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A Balance between B Cell Receptor and Inhibitory Receptor Signaling Controls Plasma Cell Differentiation by Maintaining Optimal Ets1 Levels

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Signaling through the BCR can drive B cell activation and contribute to B cell differentiation into Ab-secreting plasma cells. The positive BCR signal is counterbalanced by a number of membrane-localized inhibitory receptors that limit B cell activation and plasma cell differentiation. Deficiencies in these negative signaling pathways may cause autoantibody generation and autoimmune disease in both animal models and human patients. We have previously shown that the transcription factor Ets1 can restrain B cell differentiation into plasma cells. In this study, we tested the roles of the BCR and inhibitory receptors in controlling the expression of Ets1 in mouse B cells. We found that Ets1 is downregulated in B cells by BCR or TLR signaling through a pathway dependent on PI3K, Btk, IKK2, and JNK. Deficiencies in inhibitory pathways, such as a loss of the tyrosine kinase Lyn, the phosphatase Ssrc homology region 2 domain-containing phosphatase 1 (SHP1), or membrane receptors CD22 and/or Siglec-G, result in enhanced BCR signaling and decreased Ets1 expression. Restoring Ets1 expression in Lyn- or SHP1-deficient B cells inhibits their enhanced plasma cell differentiation. Our findings indicate that downregulation of Ets1 occurs in response to either BCR or TLR signaling, thereby allowing B cell differentiation and that the maintenance of the inhibitory Lyn → CD22/SiglecG → SHP1 pathway in B cells. *The Journal of Immunology, 2014, 193: 909–920.

B cells differentiate to Ab-secreting plasma cells to mediate the humoral arm of the immune response. Normally this process is under tight control to allow useful Abs to be produced while inhibiting the production of pathogenic autoactive Abs. However, in autoimmune diseases in humans and mouse models, B cell differentiation to plasma cells fails to be regulated correctly, resulting in autoantibody production. This can arise either through B cell-intrinsic deficiencies or by B cell–extrinsic factors such as aberrant T cell activation.

Activation of B cells can be achieved by Ag binding to the BCR and by other pathways such as triggering of TLRs. Ag binding to the BCR triggers activation of Src family kinases such as Lyn and Fyn leading to phosphorylation of Igα (CD79α) and Igβ (CD79β), recruitment of Syk kinase, and subsequent recruitment and phosphorylation of BLNK, Btk, and phospholipase Cγ1 (1). These events activate the Ras pathway, protein kinase C (PKC) pathway and calcium flux, eventually triggering the activation of NF-κB, Erk, and JNK. These positive signals are normally counterbalanced by negative signals that limit B cell activation and prevent spontaneous B cell proliferation and differentiation to plasma cells (2). Negative signals are generated by a series of membrane receptors (CD22, CD72, FcγRIIB, paired Ig-like receptor B [PIR-B], Siglec-G, and so on) that are phosphorylated by Lyn. This allows them to recruit phosphatases such as Sac homology region 2 domain-containing phosphatase 1 (SHP1) and SHP1 that reverse phosphorylation of signaling molecules in the BCR pathway and dampen BCR signaling (3–5).

Loss of negative signaling leads to increased BCR-dependent B cell activation and can result in autoimmune disease. For instance, Lyn−/- mice, which have defective negative signaling, develop severe autoimmunity (6–9). Reduced Lyn expression has been observed in PBMCs from human autoimmune patients (10, 11). Similarly, loss of SHP1, one of the main phosphatases downstream of Lyn, also results in severe autoimmunity in mice (12, 13). In contrast, loss of membrane receptors such as CD22, CD72, FcγRIIb, and Siglec-G alone leads to more modest autoreactive B cell activation, probably because of functional redundancy among these receptors (14–17). Indeed, functional redundancy exists because combined deletion of both CD22 and Siglec-G leads to a more severe autoimmune phenotype than loss of individual receptors.
of either single receptor alone (18). Interestingly, autoimmune disease in Lyn−/− mice can be ameliorated by reducing the levels of Btk, an important BCR effector kinase (19–21). This supports the idea that there is a careful balance between the positive and negative pathways.

Although much is known about the positive and negative signaling pathways that control B cell activation, less is understood about the downstream targets of these pathways or how they regulate B cell differentiation into Ab-secreting plasma cells. However, B cell differentiation is under the control of a network of transcription factors (22). Plasma cell differentiation requires the transcription factor Blimp1 as well as Irf4 and Xbp1. In contrast, the transcription factors Pax5, Bach2, and Ets1 are thought to block plasma cell differentiation.

We observed several phenotypes of mice lacking Ets1 that are in common with those of mice lacking Lyn. These include increased B cell activation, decreases in marginal zone B cells, early accumulation of IgM-secreting plasma cells, production of IgG autoantibodies with specificity classically associated with systemic lupus erythematosus, and immune complex deposition in the kidney (6–8, 23, 24). We theorized therefore that Ets1 might be an important downstream target of the negative signaling pathway regulated by Lyn. In this study, we explored a relationship between Ets1 expression and positive (BCR) and negative signaling in B cells.

Materials and Methods

Mice used

The following mouse strains were used in this paper: C57BL/6, Ets1−/− (23), Lyn−/− (8), Btk−/− (25), Btk+/+ (26), Lyn+/− (8), Btk+/+ (27), MD4 BCR transgenic (28), CD19-Cre (29), Rosa26 Stop-flox IKK2ca (30), B6.Cg-mice and either Ets1+/+ or Ets1−/− mice. Lyn−/− mice and Ets1−/− mice were generated by reconstituting lethally irradiated mice with Cre-expressing cells to excise Ets1 or Lyn alleles. Lyn−/− mice were used in all experiments except Supplemental Fig. 1C). Two sets of primers were used for qPCR: one set for Lyn (GenBank accession number XM_007394182) and the other set for Ets1 (GenBank accession number NM_012316).

Cell lines and reagents

A20 and M12 B cell lymphomas were maintained in RPMI 1640 medium supplemented with FBS, antibiotics, glucose, and 50 μM 2-ME. The retroviral packaging cell line Platinum-E was maintained in DMEM supplemented with FBS, antibiotics, glutamine, blasticidin, and puromycin. Platinum-E cells were switched to medium lacking blasticidin and puromycin a day before transfection.

