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Btk regulates localization, in vivo activation, and class switching of anti-DNA B cells

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Abstract

The autoimmune disease systemic lupus erythematosus (SLE) is characterized by loss of tolerance to nuclear antigens such as chromatin, DNA, and RNA. This focused autoreactivity is thought to arise from the ability of DNA or RNA specific B cells to receive dual signals from the BCR and TLR9 or TLR7, respectively. The Tec kinase Btk is necessary for the production of anti-DNA antibodies in several murine models of SLE. To assess the role of Btk in the fate of DNA reactive B cells, we generated Btk^{-/-} mice carrying the 56R anti-DNA Ig transgene on the C57BL/6 background. dsDNA specific B cells were present in 56R.Btk^{-/-} mice, although they were not preferentially localized to the marginal zone. These cells were able to proliferate in response to large CpG DNA containing fragments that require BCR-induced internalization to access TLR9. However, anti-DNA antibodies were not observed in the serum of 56R.Btk^{-/-} mice. A transgene expressing a low level of Btk in B cells (Btk^{lo}) restored anti-DNA IgM in these mice. This correlated with partial rescue of proliferative response to BCR engagement and TLR9-induced IL-10 secretion in Btk^{lo} B cells. anti-DNA IgG was not observed in 56R.Btk^{lo} mice, however. This was likely due, at least in part, to a role for Btk in controlling the expression of T-bet and AID in cells stimulated with CpG DNA. Thus, Btk is required for the initial loss of tolerance to DNA and the subsequent production of pathogenic autoantibodies once tolerance is breached.

Keywords

B cells; Systemic Lupus Erythematosus; Autoantibodies; Transgenic/Knockout Mice; Protein Kinases

Introduction

The autoimmune disease systemic lupus erythematosus (SLE) is characterized by loss of tolerance to nuclear antigens such as chromatin, DNA, and RNA (Plotz, 2003). This results in

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autoantibody production, immune complex deposition, inflammation, and end organ damage. Current therapy for SLE involves relatively nonspecific immunosuppression with undesirable side effects. Thus, a thorough understanding of the mechanisms controlling the development and activation of nucleic acid reactive B cells may lead to the identification of novel therapeutic targets for SLE.

The focused autoreactivity towards nuclear antigens in SLE is likely explained by the recent observation that B cells specific for DNA or RNA containing antigens can be activated by signals from both the BCR and TLR9 or TLR7, respectively (Leadbetter *et al.*, 2002; Viglianti *et al.*, 2003; Marshak-Rothstein *et al.*, 2004; Lau *et al.*, 2005). In addition to directly activating anti-DNA or anti-RNA B cells, the binding of DNA or RNA containing antigen to the BCR leads to receptor internalization and delivery of antigen to intracellular compartments containing TLR9 and TLR7. TLR signaling in B cells induces proliferation, differentiation into plasma cells, and the secretion of cytokines (Peng, 2005). In addition, dual BCR/TLR9 engagement promotes events such as production of the growth factor IL-2 that do not occur when either receptor signals alone (Busconi *et al.*, 2007).

Two related site-directed anti-DNA IgH transgenes have been widely used to generate DNA-reactive B cells in mice and study their development and regulation. The 3H9 transgene can contribute to anti-dsDNA, anti-ssDNA, and non-auto antibodies when paired with the appropriate light chains (Radic *et al.*, 1991). A second transgene, 56R, is a mutated version of 3H9 that has a stronger affinity for DNA and produces antibodies against dsDNA more frequently than 3H9 (Chen *et al.*, 1994). Tolerance to DNA is maintained in 3H9 transgenic mice on a Balb/c background such that no anti-DNA antibodies are produced. In contrast, Balb/c 56R mice generate low levels of anti-DNA IgM, while C57BL/6 56R (B6.56R) mice produce both IgM and IgG against ssDNA and dsDNA (Li *et al.*, 2002; Sekiguchi *et al.*, 2003; Fukuyama *et al.*, 2005; Sekiguchi *et al.*, 2006).

Anti-DNA B cells in 56R mice are localized preferentially to the marginal zone (Li *et al.*, 2002a; Li *et al.*, 2002b; Sekiguchi *et al.*, 2006). This was initially proposed as a mechanism of tolerance (Li *et al.*, 2002a; Li *et al.*, 2002b). However, recent reports demonstrating rapid activation and differentiation of marginal zone B cells in response to TLR ligands (Fairfax *et al.*, 2007; Genestier *et al.*, 2007) suggest that localization of anti-DNA B cells to this compartment may actually lead to autoantibody production in B6.56R mice. Once tolerance to DNA is lost in these animals, the generation of pathogenic anti-DNA IgG is promoted by TLR9 signaling (Ehlers *et al.*, 2006) and limited by the inhibitory receptor FcγRIIb (Fukuyama *et al.*, 2005).

