjugated streptavidins (Caltag Laboratories) were used to reveal biotin-coupled antibody staining.

Proliferation assays. Splenic B cells were purified by using a magnetic cell sorting system (Miltenyi Biotec, Bergisch Gladbach, Germany) following incubation with biotin-conjugated anti-CD43 (BD PharMingen) and streptavidin-coupled microbeads (Miltenyi Biotec). B cells (2×10^5) were cultivated in 0.2 ml of RPMI 1640 medium supplemented with 8% fetal calf serum, 50 μM 2-mercaptoethanol, 100 U of penicillin/ml, and 100 μg of streptomycin/ml in a 96-well plate. The cells were stimulated with $F(ab')_2$ of goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, Pa.), IL-4 (PeproTech EC, London, United Kingdom), anti-CD40 (HM40-3; BD PharMingen), or lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.). The cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 16 h of a 66-h culture period, and the incorporated [^{3}H]thymidine was measured with a MATRIX 96 direct beta counter (Packard, Meriden, Conn.).

Immunization and ELISA. Concentrations of each immunoglobulin isotype in serum were determined for 6-week-old mice by isotype-specific enzyme-linked immunosorbent assay (ELISA) (46). To measure the production of the antibodies against a TI antigen, the mice were intraperitoneally injected with 10 μg of trinitrophenol (TNP)-LPS (TI-1 antigen) or 10 μg of TNP-Ficoll (TI-2 antigen) in normal saline and bled on days 7 and 14 after injection. Serial dilutions of serum were analyzed for TNP-specific immunoglobulin isotypes by ELISA with 2,4-dinitrophenol-coupled bovine serum albumin (which cross-reacts with anti-TNP antibodies) as a capture reagent. To examine the response against a TD antigen, mice were immunized intraperitoneally with 10 μg of TNP-keyhole limpet hemocyanin (KLH) in a 1:1 homogenate of incomplete Freund's adjuvant (Nacalai Tesque, Kyoto, Japan) and normal saline. A booster dose of 10 μg of TNP-KLH in normal saline was given on day 21. The mice were bled on days 7, 14, 28, and 35, and the presence of anti-TNP antibodies of each immunoglobulin subclass was determined by using a TNP-specific ELISA.

Immunoprecipitation and immunoblotting. Purified splenic B cells were stimulated with 30 μg of $F(ab')_2$ goat anti-mouse IgM/ml and lysed with lysis buffer (19). Cell lysates were subjected to immunoprecipitation and immunoblot analysis as previously described (19). Briefly, lysates were incubated with anti-APS-N antibodies and protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) at 4°C for 1 h. Then, the Sepharose beads were washed five times with 1% NP-40–phosphate-buffered saline (PBS). The precipitated proteins were resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis under reducing conditions and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, Mass.). After blocking with 5% bovine serum albumin or low-fat dry milk–Tris-buffered saline (pH 7.6), blots were probed with anti-APS (19) and anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology, Lake Placid, N.Y.).

F-actin measurement. Purified B cells or antibody-stained splenocytes or peritoneal cells were stimulated with 30 μg of $F(ab')_2$ goat anti-mouse IgM/ml, washed with PBS, and then fixed in 3.7% formaldehyde for 30 min on ice. Following permeabilization with 0.2% Triton X-100 in PBS for 30 min, the cells were stained with rhodamine-phalloidin (Molecular Probes, Eugene, Oreg.) for 1 h and washed three times. Fluorescence intensity was measured by flow cytometry.

Confocal microscopy. Purified B cells stimulated with $F(ab')_2$ goat anti-IgM antibody were deposited on microscope slides by using a Cytospin 3 centrifuge (Shandon Scientific, Cheshire, England) and fixed in 3.7% formaldehyde. The cells were permeabilized with 0.2% Triton X-100 in PBS and stained with anti-APS and FITC-conjugated anti-rabbit IgG (for APS detection) and rhodamine-phalloidin (for actin detection). After washing with PBS, coverslips were mounted and the slides were observed with a laser scanning confocal microscope (Olympus, Tokyo, Japan).

