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Signaling by Toll-like Receptors 8 and 9 Requires Bruton's Tyrosine Kinase*

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Toll-like receptors (TLRs) are a primary surveillance system for the detection of pathogens and are crucial to the activation of host defense. TLR7 and TLR8 sense single-stranded RNA from viruses or host ribonucleoproteins and synthetic imidazoquinolines such as R848, whereas TLR9 senses unmethylated CpG motifs in viral and bacterial DNA and in host DNA. Here we report the endogenous interaction between Brutons's tyrosine kinase (Btk) and human TLR8 and TLR9 in the monocytic cell line THP1. We also show that R848, single-stranded RNA, and CpGB-DNA activate Btk in THP1 cells as shown by phosphorylation of the tyrosine 223 residue of Btk and also by increased autokinase activity. We demonstrate that Btk is required for NFκB activation, participating in the pathway to increased phosphorylation of p65 on serine 536 activated by TLR8 and TLR9. Finally we demonstrate that peripheral blood mononuclear cells from patients with X-linked agammaglobulinaemia (XLA) that have dysfunctional Btk are impaired in the induction of interleukin-6 by CpGB-DNA. This study therefore establishes Btk as a key signaling molecule that interacts with and acts downstream of TLR8 and TLR9. Lack of functioning Btk in XLA patients downstream of TLR8 and TLR9 might explain the susceptibility of XLA patients to viral infections.

Toll-like receptors (TLRs)[2] are a family of receptors in innate immunity that play an important role in the initiation of host defense. They activate signaling pathways that culminate in the induction of immune and inflammatory genes, including those encoding co-stimulatory molecules, antibodies, cytokines, chemokines, and adhesion molecules. There are 10 TLRs in humans. The best characterized is TLR4, which recognizes the Gram-negative product lipopolysaccharide (LPS). The other antibacterial TLRs are TLR2, which in combination with TLR1 recognizes triacylated lipopeptides, or in combination with TLR6, recognizes diacylated lipopeptides, and TLR5, which recognizes bacterial flagellin. The antiviral TLRs are TLR3, which recognizes double-stranded RNA, TLR7 and TLR8, which recognize single-stranded RNA (ssRNA), and TLR9, which recognizes unmethylated CpG motifs that exist in both viral and bacterial DNA (1). All TLRs contain extracellular leucine-rich repeat domains and a cytoplasmic Toll/IL-1R (TIR) domain, which is crucial for signaling downstream of the TLR (2). Once activated, TLRs trigger a cascade of cellular signals, culminating in the eventual activation of the Rel family transcription factor NFκB (3). NFκB binds to discrete nucleotide sequences in the upstream regions of genes that produce pro-inflammatory cytokines such as tumor necrosis factor-α and IL-6, thereby regulating their expression (4). Regulation of NFκB following stimulation with LPS occurs via activation of two pathways. The best characterized of these regulates the release of NFκB from its inhibitory protein IκBα, subsequently allowing NFκB to translocate to the nucleus and bind its target genes. However, this nuclear translocation of NFκB is not sufficient to activate NFκB-dependent gene transcription alone. The second pathway, which involves post-translational modifications, regulates the transactivating ability of the p65 subunit of NFκB once bound to its consensus sequence, *i.e.* the ability to recruit the transcriptional apparatus and stimulate target gene expression (5).

Bruton's tyrosine kinase (Btk), a cytoplasmic non-receptor tyrosine kinase, is a member of the Tec family of protein kinases. It was first identified in the male immune disorder X-linked agammaglobulinaemia (XLA). The XLA syndrome is manifested by severe defects in early B cell development, resulting in a nearly complete absence of peripheral B cells and immunoglobulins of all classes (6). Various mutations in the human *btk* gene were identified as being responsible for the XLA phenotype (7). These include mutations in both the kinase domain and the pleckstrin homology domain, which affect kinase activity and translocation to the membrane, respectively. The pleckstrin homology domain is important in the process of Btk activation since it localizes Btk to the membrane through its interaction with phosphatidylinositol 3,4,5-triphosphate. In mice, a naturally occurring mutation in the pleckstrin homology domain has been mapped to arginine 28 (R28C), which causes X-linked immune deficiency (Xid) in these mice. Mutated Btk in Xid mice is prevented from associating with the membrane, thus inactivating it (8).

We have previously described a key role for Btk in NFκB transactivation in response to LPS (9). The ability of Btk to associate with the TIR domain of TLR4 was also demonstrated

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[2] The abbreviations used are: TLR, Toll-like receptor; TIR, Toll/IL-1R; IL-1, interleukin-1; IL-1, IL-1 receptor; BMDM, bone marrow-derived macrophage; Btk, Bruton's tyrosine kinase; IκB, inhibitor of κB; NFκB, nuclear factor κB; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; XLA, X-linked agammaglobulinaemia; ssRNA, single-stranded RNA; Xid, X-linked immune deficiency; ISRE, interferon-stimulated response element; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay.

(10). In addition, however, we found that Btk can interact with the TIR domains of TLR8 and TLR9. This suggested a possible role for Btk in TLR8 and TLR9 signaling, although this has never been examined directly. Btk-deficient mice have exhibited high susceptibility to acute mouse adenovirus type-1 (MAV-1) in contrast to controls, and XLA patients are highly susceptible to enteroviral infections, indicating a possible role for Btk in preventing virus-induced disease (11, 12). This could be due to a role for Btk in TLR8 or TLR9 signaling. Both TLR8 and TLR9 have been shown to be expressed in B cells, and their activation has been shown to induce antibody production and proliferation (13–15). Given these aspects and the apparent interaction between Btk and TLR8 and TLR9, we have examined a role for Btk in TLR8 and TLR9 signaling. We provide the first evidence for an interaction between Btk and these TLRs endogenously and for potent activation of Btk by TLR8 and TLR9, and we demonstrate that Btk is required for transactivation by p65. We also demonstrate impaired induction of IL-6 by TLR9 in PBMCs prepared from XLA patients. Our data therefore identify for the first time a key role for Btk in TLR8 and TLR9 signaling.

