Altered Antigen Receptor Signaling and Impaired Fas-mediated Apoptosis of B Cells in *Lyn*-deficient Mice

By Jiyang Wang, Toshiki Koizumi, and Takeshi Watanabe

From the Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-82, Japan

Summary

Mice deficient in the *src*-related protein tyrosine kinase, Lyn, exhibit splenomegaly and accumulate lymphoblast-like and plasma cells in spleen as they age, resulting in elevated levels of serum IgM (10–20-fold of control) and glomerulonephritis due to the presence of immune complexes containing auto-reactive antibodies. It remains unclear, however, how antibody-producing cells are accumulated in the lymphoid tissues of $Lyn^{-/-}$ mice. To elucidate the role of Lyn in B cell function, we have studied the proliferative responses to various stimuli and Fas-mediated apoptosis in B cells from young $Lyn^{-/-}$ mice which do not yet show apparent abnormality such as splenomegaly. Compared with control B cells, $Lyn^{-/-}$ B cells were hyper responsive to anti-IgM-induced proliferation and defective in FcγRIIB-mediated suppression of B cell antigen receptor (BCR) signaling, indicating that Lyn is involved in the negative regulation of BCR signaling. In addition, the BCR-mediated signal in $Lyn^{-/-}$ B cells, unlike that in control B cells, failed to act in synergy with either CD40- or IL-4 receptor-triggered signal in inducing a strong proliferative response, suggesting that the BCR signaling pathway in $Lyn^{-/-}$ B cells is altered from that in control B cells. Furthermore, $Lyn^{-/-}$ B cells were found to be impaired in the induction of Fas expression after CD40 ligation and exhibited a reduced susceptibility to Fas-mediated apoptosis. Moreover, BCR cross-linking in $Lyn^{-/-}$ B cells suppressed Fas expression induced by costimulation with CD40 ligand and IL-4. Collectively, these results suggest that the accumulation of lymphoblast-like and plasma cells in $Lyn^{-/-}$ mice may be caused, in part, by the accelerated activation of B cells in the absence of Lyn, as well as the impaired Fas-mediated apoptosis after the activation.

The B cell antigen receptor (BCR)[1] consists of membrane immunoglobulin M (IgM) non-covalently associated with disulfide-linked heterodimers of Igα and Igβ subunits which couple mIg to several protein tyrosine kinases (PTKs) (1). Cross-linking of the BCR rapidly activates two classes of PTKs: the *src* family kinases (Lyn, Fyn, Blk, Hck, and Lck) (2–5) and the Syk kinase (6, 7), resulting in the phosphorylation of several proteins (8, 9), including PTKs themselves, Igα and Igβ (10), phospholipase C-γ (11), phosphatidylinositol (PI)-3 kinase (12, 13), the protooncogene product Vav (14–16), Ras GTPase-activating protein (17), Shc (18), and HS1 (19).

The *src*-related kinase, Lyn, is expressed preferentially in hematopoietic cells and in cells of neural tissues (20, 21). Lyn is physically associated with the BCR in B cells and is rapidly phosphorylated upon receptor cross-linking (2–5). Lyn interacts with and phosphorylates the Syk kinase (22, 23), the HS1 protein (19) and the Cbl protooncogene product (24), and associates with the p85 subunit of the PI-3 kinase after BCR cross-linking (12, 13). BCR also associates with a number of cell surface molecules, including CD45, a phosphotyrosine phosphatase that is essential for anti-IgM-induced proliferation of B cells (25, 26), and CD19, a member of Ig gene superfamily whose cross-linking inhibits B cell proliferation via anti-IgM stimulation (27, 28). Interestingly, Lyn has been shown to interact with both CD45 and CD19 (29, 30). These observations indicate that Lyn participates in BCR-mediated signal transduction through multiple pathways. The functional significance of Lyn in the BCR signaling was first demonstrated in cell lines. A *Lyn*-deficient mutant of the chicken B cell line DT40, generated through homologous recombination, exhibited delayed Ca^{2+} influx but enhanced inositol 1,4,5-triphosphate (IP_3) accumulation, indicating that Lyn regulates Ca^{2+} mobilization through a process independent of IP_3 generation (31). Moreover, using antisense oligonucleotides, Lyn was shown to be required for anti-IgM-mediated cell cycle arrest in both murine BCL1 and human

[1] *Abbreviations used in this paper:* BCR, B cell antigen receptor; CD40L, CD40 ligand; FcγIIRB, B cell receptor for IgG Fc region; IgM, membrane immunoglobulin M; NMS, normal mouse serum; PI, propidium iodide; PTK, protein tyrosine kinase; SHP-1, hematopoitic cell phosphatase; smIg, cell surface IgM.

Daudi cell lines (32). These observations suggest a role for Lyn in the negative regulation of BCR signaling.

In addition to a role of Lyn in regulating BCR signaling, biochemical analysis has suggested its involvement in the signaling pathway through CD40 (33, 34), a member of the TNF receptor family that plays a critical role in the survival, growth, differentiation and apoptosis of B lymphocytes (35–37). Lyn thus appears to be involved in the signal transduction from multiple receptors on B cells.

Lyn$^{-/-}$ mice, generated recently by gene targeting, exhibited splenomegaly with age and accumulated the lymphoblast-like and plasma cells in spleen (38, 39). There was a high concentration of serum IgM in Lyn$^{-/-}$ mice, and a significant number of Lyn$^{-/-}$ mice showed glomerulonephritis due to the presence of immune complex containing auto-reactive antibodies. It remains unclear, however, how large numbers of antibody producing cells accumulate in lymphoid tissues of *Lyn*$^{-/-}$ mice. Recent studies revealed a critical role for the CD40-mediated signal in upregulating Fas expression and inducing susceptibility to Fas-mediated apoptosis in B cells (40–42). By contrast, BCR engagement was shown to protect B cells from Fas-mediated apoptosis (43), indicating that Fas susceptibility is positively and negatively regulated by CD40- and BCR-mediated signals, respectively. It has been suggested that activated bystander B cells, which may produce various Abs including autoantibodies, are eliminated by Fas-dependent apoptosis (43). Since Lyn was implicated in the signal transduction pathways of both CD40 and BCR, we examined whether *Lyn*$^{-/-}$ B cells have defects in Fas-mediated apoptosis. We demonstrate in the present study that *Lyn*$^{-/-}$ B cells are impaired in Fas-mediated apoptosis due to both an impaired induction of Fas expression and a reduced susceptibility to Fas-mediated apoptosis. In addition, B cells from young *Lyn*$^{-/-}$ mice are hyper responsive to anti-IgM-induced proliferation and defective in FcγRII-mediated suppression of signals generated by BCR engagement. These results suggest that *Lyn*$^{-/-}$ B cells have a reduced threshold for antigen-triggered activation, and may survive for prolonged period after the activation due to impaired Fas-mediated apoptosis, resulting in the accumulation of antibody-secreting cells.