RESULTS

Generation of $APS^{-/-}$ mice. To generate APS-null mice, we deleted six exons of the *APS* gene, from the first methionine residue to the middle of the SH2 domain, and replaced this region with the neomycin phosphotransferase cassette (Fig. 1A). The successful disruption of the *APS* gene and the absence of intact or partial APS proteins were confirmed by

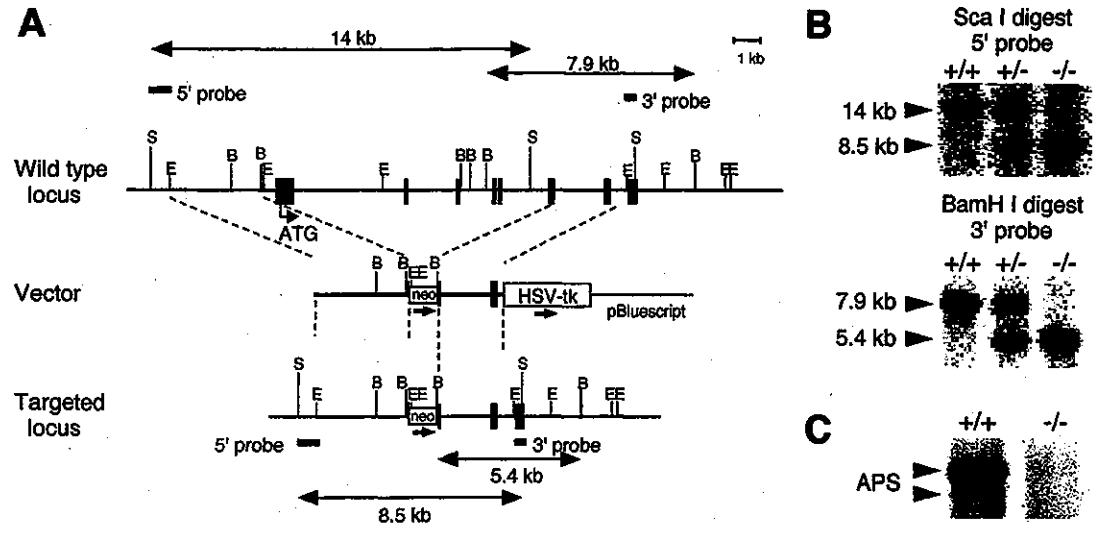

FIG. 1. Generation of *APS*-deficient mice. (A) Schematic representations of wild-type and mutant *APS* gene loci and the targeting vector. Exons are represented by black boxes. The neomycin resistance gene (neo) and the gene encoding herpes simplex virus thymidine kinase (HSV-tk) are indicated. The 5′ and 3′ probes used for Southern blotting are indicated. Restriction site abbreviations: B, *Bam*HI; S, *Sca*I; E, *Eco*RI. (B) Representative Southern blot analysis. Tail genomic DNA obtained from +/+, +/−, and −/− mice was probed with 5′ or 3′ probes. (C) Lack of APS protein expression in $APS^{-/-}$ mice. Splenocytes obtained from +/+ or −/− mice were lysed, and APS expression was determined by immunoprecipitation followed by immunoblot analysis with anti-APS C-terminal antibodies. APS proteins were detected as doublet bands (arrowheads).

Southern blot and immunoblot analyses (Fig. 1B and C). $APS^{-/-}$ mice were produced in a Mendelian ratio from heterozygous crossbreeding (+/+, 22.3%; +/−, 48.8%; and −/−, 28.9% [n = 121]) and were indistinguishable from littermates with respect to appearance, general behavior, body and organ size, and fertility.

B-1 cell numbers were increased in $APS^{-/-}$ mice. APS is highly expressed in the spleen, lymph nodes, and BM, as well as in the brain and kidney (19). It has also been shown that APS is expressed by B cells but not by T cells in the spleen and is tyrosine phosphorylated upon BCR cross-linking in B-cell lines (19). Based on these observations, we examined lympho-