EXPERIMENTAL PROCEDURES

Cell Culture—The HEK293 cell line and THP1 cell line were obtained from European Cell Culture Collection and were maintained as described previously (16). Female and Male CBA/CaHN-Btk<xid>J mice were gifts from Stephanie Vogel. We used female CBA/CaHN-Btk<xid>J that do not exhibit the disease and male CBA/CaHN-Btk<xid>J that are affected by the mutation. BMDMs were prepared from CBA/CaHN-Btk<xid>J mice and maintained as described previously (9). Two male patients diagnosed with XLA, according to the World Health Organization (WHO) classification, and two healthy age- and sex-matched controls, were included in this study after informed consent was obtained. This study was approved by the St. James's Hospital/Adelaide and Meath Hospital research ethics committee. Both patients were receiving regular intravenous immunoglobulin replacement therapy at 4-week intervals and were free of any serious infections at the time of blood sampling. Heparinized blood samples were collected prior to the infusion of intravenous immunoglobulins. PBMCs were generated as described previously (17).

Plasmids and Reagents—The following plasmids were used: pcDNA3.1 (Invitrogen), the NFκB-reporter construct (Dr. R. Hofmeister, Universitat Regensburg, Germany); BtkK430R, BtkR28C, and wild type Btk (Dr. Cornelia Escher, Universität Ulm, Germany); phRL-TK (Promega); CD4-TLR8 and CD4-TLR9 (Adrian Ozinsky, University of Washington, Seattle, WA); the Gal4-p65$^{(1-551)}$ plasmid encoding full-length p65 subunit of NFκB fused to the Gal4 DNA-binding domain (Lienhard Schmitz, German Cancer Research Centre, Heidleberg, Germany); and the Gal-luciferase reporter gene (Stratagene). The following antibodies were used: anti-Btk (C-20) and anti-p65 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); phospho-NFκB-p65 (Ser-536) and phospho-Btk (Tyr-223) antibodies (Cell Signaling Technology Inc.); anti-calnexin (Stressgen Bioreagents, Victoria, British Columbia, Canada); anti-TLR8 and anti-TLR9 (Imgenex, San Diego, CA); and anti-goat IgG-Alexa Fluor 488 and anti-rabbit IgG Alexa fluor 647 (Invitrogen, Dun Laoghaire, Ireland). The following reagents were used: CpG-ODNs (Coley Pharmaceuticals, Langenfeld, Germany); ssRNA42 (IBA GmbH, Gottingen, Germany); R848 (Invivogen, San Diego, CA); the Btk-specific inhibitor, LFM-A13 (Calbiochem, Nottingham, United Kingdom); and the IL-6 ELISA duoset (R&D Systems, Abingdon, UK). All other reagents were obtained from Sigma with the exception of the following: GeneJuice® (Novagen), coelentrazine (Argus Fine Chemicals), and passive lysis buffer (Promega).

Transient Transfections and Reporter Gene Assays—HEK293 cells were transiently transfected as described previously for both the NFκB-luciferase and the p65-luciferase assays (Doyle). For the interferon-stimulated response element (ISRE)-luciferase assay, HEK293 cells were seeded (1×10^5 ml^{-1}) onto 96-well plates 24 h prior to transfection, and 80 ng of ISRE-luciferase, 40 ng of *Renilla* luciferase, and the indicated amount of Btk-expressing plasmid and/or CD4-TLR8/9 expressing plasmid (220 ng total) were transfected using GeneJuice according to the manufacturer's instructions. Luminescence readings were assayed as described previously (9).

Western Blot Analysis—THP1 cells were centrifuged and resuspended in fresh RPMI to give a total of 1×10^7 cells/ml. BMDMs were seeded at 2×10^6/3 ml in 6-well plates. After appropriate stimulation, cells were lysed in ice-cold radioimmune precipitation buffer (phosphate-buffered saline buffer containing 1% Nonidet P40, 0.5% (w/v) sodium deoxycholate, and 0.1% SDS containing 10 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, and 1 μg/ml sodium orthovanadate). Protein estimations of cell extracts were determined by the Bradford assay. Equal amounts of protein were resolved using SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to Immobilon™ polyvinylidene difluoride membrane. After incubation with primary antibodies as indicated, blots were incubated with the appropriate peroxidase-conjugated secondary antibody. Blots were developed by enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Biosciences). Densitometric analysis was carried out using Multigauge software, and pixel intensity (absorbance unit/mm^2) was normalized to the corresponding total protein and expressed as -fold increase in intensity over the control unstimulated lane.

