Bruton’s Tyrosine Kinase Separately Regulates NFκB p65RelA Activation and Cytokine Interleukin (IL)-10/IL-12 Production in TLR9-stimulated B Cells*

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Koon-Guan Lee1†, Shengli Xu1†, Ee-Tsiong Wong9, Vinay Tergaonkar1, and Kong-Peng Lam1‡

From the 1Laboratory of Immunology, Bioprocessing Technology Institute and the 2Institute of Molecular and Cell Biology, Agency for Science, Technology, and Research (A*STAR), Singapore 138648, Singapore

B lymphocytes express both B cell receptor and Toll-like receptors (TLR). We show here that Bruton’s tyrosine kinase (Btk), a critical component in B cell receptor signaling, is also involved in TLR9 signaling in B cells. Stimulation of B cells with TLR9 ligand CpG oligodeoxynucleotide (ODN) leads to transient phosphorylation of Btk, and in the absence of Btk, TLR9-induced proliferation of B cells is impaired. Interestingly, Btk−/− B cells secrete significantly more interleukin (IL)-12 but much less IL-10 compared with wild type B cells upon TLR9 stimulation. Immunization of Btk−/− mice with CpG ODN also leads to elevated levels of IL-12 in vivo and consequently, a greater -fold increment in the production of Th1 type IgG2b and IgG3 antibodies in these mice compared with wild type controls. The addition of exogenous recombinant IL-10 could suppress IL-12 production by TLR9-activated Btk−/− B cells, suggesting that in B cells, Btk negatively regulates IL-12 through the induction of autocrine IL-10 production. TLR9 signaling also leads to the activation of NFκB, including the p65RelA subunit in wild type B cells. The lack of Btk signaling affects the activation of NFκB and impairs the translocation of the p65RelA subunit to the nucleus of B cells upon TLR9 stimulation. However, p65RelA−/− B cells could respond similarly to wild type B cells in terms of IL-10 and IL-12 secretion when stimulated with CpG ODN, suggesting that the defect in NFκB p65RelA activation is additional to the impairment in cytokine production in TLR9-activated Btk−/− B cells. Thus, Btk plays an important role in TLR9 signaling and acts separately to regulate NFκB RelA activation as well as IL-10 and IL-12 production in B cells.

Bruton’s tyrosine kinase (Btk), a member of the Tec family of protein-tyrosine kinases, has been shown to play important roles in B cell development, activation, and survival. Mutations in Btk are known to lead to X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice (1). These diseases are characterized by a block in B-lymphopoiesis and CD5+ B-1 cell generation, defects in B cell receptor (BCR) signaling, and impairment in humoral immune responses to certain types of T cell-independent antigens (2). Structurally, Btk contains multiple protein-protein interaction domains. It has a pleckstrin homology domain for membrane localization following its activation, as well as Src homology 2, Src homology 3, and proline-rich domains for binding other signaling molecules. In addition, it possesses multiple tyrosine phosphorylation sites. Hence, Btk is postulated to play a key role in signal transduction processes.

Signaling by various receptors in B cells activates Btk. These receptors include immune receptors, such as the BCR and the receptor for IgE, FcεRII (3), as well as various cytokine receptors (4, 5). B lymphocytes also express innate immune receptors, such as the Toll-like receptors (TLR). Presently, it is not quite clear whether Btk is also activated by the various TLR and whether it is involved in TLR signaling in B cells given that Btk is critically required for signaling by the BCR.

TLR are innate immune receptors that recognize molecular patterns that are unique to and invariant among entire classes of microbes. Such pathogen-associated molecular patterns are found on LPS, bacterial lipopolysaccharides and lipoteichoic acids, flagellin, and unmethylated CpG DNA of bacteria and viruses (6). Different pathogen-associated molecular patterns are recognized by different TLR (e.g. LPS is recognized by TLR4 in conjunction with other molecules, such as CD14, whereas CpG DNA is recognized by TLR9 and flagellin is recognized by TLR5). Most TLR are plasma membrane-bound receptors, but a few, such as TLR3, -7, and -9, are found in the endosomes. All TLR share a common structural feature in their cytoplasmic portions in the form of a Toll/IL-1 receptor domain that is critical for signal transduction. Engagement of TLR by ligands triggers a signaling cascade that involves the activation of adapter molecules, such as MyD88, TRIF, TIRAP, and TRAM as well as IL-1R-associated kinase and TNFR-associated factor 6, which leads ultimately to the activation of NFκB and MAP kinase signaling pathways (7).