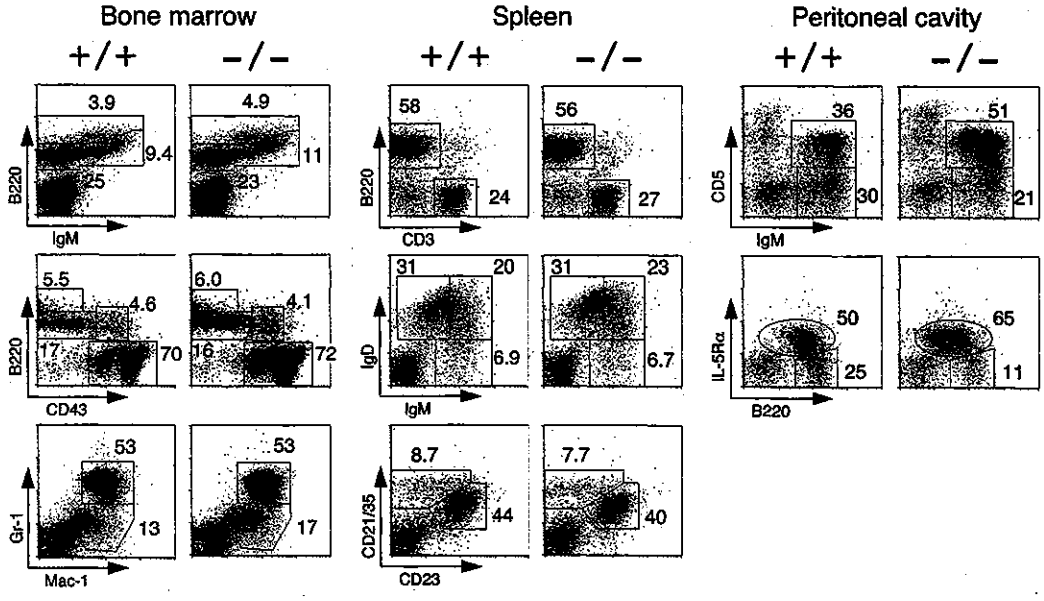

FIG. 2. Lymphocyte development in $APS^{-/-}$ mice. Representative two-color fluorescence plots show BM cell expression of B220 and IgM, B220 and CD43, and Gr-1 and Mac-1 (left panels); the splenocyte expression of B220 and CD3, IgD and IgM, and CD21/35 and CD23 (middle panels); and the peritoneal cavity lymphocyte expression of IgM and CD5 and of B220 and IL-5Rα (right panels). Numbers represent the percentages of cells that fall into the indicated boxes of total gated live cells. $CD5^+$ and IL-$5R\alpha^+$ B-1 cell numbers were increased in the peritoneal cavities of $APS^{-/-}$ mice.

TABLE 1. Increase in B-1 cell numbers in the peritoneal cavities of *APS*$^{-/-}$ mice[a]

Cells	Mean no. of B-1 cells (10^6) ± SEM in mice with genotype (n)[b]:	
	+/+ (13)	−/− (17)
Total	5.1 ± 0.31	6.1 ± 0.52
IgM$^+$ CD5$^+$	0.48 ± 0.069	0.90 ± 0.13[c]
IgM$^+$ CD5$^-$	0.41 ± 0.040	0.62 ± 0.089

[a] Cells obtained from the peritoneal cavity were labeled with the indicated antibodies and analyzed by flow cytometry.
[b] Statistical analysis was carried out by using Student's t test. n, number of mice.
[c] Significantly different from the value for +/+ mice at a P value of <0.02.

cyte and myeloid-cell development in *APS*$^{-/-}$ mice. As expected from the undetectable *APS* expression in wild-type T-lineage cells, thymocytes developed normally in *APS*$^{-/-}$ mice, and subpopulations of peripheral CD4$^+$ and CD8$^+$ T cells and CD122 (IL-2Rβ)$^+$ NK T cells were not altered in the absence of APS (data not shown).

The lymphocyte and myeloid-cell numbers in *APS*$^{-/-}$ mouse BM, spleen, and lymph nodes were also normal. B-cell precursor fractions, such as pro-B cells (B220$^+$ CD43$^+$), pro-B and pre-B cells (B220$^+$ IgM$^-$), immature B cells (B220$^+$ IgM$^+$), recirculating mature B cells (B220^{high} IgM$^+$), and myeloid cells (Mac-1$^+$ Gr-1$^+$) from *APS*$^{-/-}$ BM were also comparable to those from wild-type BM (Fig. 2). In the spleen, the ratio of B (B220$^+$) to T cells (CD3$^+$) and the percentages of B-cell subpopulations, such as immature (IgM^{high} IgD^{low}), transitional (IgM^{high} IgD^{high}), mature (IgM^{low} IgD^{high}), FO (CD21/35$^+$ CD23$^+$), and MZ (CD21/35^{high} CD23$^-$) cells, were not altered by *APS* deficiency (Fig. 2). In addition, we observed no abnormalities in terms of splenic architecture in *APS*$^{-/-}$ mice, and B-cell follicles, MZs, and T-cell areas were all well maintained (data not shown).