The Tec family kinase Btk is an important component of BCR signaling pathways (Khan *et al.*, 1995; Satterthwaite and Witte, 2000). It is necessary for the production of autoantibodies, including anti-DNA antibodies, in a number of murine models of SLE (Steinberg *et al.*, 1982; Golding *et al.*, 1983; Scribner *et al.*, 1987; Seldin *et al.*, 1987; Satterthwaite *et al.*, 1998; Takeshita *et al.*, 1998; Whyburn *et al.*, 2003). We have shown that at least some of the contribution of Btk to autoimmunity is independent of its role in transmitting BCR signals (Whyburn *et al.*, 2003). While studies in humans indicate that Btk is not required for the development of anti-DNA B cells (Ng *et al.*, 2004), the role of Btk in the determining the fate of these cells has not been studied in detail.

To study the contribution of Btk to the development, activation, and localization of anti-DNA B cells, we generated 56R.Btk^{-/-} mice on a C57BL/6 background. dsDNA-specific B cells were present in 56R.Btk^{-/-} mice, although they were not preferentially localized to the marginal zone. anti-DNA antibodies were not observed in the serum of 56R.Btk^{-/-} mice, however. A transgene expressing a low level of Btk in B cells (Satterthwaite *et al.*, 1997)

restored anti-DNA IgM, but not anti-DNA IgG, in these mice. Thus, Btk is required for both the initial loss of tolerance to DNA and the subsequent production of pathogenic autoantibodies once tolerance is breached.

Materials and Methods

Mice

Mice were on the C57BL/6 background. 56R anti-DNA IgH knock-in mice (Li *et al.*, 2001, Sekiguchi *et al.*, 2006), a gift of Dr. Martin Weigert via Dr. Chandra Mohan, were crossed to Btk^{-/-} (Khan *et al.*, 1995) and Btk^{lo} (Satterthwaite *et al.*, 1997) mice. Mice were genotyped by PCR. Anti-DNA antibodies were measured in and hybridomas generated from 6–12 month old mice in order to ensure that a lack of autoantibodies in 56R.Btk^{-/-} mice was not due simply to a delay in loss of tolerance. All other experiments used 3–6 month old mice to identify intrinsic differences in responses that might lead to or affect subsequent loss of tolerance and thus should precede the appearance of autoantibodies. All procedures were approved by the UT Southwestern Institutional Animal Care and Use Committee.

B cell purification

Splenocytes were depleted of red blood cells by a 5 min incubation in 0.15M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA. B cells were then purified using negative selection with anti-CD43 magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions.

Reagents for B cell activation

Goat-anti-mouse IgM F(ab)₂ fragments (Jackson ImmunoResearch) were used to crosslink the BCR. Plasmids containing the CG50 and HIV(CG+) fragments were a gift of Dr. Greg Viglianti (Viglianti *et al.*, 2003). They were amplified in the dam⁻/dcm⁻ E. coli strain GM2163 (New England Biolabs) to ensure that CpG sequences were hypomethylated. DNA was prepared using Qiagen Endo-Free Maxi kit to eliminate endotoxin contamination. The CG50 and HIV (CG+) fragments were excised by digesting with BamHI and EcoRI. Phosphorothioate-modified stimulatory oligodeoxynucleotide (ODN) 1826 5' TCCATGACGTTCTGACGTT 3' (Yi *et al.*, 1998) and inhibitory ODN 2088 5' TCCTGGCGGGAAGT 3' (Lenert *et al.*, 2001) were obtained from Oligo's Etc.

B cell proliferation

Purified B cells were plated in 96 well plates at 10⁶/ml in RPMI + 10% fetal calf serum. Cells were incubated with media alone, 2 or 20 ug/ml anti-IgM F(ab)₂ fragments, 0.1 or 1 ug/ml ODN 1826, 0.1 ug/ml CG50 fragment +/- 4 ug/ml ODN 2088, or 0.1 ug/ml HIV (CG+) fragment. Proliferation was measured by ³H-thymidine incorporation during the final 6–12 hours of a 36–48 hour culture.