Since signaling by the TLR is complex, it is conceivable that more molecules could be involved in their signal transduction process. Some of these molecules could be shared components with other signal transduction systems, such as the BCR signaling system. This may be especially so in the context of a B cell, which expresses both BCR and TLR. One obvious and possible shared candidate between BCR and TLR signaling would be...
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Btk. The notion that Btk might be involved in TLR signaling was suggested by the previous finding that peritoneal macrophages derived from X-linked immunodeficiency mice exhibited reduced responses to LPS (8). It was also shown recently that Btk was involved in NFκB activation in macrophages stimulated by LPS (9, 10). However, LPS binds both TLR4 and CD14 (11, 12), hence making it ambiguous whether Btk is indeed involved in TLR signaling per se. Moreover, most studies of Btk involvement in TLR signaling had been done in macrophages and dendritic cells (13–15), and it is not clear if Btk is involved in TLR signaling in B cells. Furthermore, even if Btk is involved in signaling by TLR4 (10) that is expressed on the plasma membrane, it is not clear whether Btk is involved in signaling by the endosome-located TLR, such as TLR9. To definitively assess whether Btk is involved in TLR signaling and to determine the effect of a lack of Btk in TLR engagement in B cells, we have now examined the role of Btk in signal transduction by TLR9, which is a homotrimeric receptor located in the endosomes and activated by cellular recognition of unmethylated CpG-DNA. We show here that Btk deficiency impairs TLR9-induced cellular proliferation of B cells. Furthermore, the absence of Btk signaling during TLR9 engagement also altered B cell production of pro- and anti-inflammatory cytokines, such as IL-12 and IL-10, respectively, and biased the sera antibody profile in Btk−/− mice toward that of a Th1 type, since there was greater production of IgG2b and IgG3 antibodies. At a molecular level, the lack of Btk signaling leads to defective NFκB activation and to the nuclear translocation of the p65RelA subunit in TLR9-activated B cells. This defect is additional to the impairment in cytokine production in Btk−/− B cells, since B cells lacking p65RelA could respond in a normal manner as wild type B cells in terms of IL-10 and IL-12 production when treated with CpG ODN. Thus, Btk separately regulates NFκBp65RelA activation and cytokine production during TLR9 engagement in B cells.

EXPERIMENTAL PROCEDURES

Mice—Wild type C57BL/6 and Btk−/− mice were obtained from the Jackson Laboratory (Bar Harbor, ME). NFκBp65RelA−/− mice were obtained from Dr. Amer A. Beg (University of South Florida) and bred and housed in our facilities. Experiments with mice were performed according to guidelines issued by the National Advisory Committee on Laboratory Animal Research.

Preparation and Stimulation of B Lymphocytes—Single cell suspensions were prepared from spleens of mice and treated with red blood cell lysis solution (0.15 m NH₄Cl, 0.1 mM Na₂EDTA, pH 7.2). B cells were obtained to >90% purity using a negative selection protocol with anti-CD43 mAb-coupled magnetic beads (Miltenyl Biotech). B cells were cultured in 48-well flat bottom plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and stimulated with CpG ODN 1826 (InvivoGen, San Diego, CA), LPS 0111:B4 (Sigma), or goat anti-mouse IgM F(ab′)₂ fragment (Jackson Immunoresearch Laboratories). For proliferation assays, cells were stimulated for 48 h in 96-well round bottom plates and pulsed with [³H]thymidine (1 µCi/well) for a further 6 h before harvest. Cell proliferation was quantified by a Micro-Beta TriLux scintillation counter. For cell stimulation in Western blot analyses, 1 × 10⁷ cells suspended in Opti-MEM medium (Invitrogen) were incubated with 10 µg/ml anti-IgM antibodies, 100 ng/ml LPS, or varying concentrations of CpG ODN at 37 °C for various periods of time.

Immunoprecipitations and Western Blot Analyses—Spleenic B cells were lysed on ice for 30 min in phosphorylation buffer containing 1% Nonidet P-40, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.2 mM Na₃VO₄, and a mixture of protease inhibitors (Roche Applied Science) and sonicated. Cell homogenates were centrifuged at 13,000 rpm for 15 min at 4 °C, and supernatants were recovered for protein quantification by Bayer protein assay kit (Pierce). For immunoprecipitation studies, antibodies were first coupled to Protein A/G Plus-agarose (SC-2003; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C overnight. The beads were washed twice in lysis buffer and incubated with precleared cell lysates for 1 h at 4 °C. Subsequently, beads were boiled in loading buffer for 5 min to release the proteins. For Western blot analyses, 30 µg of whole cell lysates or 5 µg of nuclear extracts were electrophoresed in 10% SDS-polyacrylamide gels and transferred onto immunoblot polyvinylidene difluoride membranes (Millipore, MA). The membranes were subsequently probed with antibodies against various signaling molecules. The anti-Btk, anti-ERK2, anti-IκBα, anti-HDAC1, and anti-RelA antibodies were from Santa Cruz Biotechnology. The anti-phosphotyrosine (4G10) antibody was from Upstate Biotechnology. Densitometric analyses of Btk phosphorylation was carried out using a Bio-Rad imaging densitometer and multianalysis software, and pixel intensity (absorbance units/mm²) was normalized to the corresponding total protein and expressed as n-fold increase in intensity over the control nonstimulated sample.