As B-1 cells have characteristics that are distinct from those of BM-derived B-2 cells in terms of development, anatomic localization, and function, we next examined peritoneal cavity B-1 cells. The total number of peritoneal lavage cells was slightly increased in *APS*$^{-/-}$ mice (Table 1). As shown in Fig. 2, IgM$^+$ CD5$^+$ B-1a cells, as well as total B220$^+$ IL-5Rα$^+$ B-1 cells, were overrepresented in *APS*$^{-/-}$ mice, and the number of IgM$^+$ CD5$^+$ B-1a cells was nearly twofold higher in *APS*$^{-/-}$ mice than in wild-type mice, while IgM$^+$ CD5$^-$ B-2 cell numbers were unchanged (Table 1). Thus, our results indicated that, while APS appeared not to be essential for the development of most lymphoid and myeloid cells, *APS* deficiency did result in a significant increase in B-1 cell numbers.

B-cell responses in the absence of APS. We then studied B-cell responses against various mitogenic stimuli to determine whether B-cell activation was affected by the absence of APS. Upon anti-IgM stimulation, *APS*$^{-/-}$ splenic B cells exhibited proliferation that was comparable to that of control B cells (Fig. 3A). *APS*$^{-/-}$ B cells also proliferated to the same extent as *APS*$^{+/+}$ cells in response to LPS, anti-CD40, and IL-4 (Fig. 3A). The patterns of total cellular protein tyrosine phosphorylation and BCR-induced calcium influx were not significantly different in *APS*$^{-/-}$ splenic B cells and splenic B cells from *APS*$^{+/+}$ littermates (Fig. 3B and data not shown). Thus, the biological and biochemical responses of splenic B cells in vitro were not significantly altered in the absence of APS.

The finding of increased B-1 cell numbers in the peritoneal cavity prompted us to examine the humoral immune responses