B cell purification and stimulation

B cells were purified from mouse spleens and lymph nodes either by negative selection with CD43 microbeads or by positive selection with B220 or CD19 microbeads (Miltenyi Biotec). Most samples were purified using positive selection with B220 microbeads to obtain better purity, except for the following samples: 1) CD43 microbeads; wild-type B cells used in signaling experiments, Pten−/− B cells and B cells from Ets1−/− mice and littersmate controls (all experiments except Supplemental Fig. 1C); and 2) CD19 microbeads; CD22−/−, SiglecG−/−, CD22−/−, SiglecG−/−, and littersmate controls. For in vitro stimulation, purified splenic B cells were allowed to rest in a tissue culture incubator at 37°C for 30 min then stimulated with one or more of the following reagents: anti-IgM F(ab′)2 (Jackson Immuno-Research Laboratories) at various doses as described in the figure legends, 5 μg/ml LPS (Sigma-Aldrich), 0.5 or 5 μg/ml CD40 ODN 1826 (InvivoGen), 50 ng/ml PMA (Sigma-Aldrich), 500 ng/ml ionomycin (Calbiochem), 10 μg/ml anti-CD40 (clone 3/23; BioLegend), 10 ng/ml IL-4 (R&D Systems), or 100 ng/ml IL-21 (R&D Systems) for the indicated times. For inhibitor experiments, a P13K inhibitor (LY294002; Cell Signaling Technology), a Btk inhibitor (PCI-32765; Selleckchem), an IKK inhibitor (inhibitor IV; Calbiochem), a MEK1 inhibitor (PD98059; Cell Signaling Technology), two JNK inhibitors (SP600125 and JNK-In-8; EMD Millipore), and a p38 inhibitor (SB203580; EMD Millipore) were used. For inhibitor studies, B cells were pretreated with inhibitors for one hour prior to stimulation.

Plasmids and transient transfections

Mouse Ets1 with a C-terminal HA tag was cloned into the retroviral plasmid MIGR1. Plasmids harboring constitutively active IKK2 (IKK2-CA) (39), kinase inactive IKK2 (IKK2-K1) (40), and constitutively active JNK (JNK-Ca) (40) were purchased from Addgene, and the kinase inserts were subcloned into MIGR1 and pCDA3.1. A retroviral plasmid harboring a constitutively active form of Akt kinase (myr-Akt) was a gift of Dr. K. Gritsman (Brigham and Women’s Hospital, Boston, MA). Myr-Akt was also subcloned from this plasmid into pCDA3.1. pCDA3.1 plasmids were transfected into A20 B cells by nucleofection and analyzed 8 h posttransfection.

Protein and mRNA stability

To measure protein and RNA stability, purified splenic B cells were treated for 0–6 h with either cycloheximide (200 μM) to inhibit new protein synthesis or with actinomycin D (4 μM) to inhibit RNA transcription. Ets1 levels were assessed by Western blot or RT-quantitative PCR (qPCR) analysis as described below.

Immunoprecipitation assay

Purified splenic B cells were lysed in a 1% Nonidet P-40 lysis buffer with protease and phosphatase inhibitors. Lysates were immunoprecipitated with either a rabbit polyclonal anti-Lyn Ab (Santa Cruz Biotechnology) or as a control rabbit IgG Ab. Immunoprecipitates were Western blotted using anti-phosphotyrosine Ab (clone 4G10; Millipore).

ELISpot assay

ELISpot analysis was performed as described previously (23, 41). Sorted virally infected splenic B cells were plated at 500, 1000, 2500, and 5000 cells/well. ELISpot plates were counted with an automated reader (Zelnet Consulting).

Retroviral production and transduction

Retrovirus production and transduction were performed as described previously (41, 42). Purified splenic B cells were stimulated with 10 μg/ml LPS for 24 h prior to infection with virus. Two days postinfection, GFP+ populations were sorted using FACSaria Cell Sorter and plated out for ELISpot assay. Virally infected cells were also analyzed for B220 and CD138 staining in the GFP+ population.

Western blot analysis

Whole B cells lysates were prepared by boiling in Laemmli sample buffer. The following Abs were used in Western blotting: rabbit monoclonal anti-mouse Ets1 Ab (clone EPR546; Epitomics), mouse monoclonal anti-GAPDH (clone 6C5; Millipore), mouse monoclonal anti-Lyn (clone LYN-01; BioLegend), rabbit monoclonal anti–phospho-Y507 Lyn (EP504Y; Epitomics), rabbit monoclonal anti–phospho-Y396 Lyn (EP503Y; Epitomics), rabbit monoclonal anti–phospho-S473 Akt (clone EP2109Y; Epitomics), rabbit monoclonal anti-ILK (clone D30C6; Cell Signaling Technology), rabbit monoclonal anti–phospho-S63-c-Jun (clone 54B3; Cell Signaling Technology), or mouse monoclonal anti-Flag (clone M2; Stratagene). Signals were quantitated by ImageJ software. Loading was normalized by GAPDH levels.

RNA isolation and RT-qPCR

Total RNA was isolated using Direct-zol RNA Purification Kit (Zymo Research). Equal amounts of RNA were reverse-transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR was performed using SYBR Green Supermix (Bio-Rad). Gene expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT) as reference gene and further normalized to control groups, which were arbitrarily set to a value of 1. The following primers were used: Ets1, 5′-
AGCTTTGTCAGTCCTTTATCAAC-3' (forward) and 5'-TTTCCCTCT-TTCCCCATCTCC-3' (reverse); Lyn, 5'-TCTTCGACATTTCCTTCCC-3' (forward) and 5'-GATCTCCGAGCTTATCTCC-3' (reverse); and HPRT 5'-CCTCATGGACTGATTATGGAG-3' (forward) and 5'-TGACAAAGAATTCTAGCCCC-3' (reverse).

**Statistical analysis**

Statistical analysis was performed with the Prism software (GraphPad Software). The p values were determined using unpaired Student t tests (two-tailed) or ANOVA with multiple comparisons test. For half-life experiments, nonlinear curve fitting was used and curves were compared with the extra sum-of-squares F test. Differences between groups were considered significant for p < 0.05.

**Results**

Both Lyn and Ets1 regulate plasma cell differentiation in a B cell–intrinsic fashion

Both Lyn and Ets1 are highly expressed in B cells (43, 44), and there are B cell abnormalities in mice lacking Lyn or Ets1 including a prominent increase in IgM- and IgG-secreting plasma cells (7, 41). However, Lyn expression is also high in myeloid cells (43), whereas Ets1 is found at high levels in T cells and NK cells (45, 46). To determine whether Lyn and Ets1 regulate B cell differentiation in a B cell–intrinsic manner, we generated mixed chimeras in which Lyn-deficient or Ets1-deficient B cells develop in the same environment as wild-type B cells (Fig. 1A).

We transferred bone marrow from Lyn+/+ or Lyn−/− mice on a C57BL/6 genetic background (and hence carrying the IgHb Ig allotype) along with wild-type congenic bone marrow carrying the IgHa allotype into irradiated congenic C57BL/6 IgMa+ recipients. As shown in Fig. 1B, Lyn−/− B cells differentiate more readily to plasma cells than do wild-type B cells developing in the same environment. Because similar numbers of Ets1+/+ and Ets1−/− derived B cells develop in chimeric mice, we quantitated allotype-specific plasma cells using ELISPOT assays. Like Lyn−/− B cells, Ets1−/− B cells showed an intrinsic propensity to differentiate to plasma cells.

