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Toll-like receptor 4–, 7–, and 8–activated myeloid cells from patients with X-linked agammaglobulinemia produce enhanced inflammatory cytokines

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Abstract

Background—Bruton tyrosine kinase (BTK) is a component of signaling pathways downstream from Toll-like receptors (TLRs) 2, 4, 7, 8, and 9. Previous work in BTK-deficient mice, cell lines, and cultured cells from patients with X-linked agammaglobulinemia (XLA) suggested defective TLR-driven cytokine production.

Objective—We sought to compare TLR-4–, TLR-7–, and TLR-8–induced cytokine production of primary cells from patients with XLA with that seen in control cells.

Methods—PBMCs from patients with XLA, freshly isolated plasmacytoid dendritic cells, monocytes, and monocytoïd dendritic cells were activated with TLR-4, TLR-7, and TLR-8 agonists. Signaling intermediates and intracellular and secreted cytokine levels were compared with those seen in control cells.

Results—Although TLR-4, TLR-7, and TLR-8 activation of nuclear factor κ B and mitogen-activated protein kinase pathways in cells from patients with XLA and control cells were comparable, TLR-activated freshly isolated monocytes and monocytoïd dendritic cells from patients with XLA produced significantly more TNF- α , IL-6, and IL-10 than control cells. TLR-7/8–activated plasmacytoid dendritic cells produced normal amounts of IFN- α . In murine models BTK regulates the degradation of Toll-IL-1 receptor domain–containing adaptor protein, terminating TLR-4–induced cytokine production. Although this might explain the heightened TLR-4–driven cytokine production we observed, Toll-IL-1 receptor domain–containing adaptor protein degradation is intact in cells from patients with XLA, excluding this explanation.

Conclusion—In contrast to previous studies with BTK-deficient mice, cell lines, and cultured cells from patients with XLA suggesting impaired TLR-driven cytokine production, these data suggest that BTK inhibits TLR-induced cytokine production in primary human cells.

Keywords

Bruton tyrosine kinase; X-linked agammaglobulinemia; Toll-like receptors; MyD88 adapter-like protein; Toll-IL-1 receptor domain-containing adapter protein

Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns, invariant structural components of bacteria and viruses. Although established as integral components of the innate and adaptive immune systems, aberrant TLR activation in T and B cells might be involved in the development or maintenance of inflammatory conditions, such as inflammatory bowel disease, rheumatoid arthritis, and systemic lupus erythematosus.^{1–4} Because TLRs are expressed in all cells of the immune system, components of the TLR signaling pathways are logical targets for pharmacologic interference in patients with these and other inflammatory diseases.^{1,5} One of these proposed targets is Bruton tyrosine kinase (BTK), which has been identified as downstream of TLR-2, TLR-4, TLR-7, TLR-8, and TLR-9.^{1,6–17} BTK is well known as a cytoplasmic protein kinase integral to B-cell receptor signaling pathways and is critical for both the growth and survival of B cells in human subjects.¹⁸ Mutations in *BTK* lead to the immunodeficiency X-linked agammaglobulinemia (XLA) in which a block in B-cell development at the pre-B-cell stage leads to a loss of B cells and agammaglobulinemia.^{19,20} Male patients with XLA are susceptible to recurrent bacterial infections unless given sufficient immunoglobulin replacement.^{21–23} Although B cells are severely impaired in patients with XLA,²⁴ the role of BTK in human neutrophils, monocytes, monocytoïd dendritic cells (mDCs), plasmacytoïd dendritic cells (pDCs), and platelets, which constitutively and abundantly also express this kinase, is less clear, possibly because of overlapping functions between BTK and other members of the Tec family of kinases.²⁵

The first suggestion that BTK might be integral to TLR signaling came from work in *Xid* mice expressing nonfunctional BTK.²⁶ *Xid* mice produce less TNF- α and IL-1 β in response to systemic LPS treatment, and isolated *Xid* macrophages and neutrophils had impaired production of reactive oxygen intermediates in response to inflammatory stimuli.^{5,14} However, TLR-4- or TLR-9-stimulated BTK-deficient murine B cells and macrophages produced increased amounts of the proinflammatory cytokines TNF- α and IL-6,^{8,15} suggesting an inhibitory role for BTK. Further studies in murine and human cell lines treated with ligands for TLR-2, TLR-4, TLR-7, TLR-8, and TLR-9 demonstrated that not only is BTK phosphorylated, it also coimmunoprecipitates with multiple TLR signaling components.^{6,10,11}

