Genetic Interaction between Lyn, Ets1, and Btk in the Control of Antibody Levels

Jessica Mayeux, Brian Skaug, Wei Luo, Lisa M. Russell, Shinu John, Prontip Saelee, Hansaa Abbasi, Quan-Zhen Li, Lee Ann Garrett-Sinha and Anne B. Satterthwaite

*J Immunol* published online 24 July 2015
http://www.jimmunol.org/content/early/2015/07/23/jimmunol.1500165

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/07/23/jimmunol.1500165.DCSupplemental

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Genetic Interaction between Lyn, Ets1, and Btk in the Control of Antibody Levels

Jessica Mayeux,* Brian Skaug,* Wei Luo,† Lisa M. Russell,† Shinu John,† Prontip Saelee,‡ Hansaa Abbasi,* Quan-Zhen Li,‡ Lee Ann Garrett-Sinha,†,1 and Anne B. Satterthwaite§,‡,1

Tight control of B cell terminal differentiation into plasma cells (PCs) is critical for proper immune responses and the prevention of autoimmunity. The Ets1 transcription factor acts in B cells to prevent PC differentiation. Ets1−/− mice accumulate PCs and produce autoantibodies. Ets1 expression is downregulated upon B cell activation through the BCR and TLRs and is maintained by the inhibitory signaling pathway mediated by Lyn, CD22 and Siglec-G, and SHP-1. In the absence of these inhibitory components, Ets1 levels are reduced in B cells in a Btk-dependent manner. This leads to increased PCs, autoantibodies, and an autoimmune phenotype similar to that of Ets1−/− mice. Defects in inhibitory signaling molecules, including Lyn and Ets1, are associated with human lupus, although the effects are more subtle than the complete deficiency that occurs in knockout mice. In this study, we explore the effect of partial disruption of the Lyn/Ets1 pathway on B cell tolerance and find that Lyn−/−Ets1−/− mice demonstrate greater and earlier production of IgM, but not IgG, autoantibodies compared with Lyn+/− or Ets1+/− mice. We also show that Btk-dependent downregulation of Ets1 is important for normal PC homeostasis when inhibitory signaling is intact. Ets1 deficiency restores the decrease in steady state PCs and Ab levels observed in Btk−/− mice. Thus, depending on the balance of activating and inhibitory signals to Ets1, there is a continuum of effects on autoantibody production and PC maintenance. This ranges from full-blown autoimmunity with complete loss of Ets1-maintaining signals to reduced PC and Ab levels with impaired Ets1 downregulation. The Journal of Immunology, 2015, 195: 000–000.
demonstrate a dramatic reduction in Ets1 levels (5). This result requires Btk and, in part, self-antigen, suggesting that the inappropriate Ets1 downregulation is due to an exaggeration of B cell activating signals because of the loss of the Lyn-dependent inhibitory signaling loop (5). Consistent with this model, BCR and TLR signals downregulate Ets1 in Lyn−/− B cells in a manner dependent on Btk (5). Thus, activating signals in B cells downregulate Ets1, whereas inhibitory signals maintain Ets1 expression.

The consequences of disrupting the balance of signals controlling Ets1 are clear in the case of complete deficiency of inhibitory pathway components Lyn, CD22 plus SiglecG, SHP-1, or Ets1 itself. The requirement for the activating pathway component Btk in the downregulation of Ets1 and the accumulation of autoreactive PCs in the absence of inhibitory signaling has also been described (5, 23). However, the physiological situation is likely more complex, both normally and in the case of autoimmune disease. In SLE and other human autoimmune diseases, expression of inhibitory signaling components is more likely to be reduced than completely eliminated (8–18, 28–32). In this context, transmission of signals via a particular pathway may be further impaired by a combination of polymorphisms, each of which confers partial loss of function to a different pathway component. This has been modeled in the mouse by compound heterozygotes of Lyn and SHP-1, which develop much more pronounced autoimmunity than do Lyn−/− or SHP-1−/− mice (33). In this study, we ask whether a similar genetic interaction can be observed between Lyn and Ets1. We also explore whether Btk-dependent downregulation of Ets1 is important for PC homeostasis under normal circumstances, when inhibitory signaling is intact. Combined with our previous work, the current results indicate that depending on the strength of the signal driving the downregulation or maintenance of Ets1, there is a continuum of effects on autoantibody production and PC maintenance. As shown previously, complete deficiency of Ets1 itself, or Ets1-maintaining signals, leads to full-blown autoimmunity. In this study, we show that partial disruption of this pathway favors a breach in checkpoint one and the production of IgM autoantibodies. In contrast, the inability to downregulate Ets1 via Btk under non-autoimmune circumstances leads to a reduction in PC frequencies and Ig levels.