Immunoprecipitation—THP1 cells were seeded at a density of 2.5×10^5 cells/ml 18 h prior to stimulation. A total of 2×10^7 cells was used per point, and cells were stimulated with either CpGB (3 μg/ml) or R848 (1 μg/ml) for the time points indicated and lysed in 800 μl of high stringency lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol (v/v), 1% Nonidet P-40 (v/v) containing 10 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, and 1 μg/ml sodium orthovanadate) or 800 μl of CHAPS buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol (v/v), 0.5% Nonidet P-40 (v/v), 0.5% CHAPS (w/v), containing 10 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, and 1 μg/ml sodium orthovanadate) for 30 min on ice. Immune complexes were immunoprecipitated by incubation for 2 h at 4 °C with gentle rotation with the appropriate antibody, which had been precoupled to protein G-Sepharose at 4 °C overnight or with phospho-tyrosine

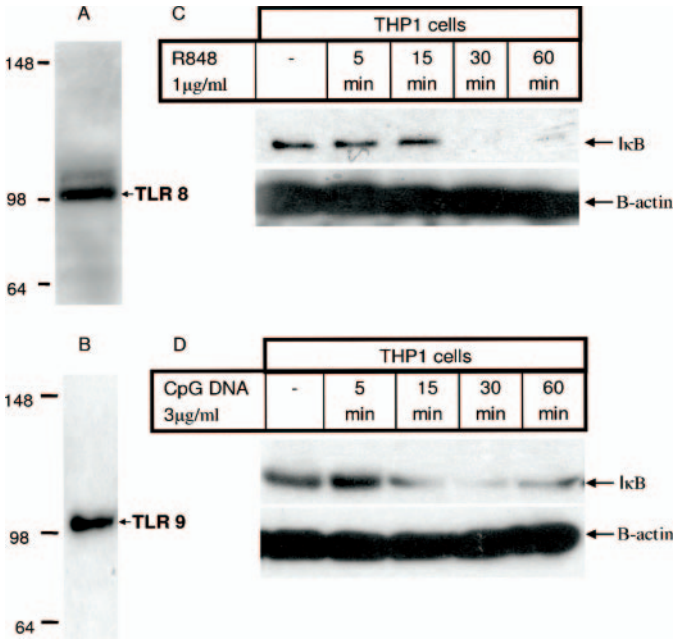


FIGURE 1. **THP1 cells express TLR8 and TLR9 and respond to R848 and CpGB DNA.** *A* and *B*, THP1 cells were lysed and immunoblotted for TLR8 or TLR9. *C* and *D*, THP1 cells were treated with R848 or CpGB-DNA for the time points indicated. Cells were lysed and immunoblotted for IκBα (*top panel*) or β-actin (*bottom panel*). Results are representative of three separate experiments.

beads. A portion of the lysate was retained to confirm that the protein of interest was expressed; this was added to the sample buffer and boiled for 5 min. Following the 2-h incubation, the immune complexes were washed three times with lysis buffer. All supernatant was removed, and beads were resuspended in 30 μl of 5× sample buffer. The samples were boiled for 5 min, separated by SDS-PAGE, and analyzed by Western blotting.

Btk Activation Assay—THP1 cells were prepared for autokinase assay as described previously (10).

IL-6 ELISA—Human PBMCs were cultured at 2×10^6 cell/ml and stimulated with CpGB or R848 of varying concentrations and time points, as indicated. Supernatants were then harvested, and the concentration of IL-6 was determined by ELISA (R&D Systems), according to the manufacturer's instructions. Absorbance at 450 nm was measured on a spectrophotometric ELISA plate reader.

RESULTS

Association of Endogenous Btk with TLR8 and TLR9—In a previous study, we found that a yeast two-hybrid screen carried out to search for possible interaction partners for Btk isolated a cDNA encoding human TLR8 (10). It was found that Btk could also interact with the TIR domains of TLR4, TLR6, and TLR9. However, the strongest interaction was found with the TIR domain of TLR8 (10). To confirm this interaction, we first carried out co-immunoprecipitation analysis. We chose THP1 monocytic cells as a model system since this cell line expressed both TLR8 (Fig. 1*A*) and TLR9 (Fig. 1*B*) and was responsive to the TLR8 ligand R848 (Fig. 1*C*) and the TLR9 ligand CpGB-DNA (Fig. 1*D*), as shown by IκBα degradation. Endogenous TLR8 or TLR9 was immunoprecipitated from lysates prepared from THP1 cells stimulated with either R848 or CpGB at various time points (Fig. 2, *A* and *B*, respectively, *top panels*). Immunopurified TLR from each time point was immunoblotted with a Btk-specific antibody. An interaction between Btk and TLR8 was detected both basally and after R848 treatment. However, we consistently found that the interaction appeared to be stronger at 15 and 30 min after R848 stimulation in comparison with basal levels of association (Fig. 2*A*,*top panel*). Similarly, an interaction between Btk and TLR9 was detected in unstimulated cells and cells treated with CpGB (Fig. 2*B*, *top panel*). Similar to the association between TLR8 and Btk, the interaction appeared to grow stronger upon CpG stimulation with an apparent increased interaction after 5 and 15 min of CpGB stimulation, decreasing again at 30 min of stimulation (Fig. 2*B*, *top panel*). In both cases, samples were blotted for Btk to ensure equal loading (Fig. 2, *A* and *B*, *second panels*). The specificity of these interactions was tested in THP1 lysates by comparing the ability of the relevant isotype-matched antibody, mouse IgG, with co-immunoprecipitate Btk to that of the TLR antibodies. No bands were detected at the same region in the IgG control lanes (Fig. 2, *A* and *B*, *third panels*). These samples were also blotted for Btk to ensure equal loading (Fig. 2, *A* and *B*, *bottom panels*).