B cell differentiation

Purified B cells were cultured at 5 × 10⁵/ml in RPMI + 10% fetal calf serum with or without 1 ug/ml ODN 1826 for 72 or 96 hours. Cultured cells and supernatants were harvested for flow cytometry and ELISA as described below.

Real time PCR

Purified B cells were cultured at 10⁶/ml in RPMI + 10% fetal calf serum with 15 ug/ml anti-IgM F(ab)₂ fragments, 1 ug/ml ODN 1826, or both for 6 or 24 hours. Total RNA was prepared using the RNeasy kit (Qiagen). cDNA was generated with a cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed in an Applied Biosystems 7300 Real Time PCR system using TaqMan reagents specific for mouse IL-2, Tbx21 (T-bet), Aicda (AID) and the

internal control GAPDH (Applied Biosystems). Data were normalized to GAPDH using the delta comparative threshold cycle (Ct) method.

Flow cytometry

Single cell suspensions of red-blood cell depleted splenocytes or cultured cells were incubated in PBS + 2 % FCS with various combinations of antibodies against the following molecules or isotype controls: B220-PerCP, CD21-FITC, CD23-PE, IgMa-PE, Igλ-biotin + streptavidin APC, CD138-biotin + streptavidin APC, CD86-biotin + streptavidin APC. All antibodies were obtained from BD Pharmingen except streptavidin APC (Caltag). Samples were run on a Becton-Dickinson FACS Calibur and analyzed using Cellquest software. Live cells were gated based on FSC and SSC.

Hybridoma production

Total splenocytes were stimulated with 20 ug/ml LPS (Sigma) for 1 to 5 days and then fused to SP2/0 myeloma cells as described in (Fukuyama *et al.*, 2005; Sekiguchi *et al.*, 2006). Hybridomas were selected via growth in HAT media.

ELISA

Autoantibodies—anti-ssDNA and dsDNA ELISAs were performed as described in (Whyburn *et al.*, 2003) using serial dilutions of serum or hybridoma culture supernatant.

Total Ig—Total IgM and IgG were measured as described in (Whyburn *et al.*, 2003) using serial dilutions of culture media from stimulated B cells or hybridoma supernatants.

IL-10—Purified splenic B cells were stimulated with media alone or 1 ug/ml ODN 1826 (Oligo's Etc.) for 24 hrs. IL-10 levels were measured in culture supernatants using a commercially available ELISA assay (Becton Dickinson).

BCR Internalization

Single cell suspensions of red-blood cell depleted splenocytes were stained on ice with FITC-labeled donkey anti-mouse Ig (H+L) F(ab)₂ fragments in PBS + 5% fetal calf serum. Stained cells were warmed to 37° to induce BCR signaling and internalization. The reaction was stopped at various time points by the addition of 20 volumes of ice cold PBS + 5% fetal calf serum + azide. The mean fluorescence intensity of FITC+ cells was determined by flow cytometry. Internalization of the BCR is accompanied by a reduction in fluorescence due to entry of the FITC label into acidic compartments (Aluvihare *et al.*, 1997).

Statistics

Groups were compared by Student's t-test.

Results

To study the role of Btk in the development, activation, and localization of anti-DNA B cells, we generated 56R.Btk^{-/-} mice on a C57BL/6 background. A similar number of B220+ cells was observed in the spleens of 3–6 month old 56R and 56R.Btk^{-/-} mice (1.47×10^7 vs. 1.09×10^7 , $p = 0.49$), and a similar frequency of B cells expressed the 56R transgene (Figure 1a). Skewing of 56R expressing B cells to the marginal zone compartment (Li *et al.*, 2002a; Li *et al.*, 2002b; Sekiguchi *et al.*, 2006) was not observed in the absence of Btk (Figure 1b,d), however. This was not due to a general requirement for Btk in the development of marginal zone B cells, as the frequency of cells in this compartment was normal in Btk^{-/-} mice in the absence of the 56R transgene (Figure 1b,d) (Martin and Kearney, 2000; Cariappa *et al.*,

2001). Nor is it due to a lack of anti-DNA B cells in Btk^{-/-} spleens, as Btk is required for the preferential localization to the marginal zone of cells expressing both the 56R transgene and Igλ, a combination known to confer DNA-reactivity (Figure 1c,d) (Chen *et al.*, 1994; Li *et al.*, 2002a; Li *et al.*, 2002b).