**FIGURE 1.** Both Lyn and Ets1 regulate plasma cell differentiation in a B cell–intrinsic fashion. (A) Lyn-deficient bone marrow chimeras were generated by mixing wild-type congenic B6.IgMa bone marrow with IgMb+ C57BL/6 genetic background Lyn+/+ or Lyn−/− bone marrow and transferring into irradiated B6.IgMa recipients. Ets1-deficient bone marrow chimeras were generated by mixing wild-type congenic B6.IgMa fetal liver cells from E16.5 d embryos with C57BL/6 IgMb+/Ets1+/+ or Ets1−/− fetal liver cells (also from E16.5 d embryos) and transferring into irradiated Rag2−/− recipients. All chimeras were allowed to reconstitute for 6–8 wk prior to analysis.

As both Ets1 and Lyn regulate B cell differentiation in a B cell–intrinsic fashion and control B cell secretion of autoantibodies, we hypothesized that they may act in the same signaling pathway. We first asked whether Ets1 can regulate Lyn expression or phosphorylation. Lyn expression was normal at both the protein and mRNA level in B cells from Ets1−/− mice (Supplemental Fig. 1A, 1B). Phosphorylation of Y508 inhibits Lyn activity, whereas phosphorylation of Y397 stimulates Lyn activity (47). Neither of these phosphorylation events was altered in resting Ets1−/− B cells (Supplemental Fig. 1A) nor was total tyrosine phosphorylation of Lyn (Supplemental Fig. 1D). Unexpectedly, Lyn Y397 phosphorylation was not significantly induced by BCR cross-linking in either wild-type or Ets1−/− B cells (Supplemental Fig. 1C) but rather showed high levels prior to stimulation. High basal levels of Y397 phosphorylation of Lyn in hematopoietic cells have also been observed in other studies (48–50). This may be relevant to its role in signaling through inhibitory pathways. Therefore, it appears that Ets1 does not regulate the expression of the Lyn gene nor other genes that control the activity of kinases or phosphatases that regulate Lyn.

After reconstitution, allotype-specific plasma cells were enumerated in Lyn+/+ and Lyn−/− chimeras using flow cytometry. Because Lyn−/− B cells compete poorly with wild-type cells in mixed chimeras (data not shown), we used flow cytometry to determine the frequency of B220+CD138+ cells among IgMa- and IgMb-expressing cells. As shown in Fig. 1B, Lyn−/− B cells differentiate more readily to plasma cells than do wild-type B cells developing in the same environment. Because similar numbers of Ets1+/+ and Ets1−/− derived B cells develop in chimeric mice, we quantitated allotype-specific plasma cells using ELISPOT assays. Like Lyn−/− B cells, Ets1−/− B cells showed an intrinsic propensity to differentiate to plasma cells.
Ets1 levels are downregulated in Lyn−/− B cells

Because Ets1 did not appear to be upstream of Lyn, we asked whether Lyn might instead regulate levels of Ets1. Ets1 protein levels were downregulated in Lyn−/− B cells compared with B cells from wild-type mice (Fig. 2A). As Lyn−/− mice age, they develop severe autoimmunity accompanied by increased levels of inflammatory cytokines such as IL-6 (21, 51, 52). To determine whether downregulation of Ets1 in Lyn−/− B cells was due to the inflammatory environment that develops in these mice as they age, we compared Ets1 protein levels in B cells isolated from young prediseseased mice (Fig. 2B). Ets1 was downregulated in B cells even in Lyn−/− mice as young as 1 mo old, suggesting that this is a primary defect and not a secondary effect of the autoimmune disease. Quantification of Western blots showed that on average Ets1 protein was expressed in Lyn−/− B cells at levels ∼27 ± 5% of the levels in wild-type B cells (p < 0.0001). We also examined Ets1 mRNA levels in Lyn−/− B cells and found that these were also decreased (Fig. 2C). In most experiments, the protein levels of Ets1 from Lyn−/− mice showed a somewhat stronger decrease than the mRNA levels in Lyn−/− B cells, indicating that Lyn might regulate not only Ets1 mRNA expression but also Ets1 protein synthesis or stability.

To better define the mechanisms that regulate Ets1 protein and mRNA levels in Lyn−/− B cells, we measured the half-life of Ets1 protein and mRNA by treating B cells with cycloheximide or actinomycin D to inhibit protein or mRNA synthesis, respectively. The half-life of Ets1 protein was short, ∼1.1 h in wild-type B cells (Fig. 2D), and was unchanged in Lyn-deficient B cells. The half-life of Ets1 mRNA was also short (∼1 h) in wild-type B cells (Fig. 2E) and slightly shorter (∼45 min) in Lyn−/− B cells, but this difference was not statistically significant. Because the absence of Lyn appears to result in very minor or no changes to the stability of Ets1 mRNA and protein, downregulation of Ets1 mRNA in Lyn−/− B cells may instead result from decreased transcription of the Ets1 gene. Downregulation of Ets1 protein may be a combined effect of decreased mRNA levels and inhibition of mRNA translation into protein.

Ets1 can be downregulated by BCR and TLR signaling

One major function of Lyn in B cells is to inhibit BCR signaling via its ability to phosphorylate inhibitory receptors. Thus, BCR signaling is hyperactive in Lyn−/− B cells. Ets1 levels can be downregulated by activation of the AgRs in B cells and T cells (53, 54). We theorized that enhanced BCR signaling in Lyn−/− B cells might cause downregulation of Ets1 in these cells, leading to enhanced plasma cell formation.

To clarify signaling cascades in the downregulation of Ets1, we treated wild-type B cells with BCR cross-linking agents and other types of stimuli. As expected, BCR cross-linking downregulated Ets1 protein and mRNA levels within a few hours after stimulation (Fig. 3A, 3B). BCR activation induces mobilization of intracellular calcium and activation of Ras and PKC enzymes. PMA can induce Ras and PKC activation, whereas ionomycin can transport calcium across the plasma membrane resulting in elevated intracellular calcium. Hence, PMA and ionomycin mimic many aspects of BCR signaling. Stimulation of splenic B cells with either ionomycin or PMA alone (Fig. 3A) or both together (not shown) downregulated Ets1 protein within 4 h, whereas treatment with vehicle (DMSO) alone had no effect. Although both PMA and ionomycin were equally effective in down-regulating the protein levels of Ets1, PMA was more effective in down-regulating the Ets1 message than was ionomycin (Fig. 3B), suggesting they may modify Ets1 expression by somewhat different mechanisms.