Although data from murine cells suggest a functional linkage between BTK and TLR pathways, experiments on cells from patients with XLA have been both inconsistent and contradictory. Three studies on mDCs derived from cytokines from patients with XLA showed impaired TLR-driven IL-6 production, TNF- α production, or both with impaired mitogen-activated protein kinase (MAPK) activation,^{9,16,17} but other experiments on fresh monocytes reported both normal intracellular IL-6 and TNF- α production and intact phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK) after LPS exposure.^{27,28} Differences in the cells examined, TLR activators, isolation methods, and/or end points might explain these differences, and some results are further confounded by the use of a potentially nonspecific BTK inhibitor to mimic the XLA condition in confirmatory studies.^{7,16,29}

On the basis of studies demonstrating the linkage between BTK and TLR pathways, BTK inhibitors are currently being developed as therapeutic interventions in patients with both autoimmune and inflammatory diseases.^{1,5,30,31} In view of the contradictory results in

human studies, a more complete understanding of the role of BTK in TLR-induced inflammation in human subjects will be essential before clinical use of these inhibitors. Here we examined TLR activation in primary nondifferentiated cells from patients with XLA to examine whether the loss of BTK promotes or inhibits TLR-induced inflammation in these cells.

METHODS

Patients and control subjects

Peripheral blood cells of 13 male patients with XLA, aged 16 months to 43 years, were studied. Each had <0.1% B cells in peripheral blood and were profoundly hypogammaglobulinemic; all were receiving replacement immune globulin in standard doses. Genetic confirmation of XLA was performed by Dr M. E. Conley. Blood samples were collected before infusions. Control subjects were healthy adult volunteers. These studies were performed by using an institutional review board–approved protocol with informed consent.

Cell isolation

PBMCs were isolated from heparinized peripheral blood by means of Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Monocytes were isolated from PBMCs through adherence to plastic, as previously described.³² These cells (85% to 95% CD14⁺, as determined by means of flow cytometry with FACSCalibur [Becton Dickinson, San Jose, Calif]) were plated at 5×10^5 cells/mL in 96-well plates and treated with TLR ligand, as described below. For studies using the BTK inhibitor LFM-A13 (Tocris Bioscience, Bristol, United Kingdom), monocytes were pretreated for 20 minutes with LFM-A13 100 μ mol/L or dimethyl sulfoxide as vehicle before treatment with TLR ligands. mDCs (>85% CD1c⁺, as determined by means of flow cytometry) were isolated from PBMCs from patients with XLA and control subjects by using magnetic bead selection (CD1c⁺ Dendritic Cell Isolation Kit; Miltenyi Biotec, Auburn, Calif) and plated in 96-well plates at 2×10^5 cells/mL. pDCs (>90% BDCA2⁺, as determined by means of flow cytometry) were isolated from PBMCs by using negative magnetic bead selection (Miltenyi Biotec) and plated in 96-well round-bottom plates at 10^5 cells/mL.

Western blotting

BTK expression in PBMCs from control subjects and patients with XLA was assessed by means of Western blotting, as previously described,³³ and found to be absent in the 13 subjects examined here (see Fig E1 in this article's Online Repository at www.jacionline.org). To examine TLR-4 and TLR-7/8 signaling pathways, PBMCs from patients with XLA or control subjects were plated at 2×10^6 cells/mL and treated with the TLR ligands LPS (1 ng/mL to 1.0 μ g/mL) or CL097 (0.125–2.5 μ g/mL; InvivoGen, San Diego, Calif). Protein was isolated, and Western blots were run as previously described³³ and developed with the primary antibodies mouse anti-phospho-I κ B- α (#9243; Cell Signaling, Beverly, Mass), rabbit anti-Toll-IL-1 receptor domain-containing adaptor protein (TIRAP; Pearl-1, Alexis Biochemicals, Axxora, San Diego, Calif), rabbit anti-phospho-38MAPK (R&D Systems, Minneapolis, Minn), rabbit anti-phospho-44–42MAPK (ERK1/2, Cell Signaling), rabbit anti-phospho-SAPK/JNK (Cell Signaling), and mouse anti- β -actin (3700, Cell Signaling). Protein was visualized with Immobilon Western HRP Substrate (Millipore, Billerica, Mass). Densitometric analyses of Western blots were compared with ImageJ software (National Institutes of Health, Bethesda, Md). Band densities were standardized to β -actin, and values were reported as a percentage of β -actin (for phosphorylation assays) or, for degradation studies, as a percentage of the untreated control value.