Materials and Methods

Reagents. Goat F(ab')^2 and intact anti-mouse IgM Abs purchased from Southern Biotechnology Associates, Inc. (SBA, Birmingham, AL) were extensively dialyzed in PBS. The Jo2 anti-mouse Fas Ab and anti-mouse CD8 Ab were purchased from PharMingen (San Diego, CA). The IC10 rat anti–mouse CD40 Ab and the biotinylated Jo2 anti-mouse Fas Ab were kindly provided by Dr. M. Howard (DNAX, Palo Alto, CA) and Dr. S. Nagata (Osaka Bioscience Institute), respectively. Culture supernatants from a myeloma cell line producing soluble CD40 ligand CD8 fusion protein (CD40L) (44), generously provided by Dr. P. Lane (Basel Institute for Immunology), were used at a fourfold dilution that was optimal for inducing proliferation of splenic B cells. Recombinant interleukin-4 was purchased from R&D Systems (Minneapolis, MN).

Cells and Cell Culture. Cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere, in RPMI 1640 containing 5×10^{-5} M 2-mercaptoethanol, 25 μg/ml of Gentamycin, supplemented with 10% heat-inactivated fetal calf serum (GIBCO BRL, Gaithersburg, MD). Splenic B cells were isolated from 4–8-wk-old mice after T cell depletion by treatment with anti-mouse Thy1.2 mAb for 30 min on ice and then with complement for 30 min at 37°C, followed by Ficoll-Hypaque density centrifugation and incubation at 37°C for 1 h to remove adherent cells. Approximately 90–95% of the isolated cells were found to express the B cell marker B220.

Proliferation Assay. Splenic B cells (5×10^5/ml, 100 μl) were placed in 96-well flat-bottom plates and cultured in the presence of various stimuli. The cultures were pulsed for the last 6 h with [^{3}H]thymidine (1 μci/ml) and the mean [^{3}H]thymidine incorporation and standard deviation were calculated for triplicate cultures.

Immunofluorescence Analysis. For CD40 staining, cells (10^6) were incubated in 50 μl of 10 μg/ml of the 1C10 antibody on ice for 20 min, followed by a phycoerythrin (PE) conjugated goat anti-rat Ig Ab (SBA). For Fas staining, cells were incubated with normal mouse serum (NMS) to block Fc receptors and then stained with the biotinylated Jo2 anti-mouse Fas Ab, followed by staining with streptavidin conjugated PE (SAPE; SBA). Cells stained with NMS and then SAPE served as controls. Cells were fixed in 1% paraformaldehyde before analyzing with a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Analysis for Cell Death. Cells were stained with 1 μg/ml of propidium iodide (PI) in PBS containing 2% FCS and 0.02% NaN_3 for 5 min on ice and the percentage of PI positive (dead) and negative (live) cells were determined with a FACScan® flow cytometer.

Results

Proliferative Responses to Various Stimuli in Lyn$^{-/-}$ *B Cells.* In the previous study, we have demonstrated that lymphoblast-like cells carrying Mac-1 antigen and cytoplasmic IgM accumulated in spleen of *Lyn*$^{-/-}$ mice (39). To find out the intrinsic defects of *Lyn*$^{-/-}$ B cells, we have investigated the function of B cells from young (4–8-wk-old) mice which do not yet show apparent abnormality such as splenomegaly and the accumulation of lymphoblast-like cells. In accordance with earlier observations (39), the proliferative responses of *Lyn*$^{-/-}$ B cells to either soluble CD40 ligand-CD8 fusion protein (CD40L or L), or CD40L plus an anti-CD8 Ab (L + anti-CD8), were considerably reduced compared with control B cells (Fig. 1 *A*). The reduced response to CD40 ligation is unlikely to reflect a low level of CD40 expression as the level of CD40 on *Lyn*$^{-/-}$ B cells was found to be similar as that on control B cells (Fig. 2 *A*). The data demonstrate that Lyn, which was reportedly activated by CD40 ligation (33, 34), is indeed critical for CD40-mediated proliferative response in B cells. Since *Lyn*$^{-/-}$ B cells could still respond to CD40 ligation to some extent, it is likely that other PTKs, such as Fyn and Syk which were also activated by CD40 ligation (33, 34), may be involved in the observed proliferative response. These PTKs, however, could not compensate for the activity of Lyn to restore a normal proliferative response induced by CD40 ligation.

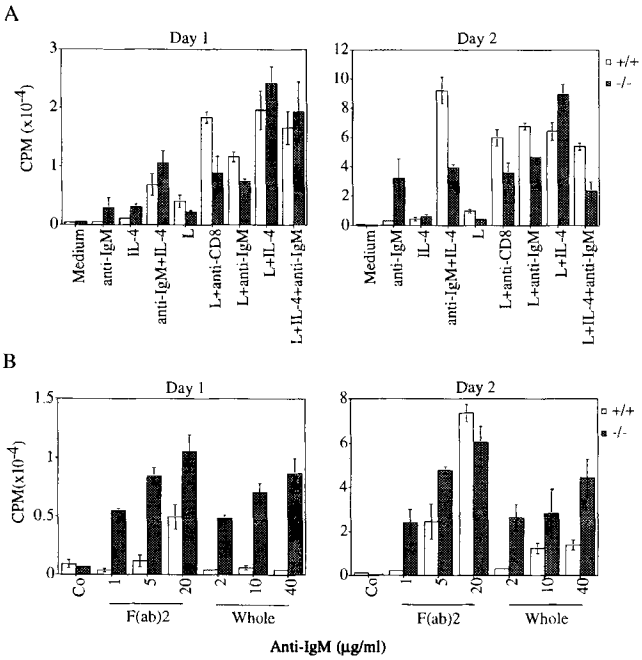

Figure 1. Proliferative responses of control and $Lyn^{-/-}$ B cells to various stimuli. (*A*) Splenic B cells were stimulated with goat $F(ab')^2$ anti-IgM Abs (5 μg/ml), IL-4 (200 U/ml), CD40 ligand (*L*), CD40 ligand plus an anti-CD8 Ab (5 μg/ml) or various combinations of these stimuli. (*B*) Splenic B cells were cultured with indicated amounts of goat $F(ab')^2$ or whole anti-IgM Abs. In both *A* and *B*, representative results of five independent experiments are shown.