FIGURE 2. Lyn regulates Ets1 levels in B cells. (A) Western blot analysis of splenic B cell lysates from two wild-type (WT) and two Lyn-deficient mice at 12 mo of age (A) or 4 mo of age (B) shows downregulation of the two most predominant isoforms of Ets1, the p54 and p42 isoforms, in Lyn−/− cells. Nine separate Western blots for Ets1 were done and included 18 WT and 15 Lyn−/− mice analyzed at ages between 1 and 12 mo. (C) RT-qPCR analysis of Ets1 mRNA in B cells from WT (Lyn+/+, n = 7) and Lyn−/− (n = 6) mice. ***p < 0.001. (D) WT or Lyn−/− splenic B cells were treated with cycloheximide for various times to inhibit protein synthesis, and Western blot was performed to measure the levels of Ets1 protein. GAPDH was used as loading control, because it has been reported to have a long half-life (84), and its levels were not changed during the treatment time. Average values of three independent experiments are shown as the quantified ratio of the major p54 isoform of Ets1 to the levels of GAPDH, normalized to the levels at time 0. (E) Equal numbers of B cells from each genotype were treated with actinomycin D for various times to suppress mRNA transcription. Ets1 mRNA levels were measured by RT-qPCR and normalized by the level at time 0. Data represent quantitation of both major RNA isoforms of Ets1 (encoding the p54 and p42 variants of the protein) from three independent experiments. No differences were observed when independently calculating the half-life of each separate isoform.
Ets1 is downregulated by BCR or TLR activation as well as PMA or Ca²⁺ flux. (A) Purified wild-type (WT) splenic B cells were rested at 37°C for 30 min in a tissue-culture incubator and then left untreated (NT) or treated with the stimuli indicated for 4 h, followed by Western blotting for Ets1 and GAPDH. Western blots with each type of stimulus were repeated a minimum of three separate times. (B) mRNA was purified from B cells stimulated for 2 h with stimuli that downregulate Ets1 protein levels as shown in (A) and analyzed by RT-qPCR. Shown is the average of three separate experiments. (C) Western blot for Ets1 and GAPDH with lysates of B cells purified from MD4 Lyn+/+ or MD4 Lyn−/− mice. Shown is representative data from one of three independent experiments (total of five MD4 Lyn+/+ and six MD4 Lyn−/− mice analyzed). (D) RT-qPCR analysis of Ets1 mRNA in B cells from WT (n = 3), MD4 Lyn+/+(n = 6), or MD4 Lyn−/−(n = 6) mice. (E) B cells isolated from WT mice were stimulated with either 1 μg/ml anti-IgM or 0.5 μg/ml Cpg ODN or a combination of both stimuli for 3.5 h. Representative data from one of two similar experiments is shown. *p < 0.05, **p < 0.001.

To test whether BCR signaling was required for the downregulation of Ets1 levels in vivo, we generated MD4 BCR transgenic Lyn−/− mice. The MD4 transgene encodes heavy and light Ig chains that confer specificity to the foreign Ag hen egg lysozyme (28). B cells from MD4 transgenic mice are thus naive since they do not encounter Ag. MD4 Lyn−/− B cells exhibited a less dramatic reduction of Ets1 levels than nontransgenic Lyn−/− B cells (Fig. 3C, 3D, compare with Figs. 2A, 2B). Quantification indicated that MD4 Lyn−/− B cells express ∼54 ± 7% as much Ets1 as do nontransgenic or MD4 Lyn−/− wild-type B cells (p < 0.0001). This suggests that BCR signaling in response to Ag binding plays a role in the downregulation of Ets1 in Lyn−/− B cells. However, fixing the BCR to a non-self Ag did not fully restore Ets1 expression to wild-type levels.

B cells can also be activated and induced to differentiate by certain TLR ligands, such as LPS and unmethylated CpG containing DNA sequences (Cpg DNA). As shown in Fig. 3A, both LPS and Cpg DNA can downregulate Ets1 levels shortly after stimulation. Furthermore, low levels of anti-IgM cooperate with low levels of Cpg DNA to induce downregulation of Ets1 (Fig. 3E). We also tested a variety of other stimuli that activate B cells including IL-4, anti-CD40, IL-21, IL-6, and TGF-β, none of which significantly affected the expression of Ets1 protein (Fig. 3A; data not shown). On the basis these results, either BCR or TLR signaling can downregulate Ets1 levels in B cells.

**PI3K and Btk, but not Akt, are required for Ets1 downregulation during B cell activation**

The BCR signaling component Btk is a target of several Lyn-dependent inhibitory signaling pathways (55, 56). To determine the role of Btk in the downregulation of Ets1, we examined Ets1 levels in Lyn−/−Btk−/− mice, which express low levels of Btk in B cells and do not accumulate plasma cells (21, 26). Interestingly, reduced Btk signaling led to a restoration of Ets1 protein expression in Lyn−/− B cells (Fig. 4A). Quantification indicated that Lyn−/−Btk−/− B cells express on average 118 ± 4% as much Ets1 as wild-type B cells (p = not significant). Ets1 mRNA levels were also partially restored in Lyn-deficient B cells by reducing Btk expression by stimulating wild-type, Btk−/− or Btk−/− mice harboring a wild-type allele of Lyn with anti-IgM. Although the Btk-deficient B cells responded to IgM cross-linking by induction of Akt phosphorylation similar to wild-type B cells, they failed to downregulate Ets1 (Fig. 4C).

Btk binds to phosphatidyl inositol 3,4,5-trisphosphate (PIP₃) produced by the activity of PI3K. Binding of Btk to PIP₃ is required for its maximal activation (57, 58), suggesting that PI3K activity may be required for downregulation of Ets1. To further validate the involvement of PI3K and Btk signaling pathways in regulating Ets1 expression, we pretreated B cells with DMSO (control), a PI3K inhibitor (LY294002), or a Btk inhibitor (PCI-32765), followed by stimulation with anti-IgM cross-linking Ab (Fig. 4D). Inhibiting either PI3K or Btk was sufficient to prevent Ets1 downregulation in response to BCR cross-linking. To test specificity of the inhibitors used above, we confirmed that the PI3K inhibitor, but not the Btk inhibitor, could block activation of Akt (Fig. 4E).