Electrophoretic Mobility Shift Assays—Cells were stimulated at 5 × 10⁶ cells in Opti-MEM medium and processed with NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The nuclear extract (5 µg) was incubated with [γ²³²P]ATP end-labeled, double-stranded probe containing two tandem NFκB-binding sites, 5′-CATGCGCTGGGAAAGTCCCCTCAACT-3′ and 5′-CATGAGTTGAGGGGACTTTCCCAGGC-3′. The reaction was performed in 20 μl of binding buffer (60 mM Hepes, pH 7.9, 20 mM Tris-HCl, pH 7.9, 300 mM KCl, 150 mM NaCl, 25 mM MgCl₂, 25 mM dithiothreitol, 62.5% glycerol, and 1 mg of poly(dl-dC)) for 30 min on ice. After incubation, samples were resolved in a 5% native gel for 3 h. The gel was dried for 1 h and exposed to autoradiography film. To verify equal amounts of proteins used in the nuclear extracts, a control oligonucleotide for NF-Y was used.

Measurement of Cytokine Production by ELISA—Untreated or CpG ODN-stimulated wild type and Btk−/− B cells were cultured in 48-well plates at 1 × 10⁶ cells/ml. At 16 h post-stimulation, supernatants were harvested and stored at −20 °C or immediately assayed for cytokine production. To measure serum IL-12 level in vivo, mice were given a single dose of 50 µg CpG ODN and bled every 2 h. The concentrations of IL-6, IL-10, IL-12p40, and IL-12p70 as well as IL-23p19 were determined using commercial ELISA kits (BD Pharmingen, San Diego, CA).
**RESULTS**

**Btk Is Transiently Phosphorylated upon CpG ODN Stimulation of B Cells**—To determine if Btk is involved in TLR9 signaling in B cells, we first examined if Btk is phosphorylated and, hence, activated upon CpG ODN stimulation of murine primary B lymphocytes. We treated purified wild type splenic B cells with either anti-IgM antibodies that cross-linked the BCR, LPS that engaged TLR4/CD14, or CpG ODN that stimulated TLR9. As expected and shown in Fig. 1A, Btk was activated, as indicated by its tyrosine phosphorylation status, upon stimulation of B cells via the BCR. LPS treatment of B cells also led to the activation of Btk, although its phosphorylation pattern appeared to be of shorter duration compared with that induced by the anti-IgM antibodies (Fig. 1B). Likewise, stimulation of B lymphocytes with CpG ODN also led to a transient phosphorylation of Btk at the 5 min time point, as evidenced by the Western blot analysis and quantification of Btk phosphorylation by densitometry (Fig. 1C). Thus, the data indicate that Btk is transiently activated upon TLR9 engagement and suggested that Btk could be involved in some aspects of TLR signaling.

**Btk Deficiency Impaired CpG ODN-induced Cellular Proliferation of B Lymphocytes**—Since Btk was transiently phosphorylated upon the engagement of TLR4 and TLR9, we were interested in elucidating the physiological role that Btk played during TLR activation in B cells. We focused on TLR9 as a model system, since this TLR had been implicated in various B cell autoimmune diseases (16–18), and the role of Btk in TLR9 signaling had not been well studied. We first examined the ability of Btk−/−B cells to up-regulate the expression of cell surface activation markers in response to TLR9 engagement. Our data indicated that wild type and Btk−/−B cells could up-regulate the expression of CD25 (IL-2Rα), CD69 (early activation marker), CD86 (B7.2), and major histocompatibility complex class II antigens after overnight treatment with CpG ODN (data not shown). This suggested that the absence of Btk did not impair TLR9-induced up-regulation of activation markers in B cells.

We next determined the ability of Btk−/−B cells to undergo cellular proliferation upon CpG ODN treatment (Fig. 2A). Wild type B cells proliferated in a dose-dependent manner upon CpG ODN stimulation, similar to their responses to treatment with LPS and anti-IgM antibodies. Interestingly, Btk−/−B cells could also proliferate in response to CpG ODN treatment; however, the extent of their cellular proliferation was much reduced compared with that of wild type B cells across the various concentrations of CpG ODN used in the stimulation assay. Thus,
Btk<sup>−/−</sup> B cells appeared to have impairment of proliferative response to CpG ODN stimulation. As control and consistent with previous studies (19), Btk<sup>−/−</sup> B cells could not respond to stimulation by anti-IgM antibodies.