Much to our surprise, $Lyn^{-/-}$ B cells from young mice were hyper responsive to anti-IgM-induced proliferation compared with control B cells. As indicated in the dose-response profiles (Fig. 1 *B*), $Lyn^{-/-}$ B cells responded greatly to even a low dose of $F(ab')^2$ anti-IgM (1 μg/ml) within 1 d of culture whereas control B cells responded poorly even after 2 d. At a higher dose of $F(ab')^2$ anti-IgM (20 μg/ml), however, the proliferative response was similar in $Lyn^{-/-}$ and control B cells (Fig. 1 *B*, compare [^{3}H]thymidine incorporation of $Lyn^{-/-}$ vs. control B cells stimulated with 20 μg/ml of $F(ab')^2$ anti-IgM for 2 d). This is likely because the response of $Lyn^{-/-}$ B cells reached near plateau at relatively high concentrations of anti-IgM Abs whereas the response of $Lyn^{+/+}$ B cells continued to increase. The hyper responsiveness of $Lyn^{-/-}$ B cells to antigen stimulation does not reflect an increased expression of cell surface IgM (sIgM) as the level of sIgM is similar in control and $Lyn^{-/-}$ B cells (Fig. 2 *C*). These results demonstrate that $Lyn^{-/-}$ B cells have a reduced threshold for anti-IgM induced proliferation with an accelerated time course than do control B cells.

Another interesting finding is that in $Lyn^{-/-}$ B cells, the whole anti-IgM Abs induced a similar, if slightly reduced, proliferative response as did $F(ab')^2$ anti-IgM Abs (Fig. 1 *B*), whereas in control B cells, the proliferative responses were significantly reduced when stimulated with whole anti-IgM Abs which have been shown to cross-link BCR with FcγRIIB, a predominant form of Fcγ receptors expressed on B cells (45–47). The results demonstrate that FcγRIIB-mediated downregulation of BCR signaling is not properly functional in the absence of Lyn, although it has not yet been studied whether Lyn is activated by cross-linking FcγRIIB.

Response to IL-4 does not seem to be impaired in $Lyn^{-/-}$ B cells as indicated by both the proliferation (Fig. 1 *A*) and upregulation of CD40 expression in response to IL-4 (Fig. 2 *B*). Notably, stimulation with CD40L in the presence of IL-4 (L + IL-4) for one or 2 d induced a normal or somewhat higher proliferative response in $Lyn^{-/-}$ B cells relative to control B cells, suggesting a possibility that IL-4 receptor-mediated signals acted in synergy with and restored the CD40-mediated proliferative signal which was otherwise reduced in $Lyn^{-/-}$ B cells (Fig. 1 *A*). Stimulation with CD40L plus both IL-4 and anti-IgM also induced a normal proliferative response in $Lyn^{-/-}$ B cells on day 1, but unlike the combination of CD40L plus IL-4, the response was significantly reduced in $Lyn^{-/-}$ B cells, as compared with control B cells, on day 2. It appears that the restored proliferative response of $Lyn^{-/-}$ B cells induced by CD40L plus IL-4, when combined with the hyper responsiveness to anti-IgM stimulation, was accelerated and decayed more rapidly than that of control B cells. Alternatively, the BCR-mediated abnormal signal in $Lyn^{-/-}$ B cells may have suppressed the restored proliferative response induced via CD40L plus IL-4.

The data shown in Fig. 1 *A* also suggested that, in the absence of Lyn, the BCR-mediated signal failed to act in synergy with CD40- or IL-4 receptor-mediated signals in inducing strong proliferative responses. In control B cells, the combinations of anti-IgM and CD40L or anti-IgM and IL-4 induced a strong proliferative response that was more than the sum of that induced by each stimulation alone

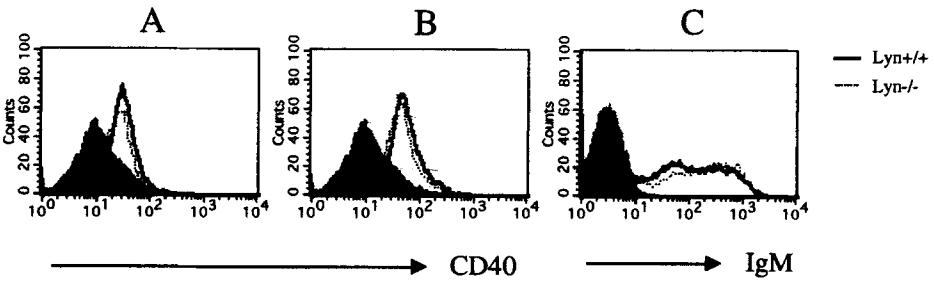

Figure 2. Expression of CD40 and IgM on control and $Lyn^{-/-}$ B cells. CD40 expression on fresh splenic B cells (*A*) or after stimulation with 200 U/ml of IL-4 for 1 d (*B*). (*C*) Cell surface IgM expression on fresh splenic B cells. Shaded areas show background staining.

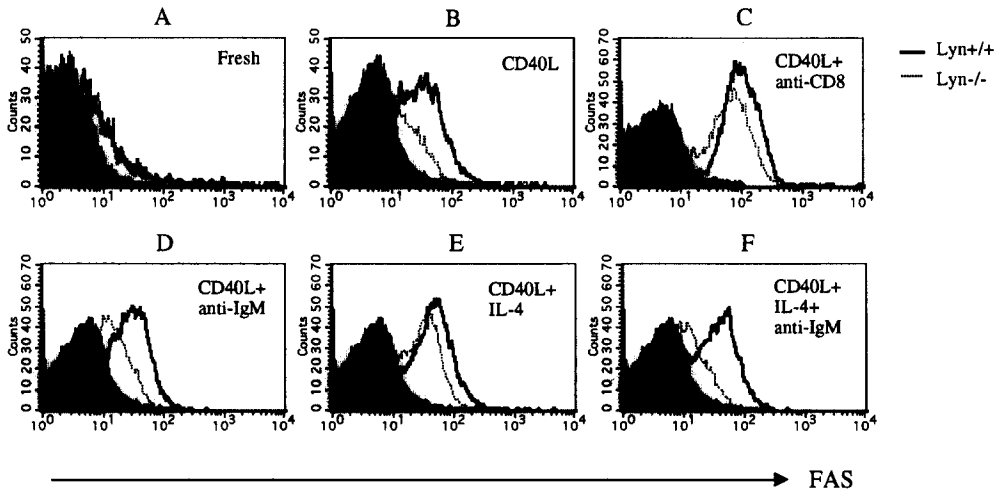

Figure 3. Fas expression after B cell activation. Splenic B cells (5×10^5/ml, 1 ml) were placed in 24-well plates and were stimulated for 2 d as in Fig. 1 *A*. Fas expression on control (*solid lines*) and $Lyn^{-/-}$ (*dotted lines*) B cells is shown. Shaded areas depict background staining.

whereas in $Lyn^{-/-}$ B cells, these combinations only acted additively, suggesting that the BCR-mediated signal transduction pathway in the absence of Lyn is clearly distinguished from that in control B cells.