Materials and Methods

Mice

Lyn−/− (20), Lyn−/−Btk−/− (34), Lyn−/−IL-6−/− (23), TCRβ−/− (35), and Btk−/− (36) mice used in this report have been described previously. The Ets1-deficient mice used in these studies were originally described by Muthusamy et al. (37) and further characterized by us (4). Lyn−/−Ets1−/− mice were generated by crossing Lyn−/− and Ets1−/− mice. Btk−/−Ets1−/− and Ets1−/− mice were generated by crossing Btk−/− or TCRβ−/− mice with Ets1−/− mice and then intercrossing the progeny. All mice were backcrossed multiple generations onto the C57BL/6 (B6) background except for the Btk−/−, Btk−/−Ets1−/−, and littermate wild type controls, which were on a mixed B6 × BALBc background, and the Ets1−/−, TCRβ−/−, Ets1−/−TCRβ−/−, and wild type littermate controls, which were on a mixed B6 × 129 background. Mixed backgrounds were used in experiments with Ets1−/− mice because Ets1 deficiency causes perinatal lethality on the C57BL/6 background (38). Lyn−/−Ets1−/− mice and controls were analyzed in two age groups (4–7 and 10–15 mo old). Btk−/−Ets1−/− mice and controls were analyzed at 2–3 mo old, and Ets1−/−TCRβ−/− mice at 4–6 mo. In some experiments, mice were prebled, immunized i.p. with 10 μg 2,4,6-trinitrophenyl (TNP)-Ficoll (Biosearch Technologies) in 100 μl PBS, and bled 8 d after immunization. Mice were housed in a specific pathogen-free barrier facility. Animal procedures were approved by the University of Texas Southwestern Medical Center and the University at Buffalo Institutional Animal Care and Use Committees.

Western blot

Splenic B cells were purified using anti-B220 beads (Miltenyi Biotec) and lysed by boiling in 2X Laemmli sample buffer. The samples were subjected to Western blotting, and membranes were incubated with rabbit monoclonal anti-mouse Ets1 (clone EPR546; Epitomics), mouse monoclonal anti-Lyn (clone LYN-01; BioLegend) or mouse monoclonal anti-GAPDH (clone 6C5; Millipore) Abs. The signals on the membranes were quantitated with ImageJ software. Loading was normalized by GAPDH levels.

Quantitative PCR

Splenic B cells were purified using anti-B220 beads (Miltenyi Biotec) and lysed in Trizol (Life Technologies). Preparation of total RNA, synthesis of cDNA, and analysis of Ets1 and Lyn expression by quantitative PCR was performed as described previously (5), except that Ets1 and Lyn expression were normalized to actin.

Flow cytometry

RBC-depleted splenocyte suspensions or peritoneal wash cells were Fc-blocked with purified rat IgG2b anti-mouse CD16/CD32 (2.4G2 Ab) for 5 min at 4˚C prior to primary staining with mAbs. The following FITC, PE, PerCP, allophycocyanin, or biotin-conjugated Abs were used in various combinations to stain for distinct populations as described in the figure legends: B220, CD19, IgM, IgD, CDS, CD11c, CD11b, CD4, CD69, CD44, and CD62L. Incubations for both primary and secondary antibodies (streptavidin allophycocyanin in the case of biotin-labeled Abs) were 15 min at 4˚C. All samples were acquired on a FACSCalibur cytometer (BD Biosciences) and analyzed using CellQuest (BD Biosciences) and FlowJo (Tree Star) software. All Abs were from BD Biosciences except for B220 PerCP (Tonbo).