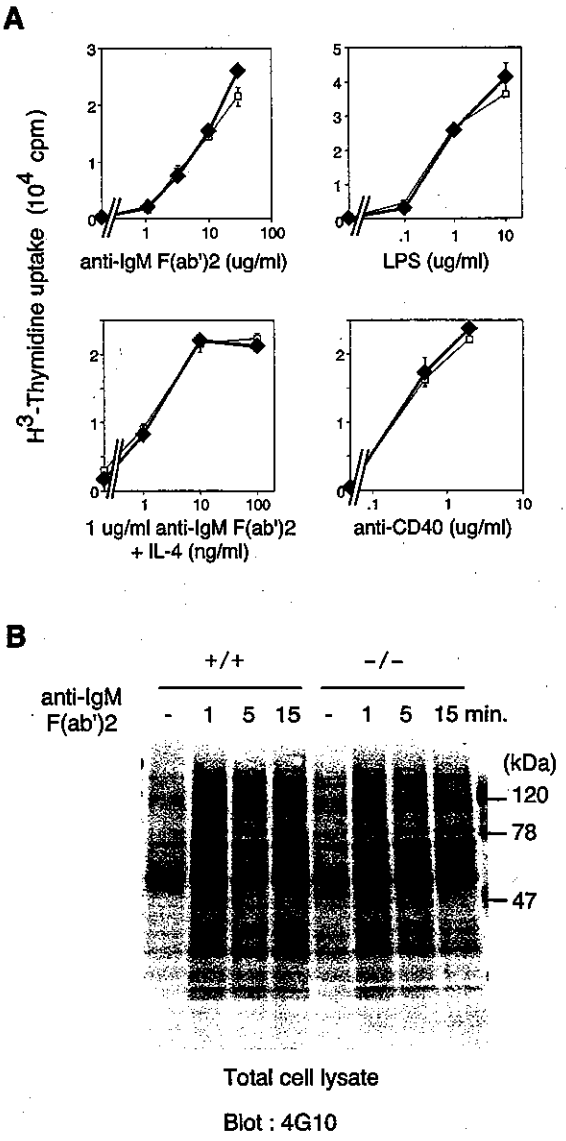

FIG. 3. Proliferative responses and protein tyrosine phosphorylation of cellular proteins in *APS*$^{-/-}$ B cells. (A) Proliferation of splenic B cells induced by anti-IgM F(ab')$_2$, LPS, anti-IgM F(ab')$_2$ plus IL-4, and anti-CD40. Splenic B cells from +/+ (□) or −/− (◆) mice were treated with the indicated stimuli, and proliferation was measured on day 3 by [^{3}H]thymidine incorporation. The values are the mean counts per minute ± standard deviations of triplicate determinations. Representative results of three independent experiments are shown. (B) Phosphorylation of intracellular proteins induced by anti-IgM F(ab')$_2$ stimulation of splenic B cells from +/+ or −/− mice. Purified splenic B cells were stimulated with 30 μg of anti-IgM F(ab')$_2$ antibody/ml for the indicated times. The total cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membrane was probed with an anti-phosphotyrosine antibody, 4G10, and visualized by enhanced chemiluminescence.

TABLE 2. Antibody production in $APS^{-/-}$ mice

Genotype (n)[a]	Mean serum Ig level (μg/ml) ± SEM for[b]:					
	IgM	IgG1	IgG2a	IgG2b	IgG3	IgA
+/+ (9)	120 ± 10	180 ± 67	90 ± 30	260 ± 42	49 ± 4.3	85 ± 9.5
−/− (12)	140 ± 13	150 ± 46	77 ± 17	200 ± 36	79 ± 6.9[c]	84 ± 7.1

[a] n, number of mice.
[b] Concentrations of immunoglobulin subclasses in serum as determined by isotype-specific ELISA. Statistical analysis was carried out by using Student's t test.
[c] Significantly different from the value for +/+ mice at a P value of <0.005.

of $APS^{-/-}$ mice. The immunoglobulin isotype levels in serum were all within their normal ranges, except for IgG3, which was slightly increased in $APS^{-/-}$ mice (Tables 2 and 3). $APS^{-/-}$ mice mounted normal humoral immune responses against the TD antigen TNP-KLH, and time courses of antibody production by $APS^{-/-}$ and $APS^{+/+}$ mice after primary and secondary immunization were indistinguishable (Table 3 and data not shown). $APS^{-/-}$ mice also showed normal responses against the TI-1 antigen, TNP-LPS. However, $APS^{-/-}$ mice displayed significantly increased IgM and IgG3 isotype antibody production after immunization with the TI-2 antigen, TNP-Ficoll (Table 3).

APS amounts affected actin assembly in activated B cells. Recently, it was proposed that Lnk family proteins contribute to the regulation of the actin cytoskeleton and that SH2-B is involved in GH-induced actin reorganization (14). The actin-binding protein ABP-280 associates with Lnk (12), and Lnk overexpression in fibroblasts results in extensive actin reorganization (S.-M. Kwon and S. Takaki, unpublished data). Therefore, we examined the roles of APS in actin reorganization in primary B cells. Because of difficulty in the detection of APS by immunostaining in wild-type primary B cells, we employed B cells from lymphocyte-specific *APS*-Tg mice. Staining with rhodamine-phalloidin and anti-APS antibodies revealed that APS colocalized with F-actins accumulated during the capping of the BCR complex in B cells stimulated by BCR cross-linking (Fig. 4A). F-actin contents increased after BCR stimulation in primary B cells, and the increase in F-actin contents was more prominent in *APS*-Tg B cells than in control B cells (Fig. 4B).

With the results observed for *APS*-Tg B cells in mind, we examined actin assembly induced by BCR cross-linking in $APS^{-/-}$ B cells. F-actin contents in peritoneal B-1 cells and splenic B-2 cells were analyzed by flow cytometry after rhodamine-phalloidin staining. Unstimulated $APS^{-/-}$ B cells tended to show slightly decreased F-actin contents compared to those of wild-type B cells (Table 4). After BCR cross-linking, $APS^{-/-}$ B-1 cells showed significantly reduced F-actin contents compared to those of wild-type B-1 cells (Fig. 5 and Table 4). The reduction in F-actin contents after BCR cross-linking was less significant in $APS^{-/-}$ B-2 cells. Thus, APS amounts affected actin assembly in activated B cells, and F-actin contents decreased in the absence of APS, especially in B-1 cells.