Reduced Fas Induction After Various Stimuli in $Lyn^{-/-}$ B Cells. We and others have recently found that CD40 ligation upregulated Fas expression and enhanced susceptibility to Fas-mediated apoptosis in B cells (40–43). Since $Lyn^{-/-}$ B cells exhibited a reduced proliferative response to CD40 ligation, Fas expression (Fig. 3) and Fas-mediated apoptosis (Fig. 4) after various stimuli were investigated. In both control and $Lyn^{-/-}$ mice, fresh splenic B cells (Fig. 3 *A*) or B cells stimulated with anti-IgM, IL-4 or anti-IgM plus IL-4 (data not shown), expressed only a low level of Fas antigen, confirming our earlier finding that Fas is not induced by BCR-cross-linking or IL-4 stimulation (42). Stimulation with CD40L upregulated Fas expression in control but not $Lyn^{-/-}$ B cells (Fig. 3 *B*). CD40L plus an anti-CD8 Ab which delivered a stronger signal as judged by the proliferative response (Fig. 1 *A*), however, induced Fas expression in $Lyn^{-/-}$ B cells to a similar level of that in control B cells (Fig. 3 *C*), suggesting that Fas induction in $Lyn^{-/-}$ B cells required a stronger CD40-mediated signal. CD40-mediated Fas induction could be restored by IL-4 (Fig. 3 *E*), in accordance with the observation that CD40L plus IL-4 restored the proliferative response (Fig. 1 *A*). The reduced Fas induction by CD40L in $Lyn^{-/-}$ B cells could not be restored by costimulation with anti-IgM (Fig. 3 *D*), in keeping with the observation that anti-IgM and CD40L did not act in synergy to restore a normal proliferative response in $Lyn^{-/-}$ B cells. Moreover, anti-IgM stimulation downregulated Fas expression of $Lyn^{-/-}$ B cells induced by CD40L plus IL-4 (compare Fig. 3, *E* and *F*). Interestingly, while a normal proliferative response was induced on day 1 by stimulation with either CD40L plus IL-4 or CD40L plus IL-4 and anti-IgM (Fig. 1 *A*, day 1), Fas expression was induced on day 1 by treatment with CD40L plus IL-4 but not CD40L plus IL-4 in the presence of anti-IgM (data not shown). Collectively, these results revealed two abnormal aspects of $Lyn^{-/-}$ B cells in the induction of Fas expression: one is that Fas induction in $Lyn^{-/-}$ B cells required a stronger CD40-mediated signal, and the other is that the abnormal BCR-triggered signal suppressed Fas induction.

Impaired Fas-mediated Apoptosis in $Lyn^{-/-}$ B Cells. Having shown the abnormality of Fas induction in $Lyn^{-/-}$ B cells, we next examined the susceptibility to Fas-mediated apoptosis in these B cells. In accordance with our prior finding, both control and $Lyn^{-/-}$ B cells activated for 1 d were resistant to Fas-triggered apoptosis (data not shown). B cells activated for 2 d were therefore examined for Fas susceptibility. Consistent with the reduced Fas expression, $Lyn^{-/-}$ B cells stimulated by CD40L alone, CD40L plus anti-IgM or CD40L plus anti-IgM and IL-4, all exhibited reduced susceptibility to Fas-mediated apoptosis after treatment with the Jo2 anti-Fas Ab for 4 h (Fig. 4 *A*) or 16 h (Fig. 4 *B*). In keeping with the observation that IL-4 acted in synergy with CD40-mediated signals to restore a normal proliferative response (Fig. 1 *A*) and upregulate Fas expression (Fig. 3 *E*), $Lyn^{-/-}$ B cells stimulated with CD40L plus IL-4 underwent Fas-triggered apoptosis in a similar dose dependent manner as did control B cells after treatment with increasing concentrations of the Jo2 anti-Fas Ab (Fig. 4 *A*), or after incubation with the anti-Fas Ab for 16 h (Fig. 4 *B*).

Although a similar level of Fas expression was observed in control and $Lyn^{-/-}$ B cells stimulated with CD40L plus the anti-CD8 Ab, $Lyn^{-/-}$ B cells exhibited a reduced susceptibility to the anti-Fas Ab-induced apoptosis relative to control B cells (Fig. 4). The data suggest that a stronger CD40-mediated signal (CD40L plus anti-CD8) upregulated Fas expression but did not completely restore the susceptibility to Fas-mediated apoptosis in $Lyn^{-/-}$ B cells. CD40 ligation has recently been shown to not only induce cell surface Fas expression, but also enhance Fas susceptibility in B cells (40–42). It is thus likely that Fas susceptibility was more affected by the Lyn-dependent, CD40-mediated signal, whereas Fas induction could be restored by a stronger CD40 ligation in the absence of Lyn. Taken together, these results demonstrate that $Lyn^{-/-}$ B cells are impaired in the induction of both Fas expression and Fas susceptibility induced by CD40L. Furthermore, the BCR-mediated sig-