ELISA

For detection of total Ig, flexible 96-well PVC plates (BD Biosciences) were coated with 2 μg/ml goat anti-mouse Ig (Southern Biotech) and blocked with 1% BSA in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM, IgG, and IgA; Sigma-Aldrich) were diluted serially, added to wells in triplicate, and incubated for 1 h at room temperature. For detection of anti-TNP Ig, flexible 96-well PVC plates (BD Biosciences) were coated with 25 μg/ml TNP-BSA. Serum was diluted serially, added to wells in triplicate, and incubated for 2 h at room temperature. For detection of anti-dsDNA autoantibodies, Immulon II plates (Nalge Nunc) were precoated with 0.1 mg/ml methylated BSA and subsequently coated with 50 μg/ml calf thymus dsDNA (Sigma-Aldrich). For detection of anti-ssDNA Abs, plates were coated as above except that the calf thymus DNA was boiled and quenched on ice immediately prior to coating. After plates were incubated overnight at 4˚C with blocking buffer (PBS, 3% BSA, 0.1% gelatin, 3 mM EDTA), serial dilutions of serum were added in duplicate and incubated for 2 h at room temperature.

In all the above cases, bound Ig was detected by an alkaline phosphatase-conjugated secondary Ab, goat anti-mouse IgM, IgG, and IgA (Southern Biotech for IgM and IgG, Mabtech for IgA), and developed with an alkaline phosphatase substrate kit (Bio-Rad Laboratories). The OD was 405 nm on an absorbance microplate reader (Bio-Tek Instruments).

Autoantigen array

Serum from 4–7-mo-old mice was analyzed with the University of Texas Southwestern Medical Center Microarray Core. Autoantibodies were detected using an autoantigen proteomic array that has been described previously (39). The array includes 95 autoantigens and 4 control proteins (https://microarray.swmed.edu/products/product/autoantigen-microarray-panel-i/); 1 μl of each sample was diluted 1:100 and added to the arrays in duplicate. Detection was with Cy3-labeled anti-mouse IgM and Cy5-labeled anti-mouse IgG (Jackson ImmunoResearch). A Genepix 4000B scanner (Molecular Devices) with laser wavelengths of 532 nm (for Cy3) and 635 nm (for Cy5) was used to generate images for analysis. Images were analyzed using Genepix Pro 6.0 software to generate a GPR file (Molecular Devices). Net fluorescence intensities were normalized using anti-mouse Ig or IgG spotted onto each array. Values obtained from duplicate spots were averaged.

The hierarchical clustering analysis of autoantibodies was performed using Cluster (currently available at http://bonsai.hgc.jp/~mdehoon/software/cluster/) and Treeview (currently available at http://sourceforge.net/projects/jtreeview/files/jtreeview/) software (originally obtained from http://rana.lbl.gov/EisenSoftware.htm).

ELISPOT

For ELISPOT analysis, Millipore MultiScreen 96-well plates with Immobilon-P membranes were coated with 5 μg/ml of a polyclonal goat anti-mouse Ig (Southern Biotech). Splenocytes were depleted of RBCs, plated in serial dilutions at 10,000, 25,000, 50,000, and 100,000 cells/well and incubated overnight at 37˚C in a tissue culture incubator. IgM Ab-secreting cells loaded was normalized by GAPDH levels.
(ASCs) were detected using a biotin-conjugated rat anti-mouse IgM (KPL Fisher) detection Ab, and IgG ASCs were detected by using a biotin-conjugated polyclonal anti-mouse IgG Ab (KPL Fisher). ELISPOT plates were counted with an automated reader (Zellnet Consulting).

**Kidney function assays**

Serum creatinine was measured using a serum creatinine colorimetric assay kit (Cayman, Ann Arbor, MI) per the manufacturer’s protocol. Serum was diluted 1:10. Protein concentration in the urine was measured using a Coomassie Protein Assay Kit (Pierce).

**B cell activation**

Splenocytes were depleted of RBCs, labeled with CFSE (Molecular Probes), and stimulated with media alone, 5 μg/ml LPS (Sigma), or 10 μg/ml anti-IgM F(ab)’2 (Jackson ImmunoResearch) fragments for 72 h. Cells were stained with Abs against B220 (BD Biosciences) and analyzed by flow cytometry for CFSE dilution and forward scatter versus side scatter.