Because PI3K activity is essential for downregulating Ets1, we sought to determine whether PI3K induced activation of Akt was also important. Akt is constitutively phosphorylated in Lyn−/− B cells (Fig. 4F), likely because of hyperactive BCR signaling.
levels of phospho-S473 of Akt (activated Akt) were measured. One of two similar experiments is shown. (A) Western blot for Ets1 and GAPDH with B cell lysates from wild-type (WT), Lyn−/− or Lyn−/−Btk−/− mice (shown is a representative example of three independent experiments, n = 4–5 mice of each genotype analyzed in total). (B) RT-qPCR analysis of Ets1 mRNA in B cells from WT, Lyn−/−, or Lyn−/−Btk−/− mice (n = 3 for each genotype). (C) Purified splenic B cells from WT, Btk−/−, or Btk−/− mice were rested at 37°C for 30 min before treatment with the indicated dose (micrograms per milliliter) of anti-IgM for 3.5 h. Western blot for Ets1, phospho-Akt, and GAPDH levels was performed with cell lysates. One of two similar experiments is shown. (D) WT splenic B cells were rested at 37°C and pretreated with either DMSO vehicle control (labeled C), 5 μg/ml of a PI3K inhibitor Ly294002 (labeled PI3), 100 ng/ml of a Btk inhibitor PCI32765 (labeled Btk) for 1 h, and then stimulated with 10 μg/ml anti-IgM for 3.5 h. Cell lysates were analyzed for Ets1 and GAPDH levels by Western blot. One of two similar experiments is shown. (E) To test the specificity of the inhibitors used in (D), cells were stimulated for 5 min, and levels of phospho-S473 of Akt (activated Akt) were measured. One of two similar experiments is shown. (F) Western blot with lysates from WT or Lyn−/− B cells to show constitutive activation of the Akt pathway in the absence of Lyn. One of three similar experiments is shown. (G) Splenic B cells from WT or Pten−/− mice were rested at 37°C for 30 min followed by treatment with the indicated dose (micrograms per milliliter) of anti-IgM for 3.5 h. Cell lysates were analyzed by Western blot. Shown is the data from one of two similar experiments. *p < 0.05, **p < 0.01.

However, Akt activation did not correlate with the levels of Ets1. Akt was activated normally in BCR-stimulated Btk-deficient B cells or when Btk inhibitor was used, but Ets1 levels were not downregulated. To further study the involvement of the PI3→Akt kinase pathway in the regulation of Ets1 levels, Pten−/− B cells (derived from CD19-Cre PtenloxP/loxP mice) were used (Fig. 4G). Pten−/− B cells produce increased PIP3 upon BCR cross-linking, resulting in increased activation of Akt and presumably other PI3K-dependent enzymes (33). In keeping with this, we detected robust Akt phosphorylation with a low dose of anti-IgM stimulation in Pten-deficient B cells but not in wild-type cells (Fig. 4G). However, Ets1 downregulation was not enhanced in Pten−/− B cells, indicating that supraphysiological levels of PIP3 are not required for the downregulation of Ets1. These data also support the idea that Akt activation does not regulate Ets1 levels, because Akt is hyperactivated upon BCR cross-linking in Pten−/− B cells, yet Ets1 levels are similar to those found in wild-type B cells under the same conditions. Taken together, these results suggested that PI3K and Btk signaling, but not Akt signaling, are involved in regulating Ets1 expression during B cell activation.

IKK and JNK signaling downstream of BCR and TLR receptors regulate Ets1 levels

Btk signaling is crucial for activating the NF-κB pathway in B cells (59, 60). NF-κB signaling is also triggered by TLR ligation and by treatment of B cells with PMA, stimuli that we have shown above to result in downregulation of Ets1 expression. We hypothesized that NF-κB may inhibit the expression of the Ets1 gene, either directly or indirectly. To test this, we pretreated primary B cells with an inhibitor of the IKK2 subunit of the NF-κB activating IKK complex, followed by cross-linking the BCR with anti-IgM or activating TLR signaling with CpG DNA. The IKK2 inhibitor was able to prevent downregulation of Ets1 by either anti-IgM or TLR agonists (Fig. 5A), confirming a role for the NF-κB signaling pathway in Ets1 regulation.

Btk has also been implicated in the activation of JNK MAPK and in some, but not all, studies in the activation of Erk MAPK (20, 61–64). We tested whether inhibition of MAPK pathways could prevent downregulation of Ets1. We pretreated primary B cells with either an inhibitor of MEKs (the upstream activators of Erk), with two different JNK inhibitors or with a p38 inhibitor. After pretreatment for 1 h, B cells were stimulated by cross-linking the BCR with anti-IgM or activating TLR signaling with CpG DNA (Fig. 5B). Both of the JNK inhibitors were able to prevent downregulation of Ets1 by either anti-IgM or CpG DNA. However, neither the MEK inhibitor nor the p38 inhibitor prevented Ets1 downregulation.

To further explore potential roles for IKK2, JNK and Akt in regulating Ets1 levels, we transfected A20 B lymphoma cells with plasmids encoding an IKK2-CA, a kinase-dead form of IKK2 (IKK2-K1), a constitutively active JNK1 (JNK-CA), or a constitutively active version of Akt (Myr-Akt). BCR cross-linking downregulates Ets1 expression in A20 B cells in a similar fashion to that seen in primary splenic B cells (data not shown),
FIGURE 5. IKK2 and JNK both contribute to downregulating Ets1. For experiments shown in (A) and (B), wild-type splenic B cells were rested at 37°C and then pretreated with either DMSO vehicle control [labeled as C in (A) and (B)] or with the inhibitors indicated below for 1 h. Cells were then stimulated for 3.5 h with anti-IgM (10 μg/ml) or CpG (5 μg/ml) for 3.5 h, followed by Western blotting for Ets1 and GAPDH. (A) Cells treated with 5 μg/ml IKK2 inhibitor IV (labeled Ikk2). Shown is data from one of three similar experiments. (B) Cells were treated with 25 μg/ml of one of two IKK inhibitors (SP600215 labeled JNK1 or JNK-In-8–labeled JNK2), 25 μg/ml of a p38 inhibitor (labeled p38), or 30 μg/ml of a MEK inhibitor (labeled MEK). One of two similar experiments is shown. (C) A20 B lymphoma cells were nucleofected with an empty vector or vectors carrying IKK2-KI, IKK2-CA, JNK-CA, or constitutively-active Akt (Myr-Akt). Eight hours postnucleofection, cells were harvested and processed for Western blot to detect the levels of Ets1, IKK2, phospho-Jun, phospho-Akt, and GAPDH. Shown are the results of one of three similar independent experiments. Also shown is a quantification of the three Western blots with statistical analysis. (D) Cells transfected as in (C) were used to prepare RNA and measure Ets1 and HPRT by RT-qPCR. Shown is the average value of three independent experiments. (E) M12 B cell lymphoma cells were infected with an empty virus or a virus carrying an IKK2-CA. Infected cells (GFP+) were sorted by FACS 48 h later. The sorted cells were returned to culture for another 24–72 h before being analyzed by Western blot for Ets1, GAPDH, and FLAG (the epitope tag on IKK2-CA). Shown are the results of one representative experiment of three replicates done (experiment shown was harvested at 72 h postsort). (F) Splenic B cells were isolated from wild-type (WT) or CD19-Cre × Rosa26 Stop-flox IKK2ca mice (labeled IKK2*) and then stimulated with indicated doses of anti-IgM for 3.5 h before being analyzed by Western blot. Shown are representative results from one of two independent experiments. *p < 0.05, **p < 0.01.