Cytokine assays

PBMCs were treated with optimum amounts of agonists, either 2.5 µg/mL CL097 or 1.0 µg/mL LPS for 6 hours along with 10 µg/mL Brefeldin A (MP Biomedicals, Solon, Ohio), to first investigate TLR-mediated intracellular cytokine production. Cells were washed, fixed in 4% paraformaldehyde, permeabilized with 1% saponin (Sigma, St Louis, Mo), and incubated with phycoerythrin-tagged mouse monoclonal anti-human IL-6 or mouse anti-human TNF-α (BD Biosciences, San Diego, Calif).³⁴ XLA and control CD14⁺ monocytes identified by using anti-CD14–fluorescein isothiocyanate were then examined for IL-6 and TNF-α by means of flow cytometry (FACS-Calibur, BD Biosciences).

Monocytes were incubated with a range of concentrations of LPS (10 ng/mL to 1.0 µg/mL) or CL097 (0.125–2.5 µg/mL) and supernatants were harvested at 6, 24, and 48 hours to quantify TLR-induced cytokine production. In other studies mDCs were treated with optimized concentrations of LPS (1.0 µg/mL) or CL097 (2.5 µg/mL), and supernatants were harvested at 24 hours. IL-6, TNF-α, and IL-10 production was quantified by using BD Opt EIA kits (BD Biosciences). pDCs were cultured with CL097 (2.5 µg/mL) or loxoribine (500 µmol/L, InvivoGen) for 24 hours, and IFN-α levels in supernatants were quantified by using ELISA (Bender MedSystems, Burlingame, Calif).

Statistical analysis

Statistical analysis was performed with PRISM 4.0 software (GraphPad Software, Inc, La Jolla, Calif). ELISA cytokine production and mean fluorescence intensity for intracellular cytokine staining for patients and control subjects were compared by using an unpaired 2-tailed *t* test. Densitometric results were also compared by using an unpaired 2-tailed *t* test.

RESULTS

TLR activation of nuclear factor κB and MAPK signaling pathways

To investigate a potential early impairment in TLR signaling in the absence of BTK, TLR-4 and TLR-8 activation of nuclear factor κB (NF-κB) and MAPKs p38, JNK, and ERK1/2 signaling pathways were compared for cells from patients with XLA and control cells. Mononuclear cells from healthy control subjects and patients with XLA demonstrated similar levels of phosphorylation of IκB-α and the MAPK p38 at all concentrations of CL097 and LPS tested; Fig 1, *A* and *B*, shows comparative data for a representative patient with XLA and a control subject. Similar levels of phosphorylation of IκB-α and p38 for 8 patients with XLA and 5 control subjects were achieved (Fig 1, *C* and *D*). Phosphorylation of the MAPK families ERK and JNK, as well as p38, was also assessed over time. TLR ligands exhibited similar activation for all 3 MAPKs in PBMCs from control subjects and patients with XLA with similar kinetics (Fig 1, *E*), although some differences were seen from sample to sample (see Fig E2 in this article's Online Repository at www.jacionline.org), which might be due to differences in cell populations.

Cytokine production by TLR-stimulated monocytes

Cytokine production in response to TLR ligands was then assessed. TLR-activated monocytes from both control subjects and patients with XLA contain large amounts of intracellular IL-6 and TNF-α as early as 6 hours after treatment (see Fig E3 in this article's Online Repository at www.jacionline.org) but with no significant differences between cells from patients with XLA and control cells, possibly because of saturation of antibody staining in the technique used to reveal intracellular cytokines. However, purified monocytes from patients with XLA cultured with LPS or CL097 for 6, 24, and 48 hours produced significantly higher amounts of both IL-6 and TNF-α compared with those seen in control cells at all time points (Fig 2, *A* and *B*). Higher levels of TNF-α and IL-6 were also found in

the supernatants of TLR-4- and TLR-7/8-activated monocytes from patients with XLA after both 24 (data not shown) and 48 hours compared with those seen in control cells (Fig 2, *C* and *D*). These differences were apparent over a range of LPS concentrations (10 ng to 1 µg/mL) and at 2.5 µg/mL CL097. In contrast, monocytes from patients with XLA and control subjects produced similar amounts of IL-10 at all time points and ligand concentrations (Fig 2, *E*).