**Results**

We previously showed that Lyn acts to maintain Ets1 expression in B cells (5). In the absence of Lyn, Ets1 levels are reduced in B cells (5) and autoreactive PCs accumulate (23) as also occurs in Ets1−/− mice (4). To define a functional, genetic interaction between Lyn and Ets1 in vivo, and to determine which of the two checkpoints controlling autoantibody production (23) are mediated by the Lyn/Ets1 pathway, we asked whether compound heterozygosity of both Lyn and Ets1 would synergize for the production of IgM and IgG autoantibodies. This approach is commonly used to define functional interactions in vivo between signaling pathway components. It may also be more reflective of the situation in SLE patients in whom combinations of subtle polymorphisms in multiple genes, rather than complete deficiency of any one, leads to disease susceptibility. We first confirmed that heterozygosity of Lyn results in reduced Lyn expression, and that heterozygosity of Ets1 results in reduced Ets1 expression in B cells (Supplemental Fig. 1). Next, we assessed autoantibody levels. Lyn+/− Ets1+/− mice produced significantly higher levels of anti-ssDNA and anti-dsDNA IgM at 4–7 mo of age than either Lyn+/− or Ets1+/− mice did as measured with ELISA (Fig. 1A, 1B). Using an autoantigen array, we found that this was reflective of widespread IgM autoreactivity against multiple self-antigens (Supplemental Fig. 2) as we previously described for Lyn−/−.
mice (23). Such synergy was not observed for IgG autoantibodies, however, as these appeared similarly late in life (10–15 mo of age), and to a similar magnitude, in Lyn−/− and Lyn−/−Ets1−/− mice (Fig. 1C, 1D). Because IgA autoantibodies have been shown to be pathogenic in the absence of IgG anti-DNA in Lyn−/− mice (40), we asked whether they might be present in aged Lyn−/−Ets1−/− mice. IgA autoantibodies were not detected (not shown). Thus, partial loss of both Lyn and Ets1 promotes a breach of checkpoint one and production of IgM autoantibodies, but is insufficient to cause increased IgG autoantibodies (checkpoint two) relative to heterozygosity of Lyn alone.

Because IgG autoantibodies are T cell dependent in Lyn−/− mice (40, 41), and because CD4+ T cells from both Ets1−/− and Lyn−/− mice are skewed toward an activated, memory/effector phenotype (7, 40, 42–44), we asked whether Lyn−/−Ets1−/− mice demonstrated enhanced T cell activation. Double heterozygous mice, but not Lyn−/− or Ets1−/− animals, had increased CD4+ T cell activation as measured by CD69 expression relative to wild type mice as early as 4–7 mo of age. Their CD4+ T cells were also skewed away from a naive and toward an effector/memory phenotype (Fig. 2A, 2B; Supplemental Fig. 3A, 3B). Although Lyn−/−Ets1−/− mice did not demonstrate elevated IgG autoantibodies, we considered the possibility that there was a T cell–dependent component to the breach in checkpoint one that leads to the increase in IgM autoantibodies. We focused on the role of Ets1 in this process, because heightened T cell activation is not required for the accumulation of PCs and IgM autoantibodies in Lyn−/− mice (23, 40, 41, 43, 45, 46). To test whether the production of PCs and IgM or IgG autoantibodies in Ets1-deficient mice was dependent in part or wholly on conventional CD4+ T cells, we examined Ets1−/−TCRβ−/− mice. Both the increase in IgM PCs and IgM autoantibodies observed in Ets1−/− mice were unaffected by TCR-β deficiency, whereas IgG PCs and IgG autoantibodies were reduced in the absence of TCR-β (Fig. 3). This finding suggests that the increased T cell activation caused by the loss of Lyn, Ets1, or both does not drive the breach in checkpoint one.