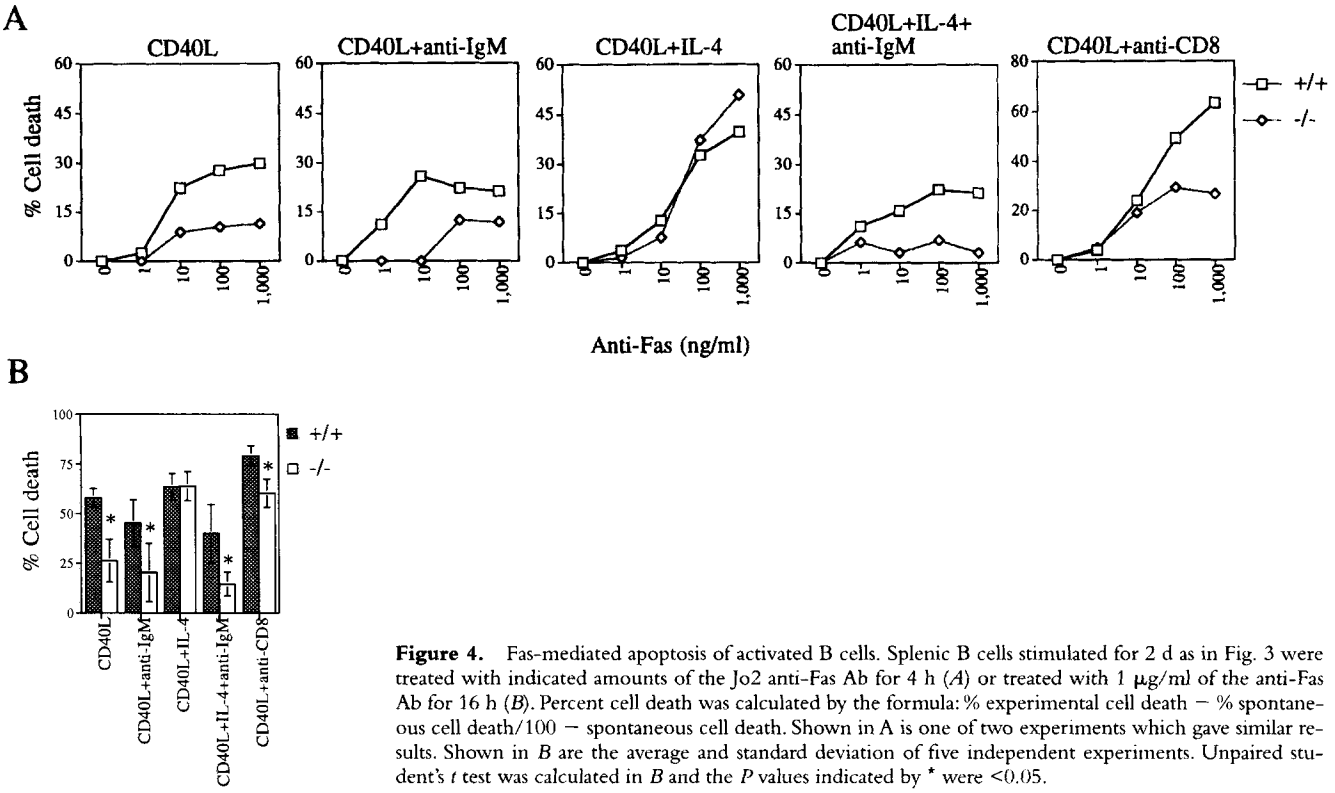

Figure 4. Fas-mediated apoptosis of activated B cells. Splenic B cells stimulated for 2 d as in Fig. 3 were treated with indicated amounts of the Jo2 anti-Fas Ab for 4 h (*A*) or treated with 1 μg/ml of the anti-Fas Ab for 16 h (*B*). Percent cell death was calculated by the formula: % experimental cell death − % spontaneous cell death/100 − spontaneous cell death. Shown in A is one of two experiments which gave similar results. Shown in *B* are the average and standard deviation of five independent experiments. Unpaired student's *t* test was calculated in *B* and the *P* values indicated by * were <0.05.

nal in the absence of Lyn interfered with the induction of Fas expression.

Discussion

To begin to elucidate the role of Lyn in B cell function, we have investigated in the present study the function of B cells from young $Lyn^{-/-}$ mice. We reasoned that while the aged $Lyn^{-/-}$ mice, which have accumulated abnormal B cells, are suitable for studying the consequence of B cell defects, young mice are better models to investigate the cause of the B cell abnormality. The results presented here demonstrate that $Lyn^{-/-}$ B cells are hyper responsive to antigen stimulation and have defects in Fas-mediated apoptosis, suggesting that $Lyn^{-/-}$ B cells are likely to be activated by low doses of antigen stimulation and may not be efficiently eliminated after the activation due to impaired Fas-mediated apoptosis, which has been implicated in the elimination of activated bystander B cells (43).

In contrast to the finding here that $Lyn^{-/-}$ B cells are hyper responsive to BCR-cross-linking, earlier studies have suggested a severe reduction in anti-IgM-induced proliferation in $Lyn^{-/-}$ B cells (38, 39). Several possibilities could be considered for the observed discrepancy. IgM^+ B cells in young mice may be characteristically distinguished from those in aged mice, for example BCR-associated signal transduction molecules including the *src* family protein tyrosine kinases or phosphatases may be differentially expressed. It is also possible that the reduced proliferative response of $Lyn^{-/-}$ B cells observed in earlier studies may reflect a decreased percentage of antigen-responsive B cells due to the accumulation of abnormal B cells which carry Mac-1 antigen and cytoplasmic IgM and therefore do not respond to BCR cross-linking (39). The hyper responsiveness to anti-Ig-induced proliferation observed in $Lyn^{-/-}$ B cells is in accordance with the previous findings that $Lyn^{-/-}$ DT40 cells exhibited enhanced IP-3 generation (31) and that Lyn is involved in the cell cycle arrest induced by anti-IgM stimulation in B lymphoma cell lines (32). It is notable that B cells from young $Lyn^{-/-}$ and control mice are phenotypically similar and express a similar level of surface IgM (Fig. 2 *B*). The hyper responsiveness observed in young $Lyn^{-/-}$ B cells thus indicates an intrinsic defect in B cell function, but not a consequence of abnormalities in other cell types.

The whole anti-IgM Ab is known to cross-link the BCR with FcγRIIB, a predominant form of the Fcγ receptors expressed on B cells, resulting in the suppression of BCR-mediated signaling (45–47). As shown in Fig. 1 *B*, $Lyn^{-/-}$ B cells apparently have defects in FcγRII-mediated suppression. It is intriguing to note that recently generated $FcγRII^{-/-}$ mice have been shown to have enhanced immune responses to both T-dependent and -independent antigens (48), a phenomenon also observed in $Lyn^{-/-}$ mice (39). These results suggest a critical role for Lyn in FcγRII-mediated downregulation of BCR-mediated proliferative

response although it remains to be examined how Lyn is involved in FcγR-mediated suppression of BCR signaling.