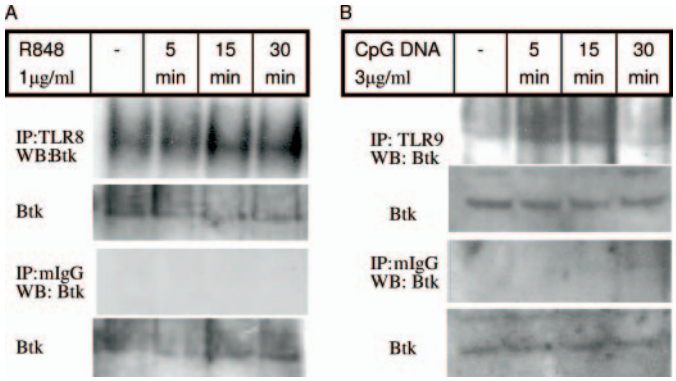


FIGURE 2. **Endogenous Btk and TLRs 8/9 interact in untreated and ligand treated THP1 cells.** *A* and *B*, THP1 cells were treated with R848 (*A*) or CpGB (*B*) for the time points indicated. Cells were lysed in CHAPs buffer, and TLR8 (*A*, *top panel*), TLR9 (*B*, *top panel*), or mouse IgG (*A* and *B*, *third panels*) was isolated by immunoprecipitation (*IP*). Immune complexes were immunoblotted (*WB*) with Btk antibody to detect an interaction (*top panels*), and whole cell lysates were also immunoblotted for total Btk (*A* and *B*, *second* and *fourth panels*). Results are representative of three separate experiments.

Stimulation of TLR8 and TLR9 Cause Btk Activation—We next examined the ability of R848 and CpGB to induce tyrosine phosphorylation on Btk, which is an indicator of Btk activation. Tyrosine phosphorylated proteins were immunoprecipitated from lysates prepared from THP1 cells treated with CpGB over various time points. Immunopurified tyrosine-phosphorylated protein from each time point was immunoblotted for Btk. Phosphorylated Btk was detected after 15 min of stimulation with CpGB (Fig. 3*A*, *upper panel*). Samples were also immunoblotted for total Btk to ensure equal loading (Fig. 3*A*, *lower panel*). An early substrate for Btk is Btk itself, which becomes phosphorylated on Tyr-223 (18); therefore the ability of immunopurified Btk to autophosphorylate *in vitro*, which is an indication of its autokinase activity, was next examined using an *in vitro* kinase assay. Treatment of THP1 cells with CpGB induced a rapid transient increase in phosphorylated Btk (Fig. 3*C*, *upper panel*). This was detected from 15 min. Samples were also blot-