Splenomegaly was also accelerated in the compound heterozygous mice, occurring earlier than in Lyn−/− mice (Fig. 2C, Supplemental Fig. 3D). Additional synergies became apparent in older mice (~1 y of age), when the Lyn−/−Ets1−/− mice demonstrated a significantly increased frequency of age-associated B cells (ABCs; CD11c+CD11b+CD19+) and myeloid dendritic cells (CD11c+CD11b+CD19−; Fig. 2D, 2E; Supplemental Fig. 3C). The former are known to be a source of autoantibodies and accumulate in autoimmune mice, perhaps because of long-term Ag exposure (47–49). The increased frequency of ABCs in Lyn−/−Ets1−/− mice was observed even in male mice, which do not normally accumulate these cells with age. Despite these increased signs of generalized immune activation often associated with autoimmuneimmunity, neither weight loss nor elevated proteinuria were observed in aged Lyn−/−Ets1−/− mice (Supplemental Fig. 3E, 3F), and a similar and low frequency of single and compound heterozygous mice demonstrated increased serum creatinine (Fig. 2F). This result was perhaps due to the lack of synergy between...
Lyn and Ets1 heterozygosity for the production of class-switched autoantibodies. However, among the few mice that did have elevated serum creatinine levels, there was a trend toward higher creatinine levels in the Lyn +/− Ets1+/− mice compared with the single heterozygotes. Taken together, these results suggest that Lyn and Ets1 work together to limit the activation of multiple cell types in the immune system, including B and T cells. In terms of B cell tolerance, maintenance of checkpoint one, which prevents the widespread differentiation of autoreactive IgM+ B cells, is particularly sensitive to disruption of the Lyn/Ets1 inhibitory pathway. Another important component of the pathway controlling checkpoint one is Btk, a critical BCR signaling component (24, 25) and target of Lyn-dependent inhibitory pathways (26, 27) that is required for Ets1 downregulation in Lyn−/− mice (5). Excessive Btk activation mediates the breach of both checkpoint one and the production of IgM autoantibodies (23, 50) via a B cell intrinsic effect (51). This suggests that Btk also acts at checkpoint one to control Ets1 levels; however, Btk has additional important functions in the autoimmune disease of Lyn−/− mice. Btk-dependent production of IL-6 by Lyn−/− myeloid cells promotes class switching of autoreactive B cells and thus drives the breach of checkpoint two (23). We asked which of these functions of Btk drives Ets1 downregulation in vivo in Lyn−/− mice. We compared Ets1 expression in B cells from Lyn−/− Btk+/− mice (in which Btk levels are reduced and its roles in both checkpoints are affected) and Lyn−/− IL-6−/− mice (which maintain Btk-dependent breach of checkpoint one, but lack IL-6 and thus class-switched Abs) (23). Ets1 levels remained low in Lyn−/− IL-6−/− B cells (Fig. 4). Thus, Btk downregulates Ets1 expression in Lyn−/− B cells independently of its role in promoting IL-6 expression.

We next asked whether Btk-dependent Ets1 downregulation is important for controlling normal PC numbers in vivo, or whether Btk plays such a role only in situations in which its activity is enhanced, such as in the absence of Lyn-dependent inhibitory signaling. Such a contribution for Btk is supported by our previous in vitro studies showing that Btk inhibitors, Btk deficiency, or reduced Btk dosage all prevent BCR-induced downregulation of Ets1 in B cells in which inhibitory signaling is intact (5).
finding suggested that the reduction in ASCs and total Ig levels observed in Btk<sup>−/−</sup> mice (36) might result from failure to downregulate Ets1 in vivo. To address this issue, we generated Btk<sup>−/−</sup>Ets1<sup>−/−</sup> mice and found that their IgM ASCs and serum IgM were normalized, and IgG ASCs and serum IgG were elevated (Fig. 5A–D). In contrast, response to the T-independent type II Ag TNP-Ficoll, which is impaired in Btk<sup>−/−</sup> mice (36), was not restored by Ets1 deficiency (Fig. 5E). However, baseline levels of anti-TNP Abs, which were reduced in the absence of Btk, were normalized in Btk<sup>−/−</sup>Ets1<sup>−/−</sup> mice (Fig. 5E). Thus, Ets1 deficiency normalizes basal Ig levels, but not the response to specific Ag, in Btk<sup>−/−</sup> mice.

Btk<sup>−/−</sup> mice have a number of defects that have been suggested to contribute to their low steady state Ig levels. These defects include a block in B cell development such that few mature IgM<sup>+</sup>IgD<sup>−</sup> B cells are present in the spleen, a reduction in CD5<sup>+</sup> B1a cells in the peritoneal cavity, failure of B cells to proliferate or survive upon BCR crosslinking, and reduced (but not completely impaired) response of B cells to LPS (36). Interestingly, none of these defects was rescued by Ets1 deficiency (Fig. 6A–C, Supplemental Fig. 4). This suggests that Btk-dependent downregulation of Ets1 is specifically important for PC differentiation and survival, but other signaling functions of Btk are required for developmental events and activation induced proliferation.