The presence of DNA reactive cells in 56R.Btk^{-/-} mice was directly demonstrated by generating hybridomas from LPS-stimulated splenocytes. 74 IgM secreting hybridomas were obtained from a total of two 56R mice, of which 66 (89%) were specific for dsDNA. 2 of 19 (11%) IgG secreting hybridomas produced anti-dsDNA antibodies. This is consistent with previous results from 56R mice on the B6 background (Fukuyama *et al.*, 2005; Sekiguchi *et al.*, 2006). Anti-DNA B cells were also present in the absence of Btk, although at a reduced frequency. 41% (27 of 65) of IgM secreting hybridomas obtained from a total of 3 56R.Btk^{-/-} mice produced antibodies against dsDNA, while none of the 10 IgG secreting hybridomas were dsDNA-specific.

Btk is required for proliferation in response to antigen engagement (Khan *et al.*, 1995). However, BCR-mediated shuttling of antigen to TLR9 is sufficient to induce TLR9-dependent proliferation of B cells in the absence of apparent BCR crosslinking (Viglianti *et al.*, 2003). This suggests that DNA containing antigens might be able to stimulate the activation of Btk^{-/-} anti-DNA B cells via TLR9 despite the lack of a mitogenic signal from the BCR. To address this issue, we first used the diffusible CpG containing ODN 1826 to assess the role of Btk in responses to TLR9 (Leadbetter *et al.*, 2002; Viglianti *et al.*, 2003; Marshak-Rothstein *et al.*, 2004). Btk^{-/-} B cells were able to proliferate, upregulate CD86, and differentiate in response to 1 μg/ml ODN 1826 (Figure 2a–d, Figure 3a). However, a six-fold reduction in the secretion of IL-10 by B cells in response to TLR9 engagement was observed in the absence of Btk (Figure 2e), consistent with recent reports published during the preparation of this manuscript (Hasan *et al.*, 2008; Lee *et al.*, 2008).

We next asked whether Btk promotes interactions between antigen and CpG DNA induced signals. Low doses of anti-IgM and ODN 1826 were unable to synergize to promote proliferation of Btk^{-/-} B cells as they did in wild type cells (Figure 3a). In addition to promoting proliferation, however, engagement of both the BCR and TLR9 results in functional consequences distinct from the sum of those elicited by stimulation of either receptor alone (Busconi *et al.*, 2007). Of particular interest is the upregulation of IL-2, which promotes the growth of both B and T cells and may serve to amplify the immune response to DNA containing antigens. Btk^{-/-} cells incubated with anti-IgM plus ODN 1826 had IL-2 mRNA levels approaching those seen in wild type cells stimulated with both reagents (Figure 3b). Synergy between BCR and TLR9 signaling was also observed visually in Btk^{-/-} B cells. Together, but not alone, anti-IgM and ODN 1826 induced homotypic adhesion of Btk^{-/-} B cells (Figure 3c). Thus, despite the lack of a mitogenic response from the BCR, Btk^{-/-} cells are able to transmit some BCR signals that interact with TLR9 signals.

Although ODN 1826 can access TLR9 directly, the larger fragments of DNA likely to activate anti-DNA B cells in vivo must be delivered to TLR9 by the BCR (Leadbetter *et al.*, 2002; Viglianti *et al.*, 2003). To determine whether Btk^{-/-} B cells are able to internalize the BCR, FITC-labeled anti-IgM was used to both induce and measure BCR internalization. Over time, the mean fluorescence intensity of IgM⁺ cells from both wild type and Btk^{-/-} B cells was reduced (Figure 4a), indicating quenching of fluorescence upon delivery of FITC to intracellular compartments with low pH (Aluvihare *et al.*, 1997).

A large DNA fragment containing tandem repeats of hypomethylated CpG sequences (CG50) was used to stimulate anti-DNA B cells. This fragment induces TLR9-dependent proliferation of B cells from mice expressing the 3H9 anti-DNA IgH transgene in a manner dependent on

the shuttling function of the BCR (Viglianti *et al.*, 2003; Marshak-Rothstein *et al.*, 2004). Similarly, CG50 stimulation induced a mitogenic response in 56R B cells that required the presence of the 56R transgene (Figure 4b). CG50 induced proliferation was prevented by the inhibitory CpG ODN 2088 (Figure 4b). A DNA fragment of similar size containing non-immunostimulatory CpG sequences (HIV-CG+) (Viglianti *et al.*, 2003) did not induce this response. CG50-induced proliferation of 56R B cells was comparable in magnitude to the response elicited by anti-IgM (Figure 4b) as was observed for 3H9 B cells (Viglianti *et al.*, 2003). Interestingly, anti-IgM induced proliferation of 56R B cells was significantly reduced compared to non-transgenic B cells (Figure 4b). This suggests that a large proportion of 56R B cells may be anergic as a result of their autoreactivity.

