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Impaired B Cell Receptor Signaling Is Responsible for Reduced TACI Expression and Function in X-Linked Immunodeficient Mice

Kadriye Uslu,*1 Adam S. Coleman,*1 Windy R. Allman,* Nora Katsenelson,*2 Richard J. Bram,† Kishore R. Alugupalli,‡ and Mustafa Akkoyunlu*

Immune response to T cell independent type 2 (TI-2) Ags, such as bacterial polysaccharides, is severely impaired in X-linked immunodeficient (XID) mice. In this study, we investigated the involvement of a proliferation-inducing ligand (APRIL) or BAFF and their receptors in the unresponsiveness of XID mouse to TI-2 Ags. We discovered that whereas serum BAFF levels were increased, the expression of the APRIL and BAFF receptor transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) was severely reduced in XID B cells. Moreover, B cells from XID mouse were unable to secrete Igs in response to APRIL or BAFF. In correlation with reduced TACI expression and impaired TACI function, APRIL or BAFF did not activate the classical NF-κB pathway in XID cells. Also correlating with the unaltered expression of BAFF receptor, BAFF stimulation induced the activation of the alternative NF-κB pathway in XID cells. Moreover, activation of MAPK pathway was ablated in APRIL-stimulated XID cells. Prestimulation of XID B cells with the TLR9 agonist, CpG led to a significant increase in TACI expression and restored TACI-mediated functions. CpG prestimulation also restored TACI-mediated signaling in APRIL- or BAFF-stimulated XID B cells. Finally, immunization of XID mouse with the prototype TI-2 Ag NP-Ficoll induced IgG and IgM Abs when CpG was given with NP-Ficoll. Collectively, these results suggest that reduced TACI expression is responsible for the unresponsivenes of XID mouse to TI-2 Ags and BCR activation controls TACI expression. The Journal of Immunology, 2014, 192: 3582–3595.

Antibodies against the capsular polysaccharides eliminate encapsulated bacteria, such as Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae, through bactericidal or opsonophagocytic mechanisms (1). Newborns and infants are especially vulnerable to systemic infections with encapsulated bacteria mostly because they are unable to mount Ab responses against bacterial polysaccharides (2). As a result, vaccines composed of polysaccharides are ineffective in this age group (3, 4). Other than neonatal age, certain B cell deficiencies also lead to poor response to polysaccharide Ags. For example, a mutation in the gene encoding Bruton’s tyrosine kinase (Btk) molecule is responsible for the unresponsiveness to polysaccharides in X-linked immunodeficient (XID) mice (5–9). Although Btk is implicated in multiple signaling pathways (10, 11), its role in mediating NF-κB activation by acting on phospholipase-γ2 has been shown to be crucial for BCR signaling (12, 13). The blockade in BCR signal transduction leads to X-linked agammaglobulinemia disorder in humans, which is mainly characterized by a defective terminal B cell differentiation (14–16). Unlike in patients with X-linked agammaglobulinemia, the reduction in mature B cells is milder in the XID mouse. The somewhat impaired Ab response in XID mice against TD Ags is largely explained with the arrest of B cell maturation in transitional B cell stage (17, 18). The cell types that are primarily responsible for mounting Ab responses to polysaccharides are marginal zone (MZ) B cells and B1 B cells (19). In XID mice, B1 B cell numbers are severely reduced, whereas MZ B cell numbers are comparable to those in wild type mice (7, 20). The fact that Ab responses to polysaccharides are entirely abrogated in XID mice suggests that although their numbers are preserved, MZ B cells are not able to compensate for the lost Ab production caused by a reduction in B1 B cells (7).

Polysaccharides are designated as T cell–independent type 2 (TI-2) Ags because T cells are not involved in the development of Ab responses, and unlike T cell–independent type 1 Ags, such as LPS, they primarily stimulate BCR without engaging a second receptor such as TLR (21). Whereas multivalent cross-linking of BCR by polysaccharide Ags is sufficient to activate polysaccharide-specific B cells, second signals enhance BCR-mediated B cell activation. For example, engagement of polysaccharide-bound complement C3d to its receptor CD21/CR2 on B cells augments polysaccharide responses (1, 22). In addition to CD21/CR2, signals mediated by the receptor, transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) are also required for mounting Abs against TI-2 Ags (23, 24). TACI has two

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Abbreviations used in this article: APRIL, a proliferation-inducing ligand; BAFF-R, BAFF receptor; BCMA, B cell maturation Ag; Btk, Bruton’s tyrosine kinase; FO, follicular; KO, knockout; MFI, mean fluorescence intensity; MZ, marginal zone; TACI, transmembrane activator and calcium-modulator and cyclophilin ligand interactor; TI-2, T cell–independent type 2; XID, X-linked immunodeficient.
ligands, namely BAFF (also known as B lymphocyte stimulator [BLyS]) and a proliferation-inducing ligand (APRIL) (25). In addition to engaging TACI, both BAFF and APRIL bind to B cell maturation Ag (BCMA), while BAFF binds to a third receptor, called BAFF receptor (BAFF-R). The importance of TACI in the development of Ab response against TI-2 Ags is well documented. For example, TACI-deficient mouse (23, 24) do not respond to TI-2 Ags and patients with combined variable immune deficiency disorder are shown to suffer from recurrent infections with encapsulated bacteria, and they respond poorly to polysaccharide vaccines (26, 27). Moreover, we recently reported that newborn mouse B cells express significantly lower levels of TACI as compared with adult B cells, and their unresponsiveness to polysaccharides is mostly due to low TACI expression (28).

In this study, we hypothesized that possible changes in the expression or function of TACI can contribute to the unresponsiveness of XID mouse to TI-2 Ags, because the deletion of the TACI gene (23, 24) or even the decrease in TACI expression (28) leads to significantly reduced response to TI-2 Ags. An investigation of the expression and function of TACI revealed that not only do XID cells express lower levels of TACI as compared with wild type CBA/CaJ mice; they also do not respond to BAFF or APRIL stimulation. Moreover, as seen in newborn B cells (28), up-regulation of TACI expression on XID B cells by CpG stimulation restored BAFF- and APRIL-induced signaling.