It was conceivable that the reduced cellular proliferation of Btk<sup>−/−</sup> B cells in response to CpG ODN was a result of reduced TLR9 expression in these mutant cells. To determine if this was the case, we directly quantified the amount of TLR9 mRNA expression in wild type and Btk<sup>−/−</sup> B cells. The amount of TLR9 mRNA expression was normalized to that of actin mRNA. Statistical significance was established by paired Student’s t test (*, p < 0.05). Data shown are representative of at least three independent experiments.

Enhanced IL-12 production by CpG ODN-stimulated Btk<sup>−/−</sup> B cells—Other than the induction of cellular proliferation, TLR engagement is also known to trigger the secretion of inflammatory cytokines, such as IL-6, IL-12, and tumor necrosis factor-α, in macrophages and dendritic cells (20, 21). To determine if Btk plays a role in TLR signaling of inflammatory cytokine production, we assayed for cytokine secretion after CpG ODN stimulation of purified wild type and Btk<sup>−/−</sup> B cells. As seen in Fig. 3A and compared with wild type B cells, Btk<sup>−/−</sup> B cells produced a higher amount of IL-6 after overnight stimulation with CpG ODN. This was in agreement with previous reports demonstrating higher IL-6 production in LPS-stimulated Btk<sup>−/−</sup> macrophages (13). Interestingly, CpG ODN-stimulated Btk<sup>−/−</sup> B cells also secreted significantly more IL-12p40 compared with similarly treated wild type B cells (Fig. 3B), suggesting that the absence of Btk signaling resulted in augmented production of this cytokine in B cells.

**FIGURE 2.** Btk-deficiency impaired CpG ODN-induced B cell proliferation. A, reduced proliferation of Btk<sup>−/−</sup> B cells in response to CpG ODN stimulation. Purified wild type (white column) and Btk<sup>−/−</sup> (black column) B cells were untreated or stimulated with anti-IgM antibodies (10 µg/ml) or with various concentrations (µg/ml) of LPS or CpG ODN, and their cellular proliferation was quantified 48 h later by thymidine incorporation. B, real time qPCR analysis of TLR9 mRNA expression in wild type and Btk<sup>−/−</sup> B cells. The amount of TLR9 mRNA expression was normalized to that of actin mRNA. Statistical significance was established by paired Student’s t test (*, p < 0.05). Data shown are representative of at least three independent experiments.

**FIGURE 3.** Enhanced IL-12 production by CpG ODN-stimulated Btk<sup>−/−</sup> B cells. Purified wild type (white columns) and Btk<sup>−/−</sup> (black columns) B cells were untreated or stimulated with various concentrations (µg/ml) of CpG ODN overnight for A and B, and 0.5 µg/ml for C, and their secretion of IL-6 (A), IL-12p40 (B), and IL-12p70 (C) was quantified via ELISA using known standards. Statistical significance was established by paired Student’s t test (*, p < 0.05). Results shown are representative of at least three independent experiments.
IL-12p40 can be secreted as homodimers with no known biological activity or as a heterodimer with IL-12p35 to form the biologically active IL-12p70 molecule (22) or with IL-23p19 to form the biologically active IL-23 (23). We subsequently assayed for the presence of bioactive IL-12p70 molecules and showed that Btk<sup>-/-</sup> cells indeed secreted larger amounts of IL-12p70 upon TLR9 engagement compared with wild type B cells (Fig. 3C). However, there was no appreciable amount of IL-23 secretion by wild type or Btk<sup>-/-</sup> cells when stimulated with CpG ODN (data not shown). Thus, Btk deficiency leads to augmented production of IL-6 and IL-12 in TLR9-activated B cells.

Since CpG ODN-stimulated Btk<sup>-/-</sup> B cells secreted significantly more IL-12, it remained possible that the production of other cytokines could also be perturbed. Hence, we assayed for IL-10 secretion in TLR9-stimulated wild type and Btk<sup>-/-</sup> B cells, since B lymphocytes were known to produce an appreciable amount of this cytokine. As shown in Fig. 4A, wild type B cells secreted IL-10 in response to CpG ODN stimulation. However, IL-10 secretion was not detectable in similarly treated Btk<sup>-/-</sup> B cells. Real time RT-PCR assays further confirmed that Btk<sup>-/-</sup> B cells did not up-regulate the expression of IL-10 mRNA upon CpG ODN stimulation, unlike the case for wild type B cells (Fig. 4B). These data suggest that in contrast to the negative regulation of IL-12 secretion, Btk positively regulates IL-10 production during TLR9 signaling in B cells.