B cells from 56R.Btk^{-/-} mice proliferated in the presence of CG50, but not CG50 + ODN 2088, HIV-CG+, or anti-IgM (Figure 4c). Btk^{-/-} B cells without the 56R transgene did not respond to CG50. The reduction in the absolute response of 56R.Btk^{-/-} B cells to CG50 compared to 56R B cells is likely explained by the two fold reduction in frequency of DNA-reactive cells and/or the lack of a mitogenic signal from the BCR in the absence of Btk. However, the stimulation index (CG50/media) for 56R.Btk^{-/-} cells was actually greater than that of 56R cells (Figure 4d). Thus, the ability of 56R.Btk^{-/-} B cells to proliferate upon stimulation with the CG50 fragment confirms the presence of anti-DNA B cells in these mice and shows that they can be at least partially activated by large DNA fragments in the absence of a mitogenic signal from the BCR.

To determine whether Btk-deficient anti-DNA B cells can be fully activated and differentiate *in vivo*, we compared levels of anti-ssDNA and anti-dsDNA IgM and IgG in 56R and 56R.Btk^{-/-} mice. Each of these anti-DNA antibodies was present in 56R mice on the C57BL/6 background (Figure 5), consistent with previous observations (Sekiguchi *et al.*, 2003; Fukuyama *et al.*, 2005; Sekiguchi *et al.*, 2006). However, there was a dramatic reduction in autoantibody levels in 56R.Btk^{-/-} mice, such that there was no significant difference between them and non-transgenic wild type mice (Figure 5). This is a more profound defect than can be explained by the approximately two fold reduction in the frequency of anti-dsDNA reactive B cells in 56R.Btk^{-/-} mice compared to 56R mice. Thus, Btk is required for the production of antibodies by anti-DNA B cells *in vivo*.

Responses to BCR crosslinking are particularly sensitive to subtle changes in Btk signal strength (Satterthwaite *et al.*, 1997). This suggested that merely reducing Btk levels, rather than eliminating Btk altogether, may also impede the production of anti-DNA antibodies. To test this hypothesis we crossed a transgene that drives approximately 25% of endogenous Btk levels (Btk^{lo}) in B cells (Satterthwaite *et al.*, 1997) to 56R.Btk^{-/-} mice. This was sufficient to significantly rescue the production of anti-ssDNA and anti-dsDNA IgM in 56R.Btk^{-/-} mice (Figure 5a,c). The Btk^{lo} transgene partially, although not completely, restored the ability of Btk^{-/-} B cells to secrete IL-10 in response to TLR9 engagement (Figure 2e). Proliferation in response to BCR crosslinking was also partially rescued, both at high doses of anti-IgM alone (Satterthwaite *et al.*, 1997) and at low doses of anti-IgM in synergy with CpG DNA (Figure 3a). This suggests that a minimal amount of mitogenic BCR signaling and/or TLR9 induced IL-10 expression is required for the activation of anti-DNA B cells *in vivo*.

Despite the presence of anti-DNA IgM in 56R.Btk^{lo} mice, there was no difference between 56R.Btk^{lo} and 56R.Btk^{-/-} mice with regard to anti-ssDNA and anti-dsDNA IgG levels (Figure 5b,d). This was not due to a general inhibitory effect of the Btk transgene on class switching, as normal levels of anti-DNA IgG were present in 56R mice expressing both the endogenous Btk gene and the transgene (Figure 5b,d, Btk^{hi}). Thus, the production of anti-DNA IgG is particularly sensitive to changes in Btk signal strength.

TLR9 signaling has been shown to contribute to class switching of anti-DNA B cells in the B6.56R model, likely via the upregulation of T-bet expression in B cells (Ehlers *et al.*, 2006; Liu *et al.*, 2003). CpG DNA also induces expression of AID, which is required for class switching (Muramatsu *et al.*, 2000; He *et al.*, 2004). We therefore asked whether these processes depend on Btk (Figure 5e). Expression levels of both T-bet and AID were 4 to 5 fold lower than normal in CpG stimulated Btk^{-/-} B cells. TLR9 engaged Btk^{lo} B cells expressed twice the amount of mRNA for T-bet and AID as Btk^{-/-} cells, but only half as much as wild type cells. Thus, Btk may regulate class switching of DNA-reactive cells at least in part by mediating TLR9-induced expression of T-bet and AID.