Materials and Methods

Mice

Eight- to twelve-week-old adult C57BL/6J, CBA/CaH/N, and CBA/CaJ-nice were purchased from Charles River Laboratories (Raleigh, NC). CBA/CaH/N mouse is designated as XID, and the control CBA/CaJ mouse is designated as CBA throughout the manuscript. TACI knock out (KO) mouse was on a C57BL/6 background (29). This study was approved by the Committee on the Ethics of Animal Experiments of the Food and Drug Administration/Center for Biologics Evaluation and Research Institutional Animal Care and Use Committee (permit number 2002-31).

Cell culture and stimulation

Complete RPMI media (RPMI 1640; Cellgro, Manassas, VA) supplemented with 10% heat-inactinated FBS, 2 mM l-glutamine, 10,000 U/ml penicillin-streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 1 mM nonessential amino acids (Invitrogen, Carlsbad, CA) and 50 µM b-ME (Sigma-Aldrich, St. Louis, MO) was used for all cell culture experiments unless otherwise stated. Spleen B cells were purified by negative selection using a B cell isolation kit (Miltenyi Biotec, Auburn, CA) according to manufacturer’s instructions. Purity of isolated B cells was greater than 97% in all experiments as determined by flow cytometry. In some experiments, TACI+ and TACI−cell populations were sorted from TACI−PE−stained (R&D Systems, Minneapolis, MN) purified B cells using a FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ). CD19-FITC, CD93-FITC, B220/CD45R-PE-Cy5, B220/CD45R-Pacific Blue, Rat IgG2b-FITC, Rat IgG2b-PE, Rat IgG2a-Biotin, Rat IgG2a-PE-Cy5, Rat IgG2a-Pacific Blue, Streptavidin-APC-CY7, purified rat anti-mouse CD16/CD32 (BD Biosciences), CD93-APC, IgM-APC-CY7, IgD-FITC (BioLegend, San Diego, CA), BAFF-R-FITC, Rat IgGl-FITC, Rat IgG2a-PE-Cy7 (eBioscience), B220/CD45R Alexa Fluor-405, and IgG2a-Alexa Fluor-405 (Invitrogen). The expression of BAFF receptors TACI, BAFF-R, and BCMA, on T1 (CD19+CD93+IgMlo), T2 (CD19+CD93+IgMlg), T2 (CD19+CD93+IgMlg+), and follicular (FO) (CD19+CD93+IgMlg) were assayed by flow cytometry. For B cell subset NF-κB experiments, B cell subsets were sorted using MEZ (B220+CD23hiCD21lo), and FO (B220+CD23hiCD21hi), using BD LSRII, FACSAria II, and FACS Aria cell sorters (BD Biosciences, Franklin Lakes, NJ), 105 mL recombinant APRIL (Axxora, San Diego, CA), 105 mL recombinant BAFF (Axxora, San Diego, CA), 105 mL LPS (Sigma-Aldrich) plus 50 ng/ml IL-4 (R&D Systems), or APRIL (1 µg/ml) in the MAPK experiments, dead cells were removed with the Dead Cell Removal Kit (Miltenyi Biotec, Auburn, CA), and equal numbers of cells were lysed in radio-immunoprecipitation assay lysis buffer with heat protease inhibitor mixture (Thermo Fischer Scientific, Rockford, IL) per the manufacturer’s instructions. To measure IκBα degradation, purified B cells were incubated overnight with either CpG 1826 or media alone. After removing dead cells as described above, equal numbers of B cells were incubated in 15–50 µg/ml cycloheximide for 30 min at 37˚C to halt protein synthesis. The cells were then stimulated with anti-IgM, BAFF, or APRIL in the presence of cycloheximide and then lysed as described above. Nuclear fractions were separated using the NE-PER Nuclear Protein Extraction Kit (Thermo Fischer Scientific, Rockford, IL), lysates were separated on 4–20% Mini-PROTEAN TGX gels and transferred to nitrocellulose using the iBlot transfer system (Invitrogen). The membranes were probed using anti-IκBα (catalog no. 9242), anti-pIκB (catalog no. 5295), anti-p100/p52 (catalog no. 9278), anti-ELK1 (catalog no. 4548), or anti-β-actin (catalog no. 5125; Cell Signaling Technology, Beverly, MA), anti-Histone H1 (ab791), anti-phospho-ERK1/2 (ab9102), (Abcam, Cambridge, MA), and anti-α-tubulin (sc-5286; Santa Cruz Biotechnology, Santa Cruz, CA). Anti-goat anti-mouse IgG and anti-rabbit IgG were used to detect Ab binding (Bio-Rad Laboratories). Blots were developed using the SuperSignal West Femto Substrate (Thermo Fischer Scientific, Rockford, IL) and visualized using α-Innotech FluorChem Imaging System (Protein Simple, Santa Clara, CA).

Measurement of BAFF production

Serum BAFF levels of XID or CBA mice were measured using Quantikine BAFF ELISA kit (R&D Systems) according to the manufacturer’s instructions.

Statistical analysis

Data were analyzed using Microsoft Excel. Student t test was used to compare groups. A p value ≤ 0.05 was considered statistically significant.

Results

Btk deficiency correlates with TACI expression and function

We started investigating the involvement of TACI in the severity of impaired immune response to TI-2 Ags in XID mouse by mea-
suring TACI levels in XID and CBA B cells. In addition to TACI, we also measured the levels of the other two BAFF receptors, BAFF-R and BCMA. Flow cytometry experiments revealed that the frequency of TACI-expressing cells was severely reduced ($p = 7.27 \times 10^{-7}$) on XID mouse (17.27% ± 5%) B cells compared with those of CBA mice (45.26% ± 10.58%; Fig. 1A). The frequency of BAFF-R–expressing cells was slightly lower on XID mouse (41.47% ± 21.29%) B cells as compared with CBA mouse cells (59.8% ± 17.20%), but this difference did not reach statistical significance ($p = 0.133$). BCMA expression was comparable in the two mouse strains ($p = 0.162$).