IL-10 is an anti-inflammatory cytokine and is known to modulate IL-12 production in dendritic cells and macrophages (24–26). The lack of IL-10 production in CpG ODN-stimulated Btk<sup>-/-</sup> B cells could be a plausible cause for the enhanced IL-12 secretion in these cells. To test this possibility, we treated CpG ODN-stimulated Btk<sup>-/-</sup> B cells with varying concentrations of recombinant IL-10 and measured their subsequent secretion of IL-12. As seen in Fig. 4C, the addition of increasing amounts of recombinant IL-10 to CpG ODN-stimulated Btk<sup>-/-</sup> B cells led to a corresponding reduction in IL-12 secretion by these cells, suggesting that IL-10 production by B cells could modulate their secretion of IL-12. Thus, taken together, the data indicate that Btk negatively regulates IL-12 secretion via the autocrine induction of IL-10 production.

**FIGURE 4.** Btk positively regulates IL-10 production in CpG ODN-stimulated B cells. A, purified wild type (white columns) and Btk<sup>-/-</sup> (black columns) B cells were stimulated with CpG ODN overnight, and their secretion of IL-10 was quantified via ELISA. Statistical significance was established by paired Student’s t test (*, p < 0.05). Results shown are representative of at least three independent experiments. B, real time qPCR analysis of IL-10 mRNA expression in wild type (white columns) and Btk<sup>-/-</sup> (black column) B cells at various time points after CpG ODN stimulation. The amount of IL-10 mRNA is normalized to that of actin mRNA. C, production of IL-12 by Btk<sup>-/-</sup> B cells is inhibited by IL-10. Wild type and Btk<sup>-/-</sup> B cells were stimulated with CpG ODN in the absence or presence of increasing concentrations (ng/ml) of recombinant IL-12p40 molecules, measured via ELISA. Results shown are representative of at least two independent experiments.

Btk<sup>-/-</sup> mice have enhanced serum IL-12 level and exhibited greater fold induction of Th1 type antibody production upon CpG ODN immunizations—CpG ODN has been proposed as an adjuvant to boost immune responses (27–29). We had shown that CpG ODN treatment of Btk<sup>-/-</sup> B cells in vitro led to their higher secretion of IL-12 (Fig. 3). Thus, CpG ODN engagement of TLR9 could have direct application in boosting cytokine or antibody levels in certain immunodeficient patients. To determine if CpG ODN could be relevant in these applications and if it could induce a higher level of proinflammatory cytokine production in vivo, we directly challenged wild type and Btk<sup>-/-</sup> mice with CpG ODN and monitored the amount of IL-12 in the sera of these mice at various time points postimmunization.

As seen in Fig. 5A, CpG ODN treatment could indeed boost the level of serum IL-12 in wild type and Btk<sup>-/-</sup> mice. Interestingly, after a single dose of CpG ODN, Btk<sup>-/-</sup> mice had significantly more IL-12 in their sera at the 4 and 8 h time points postadministration compared with wild type mice. Although various other cell types in addition to B cells could have contributed to the production of IL-12 in wild type and Btk<sup>-/-</sup> mice, the data nevertheless supported our in vitro findings with Btk<sup>-/-</sup> B cells, namely that IL-12 secretion was enhanced in TLR9-stimulated cells lacking Btk.
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IL-12 is a Th1 type cytokine and is known to induce B cell antibody class switching to that of IgG2a, IgG2b, and IgG3 (30–32). It is known that Btk−/− mice are deficient in serum IgG3 antibodies. We therefore assessed if the augmented secretion of IL-12 during CpG ODN treatment could enhance IgG3 antibody levels or lead to greater fold induction of Th1 type antibody production in Btk−/− mice compared with wild type mice. To accomplish this, we have focused on measuring the serum level of IgG2b and IgG3 in wild type and Btk−/− mice with or without CpG ODN immunization.

As shown in Fig. 5B, CpG ODN-administered Btk−/− mice produced significantly more IgG2b antibodies in their sera compared with nontreated Btk−/− and wild type mice as well as CpG ODN-treated wild type mice. This is consistent with the higher level of serum IL-12 in CpG ODN-treated Btk−/− mice and with previous findings that IL-12 could bias antibody class switching to IgG2b. Interestingly, CpG ODN immunization could also elevate the level of IgG3 antibodies in Btk−/− mice compared with nontreated Btk−/− mice, although the level did not quite reach the basal level found in wild type mice.

Upon closer examination, it was readily apparent that Btk−/− mice treated with CpG ODN had greater than 2-fold induction of class-switched IgG2b and IgG3 antibodies compared with nontreated Btk−/− mice. By contrast, similar treatment of wild type mice did not lead to a similar level of increase in the induction of class-switched IgG2b and IgG3 antibodies compared with nontreated wild type mice (Fig. 5C). Hence, the greater fold induction of Th1 type antibody production in CpG ODN-treated Btk−/− mice correlated well with the augmented production of IL-12 in these mutant mice. As control, there was no significant change in the level of serum IgM antibodies in CpG ODN-treated Btk−/− mice compared with wild type mice, nor was there any large increment in the fold induction of this antibody class in wild type and mutant animals.