suggesting that A20 cells are a suitable model for studying pathways regulating Ets1. The IKK2-CA was capable of downregulating Ets1, whereas the kinase-inactive form failed to do so (Fig. 5C, 5D). Similarly, transfection of the constitutively active JNK plasmid (denoted JNK-CA) in A20 cells also resulted in downregulation of Ets1 protein (Fig. 5C), although not Ets1 mRNA (Fig. 5D). Transfection of constitutively-active Akt resulted in a modest downregulation of Ets1 (to about ~75% of the levels in vector-transfected cells), but this was not statistically-significant. We also attempted to generate stable cell lines using viruses containing IKK2-CA and JNK. We were unable to obtain long-term, stable expression of IKK2-CA in A20 B cells, but we did obtain such expression in another B cell line, M12 B lymphoma cells, using retroviral transduction (Fig. 5E). In these cells, stable expression of IKK2-CA leads to a dramatic downregulation of Ets1. Stable expression of JNK-CA was not obtained in either B cell line, likely because of its effects on cellular apoptosis.

We also examined primary B cells purified from mice expressing IKK2-CA under the control of a CD19-Cre–responsive locus (designated IKK2+ mice) (30). B cells from IKK2+ mice had normal levels of Ets1 in the resting state, but low doses of anti-IgM that failed to induce downregulation of Ets1 in wild-type B cells were able to do so in IKK2+ B cells (Fig. 5F). Taken together, the data suggest that both IKK2 and JNK are important downstream effectors of BCR and TLR signaling that modulate Ets1 levels but that Akt plays only a minor role.

Ets1 levels are maintained in B cells by phosphatases that inhibit BCR signaling

Lyn is crucially important for negative signaling pathways that limit B cell activation, whereas other Src family kinases can substitute for Lyn in positive signaling via the BCR (8, 9). We hypothesized that loss of negative signaling in Lyn-deficient B cells, which results in enhanced positive activation signals, triggers the downregulation of Ets1. When phosphorylated by Lyn, inhibitory receptors such as CD22, CD72, PIR-B, Siglec-G, FcγRIIb, and others recruit phosphatases (SHP1 or SHIP1) to reverse the positive phosphorylation events triggered by BCR signaling (4). Loss of SHP1, SHIP1 or the inhibitory receptors results in autoantibody secretion to varying degrees (4, 13, 18, 65, 66).

We first examined the role of SHP1 in regulating Ets1 because the majority of negative receptors phosphorylated by Lyn recruit SHP1 rather than SHIP1. We purified B cells from motheaten viable mice (me+ mice), which harbor a loss of function mutation in the Ptpn6 gene (encoding SHP1) (12). Ets1 levels were dramatically...
Reduced in B cells from mev mice compared with wild-type or heterozygous mice (Fig. 6A, 6B). Quantification indicated that mev B cells express ∼9 ± 4% as much Ets1 as do wild-type B cells (p < 0.0001). mev mice develop severe inflammatory disease within weeks of birth that results in a greatly altered immune cell environment that could influence B cell differentiation (67). We therefore examined Ets1 expression in B cells from mice with a B cell–specific deletion of SHPI (mb1-cre Ptpn6floxed mice, hereafter referred to as SHPI1−−), in which B cells develop in a more normal environment. Ets1 was also very much downregulated in these B cells to an average of 12 ± 3% the levels in wild-type B cells (p < 0.0001) (Fig. 6C, 6D). The downregulation of Ets1 in B cells lacking SHPI was stronger than is its downregulation in B cells lacking Lyn, indicating that SHPI1 may function via both Lyn-dependent and Lyn-independent mechanisms to control Ets1 levels.

We next examined B cells from mice with a B cell specific deletion of the SHPI1 phosphatase (mb1-cre Inpp5d floxed mice, hereafter referred to as SHPI1−−). Ets1 expression was lower in SHPI1-deficient B cells than in wild-type B cells, but the difference was not as striking as in B cells lacking SHPI1 (Fig. 6E, 6F). Indeed, SHPI1−− B cells express ∼61 ± 5% as much Ets1 as do wild-type B cells (p < 0.001). These observations suggest that SHPI1 is the major phosphatase that controls Ets1 expression but that SHPI1 may also contribute in a smaller way.

To test the role of cell surface receptors that are phosphorylated by Lyn and that recruit phosphatases, we purified B cells from several different mouse strains lacking individual inhibitory receptors. Ets1 levels were largely normal in B cells lacking FcγRIIb (92 ± 10% of wild-type levels), PIR-B (90 ± 8% of wild-type levels), or CD72 (98 ± 8% of wild-type levels) (Fig. 7A, 7B). In contrast, loss of CD22 or SiglecG resulted in lower expression of Ets1 (Fig. 7C), although the effect was modest when compared with loss of either Lyn or SHPI1 (levels in CD22−− or SiglecG−− B cells were 74 ± 8% of wild-type [p < 0.01] and levels in SiglecG−− B cells were 55 ± 4% of wild-type [p < 0.0001]). Because CD22 and SiglecG are functionally redundant in regulating B cell responses (18), we also examined Ets1 expression in B cells lacking both CD22 and SiglecG. Ets1 expression in these cells was less than in either CD22−− or SiglecG−− B cells (levels in CD22−−/− SiglecG−− B cells were 40 ± 3% of levels in wild-type B cells [p < 0.0001]) (Fig. 7C) but still not as low as the levels Lyn−− or SHPI1−− B cells. These results suggest that CD22 and SiglecG are both involved in control of Ets1 expression but that other Lyn- and SHPI1-dependent receptors likely also play a role.