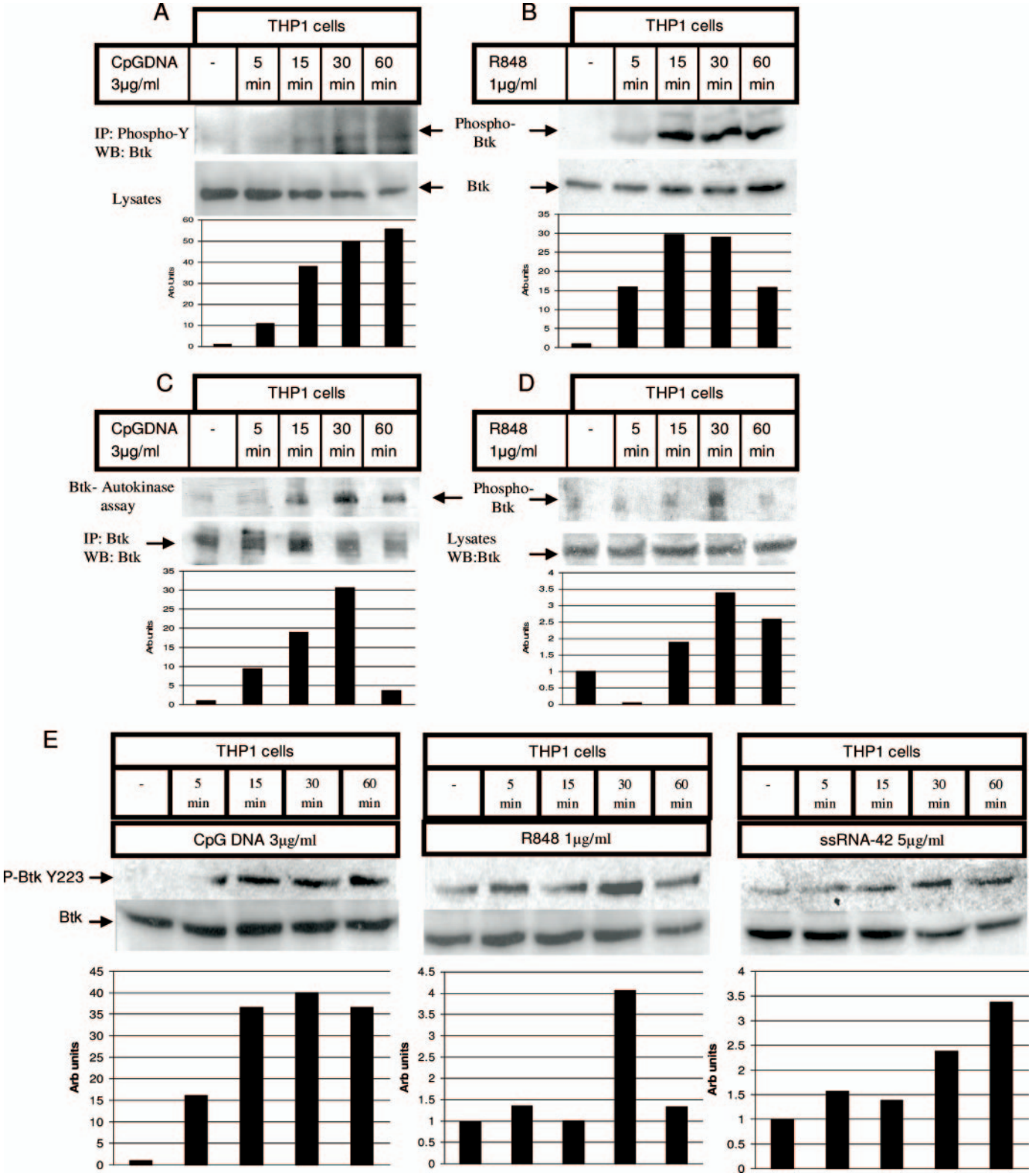


FIGURE 3. **CpGB and R848 treatment cause autophosphorylation of Btk in THP-1 cells.** THP1 cells were stimulated with CpGB, R848, or ssRNA42 for the time points indicated. Cells were lysed, and tyrosine phosphorylated protein (*A* and *B*) or Btk (*C* and *D*) was isolated by immunoprecipitation (*IP*). Immune complexes were immunoblotted (*WB*) for Btk to detect phospho-Btk (*A* and *B*, *top panels*) or assayed for Btk autokinase activity as measured by autophosphorylation of Btk in a kinase assay (*C* and *D*, *top panels*). *E*, cell lysates were immunoblotted for phospho-BtkY223 (*P-Btk Y223*, *top panels*) or total Btk (*bottom panels*). Results are representative of at least three separate experiments. Densitometry analysis was carried out for each immunoblot as shown under each figure.

ted for total Btk to ensure equal loading (Fig. 3*C*, *lower panel*). The same assays were carried out on THP1 cells treated with R848, with similar results. Tyrosine phosphorylated proteins immunoprecipitated from lysates prepared from THP1 cells treated with R848 over various time points and immunoblotted for Btk detected phosphorylated Btk after 15 min of stimulation

(Fig. 3*B*, *upper panel*). Samples were also blotted for total Btk to ensure equal loading (Fig. 3*B*, *lower panel*). Treatment of THP1 cells with R848 induced a rapid transient increase in the autophosphorylation activity of Btk, which was most evident at 30 min of stimulation (Fig. 3*D*, *upper panel*). Samples were also blotted for total Btk to ensure equal loading (Fig. 3*D*, *lower panel*).

The autophosphorylation site on Btk has been identified as Tyr-223 (8). We therefore next examined the effect of CpG and R848 on this response using a phospho-tyrosine-specific antibody raised against phosphorylated Tyr-223 on Btk. Tyr-223 phosphorylation of Btk was weakly detectable after 5 min of stimulation with CpGB, with maximum phosphorylation detected at 15, 30, and 60 min (Fig. 3*E*, *first upper panel*). R848 also increased Tyr-223 phosphorylation on Btk, the effect being evident after 5 min of stimulation with R848, with maximum phosphorylation detected at 30 min and a decrease in phosphorylation from 60 min (Fig. 3*E*, *second upper panel*). We also tested the TLR7 ligand ssRNA42 for its ability to effect Btk phosphorylation on Tyr-223. A response was detected after 15 min of stimulation with maximum phosphorylation detected at 30 min and a decrease in phosphorylation from 60 min (Fig. 3*E*, *third upper panel*). In all cases, samples were also blotted for total Btk to ensure equal loading (Fig. 3*E*, *first*, *second*, and *third lower panels*, respectively). In some experiments, as shown in Fig. 3*E*, *third* and *fifth panels*, *lane 1*, basal levels were higher, but increases were still evident.