**Impaired NFκB Activation in Btk−/− B Cells upon TLR9 Stimulation**—The NFκB signaling pathway is known to regulate inflammatory responses in many biological systems (33). It had been shown that a lack of Btk signaling led to defective NFκB activation in macrophages and monocyteic cell lines stimulated by LPS (9). Since Btk−/− B cells have reduced cellular proliferation and altered pattern of cytokine production in response to CpG ODN treatment, we examined if NFκB signaling was affected in these mutant cells.

As shown in Fig. 6A, we were unable to detect significant activation of NFκB in TLR9-stimulated Btk−/− B cells compared with wild type B cells. This was clearly demonstrated by the lack of significant induction of NFκB DNA binding activity in the nuclear extracts obtained from CpG ODN-stimulated Btk−/− B cells compared with wild type B cells.

Previous studies had indicated that LPS stimulation led to the activation of the NFκB p65RelA subunit in macrophages and that this subunit activity was affected in the absence of Btk in data point indicates one mouse analyzed. C, Btk−/− mice have greater fold induction of IgG2b and IgG3 antibodies following CpG ODN immunizations. Shown is a graphical representation of the fold-increase of serum IgM, IgG2b, and IgG3 antibodies after CpG ODN challenges in wild type (white columns) and Btk−/− (black columns) mice compared with basal levels. Statistical significance was established by unpaired Student’s t test (*, p < 0.05).
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These cells (13). To further explore if CpG ODN engagement of TLR9 in B cells also involved the activation of the NFκB p65RelA subunit, we performed supershift analysis using anti-p65RelA antibody and nuclear extracts obtained from CpG ODN-stimulated wild type B cells. As shown in the supershift assay in Fig. 6B, CpG ODN stimulation of TLR9 clearly led to the induction of p65RelA, and the intensity of the supershifted band suggested that this NFκB subunit was one of the major NFκB subunits activated in response to CpG ODN stimulation in B cells.

Since LPS-induced activation of p65RelA was defective in Btk-deficient macrophages (13) and we showed that this subunit was also involved in TLR9 signaling in B cells (Fig. 6B), we focused on analyzing NFκB p65RelA activation in TLR9-stimulated Btk-deficient B cells.

NFκB transcription factors are initially inactive and retained in the cytoplasm of cells by the IκB subunits. Upon receptor activation, the IκB subunits are phosphorylated and degraded, thus allowing the translocation of NFκB subunits to the nucleus to effect gene transcription (33). Hence, we proceeded to determine the stage in which Btk deficiency affected the activation of NFκB p65RelA during TLR9 signaling.

As shown in Fig. 6C, both wild type and Btk−/− B cells exhibited normal degradation of the IκBα subunits upon TLR9 engagement, suggesting that the absence of Btk did not affect this step of p65RelA activation.

We also performed fractionation studies to separate the cells into nuclear and cytosolic fractions after CpG ODN treatment of wild type and Btk−/− B cells. We showed that the amount of nuclear p65RelA was very much reduced in the mutant B cells upon CpG stimulation when compared with similarly treated wild type B cells (Fig. 6D), and this difference was not due to subnormal expression of this subunit, since Btk−/− B cells possess an amount of these proteins in their cytoplasm equivalent to levels wild type B cells (Fig. 6E). Moreover, we have used anti-HDAC1 and anti-tubulin antibodies to verify that we had indeed subfractionated the cell lysates clearly into nuclear and cytosolic fractions, respectively (Fig. 6F). Taken together, the data suggested that the lack of Btk signaling affected the translocation of an optimum amount of NFκB p65RelA subunit into the cell nucleus and thereby resulted in reduced NFκB transcriptional activities in the mutant cells.

B Cells Lacking NFκB p65RelA Secrete Normal Levels of IL-6, IL-10, and IL-12 upon TLR9 Activation—Since Btk deficiency affects the activation of NFκB and the production of cytokines, we asked if the defect in NFκB p65RelA translocation to the nucleus could account for the altered pattern of cytokine production in Btk−/− B cells upon TLR9 engagement. To address this possibility, we examined the cytokine response of p65RelA−/− B cells upon CpG ODN stimulation.