The hyper responsiveness to anti-IgM-induced proliferation and lack of FcγRIIB-mediated suppression of BCR signaling observed in $Lyn^{-/-}$ mice are similar to that observed in B cells from moth-eaten (*me*) and viable moth-eaten (me^v) mice (49, 50), which express no or aberrantly spliced mutant SHP-1 protein (also called PTP1C or HCP), respectively (51–53). In addition, both *me* and $Lyn^{-/-}$ mice accumulate plasma cells in their lymphoid tissues (38, 39, 54, 55). Both SHP-1 and Lyn thus negatively regulate BCR signaling for proliferation. It is conceivable that Lyn may directly or indirectly interact with SHP-1 in BCR signaling, a hypothesis that can be proved by detailed biochemical analysis. Unlike *me* mice which die shortly after birth and predominantly contain B-1 ($CD5^+$) type peripheral B cells (55, 56), $Lyn^{-/-}$ mice are viable for at least one year and contain a largely conventional B cell population with increased number of abnormal $CD5^-$, $B220^-$, $Mac\text{-}1^-$ and cytoplasmic μ^+ lymphoblast-like cells as they age (39). The less severe phenotype in $Lyn^{-/-}$ mice compared with *me* mice may suggest that Lyn and SHP-1 may regulate overlapping, but distinct B cell signaling pathways. It is also conceivable that SHP-1, which is expressed in various tissues and cell types, may regulate additional signaling pathways (55).

Stimulation with CD40L induced Fas expression in control but not $Lyn^{-/-}$ B cells, demonstrating that Lyn is involved in the CD40-mediated induction of Fas. A stronger CD40 ligation, generated by cross-linking CD40L with an anti-CD8 Ab, almost completely restored Fas induction but only partially restored the susceptibility to Fas-mediated apoptosis. The data suggest that Lyn is also involved in the enhancement of Fas susceptibility. IL-4-mediated signal could compensate for the impaired CD40 signaling in $Lyn^{-/-}$ B cells as CD40L plus IL-4 restored the proliferative response, the induction of Fas expression and the susceptibility to Fas-mediated apoptosis. B cells are activated by stimulation through multiple receptors (37). Signals through BCR, IL-4R and CD40 apparently act in synergy in normal B cells to generate strong proliferative responses since the combination of any two of the above three signals generated a much stronger growth signal that is more than the sum of that induced by each stimulation alone (Fig. 1 *A*). In the absence of Lyn, the enhanced BCR-mediated growth signal failed to cooperate with either CD40- or IL-4R-mediated signals to provide a strong synergy in inducing B cell growth. Although further studies are required to clarify at the molecular level how the abnormal signals through BCR and CD40 result in the dysfunction of B cells, the results presented here provided evidence of a critical role for Lyn in regulating not only the signal transduction through BCR and CD40 but also the interactions between these signals and with other signals.

Engagement of BCR has been shown to rescue Fas-mediated apoptosis in B cells (43). The mechanism for this protection is unclear, but our preliminary data suggest a possible role of bcl-x_L in regulating the Fas susceptibility of B cells. It is thus intriguing to examine whether the enhanced BCR signaling in $Lyn^{-/-}$ B cells would result in a rapid and sustained induction of the anti-apoptotic gene such as bcl-x_L. As is the case for B cells in *me* or me^v mice, the enhanced BCR-mediated signal in $Lyn^{-/-}$ B cells is likely to lead to accelerated activation and differentiation of B cells in response to even low doses of antigen stimulation. The impaired Fas induction and Fas-mediated apoptosis after B cell activation in $Lyn^{-/-}$ B cells, along with the observation that the abnormal BCR signaling suppressed Fas expression, indicate that activated $Lyn^{-/-}$ B cells may not be efficiently eliminated by Fas-mediated apoptosis. The accumulation of lymphoblast-like and plasma cells in $Lyn^{-/-}$ mice may be consequent to the combined effects of an accelerated activation/differentiation of B cells and an impaired Fas-dependent elimination of activated B cells.

Although apoptosis of B cells may be mediated through multiple pathways, Fas-mediated apoptosis plays an essential role in maintaining peripheral B and T cell tolerance. Mutations in gene Fas or its ligand result in autoimmune disorders of *lpr* and *gld* mice, respectively (57). While Fas is important for both T and B cell function, Lyn is not expressed in T cells and correspondingly no obvious defects have been observed in $Lyn^{-/-}$ T cells (38, 39). $Lyn^{-/-}$ mice thus provide a unique model of autoimmune disorders that are largely due to the impaired function of B cells. It remains to be investigated whether there is a human counterpart of a similar autoimmune disorder arising from the dysfunction of Lyn or other *src* family tyrosine kinases.

We wish to thank Drs. S. Nagata, M. Howard and P. Lane for providing the antibodies and the CD40 ligand, and Drs. D. Kitamura, I. Taniuchi, Y. Suzuki, T. Yamamoto, and H. Nishizumi for helpful discussions. This work was supported in part by a grant from the Ministry of Education, Science and Culture in Japan.

Address correspondence to Takeshi Watanabe, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-82, Japan.

Received for publication 30 May 1996 and in revised form 2 July 1996.