Discussion

Anti-DNA B cells can be activated via both BCR and TLR9 signals (Leadbetter *et al.*, 2002; Viglianti *et al.*, 2003; Marshak-Rothstein *et al.*, 2004). The BCR both directly activates B cells and delivers DNA to intracellular compartments containing TLR9. We now demonstrate that in the absence of Btk, which mediates mitogenic signaling from the BCR, anti-DNA B cells can develop and proliferate in response to CpG containing DNA fragments. However, 56R.Btk^{-/-} mice do not produce significant levels of anti-DNA antibodies in vivo.

One possible explanation for these results is that altered negative selection of immature B cells results in a decreased number of anti-DNA B cells in 56R.Btk^{-/-} mice. This is in part true, as a lower frequency of hybridomas recovered from the spleens of 56R.Btk^{-/-} mice were specific for anti-dsDNA compared to 56R mice. Preliminary repertoire analysis of hybridoma panels did not reveal a consistent explanation for this difference (data not shown). Nonetheless, the reduction in anti-DNA antibodies in 56R.Btk^{-/-} mice is much greater than two fold, indicating that the anti-DNA B cells that are present in these mice are not being activated in vivo.

The restoration of anti-DNA IgM in 56R.Btk^{-/-} mice by the Btk^{lo} transgene correlates with the partial rescue of a) CpG DNA-induced IL-10 secretion and b) proliferative response to BCR crosslinking, both alone and in synergy with TLR9 engagement. IL-10 secretion by B cells in response to TLR9 ligands is significantly enhanced in B cells from several murine lupus models and human lupus patients (Lenert *et al.*, 2005; Nakano *et al.*, 2008). IL-10 promotes autoantibody production by B cells from SLE patients (Llorente *et al.*, 1995), and antibodies against IL-10 have been shown to be protective in the NZB x NZW murine model of lupus (Ishida *et al.*, 1994; Balabanian *et al.*, 2003). Thus, Btk-dependent secretion of IL-10 in response to DNA-containing antigens may promote the activation and differentiation of anti-DNA B cells.

These studies also support a model in which full activation and differentiation of DNA-reactive cells requires the BCR to signal actively through Btk, not just deliver DNA to TLR9. Btk was required for the ability of low doses of anti-IgM and CpG DNA to synergize for the proliferation of B cells. Thus, a Btk-dependent BCR signal may act to sensitize B cells to respond to the limiting amounts of DNA-containing antigen that these cells are likely to encounter in vivo. Alternatively, it has recently been shown that while cyclosporin-inhibitable, Ca⁺⁺ dependent signals from the BCR synergize with CpG DNA to activate B cells, chronic BCR stimulated ERK activation can prevent CpG induced differentiation of B cells (Rui *et al.*, 2003). In primary murine B cells, Btk is required for BCR-stimulated Ca⁺⁺ signaling but not ERK activation (Forssell *et al.*, 2000). In fact, we have shown that Btk^{-/-} B cells undergo enhanced ERK phosphorylation relative to wild type cells in response to anti-IgM (Halcomb *et al.*, 2007). Thus, it is possible that in addition to not receiving a Ca⁺⁺-dependent activating signal from the BCR, Btk-deficient anti-DNA B cells are actively inhibited from differentiating in response to TLR9 engagement via BCR-induced ERK activation. In this way, they resemble anergic B cells (Rui *et al.*, 2003).

Failure to localize to the marginal zone may also contribute to reduced activation of anti-DNA B cells in vivo in the absence of Btk. Recent studies have indicated that marginal zone B cells are significantly more sensitive to TLR-induced differentiation than follicular B cells (Fairfax *et al.*, 2007; Genestier *et al.*, 2007). Reduced skewing of 56R.Btk^{-/-} B cells to the marginal zone population is consistent with previous observations demonstrating that Btk-mediated BCR signals are required for the enrichment of certain B cell clones into this compartment (Martin and Kearney, 2000). Interestingly, however, Btk is not required for the development of marginal zone cells (Martin and Kearney, 2000), and in some cases Btk signaling even decreases the frequency of this population (Cariappa *et al.*, 2001). This suggests that either 1) not all marginal zone B cells are positively selected by BCR signaling or 2) Btk is required only for the entry or maintenance of particular B cell clones within the marginal zone.