p65RelA−/− mice were reported to suffer from embryonic lethality due to massive inflammation (34). However, we were able to generate viable homozygous p65RelA−/− mice in our SPF mouse facility, although they were not obtained in a Mendelian ratio. We confirmed that the mutant mice indeed lack p65RelA by assaying for RelA transcripts by RT-PCR (Fig. 7A) and examining its protein expression by Western blot analysis (Fig. 7B). Flow cytometry analyses of the B cell populations in
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FIGURE 7. p65RelA−/− B cells secrete normal levels of IL-6, IL-10, and IL-12 in response to CpG ODN stimulation. A, RT-PCR analysis of p65RelA transcripts in wild type and p65RelA−/− B cells. RT-PCR of the hprt gene expression was included as a control. B, Western blot analysis of p65RelA protein expression in wild type and p65RelA−/− mice. Whole cell lysates obtained from wild type and p65RelA−/− splenocytes were examined with anti-p65 antibodies and later reprobed with anti-ERK2 antibody to determine equal loading of protein. C, normal splenic B cell populations in p65RelA−/− mice. Shown is flow cytometry analysis of B cell populations in the spleen of wild type and p65RelA−/− mice using fluorochrome-conjugated anti-IgM and anti-IgD antibodies. The numbers indicate percentage of total lymphocytes, and results shown are representative of three independent analyses. D, normal production of IL-6, IL-10, and IL-12 by purified splenic p65RelA−/− B cells upon CpG ODN stimulation. Wild type and p65RelA−/− B cells were stimulated with CpG ODN, and their secretion of various cytokines was quantified as in Fig. 3. Results shown are representative of two independent experiments.

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The bone marrow of p65RelA−/− mice also indicated that the development of B cells was not affected in these mice (data not shown) and that p65RelA−/− splenic B cells were indistinguishable from their normal counterparts in terms of phenotype (Fig. 7C) or cell numbers (data not shown).

Interestingly, when purified p65RelA−/− B cells were stimulated with CpG ODN, they were able to secrete IL-6, IL-10, and IL-12 at levels comparable with those produced by similarly treated wild type B cells (Fig. 7D). This indicated that the altered pattern of IL-6, IL-10, and IL-12 production in CpG ODN-treated Btk−/− B cells was not due entirely to the impairment in p65RelA activation in these cells. Thus, the impairment in NFκB p65RelA activation and altered production of IL-6, -10, and -12 cytokines in TLR9-stimulated B cells could be uncoupled.

DISCUSSION

B cells possess both BCR and TLR, and it is not clear if the two receptor systems would share downstream signaling components. We show here that BCR, TLR4, and TLR9 stimulations could activate Btk, as indicated by its tyrosine phosphorylation status, in B lymphocytes. However, the pattern of Btk activation during the engagement of TLR4 and -9 is more transient compared with that activated by the BCR (Fig. 1). This could imply that TLR have a lesser requirement for Btk in their signal transduction as compared with the BCR, where it plays a more central role. Consistent with this, TLR9-induced cellular proliferation is partially impaired but not completely abrogated in Btk−/− B cells (Fig. 2), and this is in contrast to the absolute requirement for Btk signaling in BCR-induced B cell proliferation (19).

Interestingly, the lack of Btk signaling during TLR9 stimulation of B cells altered significantly their cytokine secretion profiles. Compared with wild type B cells, Btk−/− B cells produce elevated levels of IL-12 but much less IL-10 upon CpG ODN treatment (Figs. 3 and 4). In the absence of Btk signaling, B cells do not produce a detectable level of IL-10 mRNA, and the addition of exogenous recombinant IL-10 protein to CpG ODN-treated Btk−/− B cells could modulate their production of IL-12. Taken together, the data indicate that Btk is required for TLR9-induced IL-10 biosynthesis and that the production of IL-10 by B cells could modulate their secretion of IL-12. Thus, the in vitro data implied the existence of an autocrine feedback loop in which B cells modulate its own production of IL-10 and IL-12 during TLR9 signaling.

The elevated level of IL-12 production in the absence of Btk signaling can be recapitulated in vivo, since Btk−/− mice have higher serum IL-12 level upon CpG ODN administration. Since Btk−/− mice have lower levels of serum IgG3 (33) antibodies and IL-12 has been known to induce Th1 type Ig class switching (30, 31), it would appear that CpG ODN could function as an adjuvant to boost Th1 type Ig levels in Btk immunodeficiency syndromes. Indeed, we showed here that CpG ODN treatment of Btk−/− mice could enhance their serum level of IgG2b and IgG3 antibodies. Thus, CpG ODN through its engagement of TLR9 appeared to be a good stimulant to boost Th1 type immune responses in Btk immunodeficiency syndromes. The alteration in IL-10/IL-12 production in CpG ODN-stimulated Btk−/− B cells and mice would imply that these mutant mice would have very different immune responses and outcomes to pathogenic challenges compared with normal mice. The finding in this paper indicates that Btk−/− mice would bias toward Th1 type immune responses due to their higher production of IL-12 upon encounter with pathogens. Indeed, X-linked agammaglobulinemia patients are reported to have a skewing toward Th1 type immune responses (35).