It is well established that BAFF- and APRIL-induced Ig secretion directly correlates with the level of TACI expression on B cells (28–32). To assess whether decreased TACI expression would also lead to a diminished TACI-dependent responses in XID mouse, we measured Ig secretion following BAFF and APRIL stimulation. As anticipated, low TACI expression resulted in severely reduced IgG, IgA, and IgM secretion from XID B cells in response to BAFF or APRIL stimulation. Culture supernatant IgG ($p = 0.0013$), IgA ($p = 0.0008$), and IgM ($p = 0.0008$) levels in BAFF stimulated XID cells were significantly lower than those from CBA cells (Fig. 1B). Similarly, APRIL stimulation resulted in significantly lower levels of IgG ($p = 0.0049$), IgA ($p = 0.0069$), and IgM ($p = 0.0245$) in XID cells as compared with CBA cells. Interestingly, while XID and CBA cells secreted comparable levels of IgG in response to LPS plus IL-4 stimulation, IgM levels in XID cells remained significantly lower ($p = 0.0292$) than those secreted from CBA mouse. The concentration of IgA was also significantly lower ($p = 0.0178$) in XID B cells than the CBA cells following LPS plus TGF-β stimulation.

Because Btk insufficiency primarily affects BCR signaling and previous reports showed that BCR engagement leads to increased TACI expression (28, 29), low TACI expression in XID B cells may be due to ablated Btk signaling. Indeed, whereas the stimulation of CBA B cells with anti-IgM Abs led to a significant increase in TACI expression, XID B cell TACI levels remained unchanged after incubation with anti-IgM Abs (Fig. 1C).

**Analysis of TACI expression and function in B-cell subsets**

We next determined the expression levels of TACI on splenic B cell subsets because it has been shown that XID mouse spleens have reduced numbers of mature B cells (7, 33), and the decrease in TACI$^+$ cell frequency may be a result of altered composition of B cell subsets. We first identified CD93$^+$ (immature) and CD93$^-$ (mature) on CD19$^+$ cells (Fig. 2A). T1 and T2 immature subsets were identified as IgM$^+$IgD$^-$ and IgM$^-$IgD$^+$ cells, respectively. Mature B cell subsets were further identified as MZ (IgM$^{hi}$IgD$^{lo}$) and FO (IgM$^{lo}$IgD$^{hi}$) cells. As reported previously, we found that XID mouse B cells have significantly reduced the percentage of CD93$^-$ mature B cells ($p < 0.001$) and significantly increased percentage of CD93$^+$ immature B cells ($p < 0.001$). Analysis of absolute cell numbers in spleens revealed that XID mice had significantly reduced numbers of the mature B cell subsets, FO, and MZ B-cells than the CBA mice. Next, we determined TACI expression in each subset and found that the percentage of TACI$^+$ cell frequency was significantly less in XID mice than in CBA mice.

**FIGURE 1.** Expression and function of BAFF/APRIL receptors on XID and CBA B cells. (A) Purified splenic B cells from XID or CBA mice were stained with fluorescence-labeled Abs against TACI, BAFF-R, and BCMA together with B220 Ab. Numbers in B220$^+$ TACI$^+$ quadrant indicate mean percentages ± SD from six to ten experiments. (B) Purified splenic B cells of XID or CBA mice were stimulated with media, BAFF, APRIL, LPS plus IL-4, or LPS plus TGF-β for 6 d and culture supernatant IgG, IgA, and IgM concentrations were measured with ELISA. Experiments were repeated three to four times. (C) Purified splenic B cells from XID or CBA mice were stimulated with anti-IgM Abs (10 μg/ml) or left in media for 24 h. The next day, propidium iodide negative (live cells) were stained with fluorescence-labeled Abs against TACI and B220. In each experiment, B cells from one CBA mouse and pooled B cells from three XID mice were used. Numbers in the B220$^+$TACI$^+$ quadrant indicate mean percentages ± SD from three experiments. *$p = 0.05$, **$p = 0.01$, ***$p = 0.001$. Downloaded from http://www.jimmunol.org/ by guest on April 16, 2017
mice regardless of the B cell subset (Fig. 2B). Similarly, TACI mean fluorescence intensity (MFI) values were significantly reduced in all subsets of XID B cells as compared with CBA mouse subsets.

**XID mouse have increased levels of circulating BAFF**

Our previous results showed that newborn BALB/c macrophages express higher levels of BAFF as compared with adult mice (28). Consistent with recent reports (34), serum BAFF levels were higher ($p < 0.002$) in TACI KO mice as compared with the wild type C57BL/6 mouse also (Fig. 3). These observations suggested that there may be an inverse correlation between TACI and BAFF levels. Because we have determined that XID mouse TACI levels are severely reduced compared with wild type mice, we hypothesized that XID mice have increased levels of BAFF. Indeed, measurement of serum BAFF levels in XID and CBA mice demonstrated that XID mouse BAFF levels were significantly higher ($p < 0.0054$) than those of CBA mouse (Fig. 3).

**CpG increases the expression of TACI on XID B cells and renders them sensitive to BAFF and APRIL-stimulation**

Previous experiments have shown that CpG is a strong inducer of TACI expression on mouse B cells (28–30, 35, 36). We therefore investigated whether CpG could increase the frequency of TACI-expressing B cells in XID mouse as well. Incubation of XID mouse splenic B cells for 24 h with CpG resulted in a significant increase ($p < 0.0231$) in the percentage of TACI-expressing cells compared with CBA mice (Fig. 4A). We next measured TACI expression on T1 (CD19$^+$CD93$^+$IgM$^+$IgD$^-$), T2 (CD19$^+$CD93$^+$IgM$^+$IgD$^+$), MZ (CD19$^+$CD93$^-$IgM$^+$IgD$^-$) and FO (CD19$^+$CD93$^-$IgM$^-$IgD$^+$) B cell subsets in CpG treated XID cells and determined that TACI was significantly higher in T1 (67.6% ± 7.7%; $p = 2.89 \times 10^{-5}$), T2 (56.78% ± 20.16%; $p = 0.00176$), MZ (81.93% ± 12.35; $p < 1.38 \times 10^{-5}$), and FO (46.35% ± 24.71%; $p < 5.59 \times 10^{-5}$) subsets as compared with T1 (24.23% ± 3.54%), T2 (9.7% ± 2.09%), MZ (24.9% ± 3.56%), and FO (3.5125% ± 0.910032%) subsets of cells incubated with media (Fig. 4B). Calculation of MFIs of TACI$^+$ cells showed that there also was a statistically significant increase ($p < 0.001$) in the MFI values of TACI$^+$ cells in CpG treated XID cells compared with media treated cells (Fig. 4C).