References

1. Bolen, J.B. 1995. Protein tyrosine kinases in the initiation of antigen receptor signaling. *Curr. Opin. Immunol.* 7:306–311.
2. Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima. 1991. Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science (Wash. DC).* 251:192–194.
3. Burkhardt, A.L., M. Brunswick, J.B. Bolen, and J.J. Mond. 1991. Anti-immunoglobulin stimulation of B lymphocytes activates src-related protein-tyrosine kinases. *Proc. Natl. Acad. Sci. USA.* 88:7410–7414.
4. Campbell, M.-A., and B.M. Sefton. 1992. Association between B-lymphocyte membrane immunoglobulin and multiple members of the src family of protein tyrosine kinases. *Mol. Cell. Biol.* 12:2315–2321.
5. Li, Z-.H., S. Mahajan, M.M. Prendergast, J. Fargnoli, X. Zhu, S. Klages, D. Adam, G.L. Schieven, J. Blake, J.B. Bolen, and A.L. Burkhardt. 1992. Cross-linking of surface immunoglobulin activates src-related tyrosine kinases in WEHI231 cells. *Biochem. Biophys. Res. Commun.* 187:1536–1544.
6. Hutchcroft, J.E., M.L. Harrison, and R.L. Geahlen. 1992. Association of the 72-kD protein-tyrosine kinase PTK72 with the B cell antigen receptor. *J. Biol. Chem.* 267:8613–8619.
7. Yamada, T., T. Taniguchi, C. Yang, S. Yasue, H. Saito, and H. Yamamura. 1993. Association with B-cell-antigen receptor with protein-tyrosine kinase p72syk and activation by engagement of membrane IgM. *Eur. J. Biochem.* 213:455–459.
8. Gold, M.R., D.A. Law, and A.L. DeFranco. 1990. Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. *Nature (Lond.).* 345:810–813.
9. Campbell, M.-A., and B.M. Sefton. 1990. Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2125–2131.
10. Gold, M.R., L. Matsuuchi, R.B. Kelly, and A.L. DeFranco. 1991. Tyrosine phosphorylation of components of the B-cell antigen receptor following receptor crosslinking. *Proc. Natl. Acad. Sci. USA.* 88:3436–3440.
11. Coggeshall, K.M., J.C. McHugh, and A. Altman. 1992. Predominant expression and activation-induced tyrosine phosphorylation of phospholipase C-γ2 in B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 89:5660–5664.
12. Yamanashi, Y., Y. Fukui, B. Wongsasant, Y. Kinoshita, Y. Ichimori, K. Toyoshima, and T. Yamamoto. 1992. Activation of src-like protein-tyrosine kinase Lyn and its association with phosphatidylinositol 3-kinase upon B-cell antigen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA.* 89: 1118–1122.
13. Gold, M.R., V.W.-F. Chan, C.W. Turck, and A.L. DeFranco. 1992. Membrane Ig cross-linking regulates phosphatidylinositol 3-kinase in B lymphocytes. *J. Immunol.* 148: 2012-2022.
14. Bustelo, X.R., and M. Barbacid. 1992. Tyrosine phosphorylation of the *vav* proto-oncogene product in activated B cells. *Science (Wash. DC).* 256:1196–1199.
15. Bustelo, X.R., J.A. Ledbetter, and M. Barbacid. 1992. Product of *vav* proto-oncogene defines a new class of tyrosine protein kinase substrates. *Nature (Lond.).* 356:68–71.
16. Margolis, B., P. Hu, S. Katzav, W. Li, J.M. Oliver, A. Ullrich, A. Weiss, and J. Schlessinger. 1992. Tyrosine phosphorylation of *vav* proto-oncogene product containing SH2 domain and transcription factor motifs. *Nature (Lond.).* 356:71–74.
17. Gold, M.R., M.T. Crowley, G.A. Martin, F. McCormick, and A.L. DeFranco. 1993. Targets of B lymphocyte antigen receptor signal transduction include the $p21^{ras}$ GTPase-activating protein (GAP) and two GAP-associated proteins. *J. Immunol.* 150:377.
18. Saxton, T.M., I. van Oostveen, D. Bowtell, R. Aebersold, and M.R. Gold. 1994. B cell antigen receptor cross-linking induces phosphorylation of the p^{21} ras oncoprotein activator SHC and mSOS1 as well as assembly of complexes containing SHC, GRB-2, mSOS1, and a 145-kDa tyrosine-phosphorylated protein. *J. Immunol.* 153:623–636.
19. Yamanashi, Y., M. Okada, T. Semba, T. Yamor, H. Uemori, S. Tsunasawa, K. Toyoshima, D. Kitamura, T. Watanabe, and T. Yamamoto. 1993. Identification of HS1 protein as a major substrate of protein-tyrosine kinase(s) upon B-cell antigen receptor-mediated signaling. *Proc. Natl. Acad. Sci. USA.* 90:3631–3635.
20. Yamanashi, Y., S. Mori, M. Yoshida, T. Kishimoto, K. Inoue, T. Yamamoto, and K. Toyoshima. 1989. Selective expression of a protein-tyrosine kinase, $p56^{lyn}$, in hematopoietic cells and association with production of human T cell lymphotropic virus type I. *Proc. Natl. Acad. Sci. USA.* 86:6538–6542.
21. Umemori, H., A. Wanaka, H. Kata, M. Takeuchi, M. Tohyama, and T. Yamamoto. 1992. Specific expression of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine kinases, in the central nervous system. *Mol. Brain Res.* 16:303–310.
22. Sidorenko, S.P., C-L. Law, K.A. Chandran, and E.A. Clark. 1995. Human spleen tyrosine kinase $p72^{syk}$ associates with the src-family kinase $p53/56^{lyn}$ and a 120-kDa phosphoprotein. *Proc. Natl. Acad. Sci. USA.* 92:359–363.
23. Aoki, Y., Y-T. Kim, R. Stillwell, T.J. Kim, and S. Pillai. 1995. The SH2 domains of src family kinases associate with Syk. *J. Biol. Chem.* 270:15658–15663.
24. Tezuka, T., H. Umemori, N. Fusaki, T. Yagi, M. Takata, T. Kurosaki, and T. Yamamoto. 1996. Physical and functional association of the cbl proto-oncogene product with a *src*-family protein tyrosine kinase, $p53/56^{lyn}$, in the B cell antigen receptor-mediated signaling. *J. Exp. Med.* 183:675–680.
25. Justement, L.B., K.S. Campbell, N.C. Chien, and J.C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science (Wash. DC).* 252:1839–1842.
26. Kishihara, K., J. Penninger, V.A. Wallace, T.M. Kundig, K. Kawai, A. Wakeham, E. Timms, K. Pfeffer, P.S. Ohashi, M.L. Thomas et al. 1993. Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell.* 74:143–156.
27. Pesando, J.M., L.S. Bouchard, and B.E. McMaster. 1989. CD19 is functionally and physically associated with surface immunoglobulin. *J. Exp. Med.* 170:2159–2164.
28. Pezzutto, A., B. Dorken, P.S. Rabinovitch, J.A. Ledbetter, G. Moldenhauer, and E.A. Clark. 1987. CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. *J. Immunol.* 138:2793-2799.
29. Brown, V.K., E.W. Ogle, A.L. Burkhardt, R.B. Rowley, J.B. Bolen, and L.B. Justement. 1994. *J. Biol. Chem.* 269: 17238-17244.
30. van Noesel, C.J.M., A.C. Lankester, G.M.W. van Schijndel, and R.A.W. van Lier. 1993. The CR2/CD19 complex on human B cells contains the src-family kinase *Lyn. Int. Immu-*

nol. 5:699–705.