While this work was in progress, several reports were published that implicated a role for Btk in TLR signaling. It was shown that Btk was phosphorylated after LPS stimulation of
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human monocytic cell lines (10) and that it could bind TLR-signaling adaptor proteins MyD88 (10), Mal (10, 36), and the IL-1R-associated kinase 1 (10). However, most of these studies were either mechanistic in nature or were carried out on macrophages and dendritic cells and used LPS, which binds TLR4 and CD14 as the agonist. Prior to this current study, the physiological effect of Btk mutation on TLR signaling in B cells has not been well examined, and in particular, a role for TLR9 signaling in B cells has not been established. Our current report extended these previous studies to B cells and yielded new and complementary data to these published reports. We show here that Btk is required for IL-10 production in TLR9-stimulated B cells, as was the case for dendritic cells (15) and macrophages (13) stimulated by LPS, which engages TLR4 and CD14. In addition, our analysis of IL-10 mRNA level in TLR9-stimulated Btk−/− B cells indicated that Btk activates the transcription of the IL-10 gene during TLR signaling. Thus, Btk is required for TLR-induced IL-10 production. Our current paper also establishes a critical role for Btk in the negative regulation of IL-12 via production of IL-10 during TLR signaling in B cells, which had not been reported previously.

We also noted some differences in the responses of B cells and dendritic cells to TLR stimulation in the absence of Btk. We reported here that CpG ODN-stimulated Btk−/− B cells secrete higher level of IL-12 due to their lack of IL-10 production. However, it was reported that there was no difference in IL-12 production in LPS-stimulated Btk−/− dendritic cells (15). Thus, there might exist cell type or TLR-specific differences in the innate immune responses. Similarly, differences in IL-6 production by Btk-deficient cells were also observed. Our current report suggested that the production of IL-6 was also enhanced in the absence of Btk signaling in TLR9-stimulated B cells (Fig. 3). In agreement, Schmidt et al. (13) also reported increased IL-6 production in LPS-stimulated Btk−/− macrophages. However, several reports indicated otherwise and showed either no change (36) or decrease (37) in IL-6 production in LPS and/or single-stranded RNA-stimulated Btk−/− cells. These differences will need to be resolved in future studies.

Data presented in this paper also indicated that TLR9 signaling in B cells involved the activation of NFκB, and this extends to the activation of the p65RelA subunit (Fig. 6). And in the absence of Btk, TLR9-induced NFκB activation was impaired as there was reduced translocation of p65RelA to the nucleus of Btk−/− B cells upon CpG ODN treatment (Fig. 6). However, IkBα degradation was intact in CpG ODN-stimulated Btk−/− B cells. This contrasted with the absolute requirement for Btk in signaling NFκB mobilization during BCR signal transduction (19). In the case of Btk−/− B cells stimulated via the BCR, the defect in NFκB activation was much more severe, since IkBα degradation did not occur, and the nuclear translocation of p65RelA was completely abrogated (38).

Our finding of reduced NFκB translocation in TLR9-stimulated Btk−/− B cells was also consistent with a previous report that indicated reduced nuclear translocation of p65RelA in Btk-deficient macrophages treated with LPS (13). Here, we have taken a step further to address if the defective nuclear translocation of p65RelA in Btk−/− B cells could account for the altered pattern of cytokine production in these cells upon TLR9 stimulation. To this end, we examined the secretion of cytokines by p65RelA−/− B cells upon CpG ODN stimulation and, interestingly, found that p65RelA−/− B cells were able to produce normal levels of IL-6, IL-10, and IL-12 upon TLR9 engagement. This suggested that the defective nuclear translocation of p65RelA in CpG ODN-stimulated Btk−/− B cells could not explain the altered cytokine production in these cells.

Other studies have indicated that NFκB p65RelA is important in LPS-induced production of IL-6 in dendritic cells (39). This contrasted with our current data, in which IL-6 production is normal in CpG ODN-stimulated p65RelA−/− B cells. This difference could be due to the use of a different cell type or TLR in the current study. In addition to p65RelA, B cells also express c-Rel and p50, and they may substitute for the lack of p65RelA during specific TLR signaling. Future studies using c-Rel−/− and p50−/− B cells may help to address this discrepancy.

Our present study is also consistent with two recent studies that demonstrated a role for Btk in TLR9 signaling (40, 41). Although our current report and other recent studies (9, 13, 15, 40, 41) clearly indicated a role for Btk in TLR signaling of cytokine production, much work remains to be accomplished. For example, the identities of the immediate downstream molecules that interacted with Btk in TLR signaling are not known. Moreover, the subcellular localization of Btk following TLR stimulation is also not examined, and this is particularly interesting, since some TLR are endosome-located whereas other TLR are plasma membrane-bound.

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