DISCUSSION

***APS* deficiency results in increase of B-1 cells.** In this study, we investigated the in vivo functions of APS, with a particular focus on the immune system, by generating $APS^{-/-}$ mice. Our results showed that, while the development and distribution of B-2 cells was normal, the numbers of B-1 cells in the peritoneal cavity and the humoral immune responses against a TI-2 antigen were significantly enhanced in $APS^{-/-}$ mice. Although various cellular responses induced by BCR stimulation were normal, assembled F-actins in activated B-1 cells were significantly reduced in the absence of APS. These results indicate that APS appears to play a role in B-1 cell development, maintenance, or activation and that APS may regulate actin reorganization and modulate the number of B-1 cells.

B-1 cells undergo positive selection and, compared to B-2 cells, require stronger BCR-mediated signaling or higher BCR expression levels for development and/or maintenance (10). A deficiency of signaling molecules that attenuate BCR signals often results in the selective reduction of B-1 cells in mice. For example, mice lacking the p85α subunits of phosphatidylinositol 3-kinase, Btk, or phospholipase C-γ2 all show severely reduced B-1 cell numbers, while B-2 cell numbers remain rel-

TABLE 3. Antibody responses against the TI-1, TI-2, and TD antigens

Antigen and genotype (n)[a]	Mean antibody production (relative titer [10^2]) ± SEM[b]						
	IgM	IgG	IgG1	IgG2a	IgG2b	IgG3	IgA
TI-1[c]							
+/+ (8)	11 ± 1.6	0.66 ± 0.15					
−/− (7)	16 ± 2.6	0.89 ± 0.21					
TI-2[d]							
+/+ (9)	11 ± 1.1					4.2 ± 1.1	
−/− (10)	16 ± 1.7[e]					9.5 ± 2.0[e]	
TD[f]							
+/+ (3)	4.7 ± 0.32		93 ± 6.8	16 ± 5.5	74 ± 22	9.4 ± 2.3	8.9 ± 2.2
−/− (5)	3.9 ± 0.80		120 ± 33	11 ± 3.1	46 ± 14	7.9 ± 1.7	10 ± 2.8

[a] n, number of mice.
[b] Statistical analysis was carried out by using Student's t test.
[c] Mice were injected with the TI-1 antigen TNP-LPS, and the levels of hapten-specific IgM and IgG antibodies were measured at day 14 by ELISA.
[d] Mice were injected with the TI-2 antigen TNP-Ficoll, and the levels of hapten-specific IgM and IgG3 antibodies were measured at day 14 by ELISA.
[e] Significantly different from the value for +/+ mice at a P value of <0.05.
[f] Mice were immunized with the TD antigen TNP-KLH, and the TNP-specific immunoglobulin subclasses were measured 7 days after the second immunization.