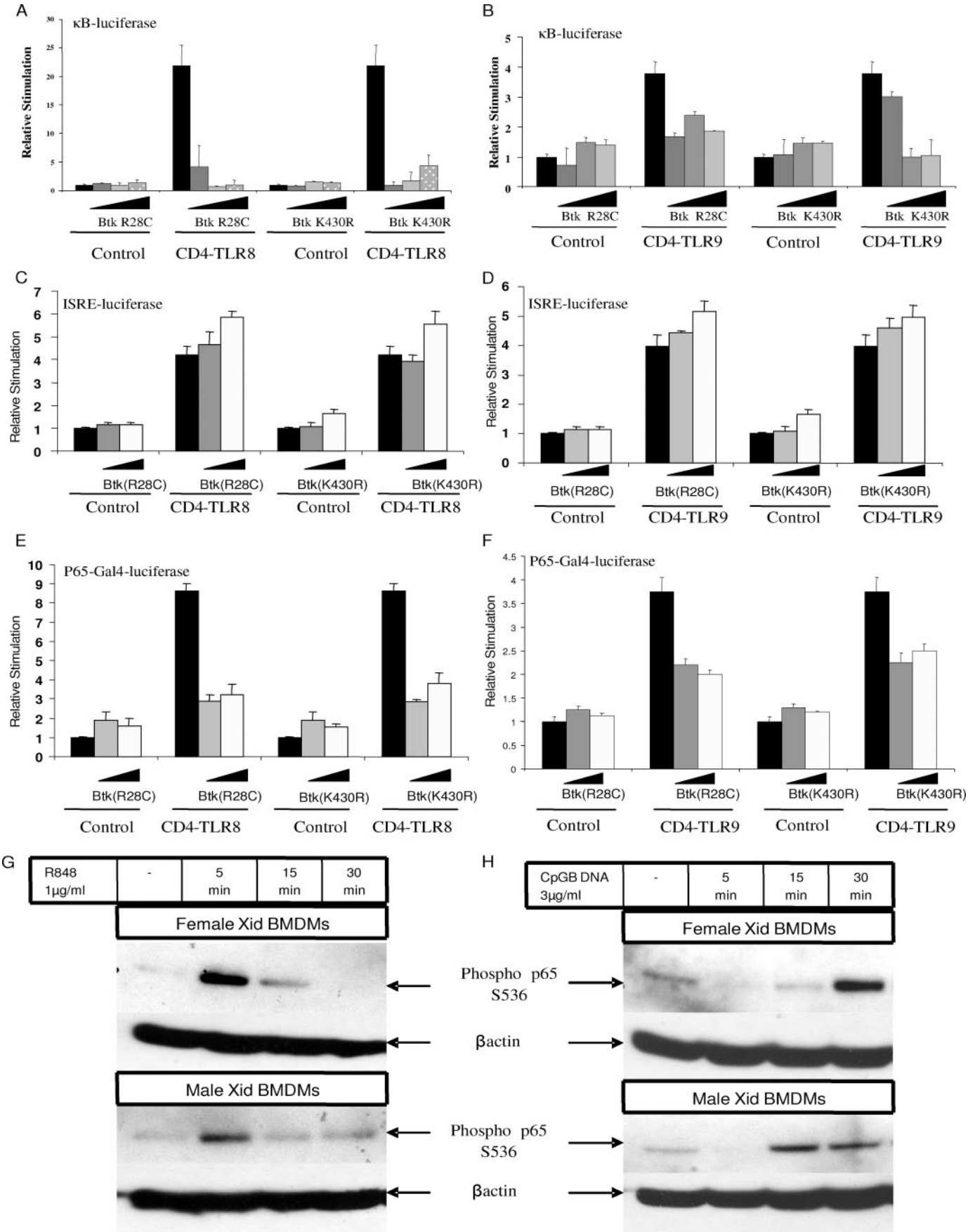
Btk Is Involved on the Signaling Pathway from TLR8 and TLR9 to NFκB—The ability of both R848 and CpGB to activate Btk implied that Btk might be an important signaling modulator downstream of TLR8 and TLR9. We have previously shown that Btk is activated downstream of TLR4 and plays a role in the phosphorylation of the NFκB p65 subunit on Ser-536, which is essential for the efficient transactivation of NFκB in response to LPS (9). We therefore determined whether Btk played a role in TLR8 and TLR9 signaling to NFκB. To test this, we examined the effect of two mutant forms of Btk, Btk R28C, which is unable to localize to the membrane, and Btk K430R, a kinase-inactive form of Btk, on the induction of an NFκB-dependent reporter gene (NFκB-luciferase) by TLR8 and TLR9. The chimeric versions of TLR8 and TLR9, CD4-TLR8, and CD4-TLR9, respectively, comprising the murine extracellular CD4 domain fused to the cytoplasmic domains of the TLRs, are constitutively active. As can be seen in Fig. 4*A*, overexpression of CD4-TLR8 in HEK293 cells led to the induction of the NFκB reporter gene construct. This activation was dramatically inhibited by the two mutant forms of Btk, Btk R28C and Btk K430R. However, whereas overexpression of CD4-TLR8 in HEK293 cells also led to the induction of the ISRE-reporter gene construct, this activation was unaffected by overexpression of either Btk K430R or Btk R28C (Fig. 4*C*). Similarly, overexpression of CD4-TLR9 in HEK293 cells led to the induction of the NFκB reporter gene construct. This activation was also inhibited by the two mutant forms of Btk (Fig. 4*B*). However, as in the case of TLR8, whereas overexpression of CD4-TLR9 in HEK293 cells also led to the induction of the ISRE-reporter gene construct, this activation was unaffected by overexpression of either Btk K430R or Btk R28C (Fig. 4*D*). The lack of effect on the ISRE construct indicates an important specificity in the role of Btk in TLR8 and TLR9 signaling. We next examined the effect of Btk R28C and Btk K430R on the Gal4-p65$^{(1-551)}$ trans-reporting system using HEK293 cells to test for a role for Btk downstream of TLR8 and TLR9 in the p65 transactivation pathway. HEK293 cells transiently transfected with a plasmid encoding CD4-TLR8 displayed activation of the Gal4-p65$^{(1-551)}$ trans-reporting system (Fig. 4*E*). This ability of CD4-TLR8 to activate Gal4-p65$^{(1-551)}$ was abolished when cells were transfected with a plasmid encoding either Btk R28C or Btk K430R. Similarly, when CD4-TLR9 was transfected into HEK293 cells, the Gal4-p65$^{(1-551)}$ trans-reporting system was induced. This ability of CD4-TLR9 to activate Gal4-p65$^{(1-551)}$ was diminished when cells were transfected with a plasmid encoding either Btk R28C or Btk K430R (Fig. 4*F*).

As we had previously shown that Btk is involved in the phosphorylation of p65 on Ser-536 in response to LPS, we next investigated whether Btk played a similar role in response to CpGB or R848. The phosphorylation of serine 536 in response to CpGB was tested in BMDMs from female and male Xid mice. Xid mice express a mutant form of Btk, Btk(R28C). In this case, the female BMDMs act as the control due to Xid being an X-linked disease with only one X chromosome being defective. CpGB increased p65 phosphorylation at 30 min (Fig. 4*G*, *top panel*). This response was significantly impaired in BMDMs from male Xid mice (Fig. 4*G*, *bottom panel*). When Xid BMDMs were stimulated with R848, there was also increased p65 phosphorylation, which occurred from 5 min, decreasing by 30 min (Fig. 4*H*, *top panel*). This response was also impaired in BMDMs from male Xid mice (Fig. 4*H*, *bottom panel*).