Cytokine production by TLR-stimulated dendritic cells

In previous studies IL-4 plus GM-CSF-differentiated mDCs from patients with XLA showed impaired production of TNF-α and IL-6.^{16,17} More in accordance with physiologic conditions, we assessed CLO97- or LPS-induced cytokine production in primary mDCs and CD1c⁺ mDCs from patients with XLA and control subjects. mDCs from patients with XLA produced significantly higher levels of IL-6 and TNF-α, as well as IL-10, compared with control mDCs (Fig 3, *A–C*). Interestingly, even nonstimulated mDCs from patients with XLA in culture displayed heightened production of cytokines compared with that seen in control cells, suggesting an intrinsic baseline difference between monocytes from patients with XLA and control monocytes.

We had previously shown that IFN-α production by pDCs from patients with XLA in response to the TLR9 ligand ODN-2006 was normal.³⁴ pDCs from patients with XLA were treated with loxoribine or CL097 to examine responses to TLR7 and TLR7/8 agonists. Production of IFN-α by pDCs from patients with XLA was again found to be comparable with that seen in the pDCs of healthy control subjects (Fig 3, *D*).

LFM-A13 is a nonspecific BTK inhibitor

Previous studies have used LFM-A13, a leflunomide metabolite, as a BTK inhibitor to mimic the XLA condition.^{7,16,29} To examine the specificity of LFM-A13, monocytes from control subjects and patients with XLA were pretreated with inhibitor or the vehicle dimethyl sulfoxide and then cultured with LPS or CL097 to examine cytokine production. Surprisingly, LFM-A13 increased TNF-α production and decreased IL-6 and IL-10 production for both monocytes from control subjects and those from patients with XLA (see Fig E4 in this article's Online Repository at www.jacionline.org). Because this inhibitor should have no effect on cells from patients with XLA, the nonspecific effects we have demonstrated here show that LFM-A13 should not be used to model the loss of BTK in other studies.

BTK is not required for degradation of TIRAP through TLR4 signaling

As a possible explanation for the mechanistic action of BTK in the TLR pathways, BTK had been shown to phosphorylate the TLR-2 and TLR-4 adaptor protein TIRAP (Mal), leading to polyubiquitination and degradation.^{29,35} Because TIRAP degradation disrupts the TLR-4 signaling complex, BTK could limit cytokine production and explain some of the enhanced cytokine production we describe here. However, TIRAP was rapidly degraded in LPS-treated cells from patients with XLA and control cells (Fig 4, *A*) and remained degraded for up to 4 hours (Fig 4, *B*), demonstrating that BTK was not essential for this process and that redundant regulatory mechanisms exist.

DISCUSSION

The role of BTK in adaptive immunity is well established because without a functional kinase, human B cells do not develop in human subjects. On the basis of previous work in the *Xid* mouse and *BTK*^{-/-} mice and studies on murine and human cell lines, an integral role for BTK in a number of TLR pathways has been proposed.^{6–8,10–13,15–17,29,35} This

intriguing hypothesis has led to speculation that inhibitors of BTK might be therapeutically useful in patients with inflammatory or autoimmune diseases in which aberrant activation or hyperactivation of TLRs might be implicated. In these studies we investigated selected pathways and functional outcomes of TLR signaling in primary cells of patients with XLA to determine what role BTK might play in signaling through TLRs.

The responses of BTK-deficient mononuclear cells, mDCs, and pDCs to TLR-4, TLR-7, and TLR-8 were assessed for the induction of the MAPKs, as well as the NF- κ B signaling pathways downstream of TLRs. In contrast to one study that found low levels of p38 activation after LPS treatment,⁹ we found that phosphorylation of p38, ERK, and JNK, as well as I κ B- α , occurred normally in activated cells from patients with XLA. In addition, we found that TLR-4-stimulated monocytes from patients with XLA and freshly isolated mDCs produced substantially greater amounts of IL-6 and TNF- κ than control cells, starting at 6 hours and extending for 48 hours, over a wide range of LPS concentrations. These data are more in accordance with a number of murine studies^{8,15,36} and contradict a study that examined TLR-activated IL-4 plus GM-CSF-derived mDCs from patients with XLA.¹⁶ However, the comparability of GM-CSF-derived mDCs from patients with XLA with control dendritic cells has been questioned. Although mDCs from patients with XLA appeared to differentiate and mature in response to LPS,³⁷ blunted CD1a, CD83, CD80, and CD86 surface expression of cultured mDCs from patients with XLA suggests that these cells are not comparable with control mDCs.^{16,28,38}