The Btk^{lo} transgene significantly increased IgM anti-DNA levels in 56R.Btk^{-/-} mice, suggesting that a minimal amount of BCR signaling and/or TLR9-induced IL-10 expression is required for the activation and differentiation of anti-DNA B cells. However, there was no difference between 56R.Btk^{lo} and 56R.Btk^{-/-} mice with respect to anti-DNA IgG levels. 56R.Btk^{lo} mice thus resemble 56R.Balb/c mice in the production of intermediate levels of anti-DNA IgM and no anti-DNA IgG (Fukuyama *et al.*, 2005). The Btk^{lo} transgene did not impair anti-DNA IgG production in the presence of a normal endogenous Btk gene, however, ruling out linkage of the Btk^{lo} transgene to a Balb/c-like allele of a gene controlling anti-DNA IgG levels. We did not observe significant impairment of class switching in Btk^{lo} mice in response to T-independent or T-dependent viral antigens (Pinschewer *et al.*, 1999), suggesting that the current observation may be specific for anti-DNA B cells.

Consistent with this idea, we demonstrate that Btk mediates CpG DNA induced expression of AID and T-bet in B cells. AID is required for class switching (Muramatsu *et al.*, 2000), and T-bet deficient MRL.lpr mice have dramatic reductions in pathogenic autoantibody levels (Peng *et al.*, 2002). While AID and T-bet levels were partially restored in Btk^{lo} B cells, they remained lower than normal and may be insufficient to promote class switching of anti-DNA B cells. Additionally, the balance between Btk-dependent activating signals and FcγRIIb-mediated inhibitory signals may regulate the terminal differentiation of switched anti-DNA B cells. A major function of FcγRIIb is to inhibit BCR-induced membrane association and activation of Btk (Bolland *et al.*, 1998; Scharenberg *et al.*, 1998). 56R.FcγRIIb^{-/-} mice have similar levels of anti-DNA IgM to 56R mice but significantly increased levels of anti-DNA IgG due to an increase in the frequency of IgG secreting plasma cells (Fukuyama *et al.*, 2005). Taken together, these observations suggest that Btk signal strength is a critical determinant of whether pathogenic IgG autoantibodies will be produced.

In summary, Btk plays an important role at multiple checkpoints during the development, localization, activation, and class switching of DNA-reactive B cells. The particular sensitivity of anti-DNA IgG to subtle changes in Btk signal strength suggests that Btk, its activators or effectors may be points of potential therapeutic intervention to prevent pathogenic autoimmunity in SLE.

Abbreviations

AID	activation induced cytidine deaminase
BCR	B cell antigen receptor
Btk	Bruton's tyrosine kinase
ODN	oligodeoxynucleotide
SLE	Systemic lupus erythematosus

TLR Toll-like receptor

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Figure 1. B cells with potential for anti-DNA reactivity are present in 56R.Btk^{-/-} but are not preferentially localized to the marginal zone

Splenocytes from 3–6 month old mice were stained with antibodies against A) B220 and IgMa and B,C) B220, CD21, CD23, and Igλ. All B220+ cells (B) or B220+ Igλ+ cells (C) were analyzed for expression of CD21 and CD23. The frequency of gated cells in the marginal zone (CD23-CD21+) population is indicated. D) The mean $\pm$ SD percentage of marginal zone cells among B220+ cells in mice without the 56R transgene and among B220+ and B220+Igλ+ cells in mice with the 56R transgene (D) is shown. $n = 3$. * = $p < 0.05$, wt vs. Btk^{-/-}.

Figure 2. Btk^{-/-} B cells proliferate and differentiate in response to CpG DNA but demonstrate impaired upregulation of IL-10

A) Purified B cells from 3–6 month old mice were stimulated with 1 $\mu\text{g}/\text{ml}$ ODN 1826. Proliferation was measured by ^3H -thymidine incorporation during the final 6 hrs of a 48 hr culture. Data represent mean $\pm$ SEM, $n = 6-8$, $p = 0.5$ (not significant). B) Purified B cells were stimulated with media alone (thin line) or 1 $\mu\text{g}/\text{ml}$ ODN 1826 (bold line) for 72 hours and stained with antibodies against CD86. Data are representative of 3 independent experiments. C) Purified B cells were stimulated with 1 $\mu\text{g}/\text{ml}$ ODN 1826 for 72 hours and stained with antibodies against B220 and CD138, a plasma cell marker. The frequency of CD138^{hi} cells is indicated. Data are representative of 4–7 individual mice per genotype. D) Purified B cells were stimulated with 1 $\mu\text{g}/\text{ml}$ ODN 1826 for 72 or 96 hours. Total IgM levels in the culture supernatants were measured by ELISA. The 72 hr. and 96 hr. time points were obtained in separate experiments, and each time point represents the mean $\pm$ SD of 2 mice per genotype, for a total of 4 mice analyzed per genotype. E) Purified splenic B cells were stimulated with 1 $\mu\text{g}/\text{ml}$ ODN 1826 for 24 hours. Culture supernatants were analyzed for IL-10 by ELISA. Data represent mean $\pm$ SD, $n = 4-5$. * = $p < 0.05$, ** = $p < 0.01$. No IL-10 was detected in supernatant from cells stimulated with media alone.