**FIGURE 2.** Expression of TACI on XID and CBA mice splenic B cell subsets. (A) Purified B cells were stained with CD19, CD93, IgM, and IgD Abs to gate T1 (CD19$^+$CD93$^+$IgM$^+$IgD$^-$), T2 (CD19$^+$CD93$^+$IgM$^+$IgD$^+$), MZ (CD19$^+$CD93$^-$IgM$^+$IgD$^-$) and FO (CD19$^+$CD93$^-$IgM$^-$IgD$^+$) B cells in flow cytometry. Average percentages of mature (CD93$^+$) and immature (CD93$^-$) cell populations are plotted. Absolute numbers of T1, T2, MZ, and FO B cells in XID and CBA mouse spleens are shown. The data are plotted from three CBA and six XID mice. *** $p < 0.001$, showing differences between XID and CBA mice values. (B) Percentage ± SD of TACI$^+$ cells on gated B cell subsets are shown in a representative histogram. The percentages in each subset are average frequencies of TACI$^+$ cells from three CBA and six XID mice. Average TACI MFI values from three CBA and six XID mice are plotted. **$p < 0.001$ showing differences between XID and CBA mice MFI values.

**FIGURE 3.** Serum BAFF levels in XID, CBA, C57BL/6, and TACI KO mice. Serum BAFF levels were measured with ELISA. Average values from three to six mice in each group are plotted, and $p$ value is calculated comparing BAFF levels in XID and CBA mice. **$p < 0.01$. 
cally significant difference between the XID and CBA B cell subsets (Supplemental Fig. 1). As with other immunocompetent mice (28–30), CpG induced a significant increase \((p \leq 0.0414)\) in CBA mouse TACI expression (45.28% ± 12.1%) compared with media-treated B cells (27.9% ± 6.8%; Fig. 4A). Moreover, TACI MFIs (Fig. 4B) and the percentage of TACI+ B cells (Supplemental Fig. 1) in each B cell subset was statistically significantly higher in CpG-stimulated cells as compared with cells left in media. To determine whether CpG induced the proliferation of existing TACI+ cells or increased the expression of TACI on TACI− cells, we performed CpG stimulation experiments on sorted TACI+ and TACI− B cells. Analysis of TACI expression on sorted TACI− cells revealed that the frequency of TACI+ cells (Fig. 5A, 5B) and the MFIs (Fig. 5C) increased significantly in both XID and CBA after CpG stimulation. The increase in TACI− cells was 32-fold in XID mouse and 20-fold in CBA mouse (Fig. 5D). CpG treatment led to a statistically significant increase in the TACI levels of sorted TACI+ XID and CBA cells also (Fig. 5A–5C). This increase was 7.1-fold in XID mouse and 3.2-fold in CBA mouse (Fig. 5D). Collectively, these experiments suggested that CpG treatment resulted in TACI expression on TACI− cells.

Having shown that CpG increases TACI expression on XID B cells, we next investigated whether CpG preexposure sensitizes XID B cells to BAFF and APRIL stimulation. Measurement of Ig concentrations in the culture supernatants of BAFF or APRIL-stimulated B cells that had been pretreated with CpG showed that all three Ig isotypes were increased in XID B cells (Fig. 6). BAFF-induced IgG \((p \leq 0.0285)\), IgA \((p \leq 0.0029)\), and IgM \((p \leq 0.0295)\) levels were significantly higher in CpG-pretreated XID cells as compared with media-preincubated cells. CpG pretreatment also led to a significantly higher levels of IgG \((p \leq 0.0443)\), IgA \((p \leq 0.0003)\), and IgM \((p \leq 0.0191)\) than the media preincubated cells after APRIL stimulation. Wild type CBA mouse cells also secreted higher levels of Igs after CpG pretreatment compared with media-pretreated cells.

FIGURE 4. CpG increases TACI expression on XID and CBA B cells. (A) Purified XID or CBA splenic B cells were pretreated with CpG or media for 24 h, after which the expression of TACI was measured with flow cytometry. The average percentage ± SD of TACI+ cells is shown on a representative histogram from three to four experiments with similar results. (B) Expression of TACI on B cell subsets was also determined on CpG- or media-treated cells. Average MFI ± SD of TACI+ cells is shown on gated populations of T1 (CD19+CD93+IgM+IgD−), T2 (CD19+CD93+IgM+IgD+), MZ (CD19+CD38+IgM+IgD−) and FO (CD19+CD38+IgM−IgD+). Experiments were repeated six times, and mean percentages of TACI+ cells in media- and CpG-treated cells were compared for statistical evaluation. **\(p \leq 0.01\), ***\(p \leq 0.001\).
BAFF and APRIL induced classical NF-κB pathway is ablated in XID B cells

Discovery of impaired TACI-mediated functions in XID B cells led us to investigate the signaling pathways downstream of TACI. Stimulation of B cells through TACI and BCMA results in the activation of classical NF-κB pathway (37–40), and BAFF-R preferentially mediates signals that activate the alternative NF-κB pathway (38, 41–43). Thus, BAFF induces the activation of the alternative and classical NF-κB pathways, whereas APRIL activates only the classical NF-κB pathway. Because we have determined that TACI expression, but not BAFF-R, is severely reduced in XID mouse, we anticipated normal activation of the alternative NF-κB pathway and an impaired activation of the classical NF-κB pathway in BAFF-stimulated XID mouse B cells. APRIL-stimulated cells, on the other hand, are expected to exhibit diminished levels of classical NF-κB pathway activation. Activation of the classical NF-κB pathway was assessed by examining IκBα degradation in BAFF- or APRIL-stimulated XID mouse B cells. APRIL-stimulated cells, on the other hand, are expected to exhibit diminished levels of classical NF-κB pathway activation. Activation of the classical NF-κB pathway was assessed by examining IκBα degradation in BAFF- or APRIL-stimulated XID mouse B cells (12, 13). As predicted, stimulation of CBA B cells with BAFF or APRIL resulted in the degradation of IκBα in the absence of TACI expression (39). Conversely, IκBα degradation was not detected in BAFF- or APRIL-stimulated XID cells (Fig. 7A). Despite the failure of BAFF, APRIL, and anti-IgM to activate the classical NF-κB pathway, the machinery for this pathway was intact because PMA, which activates classical NF-κB pathway independently of Btk or TACI, successfully induced the degradation of IκBα.