31. Takata, M., H. Sabe, A. Hata, T. Inazu, Y. Homma, T. Nukada, H. Yamamura, and T. Kurosaki. 1994. Tyrosine kinases Lyn and Syk regulate B cell receptor-coupled Ca^{2+} mobilization through distinct pathways. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1341–1349.
32. Scheuermann, R.H., E. Racila, T. Tucker, E. Yefenof, N.E. Street, E.S. Vitetta, L.J. Picker, and J.W. Uhr. 1994. Lyn tyrosine kinase signals cell cycle arrest but not apoptosis in B-lineage lymphoma cells. *Proc. Natl. Acad. Sci. USA.* 91: 4048–4052.
33. Ren, C.L., T. Morio, S.M. Fu, and R.S. Geha. 1994. Signal transduction via CD40 involves activation of Lyn kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase Cγ2. *J. Exp. Med.* 179:673–680.
34. Faris, M., F. Gaskin, J.T. Parsons, and S.M. Fu. 1994. CD40 signaling pathway: anti-CD40 monoclonal antibody induces rapid dephosphorylation and phosphorylation of tyrosine-phosphorylated proteins including protein tyrosine kinase Lyn, Fyn, and Syk and the appearance of a 28-kD tyrosine phosphorylated protein. *J. Exp. Med.* 179:1923–1931.
35. Liu, Y.-J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C.M. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centers. *Nature (Lond.).* 342: 929–931.
36. Banchereau, J., P.D. Paoli, A. Valle, E. Garcia, and F. Rousset. 1991. Long-term human B cell lines dependent on Interleukin-4 and antibody to CD40. *Science (Wash. DC).* 251:70–72.
37. Kehry, M.R. 1996. CD40-mediated signaling in B cells: balancing cell survival, growth, and death. *J. Immunol.* 156: 2345-2348.
38. Hibbs, M.L., D.M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglitto, S.A. Stacker, and A.R. Dunn. 1995. Multiple defects in the immune system of *Lyn*-deficient mice, culminating in autoimmune disease. *Cell.* 83:301–311.
39. Nishizumi, H., I. Taniuchi, Y. Yamanashi, D. Kitamura, D. Ilic, S. Mori, T. Watanabe, and T. Yamamoto. 1995. Impaired proliferation of peripheral B cells and indication of autoimmune disease in *lyn*-deficient mice. *Immunity.* 3:549–560.
40. Garrone, P., E.-M. Neidhardt, E. Garcia, L. Galibert, C. van Kooten, and J. Banchereau. 1995. Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.* 182:1265–1273.
41. Schattner, E.J., K.B. Elkon, D.-H. Yoo, J. Tumang, P.H. Krammer, M.K. Crow, and S.M. Friedman. 1995. CD40 ligation induces Apo-1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. *J. Exp. Med.* 182:1557–1565.
42. Wang, J., I. Taniuchi, Y. Maekawa, M. Howard, M.D. Cooper, and T. Watanabe. 1996. Expression and function of Fas antigen on activated murine B cells. *Eur. J. Immunol.* 26:92–96.
43. Rothstein, T.L., J.K.M. Wang, D.J. Panka, L.C. Foote, Z. Wang, B. Stanger, H. Cui, S.-T. Ju, and A. Marshak-Rothstein. 1995. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature (Lond.).* 374:163-165.
44. Lane, P., T. Brocker, S. Hubele, E. Padovan, A. Lanzavecchia, and F. McConnell. 1993. Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T cell-dependent activation. *J. Exp. Med.* 177:1209–1213.
45. Phillips, N.E., and D.C. Parker. 1983. Fc-dependent inhibition of mouse B cell activation by whole anti-μ antibodies. *J. Immunol.* 130:602–606.
46. Fridman, W.H., C. Bonnerot, M. Daeron, S. Amigorena, J.-L. Teillaud, and C. Sautes. 1992. Structural bases of Fcγ receptor functions. *Immunol. Rev.* 125:49–76.
47. D'Ambrosio, D., K.L. Hippen, S.A. Minskoff, I. Mellman, G. Pani, K.A. Siminovitch, and J.C. Cambier. 1995. Recruitment and activation of PTP1C in negative regulation of antigen receptor signaling by FcγRIIB1. *Science (Wash. DC).* 268:293–297.
48. Takai, T., M. Ono, M. Hikida, H. Ohmori, and J.V. Ravetch. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature (Lond.).* 379:346–349.
49. Pani, G., M. Kozlowski, J.C. Cambier, G.B. Mills, and K.A. Siminovitch. 1995. Identification of the tyrosine phosphatase PTP1C as a B cell antigen receptor-associated protein involved in the regulation of B cell signaling. *J. Exp. Med.* 181: 2077–2084.
50. Cyster, J.G., and C.C. Goodnow. 1995. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines threshold for negative selection. *Immunity.* 2:13–24.
51. Kozlowski, M., I. Mlinaric-Rascan, G.S. Feng, R. Shen, A. Pawson, and K.A. Siminovitch. 1993. Expression and catalytic activity of the tyrosine phosphatase PTP1C is severely impaired in motheaten and viable motheaten mice. *J. Exp. Med.* 178:2157–2163.
52. Tsui, H.W., K.A. Siminovitch, L. de Souza, and F.W.L. Tsui. 1993. Motheaten and viable motheaten mice have mutations in the hemopoietic cell phosphatase gene. *Nature Genet.* 4:124–129.
53. Shultz, L.D., P.A. Schweitzer, T.V. Rajan, T. Yi, J.N. Ihle, R.J. Matthews, M.L. Thomas, and D.R. Beier. 1993. Mutations at the motheaten locus are within the hemopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell.* 73:1445–1454.
54. Davidson, W.F., H.C. Morse III, S.O. Sharrow, and T.M. Chused. 1979. Phenotypic and functional effects of the motheaten gene on murine B and T lymphocytes. *J. Immunol.* 122:884–891.
55. Okumura, M., and M.L. Thomas. 1995. Regulation of immune function by protein tyrosine phosphatases. *Curr. Opin. Immunol.* 7:312–319.
56. Sidman, C.L., L.D. Shultz, R.R. Hardy, K. Hayakawa, and L.A. Herzengerg. 1986. Production of immunoglobulin isotypes by $Ly\text{-}1^+$ B cells in viable motheaten and normal mice. *Science (Wash. DC).* 232:1423–1425.
57. Nagata, S., and T. Suda. 1995. Fas and Fas ligand: *lpr* and *gld* mutations. *Immunol. Today.* 16:39–43.