**Discussion**

In this study, we have defined in vivo genetic interactions between Ets1 and two of its important regulators: Lyn, which maintains Ets1 expression to limit the differentiation of autoreactive PCs, and Btk, a BCR signaling component which downregulates Ets1 expression. The results presented in this study, taken together with our previously published work (4, 5, 23), suggest a model in which a tight balance between inhibitory signals that maintain Ets1 expression in B cells and activating signals that downregulate it controls PC homeostasis (Fig. 6D). Disruption of this balance in either direction can have pathologic consequences. For example, in Lyn<sup>−/−</sup> mice, inhibitory signals that maintain Ets1 expression are non-functional, and excessive activating signals through Btk downregulate Ets1 inappropriately (5). This downregulation results in a dramatic accumulation of PCs and the production of IgM and IgG autoantibodies (23), as is also seen in Ets1<sup>−/−</sup> mice (4). When the ability of Lyn and Ets1 to inhibit B cell differentiation is partially impaired, as occurs in Lyn<sup>−/−</sup>Ets1<sup>−/−</sup> mice, checkpoint one is breached and large amounts of IgM autoantibodies are produced, but sufficient inhibitory signaling remains to limit the class switching of autoreactive B cells and the production of pathogenic autoantibodies. Conversely, when Ets1 cannot be downregulated by activating signals as in Btk<sup>−/−</sup> mice, PC numbers are inappropriately low. This suggests that therapeutic approaches that modulate Ets1 levels or target pathways that control Ets1 levels could be efficacious for Ab-mediated autoimmune diseases and for situations in which Ab production is impaired.

Ets1 expression is maintained in B cells by an inhibitory pathway involving both Lyn and SHP-1 (5). We observed a significant genetic interaction between Lyn and Ets1 in B cells, resulting in a greater and more rapid production of IgM autoantibodies in Lyn<sup>−/−</sup>Ets1<sup>−/−</sup> mice than in Lyn<sup>−/−</sup> or Ets1<sup>−/−</sup> mice. However, this effect was less dramatic than the interaction previously shown by Tsantikos et al. (33) between Lyn and SHP-1. Lyn<sup>−/−</sup>SHP-1<sup>−/−</sup> mice have a number of defects that have been suggested to contribute to their low steady state Ig levels. These defects include a block in B cell development such that few mature IgM<sup>+</sup>IgD<sup>−</sup> B cells are present in the spleen, a reduction in CD5<sup>+</sup> B1a cells in the peritoneal cavity, failure of B cells to proliferate or survive upon BCR crosslinking, and reduced (but not completely impaired) response of B cells to LPS (36). Interestingly, none of these defects was rescued by Ets1 deficiency (Fig. 6A–C, Supplemental Fig. 4). This suggests that Btk-dependent downregulation of Ets1 is specifically important for PC differentiation and survival, but other signaling functions of Btk are required for developmental events and activation induced proliferation.