Because initial observations suggested that CpG pretreatment restores XID mouse B cell responses to BAFF or APRIL (Figs. 4, 5, and 6), we investigated whether CpG pretreatment would also restore the classical NF-κB pathway in XID cells stimulated with BAFF or APRIL. Indeed, both BAFF and APRIL induced a significant degradation of IκBα in CpG-pretreated XID cells (Fig. 7A). Moreover, CpG pretreatment led to a further increase in the degradation of IκBα in BAFF- and APRIL-stimulated control CBA cells, a likely consequence of its upregulatory effect on TACI-expression (Fig. 7A). Anti-IgM–induced activation of classical NF-κB pathway was also restored in XID B cells after CpG pretreatment (Supplemental Fig. 2). Densitometric analysis of Western blot bands demonstrated that the decrease in IκBα was statistically significant at 45, 90, and 180 min after stimulation of XID B cells with BAFF or APRIL compared with those that were not pretreated with CpG (Fig. 7B). Similarly, CpG pretreatment of XID cells led to a statistically significant decrease in IκBα at 90 and 180 min in anti-IgM stimulated cells.

Although XID and CBA mice have comparable numbers of MZ B cells, XID mouse spleens significantly reduced numbers FO B cells than those of CBA mouse (Fig. 2A). To exclude a possible contribution of this altered B cell composition to the signaling defect observed in total B cell analysis, we assessed classical NF-κB activation in purified MZ and FO B cells stimulated with BAFF. As predicted, APRIL stimulation induced a significant reduction in the band-density of MZ (p ≤ 0.03) and FO (p ≤
0.006) B cell subsets of CBA mice. Confirming the results obtained in total splenic B cells, there was no change in the band density of IκBα protein in MZ (p = 0.9) or FO (p = 0.4) XID B cells after APRIL stimulation. Thus, in XID B cells, even the mature B cell subsets, MZ and FO cells do not respond to APRIL.

BAFF-induced alternative NF-κB pathway signaling is intact in XID B cells

Unlike APRIL, BAFF activates both the classical and alternative NF-κB pathways because it binds to BAFF-R in addition to the APRIL receptors TACI and BCMA (25, 38, 41, 43). Given the fact that our flow cytometry analysis showed severely reduced expression of TACI while BAFF-R expression was maintained in XID B cells, we anticipated a preserved alternative NF-κB signaling downstream of BAFF-R. We measured total p100 and p52 levels as well as nuclear RelB levels in BAFF-stimulated XID or CBA B cells Western blot. Confirming previous reports, in CBA mice, BAFF stimulation induced a significant decrease in p100 and an increase in p52 (p52/p100 ratio; p ≤ 0.02), and a significant increase in RelB protein (p = 0.00497) as compared with cells left in media (Fig. 8A). Stimulation of XID cells with BAFF led to a significant change in p52/p100 ratio (p = 0.02) along with an increase in RelB protein (p = 0.0369) in the nuclear fraction. In addition to assessing alternative NF-κB signaling in total splenic B cells, we also measured p100, p52, and RelB levels in BAFF-stimulated MZ and FO cells from both the strains to exclude the possibility of a contribution of reduced mature B cell profile in XID cells. As expected, BAFF induced a statistically significant p52/p100 band density change in CBA mice MZ (p = 0.0002) and FO (p = 0.0003) subsets after BAFF stimulation and RelB band density was higher in BAFF stimulated MZ (p = 0.0043) and FO (p = 0.0016) subsets than the media exposed cells. Thus, it appears that either TACI downregulation or further blockage of TACI signaling are responsible for the ablation of BAFF- or APRIL-induced classical NF-κB pathway activation in XID mouse, whereas the deficiency in Btk does not change BAFF-R expression and does not impair BAFF-R–mediated alternative NF-κB activation.

BAFF and APRIL fail to activate MAP kinase pathway in XID mouse

Although the data obtained thus far are in support of a correlation between CpG-induced upregulation of TACI and an increase in BAFF- or APRIL-induced NF-κB activation in XID mice, we cannot exclude the possibility of a role for Btk downstream of TACI. Previous reports have shown that anti-IgM and BAFF activate the MAPK pathway in B cells (44, 45). It has also been shown that although anti-IgM–induced NF-κB activation is Btk dependent, ERK1/2 phosphorylation is not (44, 45). Because our results with BAFF- or APRIL-induced classical NF-κB pathway activation in XID mouse does not distinguish between a signaling block owing to a possible involvement of Btk downstream of TACI and decreased TACI levels, investigation of MAPK pathway could help to dissect these two possibilities because its signaling does not depend on Btk. In line with published results (44, 45), we also detected the phosphorylation of ERK1/2 in CBA and XID mice 5 min after anti-IgM stimulation (Fig. 9A). BAFF-induced ERK1/2 was demonstrated in normal mouse B cells previously, and BAFF-R was implicated in transducing these signals (46). APRIL-induced ERK1/2 phosphorylation has been shown in monocytes and adipocytes (47, 48), but its effect on B cell–ERK1/2 phosphorylation has not been reported. Therefore,
we first analyzed the ERK1/2 phosphorylation in APRIL-stimulated CBA B cells and showed that APRIL also activates the MAPK pathway in B cells (Fig. 9A). Detection of ERK1/2 phosphorylation at 24 h indicated that APRIL induces the activation of MAPK pathway significantly later than anti-IgM does. These observations also suggested that APRIL and BAFF have similar kinetics because Otipoby et al. (46) reported ERK1/2 phosphorylation 24 h after BAFF stimulation. Contrary to CBA cells, stimulation of XID B cells with APRIL did not lead to a detectable ERK1/2 phosphorylation (Fig. 9B). Because ERK1/2 phosphorylation is independent of Btk, the absence of ERK1/2 phosphorylation in APRIL-stimulated B cells from XID mice suggests that decreased TACI expression is likely to play a role in impaired TACI signaling. We further investigated this possibility by assessing ERK1/2 phosphorylation in CpG-pretreated XID cells because, as shown in Figs. 3, 4, and 5, CpG prestimulation results in increased TACI expression along with increased TACI-mediated functions. Indeed, stimulation of CpG-preincubated XID B cells with APRIL resulted in the phosphorylation of ERK1/2 at 24 h (Fig. 9B). We ruled out the involvement of BCMA in APRIL-induced ERK1/2 phosphorylation, because stimulation of TACI KO B cells with APRIL did not lead to ERK1/2 phosphorylation even after CpG pretreatment (Fig. 9C). Although slightly decreased as compared with phosphorylation seen in CBA mouse, ERK1/2 phosphorylation was detected in XID B cells stimulated with BAFF (Fig. 9B). This slight decrease in ERK1/2 phosphory-