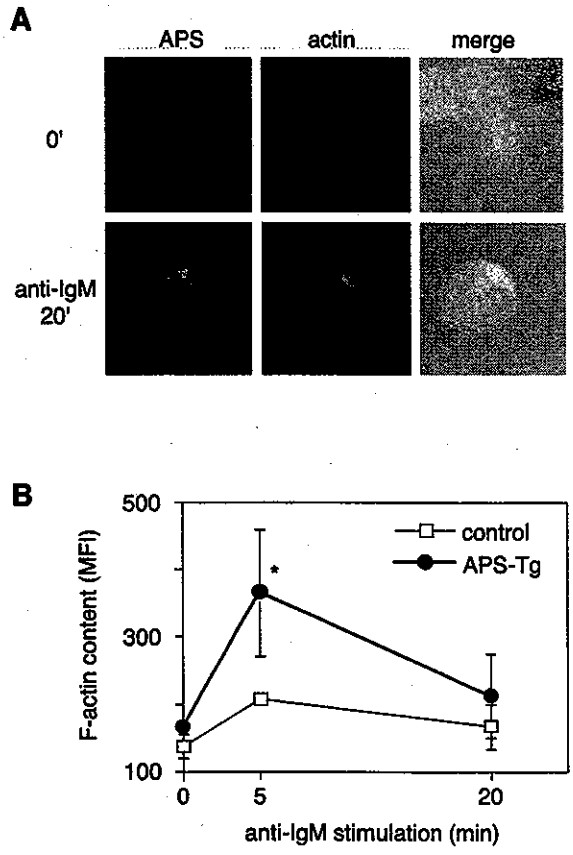

FIG. 4. APS colocalizes with actin and regulates its reorganization. (A) APS colocalizes with polymerized actin fibers. *APS*-Tg B cells stimulated with goat anti-IgM $F(ab')_2$ fragments for 20 min were fixed, permeabilized, and stained with rabbit anti-APS and FITC-conjugated anti-rabbit IgG (left column) or rhodamine-phalloidin (center column). Merged images of anti-APS staining, actin staining, and a phase-contrast image are also shown (right column). (B) Analysis of F-actin content by flow cytometry. Purified splenic B cells were stimulated with anti-IgM, fixed, permeabilized, stained with rhodamine-phalloidin, and analyzed by flow cytometry. The average F-actin content is represented as the mean fluorescence intensity (MFI) ± standard deviation of results from three independent experiments at each time point, as shown on the lower axis. Statistical analysis was carried out by using Student's *t* test. *, statistically significant at a *P* value of <0.05 compared with control.

atively normal (7, 23, 24, 41, 48). *APS* transcripts were comparably expressed in both B-1 and B-2 cells (data not shown). Our cellular and biochemical analyses of $APS^{-/-}$ splenic B cells failed to detect any obvious enhancement of BCR-mediated responses. However, it may be hypothesized that B-1 cells, which are more sensitive to BCR-mediated signal strength than B-2 cells, are likely to be selectively expanded in $APS^{-/-}$ mice due to the high level of BCR expression combined with slightly enhanced BCR-mediated signaling in the absence of APS. The reduction of actin assembly in stimulated B-1 cells might provide a certain advantage in signaling through BCRs. Alternatively, *APS* deficiency or the resulting reduced F-actin contents after BCR cross-linking may provide better signaling through growth factor or cytokine receptors that mediate the growth or survival responses of B-1 cells.

TABLE 4. Decreased F-actin contents in $APS^{-/-}$ B-1 cells

Cell type	Anti-IgM	Mean F-actin content (%) ± SEM[a]	
		+/+	−/−
B-1	−	100	92 ± 4.5
	+	100	88 ± 2.8[b]
B-2	−	100	97 ± 1.9
	+	100	96 ± 2.9

[a] Antibody stained splenocytes or peritoneal cells were stimulated with anti-IgM (+) or left unstimulated (−), fixed, permeabilized, stained with rhodamine-phalloidin, and analyzed by flow cytometry. The average F-actin contents for −/− mice measured by fluorescence intensity for rhodamine-phalloidin are represented as the values relative to those for +/+ mice from four independent experiments. Statistical analysis was carried out by using Student's *t* test.
[b] Significantly different from the value for +/+ cells at a *P* of <0.01.

APS in signals mediated through growth factor receptors. It has been demonstrated that APS can be phosphorylated by various tyrosine kinase receptors, including c-Kit, the NGF receptor, the platelet-derived growth factor receptor, cytokine receptors such as IL-3R, and the GH receptor (19, 39, 50, 51). Indeed, APS was originally isolated as a potent c-Kit substrate and has been shown to interact with c-Kit in a tyrosine phosphorylation-dependent manner (50). It has been shown that Lnk, another member of the adaptor protein family, negatively regulates c-Kit signaling (32, 42, 43). However, $APS^{-/-}$ mice showed neither developmental abnormalities nor growth retardation. In addition, $APS^{-/-}$ mice manifested no anemia, and mast cells established from $APS^{-/-}$ BM cells displayed normal c-Kit-mediated proliferation and adhesion (C. Kubo, M. Iseki, and S. Takaki, unpublished data). Despite APS phosphorylation induced via receptors for growth factors and cytokines, we found no evidence that APS functions in c-Kit, NGF receptor, platelet-derived growth factor receptor, IL-3R, or GH receptor signaling. Thus, APS may be dispensable for signals mediated through tyrosine receptor kinases or cytokine receptors.