**Restoration of Ets1 limits the differentiation of Lyn or SHPI1-deficient B cells into plasma cells in vitro**

B cells lacking Lyn or SHPI1 show a major defect in expression of Ets1, which is known to control plasma cell differentiation. However, Lyn and SHPI1 likely also control other pathways that influence B cell differentiation. To determine whether the downregulation of Ets1 in Lyn−− or SHPI1−− B cells was important for their increased propensity to differentiate to plasma cells, we restored Ets1 expression in these cells using a retroviral construct and measured their differentiation in response to LPS. Both Lyn−− and SHPI1−− B cells showed increased differentiation to plasmablasts as compared with wild-type B cells. Enforced expression of Ets1

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**FIGURE 6.** SHPI1 is the major phosphatase regulating Ets1 levels in B cells. (A) Western blot for Ets1 and GAPDH with B cell lysates from wild-type or moth-eaten viable mice with heterozygous (me+/me−) or homozygous (me−/me−) mutations in the SHPI1 phosphatase (shown are representative results from one of three separate experiments, n = 4 mice of each genotype in total analyzed). (B) RT-qPCR analysis of Ets1 mRNA in B cells from me−/me mice (n = 3 for each genotype). (C) Western blot for Ets1 and GAPDH with B cell lysates from wild-type, Ptpn6floxed (SHPI1 floxed), and Ptpn6floxed mb1-Cre (SHPI1 knockout) mice. Shown are the results of one of three independent experiments (with a total of six mice of each genotype analyzed). (D) RT-qPCR analysis of Ets1 mRNA in B cells from SHPI1−− and SHPI1−−/− mb1-Cre mice (n = 4 mice of each genotype). (E) Western blot for Ets1 and GAPDH with B cell lysates from wild-type mice, Inpp5d floxed (SHPI1 floxed), or mb1-Cre Inpp5d floxed (SHPI1 knockout) mice. Shown are the results of one of two independent experiments (with a total of four mice of each genotype analyzed). (F) RT-qPCR analysis of Ets1 mRNA in B cells from Inpp5d floxed or Inpp5d floxed mb1-Cre mice (n = 4 for each genotype). **p < 0.01, ***p < 0.001.
could limit plasmablast differentiation in wild-type as well as Lyn−/− and SHP1−/− B cells. This was evident both in flow cytometry (as measured by a B220lowCD138+ phenotype; Fig. 8A, 8C) as well as in ELISPOT assays to measure IgM- and IgG-secreting cells among retrovirally transduced cells (Fig. 8B, 8D). Although loss of Lyn or SHP1 presumably alters the expression of a large number of genes, our results suggest that maintenance of Ets1 is a key event under the control of a Lyn→

FIGURE 7. CD22 and SiglecG both contribute to maintaining Ets1 expression in B cells. (A) Western blot for Ets1 and GAPDH with B cell lysates from wild-type (WT), CD72−/−, or PIR-B−/− mice. Shown are results of one of two independent experiments with a total of four mice of each genotype being analyzed. (B) Western blot for Ets1 and GAPDH with B cell lysates from WT or FcγRIIb−/− mice. Shown are results of one of two independent experiments with a total of three mice of each genotype. (C) Western blot for Ets1 and GAPDH with B cell lysates from WT, CD22−/−, SiglecG−/−, or CD22−/− SiglecG−/− mice. Shown are the results of one of two independent experiments (n = 8 mice of each genotype). Below is a quantification of multiple Western blots. (D) RT-qPCR analysis of Ets1 mRNA in B cells from WT, CD22−/−, SiglecG−/−, or CD22−/− SiglecG−/− B cells (n = 5 for each genotype). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

FIGURE 8. Ets1 prevents excess differentiation of Lyn−/− or SHP1−/− B cells. Total splenocytes from Lyn-deficient (A and B) or B220+ splenic B cells from SHP1-deficient mice (C and D) were stimulated overnight with LPS to induce cell cycle progression. Cells were infected with an empty retrovirus (empty) or with a virus-containing mouse Ets1. (A and C) Flow cytometry was performed after 2 additional days in culture with LPS to determine the frequency of B220lowCD138high plasma cells (plasmablasts) among GFP+ virally infected cells. Shown is a representative flow plot as well as quantification of four (Lyn−/−) or five (SHP1−/−) separate experiments in the bar graphs. (B and D) GFP+ cells were also sorted out and replated in LPS-containing medium for an additional day on ELISPOT plates for enumeration of IgM- and IgG-secreting plasma cells. Three independent experiments were performed with similar results. **p < 0.01, ***p < 0.001.
CD22/SiglecG → SHP1 pathway that is crucial for limiting B cell differentiation in response to TLR stimulation.

Discussion

B cells can be triggered to differentiate into plasma cells that secrete Abs, a process relevant to both normal immune responses and autoimmune diseases. BCR signaling pathways can promote B cell differentiation to Ab-secreting plasma cells (68, 69) and are normally counterbalanced by inhibitory signaling pathways such that B cell differentiation is limited (4). The crucial targets whose expression is regulated by the BCR pathway and inhibitory signaling pathways have remained ill-defined. In this report, we have determined that the transcription factor Ets1 and the tyrosine kinase Lyn both play B cell–intrinsic roles in regulating plasma cell formation. Consistent with these findings, B cell-specific deletion of Lyn results in increased plasma cells and autoantibody secretion (70). We have further demonstrated that Ets1 is downregulated by the BCR signaling cascade and that its expression is maintained in naïve B cells by Lyn-dependent inhibitory signaling pathways. We also found that PMA or ionomycin, which mimic some aspects of BCR signaling, or TLR ligands (LPS or CpG DNA) can also downregulate Ets1 expression in B cells. Interestingly, low levels of anti-IgM stimulation cooperated with low levels of CpG stimulation to downregulate Ets1. This may be relevant to autoimmune disease where combined BCR/TLR ligation can drive autoantibody production (71). In contrast to BCR and TLR signaling, signaling via CD40 or a number of different cytokine receptors that activate Stat1, Stat3, or Stat6 failed to alter Ets1 levels in B cells.

Ets1 inhibits B cell differentiation to Ab-secreting plasma cells by both stimulating the expression of Pax5 and by blocking Blimp1 DNA binding (41, 42). Thus, downregulation of Ets1 can explain the ability of the BCR and TLRs to promote B cell differentiation. It is likely that the inhibitory Lyn → CD22/SiglecG → SHP1 pathway that normally counterbalances B cell activation regulates a number of different transcriptional programs to control B cell differentiation. However, the key role of Ets1 as an important downstream target of this pathway is demonstrated by the ability of virally expressed Ets1 to suppress the excessive plasma cell formation characteristic of both Lyn-/- and SHP1-/- B cells in response to LPS stimulation.

Ets1-/- B cells expressed normal levels of Lyn and showed normal phosphorylation of Lyn in basal and stimulated states. However, both Ets1 protein and mRNA levels were downregulated in B cells from Lyn-deficient mice. Ets1 levels are low in B cells from both young and aged Lyn-/- mice, indicating that development of frank autoimmune disease or of an inflammatory environment is not necessary for the down-modulation of Ets1.

Analysis of Ets1 protein levels in Lyn-/- B cells showed that they were dramatically downregulated (~25–30% of the level in wild-type B cells). The level of Ets1 mRNA was also reduced in Lyn-/- B cells but not by as much (~50% of the level of wild-type B cells). Yet, the half-lives of Ets1 protein and mRNA were not significantly altered in Lyn-/- B cells. The most likely explanation for these observations is that Lyn promotes both the transcription of the Ets1 gene and also the translation of Ets1 mRNA into protein. Lyn may control translation of Ets1 mRNA by altering levels of microRNAs that bind to long and well-conserved 3′-untranslated region of the major Ets1 mRNA species (72).