XLA-PBMCs Have Impaired Capacity to Produce IL-6 in Response to TLR8 and TLR9 Stimulation—Finally we examined the induction of IL-6, a pro-inflammatory cytokine known to be under the transcriptional regulation of NFκB, in PBMCs from XLA patients. Stimulation with CpGB failed to induce any IL-6 production by XLA-PBMCs after both 18 and 24 h treatments when compared with control PBMCs (Fig. 5*A*). We also examined control PBMCs pretreated with a Btk inhibitor LFM-A13 before CpGB stimulation. IL-6 production induced by CpGB was impaired in cells treated with LFM-A13 when compared with cells not treated with the inhibitor (Fig. 5*B*). We were unable to examine R848 in XLA PBMCs since no response was detected in normal PBMCs, which is likely to be due to low level expression of TLR8. Taken together, our data therefore indicate an important role for Btk in signaling by TLR8 and TLR9.

DISCUSSION

In this study, we demonstrate that endogenous TLR8 and TLR9 interact with Btk by co-immunoprecipitation and that ligation of TLR8 or TLR9 results in the activation of Btk in a time-dependent manner. TLR7, TLR8, and TLR9 belong to a subfamily of nucleic acid-sensing TLRs. Activation of TLR7, TLR8, and TLR9 in response to their respective ligands depends on acidification and maturation of endosomes and targets MyD88 to vesicular structures with lysosomal characteristics (19). Therefore TLR7, TLR8, and TLR9 form a functional subgroup within the TLR family that recognizes nucleic acids in the acidic environment of an endosome/lysosome. Specifically,



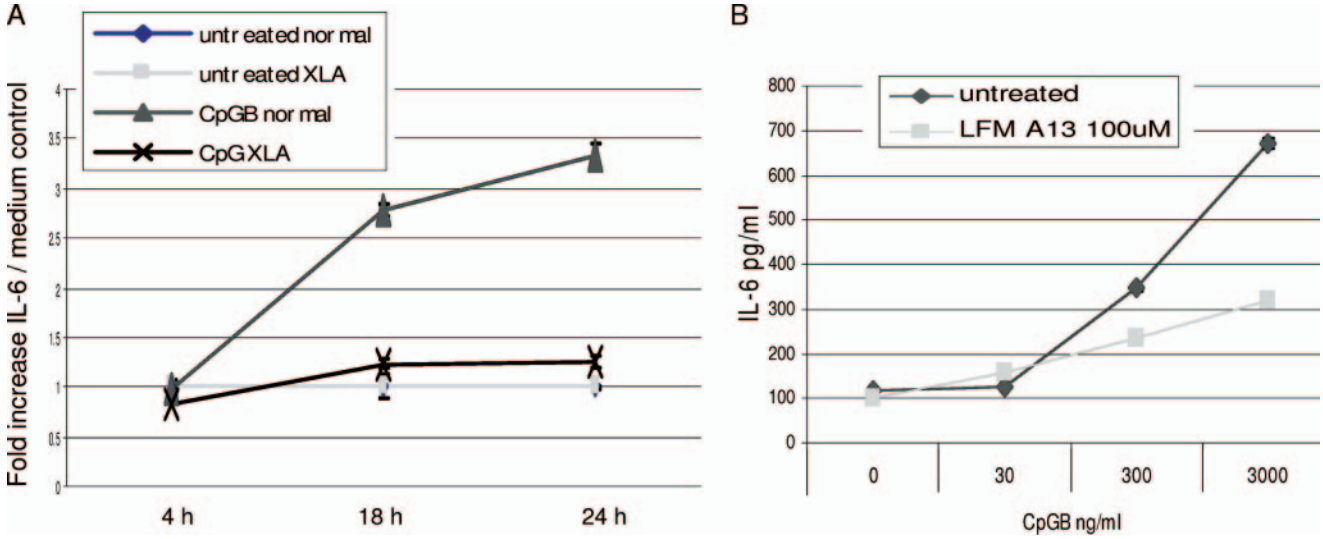


FIGURE 5. **Btk is required for IL-6 secretion from PBMCs stimulated with CpGB.** *A*, PBMCs were isolated from male XLA patients and age- and sex-matched controls. PBMCs were treated with CpGB for 4, 18, and 24 h. Cell supernatants were removed and assayed for IL-6 by ELISA. IL-6 concentrations were calculated by comparison with a standard curve of IL-6 concentrations and measured as pg/ml. The measurements taken from the CpGB-stimulated samples were then compared with those observed in the relative untreated samples for each time point and plotted as the -fold increase of IL-6 production over the unstimulated controls. *B*, PBMCs were pretreated with Me_2SO or LFM-A13 100 μM for 1 h and then treated with varying doses of CpGB for 18 h. Cell supernatants were then removed and assayed for IL-6 by ELISA. Results are mean ± S.D. for two experiments both performed in triplicate.

unmethylated bacterial and viral CpG-DNA is recognized by TLR9, and the natural ligands for TLR7 and TLR8 are guanosine- and uridine-rich ssRNA (20–22). Using confocal microscopy and counterstaining with endoplasmic reticulum and early endosomal marker proteins, Latz *et al.* (23) have identified the membranes that TLR7, TLR8, and TLR9 are expressed in as the endoplasmic reticulum, which then localizes to endosomes. Latz *et al.* (23) and others (19, 24) have also shown that signaling is initiated in these compartments as evidenced by the concomitant recruitment of MyD88 to vesicular structures in response to loxoribine and R848 (ligands for TLR7 and TLR7/8, respectively) or CpG-DNA. We have demonstrated by co-immunoprecipitation that Btk interacts with TLR8 and TLR9 endogenously and that ligation increased the association. Given that we have also shown that Btk can interact with MyD88 (10), it is possible that Btk localization to the endoplasmic reticulum compartment is via MyD88.

We next examined a role for Btk in TLR8 and TLR9 signal transduction. We had previously shown that Btk is activated upon LPS stimulation of TLR4 (10). Our present data clearly demonstrate that Btk is also tyrosine phosphorylated and subsequently activated in response to both R848 and CpG-DNA treatment in the human monocytic THP1 cell line. Our data identify Btk as an important tyrosine kinase activated by TLR8 and TLR9.