Independent from its identification as a possible c-Kit substrate, APS has also been isolated by virtue of its ability to associate with a cytoplasmic domain of the insulin receptor

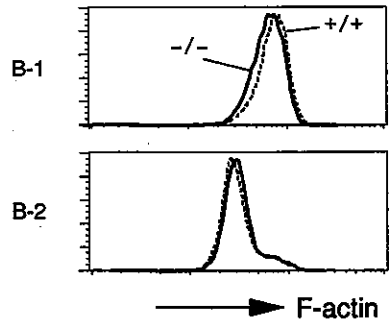

FIG. 5. Reduced F-actin contents in $APS^{-/-}$ B-1 cells stimulated by BCR cross-linking. Antibody-stained splenocytes or peritoneal cells were stimulated with 30 μg of $F(ab')_2$ goat anti-mouse IgM/ml, washed, fixed, permeabilized, stained with rhodamine-phalloidin, and analyzed by flow cytometry. The F-actin contents of anti-IgM-stimulated B-1 or B-2 cells from +/+ (dashed line) or −/− (solid line) mice are shown. Representative results of four independent experiments are shown.

(31). APS binds to phosphotyrosines within the activation loop of the insulin receptor via the SH2 domain and undergoes phosphorylation of a tyrosine residue upon stimulation with insulin. Interestingly, $APS^{-/-}$ mice exhibited increased sensitivity to insulin and enhanced glucose tolerance (30). Although the mechanisms that lead to insulin hypersensitivity are not clear at present, it does appear that APS plays a negative regulatory role in insulin function and glucose metabolism. As discussed above, it cannot be ruled out at this point that an increase of peritoneal B-1 cells is a secondary effect of insulin hypersensitivity.

Possible molecular mechanisms for APS to function in B-lineage cells. Despite the observed interaction between APS and c-Cbl in transfected cell lines (1, 47, 51), APS probably exerts its function in a c-Cbl-independent manner. We found no association between APS and c-Cbl in wild-type B cells (data not shown). The time courses of phosphorylation for various cellular proteins were not affected in $APS^{-/-}$ B cells. Interestingly, it has been shown that Lnk does not down-regulate or affect c-Kit expression levels but negatively regulates c-Kit-mediated growth signals by attenuating Gab2 phosphorylation following activation of the mitogen-activated protein kinase pathway in a mast cell line (42). The C-terminal tyrosine residue of Lnk that corresponds to the c-Cbl binding site of APS is not required for the inhibitory function of Lnk either in the mast cell line or in B progenitors in BM (42, 44). Thus, both APS and Lnk function as negative regulators of signaling in B-lineage cells and work in a c-Cbl-independent manner, although they seem to act at different stages of lymphocyte development and in different signaling pathways. In contrast, SH2-B seems to act as a positive regulator of signal transduction. $SH2\text{-}B^{-/-}$ mice show mild growth retardation and infertility due to impaired gonad organ maturation (33). Despite the significant structural similarities between APS, Lnk, and SH2-B, these proteins appear to have diverse functions. However, possible overlapping functions of these adaptor proteins in vivo should be examined by generating mutant mice that lack various combinations of APS, Lnk, and SH2-B.

Vav3 has been shown to interact with APS (49) and to play a role in BCR signaling (17). Vav1 and Vav2 are expressed in B cells and regulate BCR signals (5, 45). Although we found no clear evidence for a relationship between APS and Vav family proteins, it is still possible that Vav proteins are involved in the signal transduction through APS. Vav1, the hematopoietic-specific guanine nucleotide exchange factor for Rho/Rac/Cdc42, controls the actin cytoskeleton in primary T cells (6, 16). F-actin contents were reduced in activated $APS^{-/-}$ B-1 cells. The involvement of microfilament assemblies in BCR signaling events that lead to mitogenesis or apoptosis has been demonstrated previously (27, 28). Although the molecular mechanisms that connect APS with actin polymerization remain to be elucidated, it is possible that APS may regulate the strength of signals by controlling the functions of Vav family proteins and actin assemblies.

In conclusion, our studies describe a novel regulatory role for APS in controlling B-1 cell compartment size in vivo. B-1 cells were increased and antibody production against a TI-2 antigen was enhanced in $APS^{-/-}$ mice. APS colocalized with F-actin, and actin reorganization was augmented in B cells overexpressing APS. In contrast, F-actin contents were decreased in $APS^{-/-}$ B cells, especially in B-1 cells. We propose that APS may modulate the strength of the signals, which control B-1 cell development or maintenance by controlling actin polymerization.

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