FIGURE 7. CpG pretreatment restores the activation of the classical NF-κB pathway in BAFF- or APRIL-stimulated XID B cells. (A) Activation of classical NF-κB pathway was assessed in Western blot analysis by using Abs against IκBα in BAFF- or APRIL-stimulated CBA or XID cells. Purified B cells were incubated for 24 h in media or CpG prior to stimulation in the presence of 15–50 μg/ml cycloheximide. PMA and anti-IgM–stimulated cells’ IκBα degradation in XID mouse B cells is shown as a control. Cycloheximide was present in the culture media of stimulated cells when measuring IκBα degradation. Protein loading is assessed by using Abs against α-tubulin. One experiment out of three to six experiments with similar results is shown. (B) Band density analysis of IκBα degradation in BAFF-, APRIL-, and anti-IgM–stimulated XID B cells with and without CpG prestimulation. Average data from four to six experiments are plotted. Statistical evaluation is done in conditions with and without CpG. (C) Pooled B cells from three to five CBA or XID spleens were sorted by flow cytometry into MZ and FO subsets. After a 30-min incubation with 15–50 μg/ml cycloheximide, the subsets were then incubated in media or stimulated with 1 μg/ml of APRIL for 180 min in the presence of cycloheximide. Changes in IκBα protein were analyzed in Western blot analysis. Average ratio of IκBα/α-tubulin band density measurements from five to seven replicates were plotted, and the values of media exposed and APRIL-stimulated subsets were compared for statistical evaluation. *p ≤ 0.05, **p ≤ 0.01.
The alternative NF-κB pathway is activated in BAFF-stimulated XID B cells. (A) Alternative NF-κB pathway activation in CBA or XID B cells is assessed by determining the total p100, p52, and nuclear Rel-B proteins 24 h after BAFF stimulation. The ratio of p52 to p100 was detected by Western blot, and α-tubulin is shown as a loading control. Nuclear translocation of RelB is detected in the nuclear extract and Histone [3H] is shown as a loading control. Average p52/p100 ratio in media-exposed cells from three separate experiments was compared with p52/p100 ratio in BAFF-stimulated cells for statistical evaluation. To quantify the change in RelB band density, the average ratio of RelB band density to Histone [3H] band density (RelB/Histone [3H]) from three separate experiments is plotted. (B) Alternative NF-κB pathway activation was also analyzed in BAFF-stimulated sorted FO and MZ B cell subsets. One representative Western blot analysis out of four experiments with similar results is shown. Average p52/p100 ratio in media-exposed or BAFF-stimulated B cell subsets from four separate experiments is plotted. Similarly, RelB/Histone [3H] ratio in media-exposed and BAFF-stimulated B cell subsets is plotted. The values in media-exposed cells were compared with those of BAFF-stimulated cells for statistical evaluation. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

BAFF-R. Finally, preincubation of XID cells with CpG also led to increased ERK1/2 phosphorylation in BAFF-stimulated cells. Upregulated TACI expression is likely to be responsible for the increase in signaling induced by BAFF.
CpG renders XID mouse responsive to NP-Ficoll

Results obtained thus far suggest that impaired TACI expression and signaling may be, at least in part, responsible for the unresponsiveness of XID mouse to TI-2 Ags. Because we demonstrated that CpG exposure increases TACI expression on XID B cells and renders them susceptible to BAFF or APRIL stimulation, we next sought to determine the in vivo effect of CpG on the development of Abs against the prototype TI-2 Ag NP-Ficoll. As expected, XID mice immunized with NP-Ficoll alone manifested a modest increase in IgG or IgM Abs (Fig. 10). Immunization of XID mice with CpG and NP-Ficoll, on the other hand, led to the development of significantly increased levels of serum anti-NP IgG Abs. The level of IgG Abs in XID mice immunized with NP-Ficoll alone manifested a modest increase in IgG or IgM Abs (Fig. 10). Immunization of XID mice with CpG and NP-Ficoll, on the other hand, led to the development of significantly increased levels of serum anti-NP IgG Abs. The level of IgG Abs in XID mice immunized with CpG containing NP-Ficoll was significantly higher than in the NP-Ficoll immunized mice on days 7 (p ≤ 0.0194), 15 (p ≤ 0.0058), and 30 (p ≤ 0.0120). XID mice immunized with CpG and NP-Ficoll had higher levels of IgM Abs than the NP-Ficoll immunized mice on day 7 (p ≤ 0.0295), after which Ab levels dropped. The kinetics of IgG and IgM Ab responses in CBA mice paralleled those of XID mice, although their magnitudes were significantly higher.

Discussion

Experiments were designed to test whether a change in TACI expression and function is responsible for the unresponsiveness of XID mice to TI-2 Ags. A point mutation in the pleckstrin homology domain of \textit{btk} in an XID mouse leads to ablated BCR signaling and a decrease in mature B cells in the periphery. A more severe form of XID-phenotype is observed in a Btk KO mice generated on a 129/Sv X C57BL/6 background (12, 13). Total gene analysis of B cells from Btk KO and XID mice revealed that a subset of genes differentially expressed as a result of Btk insufficiency were significantly more altered in Btk KO cells than in the XID cells (49). Nevertheless, NF-κB activation is severely impaired in B cells from both the strains upon BCR stimulation (12, 13). More importantly, the profiles of Ab responses to TD and TI-2 Ags are similar in XID and BTK KO mice (7, 33). Both the strains fail to mount a measurable Ab response to TI-2 Ags, and they mount comparable levels of Abs against TD Ags. The diminished BCR signaling and the decrease in mature B cell populations appear particularly to affect immune responses to TI-2 Ags, whereas Ab response to TD Ags is partially compensated with CD40-CD40L-mediated costimulation (6, 50, 51). It is not entirely clear why impairment in BCR signaling primarily affects immune response to TI-2 Ags. We hypothesized that, as with newborn mouse (28), unresponsiveness of XID mouse to TI-2 Ags may be due to low TACI expression. Indeed, analysis of XID B cells revealed that not only their B-cell TACI levels were significantly reduced as compared with CBA B cells but they also responded poorly to BAFF and APRIL stimulation (Fig. 1). The decrease in mature B cells in XID mice cannot alone explain the reduced TACI+ cell frequency, because we measured reduced TACI expression on T1, T2, MZ,
and FO subsets of XID B cells. Thus, TACI expression was lower regardless of the maturation stage of B cells in XID mouse.