Lyn is a crucial mediator of inhibitory signaling pathways in B cells but is redundant with other Src family kinases in mediating positive BCR signaling (6, 8, 9). Therefore, Lyn-deficient B cells have alterations in BCR signaling with some downstream proteins showing impaired phosphorylation, but with an overall increased Ca2+ flux and MAPK activation (6, 8, 9). Our data suggest that the downregulation of Ets1 in Lyn-/- B cells is due to enhanced BCR signaling in these cells. This is supported by the fact that crossing Lyn-/- mice to mice with low levels of Btk (Lyn-/- Btk+/+) largely restores Ets1 expression. In addition, fixing the specificity of the BCR on Lyn-deficient B cells to a foreign Ag (hen egg lysozyme) also partially restores Ets1 expression, although it does not reach the levels of Lyn+/+ B cells. Two different models can explain the fact that fixing the BCR fails to completely restore Ets1 expression in Lyn-/- B cells. First, it is possible that tonic low level and Ag-independent signaling via the BCR also controls Ets1 expression.

Second, Ets1 may be downregulated in Lyn-/- mice not only because of enhanced BCR signaling, but also because of enhanced signaling via other pathways. TLR-mediated signaling pathways can also be negatively regulated by Lyn (73) and are potential contributors to the downregulation of Ets1 in the second model.

Downregulation of Ets1 in B cells is dependent on the BCR signaling components PI3K, Btk, IKK2, and JNK. PI3K generates PIP3 that recruits Btk to the membrane. Btk then triggers a pathway that eventually activates the IKK complex and JNK. Another effector of PI3K signaling, Akt, does not appear to contribute. This is shown by (1) the fact that Akt activation is normal in both Btk-deficient B cells and in wild-type B cells treated with a Btk-specific inhibitor, yet Ets1 downregulation is blocked (2), that Pten-/- B cells, which have hyperactive Akt, do not show enhanced downregulation of Ets1 upon BCR cross-linking and (3) that transfection of constitutively active Akt into A20 B lymphoma cells results in only a very modest downregulation of Ets1. Downstream of Btk, both JNK and IKK2 appear to regulate Ets1 levels. Studies with an inhibitor that blocks IKK2 activity showed that IKK2 signaling is essential in downregulating Ets1 in response to BCR ligation. However, activation of IKK2 alone (as found in IKK2+/- mice) was not sufficient to downregulate Ets1 in primary B cells but rather cooperated with low levels of BCR ligation to inhibit Ets1 expression. In contrast, activation of IKK2 alone was sufficient to downregulate Ets1 in A20 and M12 B lymphoma cells. One or more signaling pathways that cooperate with IKK2 activation to downregulate Ets1 may have become activated in the B lymphoma cells during their oncogenic conversion. Similarly, JNKs also appear to be involved in regulation of Ets1 expression.

Two separate inhibitors of JNKs that function by different mechanisms blocked the downregulation of Ets1 in stimulated primary B cells. Furthermore, expression of a constitutively active form of JNK1 downregulated Ets1 in A20 B lymphoma cells, although a caveat to this result is that the A20 cells may have activated other pathways that cooperate with JNK to downregulate Ets1 as we determined for IKK2. The targets of the IKK2 and JNK pathways that regulate Ets1 expression remain to be defined.

A series of inhibitory signaling pathways counterbalance the BCR signaling pathway to limit B cell activation. Lyn-mediated phosphorylation of inhibitory membrane receptors results in the recruitment of phosphatases to these receptors, either SHIP1 to CD22, CD72, Siglec-G, PIR-B, and FcyRIIb or SHIP1 to FcyRIIb. SHIP1 suppresses BCR signaling by dephosphorylating key substrates such as Iga, Igβ, Syk, and Lyn (74, 75), whereas SHIP1 functions to convert PIP3 to PI(3,4)P2 thereby suppressing PI3K-dependent signaling pathways. The absence of SHIP1 or SHIP1 leads to autoimmune symptoms, presumably due in part to increased BCR signaling (12, 13, 17, 65). Ets1 is dramatically downregulated in B cells lacking SHIP1, but only modestly downregulated in B cells lacking SHIP1. Restoration of Ets1 to SHIP1-deficient B cells by retroviral transduction reversed their enhanced differentiation into plasma cells in response to LPS.
stimulation. This indicates the SHP1 is the primary Lyn-dependent phosphatase controlling Ets1 levels in B cells.

Mice lacking any one of the inhibitory receptors showed relatively weak (CD22 and Siglec-G single knockouts) or no (FcyRIIB, CD72, and PIR-B single knockouts) downregulation of Ets1. The strongest downregulation of Ets1 was obtained in mice doubly deficient in both CD22 and Siglec-G, although the level of Ets1 in B cells from these mice is still higher than that found in B cells lacking Lyn or SHP1. This suggests that multiple inhibitory receptors on the B cell surface, including CD22 and Siglec-G, cooperate to maintain Ets1 expression under physiological conditions.

Disease-associated SNPs in the LYN and ETS1 gene loci have been identified in human autoimmune patients (76–80). Furthermore, both Lyn and Ets1 levels have been shown to be down-regulated in PBMCs and B cells of autoimmune disease patients (10, 11, 81–83). These studies support the involvement of LYN and ETS1 in human autoimmune disease processes. We propose that in human B cells, like in mouse B cells, Lyn is an upstream regulator of Ets1 expression and that mutations in either gene can promote susceptibility to autoimmune disease. Thus, the Lyn-Ets1 axis could be fruitful target for new therapeutics for autoimmune diseases.

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Disclosures
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Supplemental Figure 1: Lyn expression and phosphorylation is not changed in Ets1-deficient B cells. (A) Western blot for Lyn, phospho-Lyn and GAPDH with B cell lysates from wild-type (WT) and Ets1 deficient mice. Each Western blot was done either 2 (Y397 Lyn) or 3 (total Lyn and Y508 Lyn) times with separate sets of wild-type and Ets1-/- mice. (B) RT-qPCR analysis of Lyn mRNA in B cells from wild-type and Ets1-/- mice (n=2 for each genotype). (C) Time-course of anti-IgM stimulation of wild-type (WT) or Ets1-deficient B cells followed by Western blotting for phospho-Y397 of Lyn. One of two similar experiments is shown. (D) Splenic B cells from wildtype (WT) or Ets1-deficient mice were immunoprecipitated with anti-Lyn antibody or control IgG, then Western blotted with an antibody against phosphotyrosine (4G10). One of two similar experiments is shown.