Figure 3. Btk is required for some, but not all, interactions between BCR and TLR9 signals

A) Purified B cells were stimulated with 1 ug/ml ODN 1826, 0.1 ug/ml ODN 1826, 2 ug/ml anti-IgM F(ab)₂ fragments, or 0.1 ug/ml ODN 1826 plus 2 ug/ml anti-IgM F(ab)₂ fragments for 48 hours. Proliferation was measured by ³H-thymidine incorporation. Data represent mean $\pm$ SD of triplicate wells and are representative of 4 independent comparisons. * = $p < 0.01$ vs. **both** 0.1 ug/ml ODN 1826 alone **and** 2 ug/ml anti-IgM alone. B) Purified B cells were stimulated with 15 ug/ml anti-IgM F(ab)₂ fragments, 1 ug/ml ODN 1826, or both for 6 hours. cDNA was prepared and subjected to quantitative real time PCR for IL-2, using GAPDH as a loading control. Data are presented as relative IL-2 expression compared to wild type cells stimulated with anti-IgM + ODN 1826, mean $\pm$ SD, $n=2-3$. * = $p < 0.05$ vs. wt cells treated

with anti-IgM + ODN 1826. C) Purified Btk^{-/-} B cells were stimulated with 15 ug/ml anti-IgM F(ab)₂ fragments, 1 ug/ml ODN 1826, or both for 48 hours. Cultures were photographed with a Zeiss Axiovert microscope at 10X magnification.

Figure 4. 56R B cells proliferate in response to CpG-containing DNA fragments independently of Btk

A) Total splenocytes were stained on ice with FITC-labeled donkey anti-mouse Ig F(ab)₂ fragments. Cells were then incubated at 37°C for the indicated times to induce BCR signaling and internalization and analyzed by flow cytometry. The mean fluorescence intensity of FITC + cells was determined and is presented as % of the starting value. Internalization of the BCR is accompanied by a reduction in fluorescence due to entry of the FITC label into acidic compartments (Aluvihare *et al*, 1997). Data represent the mean $\pm$ SD for two experiments. Similar results were also obtained with xid mice, which have an inactivating point mutation in Btk (data not shown). B, C) Purified B cells were stimulated with media alone, 20 μ g/ml anti-

IgM F(ab)₂ fragments, 0.1 ug/ml CG50 fragment +/- 4 ug/ml inhibitory ODN 2088, or 0.1 ug/ml HIV (CG+) fragment. Proliferation was measured by ³H-thymidine incorporation during the final 12 hrs of a 36 hr culture. Cpm are presented as mean +/- SEM, n = 3-6. * = p < 0.05, ** = p < 0.01 56R vs. no 56R. D) Stimulation index = avg cpm stimulated/avg cpm media from the data in B and C. Note that the response of wt cells to anti-IgM is off scale in B and D. The legend in panel C is also for panels B and D.

Figure 5. Differential effects of reduced Btk signaling on anti-DNA IgM and IgG production
 6–12 month old wild type or 56R mice with the indicated Btk genotypes were bled and the serum levels of anti-ssDNA (A,B) and anti-dsDNA (C,D) IgM (A,C) and IgG (B,D) measured by ELISA. The OD405 for a 1:100 dilution of serum is indicated. Each diamond represents an individual mouse. Horizontal bars represent the mean. * = $p < .02$ vs wild type and 56R. Btk^{-/-}. Btk^{hi} mice express both the endogenous Btk gene and the Btk^{lo} transgene. E) Purified splenic B cells were stimulated with 1 μ g/ml ODN 1826 for 24 hours. Cells were harvested for RNA and analyzed by real time PCR for expression of Tbx21 (Tbet) and Aicda (AID), using GAPDH as a loading control. Data represent the percentage of the average wild type level in

ODN 1826 stimulated cells and are plotted as mean $\pm$ SD, n = 2–4. * = $p < 0.05$, ** = $p < 0.01$.