Similar to what was observed in newborn mouse (28), low TACI expression correlated with a decrease in TACI-mediated functions in XID mice. B cells from XID mice did not secrete IgGs in response to BAFF or APRIL. In addition to a decrease in TACI expression and an ablated BAFF- and APRIL-induced Ig secretion, signaling mediated by TACI was also impaired in XID mice. Both the classical and alternative NF-κB activation pathways are induced by BAFF, whereas APRIL only induced classical NF-κB pathway (37–43). Although BCMA is also implicated, TACI is mostly responsible for APRIL-induced activation of the classical NF-κB pathway (37–40). Western blot analysis demonstrated that the classical NF-κB pathway was activated by APRIL and BAFF in CBA mice. In contrast, both APRIL and BAFF failed to activate the classical NF-κB pathway in XID B cells. In APRIL-stimulated cells, TACI is likely to be responsible for the IkBα degradation because the percentage of BCMA-expressing cells is extremely low in the spleen (<5%) and APRIL cannot induce IkBα degradation in TACI KO mouse. We eliminated a reduced number of mature B cells as a reason for ablated IkBα degradation in XID mice because analysis of the classical NF-κB pathway in purified MZ and FO B cell subsets demonstrated that APRIL was unable to activate this pathway, even in mature B cell subsets. Next, we reasoned that if increased expression of TACI in XID cells resulted in the restoration of TACI-mediated signaling, reduced TACI levels were likely to be responsible for the impairment of TACI-mediated signaling. To test this hypothesis, we stimulated XID B cells with CpG because TLR agonists and especially CpG can strongly upregulate TACI expression (28, 29). Indeed, CpG-stimulated XID B cells not only had increased TACI expression; they also secreted IgGs in response to APRIL stimulation. Moreover, signaling through the classical NF-κB pathway was restored in APRIL-stimulated XID cells after CpG prestimulation. The ability of CpG to boost TACI expression in XID B cells is noteworthy because previous reports have indicated that Btk is involved in TLR9 signaling (10, 52).

Interestingly, despite a reduction in p65 subunit of NF-κB in TLR9-activated B cells, IkBα degradation and the activity of IFN-stimulated response elements were preserved (10, 52). Moreover, CpG induced higher levels of IL-12 and IL-6 from XID and Btk KO B cells (11, 52). Our results complement these reports and show that the TACI-upregulatory effect of CpG is also independent of Btk.

The fact that CpG prestimulation increased TACI expression and restored TACI signaling despite the presence of the btk mutation strengthens reduced TACI expression as the reason for the impaired TACI signaling in XID mouse. However, this observation still does not eliminate the possibility of a role for Btk downstream of TACI because others have previously shown that anti-IgM–induced B cell proliferation can be restored in XID mice in the presence of second signals such as CD40L, LPS, and CpG through a mechanism called “receptor cross-talk” (53–55). According to Guo et al. (56), in addition to the signalosome-dependent (classical) pathway, a signalosome-independent (alternative) pathway is activated by second signals (such as CpG) in BCR-stimulated cells. We therefore sought to explore whether CpG allows the bypassing of Btk downstream of TACI in APRIL-stimulated XID B cells. To differentiate the role of TACI expression levels from downstream signaling blockages in APRIL-stimulated XID B cells, we investigated the MAPK pathway because it has been shown that activation of this pathway in anti-IgM–stimulated B cells is independent of Btk (44, 45). We first determined that the MAPK pathway is involved in TACI-mediated signaling by demonstrating the phosphorylation of ERK1/2 in APRIL-stimulated CBA B cells. Stimulation of XID B cells with APRIL confirmed the involvement of TACI in APRIL-induced MAPK pathway because APRIL failed to phosphorylate ERK1/2 in XID mice. Because the MAPK pathway is known to be independent of Btk, the absence of ERK1/2 phosphorylation is likely to be due to the low TACI expression in XID mouse. Supporting this hypothesis, we found that ERK1/2 phosphorylation was restored after the stimulation of CpG-pretreated XID B cells with APRIL.
Unlike APRIL-stimulated cells, the interpretation of results in BAFF-stimulated cells is more complex because, whereas it is well established that BAFF-R is responsible for the transduction of signals leading to the alternative NF-κB pathway (41–43), the receptors transducing BAFF-induced classical NF-κB is less clear. By demonstrating IκBα degradation in BAFF-stimulated TACI KO B cells, Shinners et al. (57) suggested that only BAFF-R is responsible for BAFF-induced classical NF-κB activation. Similarly, two other studies suggested a role for BAFF-R in BAFF-induced activation of classical NF-κB pathway (58, 59), Enzler et al. (37), however, reported that although all three receptors can transduce signals that lead to BAFF-induced classical NF-κB activation, the involvement of BAFF-R is minimal. Finally, Morrison et al. (43) demonstrated that the alternative but not the classical NF-κB pathway is activated through BAFF-R. We found that XID BAFF-R levels are comparable to those of CBA, whereas TACI expression is significantly reduced. Like us, Shinners et al. (57) also showed that BAFF-R levels were not reduced in XID; however, they did not report TACI levels. In the same study, Shinners et al. (57) also showed that activation of the classical NF-κB pathway is ablated in BAFF-stimulated XID B cells. Based on these observations, they concluded that the blockage of the classical NF-κB pathway indicates a role for Btk downstream of BAFF-R (57). We believe that Btk is unlikely to be downstream of BAFF-R because by analyzing p100, p52, and Rel-B levels, we found that BAFF-induced activation of alternative NF-κB pathway was not affected in XID cells. We also analyzed the alternative NF-κB signaling in mature B cell subsets (MZ and FO cells) of XID mice to exclude the possibility of a skewed response caused by reduced numbers of mature B cells in the XID splenic pool. As in total B cells, subset analysis also showed intact NF-κB signaling in BAFF-stimulated XID cells.