**FIGURE 5.** Ets1-deficiency increases basal Ig levels in Btk<sup>−/−</sup> mice. (A and B) ELISPOT analysis of IgM (A) and IgG (B) secreting cells per 25,000 splenocytes from wt, Btk<sup>−/−</sup>, and Btk<sup>−/−</sup>Ets1<sup>−/−</sup> mice. Data are shown as mean ± SEM (n = 4–6). **p < 0.01, Student t test. (C and D) ELISA analysis of total IgM (C) and IgG (D) in the indicated dilution of serum from wt, Btk<sup>−/−</sup>, and Btk<sup>−/−</sup>Ets1<sup>−/−</sup> mice. Data are shown as mean ± SEM (n = 5–6). *p < 0.05, **p < 0.01, ***p < 0.001, Student t test. (E) wt, Btk<sup>−/−</sup>, and Btk<sup>−/−</sup>Ets1<sup>−/−</sup> mice were prebled (pre), immunized with 10 μg/ml TNP-Ficoll in PBS, and bled 8 d later (post). Anti-TNP IgM was measured with ELISA using a 1:100 dilution of serum. Data represent mean ± SD (n = 2–3). **p < 0.01, Student t test.
FIGURE 6. Ets1 deficiency does not rescue B cell development or proliferation in Btk−/− mice. (A) Splenocytes from wt, Btk−/−, and Btk−/−Ets1−/− mice were stained with Abs against B220, IgM, and IgD. The frequency of IgMloIgDhi mature B cells is indicated. Each symbol represents a mouse; the bar is the mean. *p < 0.05, ***p < 0.001, Student t test. (B) Peritoneal wash cells from wt, Btk−/−, and Btk−/−Ets1−/− mice were stained with Abs against B220 and CD5. The frequency of B220+CD5+ B-1 cells is shown among lymphocytes and total cells (all). Each symbol represents a mouse; the bar is the mean. *p < 0.05, ***p < 0.01, ****p < 0.001, Student t test. (C) Splenocytes from mice of the indicated genotypes were labeled with CFSE, stimulated with media alone (black), 5 μg/ml LPS (blue), or 10 μg/ml anti-IgM F(ab)2 fragments (pink) for 72 h, and stained with Abs against B220. CFSE levels in B220+ gated cells are shown. Data are representative of two independent experiments. (D) Model for the role of Btk-mediated activating signals and Lyn-dependent inhibitory signals in the control of PC numbers via Ets1.

Consistent with breach of checkpoint one being more sensitive to loss of the Lyn/Ets1 inhibitory pathway than checkpoint two is, modulation of Ets1 expression is not controlled by checkpoint two events. We previously showed that Ets1 levels are low in B cells from Lyn−/− mice as young as 1 month of age, prior to the production of IgG autoantibodies or the development of proinflammatory feedback loops (5). In this study, we show that Ets1 levels remain low in Lyn−/− IL-6−/− mice, which do not develop autoreactive IgG, T cell hyperactivation, or kidney damage (23, 45, 54). Consistent with this observation, inflammatory factors (e.g., BAFF and IL-6) that increase with age and drive class switching of autoreactive B cells in Lyn−/− mice (23, 43, 45) do not cause Ets1 downregulation in wild type cells in vitro, whereas stimuli that induce B cell activation and differentiation, such as BCR and TLRs, do (5). Taken together, these observations indicate that decreases in Ets1 levels occur prior to, and are not a consequence of, the development of autoimmune disease and are likely an important event in the initiation of the loss of B cell tolerance.

In the absence of Lyn-dependent inhibitory signals, the excessive downregulation of Ets1 in B cells and the accumulation of autoreactive PCs are driven by Btk (5, 23). In this study, we show that Btk-dependent downregulation of Ets1 is also important for normal steady state numbers of PCs. Btk−/− mice, whose B cells maintain high levels of Ets1 after activation (5), have a significant decrease in IgM ASCs and serum IgM that is normalized by Ets1 deficiency. This was due to a specific effect of Ets1 deficiency on PC differentiation, as Btk−/−Ets1−/− resembled Btk−/− mice in terms of reduced mature B cells, reduced B1a cells, and impaired proliferation and survival in response to BCR cross-linking. A possible mechanism for this observation is suggested by a study in which xid B cells (which carry a loss of function point mutation in Btk) were found to be capable of upregulating the PC transcription factor Blimp1 in vivo in response to Ag, but did not differentiate into PCs (55), possibly because they failed to downregulate Ets1, which prevents PC differentiation by...
inhibiting Blimp1 (2). Restoration of differentiation but not proliferative expansion could explain the rescue of basal IgM levels, but not in response to specific immunization with TNP-Ficoll, in Btk−/− Ets1−/− mice. In any given response, even enhanced differentiation without extensive proliferation could result in lower than normal levels of Abs, but overall, as animals respond continuously to environmental Ags, IgM levels could be normalized. An alternative, and not mutually exclusive, explanation is that the impaired response to TNP-Ficoll may be secondary to the reduction in marginal zone B cells (56) that occurs in the absence of Btk−/− and Btk−/− Ets1−/− mice. In any event, the finding that Btk has separate effects on Ets1 downregulation (and hence Lyn in signal initiation) and B cell development or activation.