Given that TLR7, TLR8, and TLR9 activate both Btk and NFκB and having previously shown a role for Btk in LPS-induced NFκB activation (9), we investigated whether Btk played a similar role in TLR7-, TLR8-, and TLR9-induced NFκB activation. Activation of an NFκB-linked reporter gene by TLR8 and TLR9 was inhibited by dominant negative forms of Btk. Interestingly the dominant negative forms of Btk had no effect on ISRE activation by TLR8 or TLR9, indicating that Btk acts selectively upstream of NFκB activation. It is now generally accepted that phosphorylation of the p65 subunit controls the nuclear transcriptional potential of NFκB and is required for efficient transcriptional activation. This phosphorylation of p65 is known to be required for transactivation of gene expression because mutating serine residues in the transactivation domains of p65 greatly impairs NFκB-dependent gene transcription (reviewed in Ref. 5). As we had previously placed Btk downstream of TLR4 in the transactivation of the p65 subunit of NFκB, we next examined whether Btk was similarly involved in TLR8- and TLR9-mediated p65 transactivation. TLR8 and TLR9 activated a p65 transactivation assay, and dominant negative forms of Btk diminished this response. Furthermore, we show here for the first time that CpGB and R848 induced phosphorylation of p65 on Ser-536 and that BMDMs defective in Btk were attenuated in this response. Although the roles of individual phospho-serines have not been fully determined, phosphorylation of the p65 subunit of NFκB has emerged as a mechanism by which transcriptional activity of NFκB complexes is regulated in an IκB-independent manner. However, it is suggested that phospho-Ser-536 may contribute to promot-

FIGURE 4. **Btk participates in NFκB activation by TLR8 and TLR9.** *A* and *B*, HEK293 cells were transfected with κB-luciferase (80 ng) and *Renilla*-luciferase (40 ng) and plasmids expressing CD4-TLR8 or CD4-TLR9 (50 ng) or BtkR28C or BtkK430R in amounts of 1, 5, or 10 ng. *C–F*, HEK293 cells were transfected with plasmids expressing CD4-TLR8 (100 ng) or CD4-TLR9 (100 ng) as indicated and a plasmid expressing either Btk(K430R) or Btk(R28C) (1 or 5 ng), as indicated, and either ISRE-luciferase (80 ng) and *Renilla*-luciferase (40 ng) (*C* and *D*) or Gal-luciferase (10 ng), *Renilla*-luciferase (10 ng), and a plasmid expressing Gal4-p65 (10 ng) (*E* and *F*). 18 h after transfection, the cells were harvested, and luciferase activity was subsequently measured. Results are normalized for *Renilla* luciferase activity and represented as relative stimulation over the non-stimulated empty vector control. Results are expressed as mean ± standard deviation for triplicate determinations. *G* and *H*, female (*top panel*) and Male xid (*bottom panel*) murine BMDMs were stimulated with R848 (*G*) or CpG (*H*) for the time points indicated. Cells were then lysed and immunoblotted for phospho-serine 536 on p65 and for β-actin. Luciferase assays are representative of at least three separate experiments, and each was carried out in triplicate. *G* and *H* are representative of two separate experiments.

er-specific assembly of the basal transcriptional machinery and/or coactivators. Indeed, the role of Ser-536 phosphorylation in coupling p65 to coactivators, corepressors, or components of the basal transcriptional machinery has been reported (25, 26).

Considering that Btk appears to play a role downstream of TLR8 and TLR9 in the transactivating potential of NFκB, it seemed likely that a lack of Btk might impact on the induction of NFκB-dependent genes in response to R848 or CpG-DNA. We found a clear impairment in IL-6 induction in response to CpGB in PBMCs from XLA patients relative to control PBMCs. The Btk inhibitor LFM-A13 also blocked induction of IL-6 by CpG in PBMCs. We were therefore able to confirm a key role for Btk in TLR9 signaling in human PBMCs. We found that PBMCs did not respond adequately to R848, and so we were unable to examine TLR8 in a similar fashion. However, impaired ssRNA-mediated IL-6 and tumor necrosis factor-α production has been reported in PBMC-derived dendritic cells from patients with XLA (27). From our data, it now seems likely that this impairment is due to attenuated TLR8 signaling in cells with defective Btk.

These findings are important in relation to XLA patients because although regular intravenous immunoglobulin treatment leads to satisfactory levels of circulating antibodies, XLA patients frequently present with chronic and potentially severe and commonly fatal enteroviral infections, caused by ssRNA viruses. In fact, enteroviral infections are the most common cause of mortality in XLA patients (12, 28). Additionally, patients with XLA are known to be susceptible to herpes viruses such as HHV-6, varicella-zoster, herpes simplex, and cytomegalovirus (29). Herpes viruses have been shown to contain unmethylated CpG motifs (30), and according to one study carried out on 96 XLA patients, herpes simplex virus infections represented 37% of all viral infections (31, 32). An impairment in TLR9 signaling due to the defect in Btk may help explain this susceptibility. Our data are also interesting in relation to certain systemic inflammatory diseases such as the autoimmune disease systemic lupus erythematosus. Aberrant B cell receptor signaling combined with inappropriate or overly active stimulation of TLR7/8 or TLR9 with host nucleic acids in immune complexes have been hypothesized to contribute dramatically toward the onset and severity of systemic lupus erythematosus (33). Given the role of Btk in signaling by TLR8 and TLR9 pharmacologically targeting Btk might be useful in the treatment of systemic lupus erythematosus.

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