As in APRIL-stimulated cells, low TACI expression is likely to be responsible for the amination of the classical NF-κB pathway in BAFF-stimulated XID B cells. At the same time, both TACI and BAFF-R are likely to mediate MAPK pathway-inducing signals because, although lower than the CBA cells, XID mouse ERK1/2 phosphorylation was detected after BAFF stimulation. This decrease in ERK1/2 phosphorylation may be caused by the absence of TACI signaling in XID mice. Consequently, upregulation of TACI B cell TACI expression by CpG is likely to be responsible for the restoration of BAFF-induced classical NF-κB pathway and increased MAPK pathway activation.

In this study, we also demonstrated that CpG containing NP-Ficoll vaccine is able to induce an anti-NP IgG and IgM Ab response in otherwise unresponsive XID mice (5, 60). The generation of an Ab response to a TI-2 requires the engagement of a BCR on an Ag-specific B cell with Ag and co-stimulation through a second signal such as TACI. We have shown previously that in normal newborn (28) and adult (29) mice, the adjuvant effect of CpG on TI-2 Ags depends on TLR9 and TACI. In adult XID and normal newborn mice, it is likely that BCR signaling is restored by CpG because of the receptor crosstalk phenomenon (53, 54). At the same time, CpG-induced upregulation of TACI allows BAFF and APRIL to provide the second signal. These results resemble immunization studies with polysaccharide and CD40L in which simultaneous injection of CD40L with pneumococcal polysaccharides or NP-Ficoll augmented Ab response in normal mouse and restored Ab response in XID mice (61, 62). Like CpG, CD40L also provides receptor crosstalk signals (54) and induces the upregulation of TACI (29). We should also point out that CpG-mediated restoration of anti-NP IgG and IgM response was partial. This is probably due to a severe reduction in the B1 cell subset in XID mice.

In contrast to the impaired B cell TACI expression, and in agreement with a recently reported study (34), we determined that the TACI ligand BAFF was significantly increased in the sera of XID mice. This inverse relationship between BAFF and TACI levels is also seen in newborn mice (28) and TACI KO mice (Fig. 3). Supporting the observations in mice, increased BAFF expression is reported by Jin et al. (63) in patients with X-linked agammaglobulinemia who, like XID mice, also have a mutation in the pleckstrin homology domain of the btk gene. Another case in which impaired TACI signaling is accompanied by high serum BAFF is patients with combined variable immune deficiency who have mutations in their TACI genes (63, 64). Kreuzaler et al. (65)—who studied primary B cell defects such as Btk, BAFF-R, or TACI deficiencies—suggested a connection between low B cell numbers or decreased BAFF/APRIL receptors and high serum BAFF levels. We believe that it is the decreased TACI expression rather than the low B cell numbers that is responsible for the increased serum BAFF levels, because TACI KO mice have significantly increased levels of circulating BAFF despite having an increased number of B cells (23, 24). Because TACI is mostly expressed on B cells, a consequence of decreased B cell numbers will be a reduction in available TACI. Thus, the increase in circulating BAFF resulting from low B cell numbers is likely due to a simultaneous decrease in the number of TACI molecules.

Unlike adult B cells, B cells from newborns are known to proliferate poorly in response to BCR engagement (66). In many ways, XID B cell responses resemble those of newborn mice (67). For example, anti-IgM–induced B cell proliferation can be restored in both age groups by second signals such as LPS, IL-4, CD40L, or CpG (53, 54, 68–70). Because Btk deficiency primarily affects the NF-κB pathway, and because cells from XID mice and newborns proliferate poorly in response to BCR stimulation, it is likely that the newborn NF-κB pathway is also impaired. Moreover, similar to results obtained in the current study with XID B cells, we have shown previously that unlike adult B cells, crosslinking of the BCR on newborn B cells does not increase TACI expression (28). Consequently, as in XID mice, impaired BCR-induced NF-κB pathway activation may be responsible for the severely reduced TACI expression in newborn mice (66, 69, 71, 72). Similarly, both newborn and XID B cell BAFF-R levels are comparable to their respective control mice, suggesting that in both mice, the pathway responsible for controlling BAFF-R expression remains functional and is not dependent on NF-κB. In a recently published study, Rowland et al. (73) showed that immature B cells with reduced surface IgM express lower levels of BAFF-R. They showed similarly reduced BAFF-R levels in a transgenic mouse strain (hypomorphic Ig-α strains) expressing reduced amounts of surface IgM. Based on these observations, they concluded that surface IgM levels and tonic BCR signaling controls BAFF-R expression. Their study did not include the analysis of reduced IgM levels and tonic BCR signaling in TACI expression. The engagement of BCR results in the activation of four signaling pathways: NF-κB, NF of B cells, MAPK, and mammalian target of rapamycin pathways (74). Because the btk mutation primarily affects the NF-κB pathway and TACI is severely reduced in XID mouse, BCR-induced NF-κB activation is likely to control TACI expression. The fact that BAFF-R levels are normal in XID mice suggests that pathways not affected by Btk insufficiency are likely to mediate BCR-induced control of BAFF-R expression.
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental figure 1. The effect of CpG on TACI expression in XID and CBA B cells. Purified XID or CBA splenic B cells were pretreated with CpG or media for 24 hrs, after which, the expression of TACI on of T1 (CD19^+CD93^+IgM^+IgD^-), T2 (CD19^+CD93^+IgM^+IgD^+), MZ (CD19^+CD93^+IgM^{hi}IgD^{lo}) and FO (CD19^+CD93^- IgM^{lo}IgD^{hi}) B cell subsets were measured in flow cytometry. Average frequency of TACI+ cells from six experiments for media and CpG treated cells were compared for statistical evaluation. **p ≤ 0.01, and ***p ≤ 0.001.
Supplemental figure 2. CpG restores the activation of classical NFκB pathway in anti-IgM stimulated XID B cells. The effect of CpG to restore anti-IgM induced classical NFκB pathway in XID B cells was assessed as a control for the evaluation of the effect of CpG on BAFF and APRIL induced classical NFκB pathway activation (Figure 7). (A) Activation of classical NFκB pathway was assessed in Western blot analysis by using Abs against IκBα in anti-IgM stimulated CBA or XID cells. In some experiments purified B cells were pre-stimulated with CpG for 24 hrs prior to stimulation with anti-IgM. Cyclohexamide was present in the culture media of stimulated cells when measuring IκBα degradation. Protein loading is assessed by using Abs against α-tubulin. One experiment out of three experiments with similar results is shown.