A number of previous studies have relied on LFM-A13 as a BTK inhibitor on control cells to confirm that the loss of BTK impairs cytokine production.^{7,11,16,29} However, LFM-A13 also inhibits phosphorylation and activation of the other Tec kinase family members Tec and Bmx.³⁹ These kinases, along with BTK, intersect with signals from cytokine receptors, such as the IL-6 and IL-10 receptors, potentially altering autocrine pathways.^{40,41} LFM-A13 also inhibits Janus-associated kinase 2⁴² downstream of both the IL-6 and GM-CSF receptors and other cell-surface receptors. Perhaps most conclusive, we show that LFM-A13 alters the cytokine responses of monocytes from patients with XLA, clearly excluding its use in modeling BTK deficiency in human cells.

To explore why TLR-stimulated monocytes and mDCs from patients with XLA might produce significantly increased IL-6 and TNF- κ levels, we investigated the LPS-induced degradation of TIRAP. TIRAP is the TLR adaptor responsible for recruitment of MyD88 and TNF receptor associated factor (TRAF) protein family-6 (TRAF6) to the cytoplasmic tail of activated TLR-4; phosphorylation promotes degradation and termination of TLR-4 signaling. Loss of BTK could enhance or prolong signaling^{29,35,43} according to previous studies of TLR-4 signaling in *Xid* cells and the monocytic leukemia cell line THP-1 treated with LFM-A13. We show here that TIRAP degradation is intact in mononuclear cells from patients with XLA after LPS treatment. TLR activation of IL-1 receptor-associated kinase (IRAK) 1 and IRAK4 also results in phosphorylation and degradation of TIRAP,⁴⁴ which could provide an alternate means for TIRAP degradation in cells from patients with XLA. Additionally, defective BTK-mediated degradation of TIRAP would not account for the increased cytokine production by cells from patients with XLA treated with CL097, a ligand for endosomal TLR-7 and TLR-8, which signal independently of TIRAP.³⁵

Another possibility is that BTK, although involved in TLR signaling, might also restrict the inflammatory response to TLR ligands through other receptors, such as the IL-6 and IL-10 receptors.^{40,41} Expression profiling of EBV-transformed B cells from patients with XLA demonstrated significantly higher levels of signaling intermediates downstream of both TLRs and many cytokine receptors: Hck, Lyn, Fyn, and signal transducer and activator of transcription 5A.⁴⁵ Although the mechanistic explanations for these data remain to be

elucidated, our results are in accord with clinical observations that patients with XLA are not prone to the infections found in patients with IRAK4 or MyD88 defects, although one might argue that the standard and early use of intravenous immunoglobulin could prevent such infections.

In contrast to XLA, a different pattern of TLR-7, TLR-7/8, and TLR-9 responses are found in patients with common variable immunodeficiency (CVID), with pronounced B-cell proliferation and maturational defects. However, here, PBMCs from patients with CVID produce normal amounts of TNF- α and IL-6 in response to TLR-2, TLR-4, and TLR-5,⁴⁶ and LPS-activated mDCs from patients with CVID produce normal levels of TNF- α , IL-6, and IL-10 (data not shown).

In summary, primary hematopoietic cells from patients with XLA have more robust cytokine responses than cells of healthy control subjects, suggesting that BTK plays an as yet unclarified role in inhibiting TLR-induced responses in human subjects. We noted with interest that increased levels of IL-6, IL-1 β , and TNF- α were found in the sera of patients with XLA in another study,⁴⁷ which might correlate with our data. However, patients with XLA receiving adequate immunoglobulin replacement are generally healthy,⁴⁸ with the possible exception of uncommon inflammatory rheumatologic disorders potentially caused by *Mycoplasma* species^{49–53} and a few other rare infectious diseases.^{54,55} Whether the loss of BTK contributes to the inflammatory sequelae of these conditions is unknown.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

BTK	Bruton tyrosine kinase
CVID	Common variable immunodeficiency
ERK	Extracellular signal-regulated kinase
IRAK	IL-1 receptor–associated kinase
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
mDC	Monocytoid dendritic cell
NF-κB	Nuclear factor κ B
pDC	Plasmacytoid dendritic cell
TIRAP	Toll–IL-1 receptor domain–containing adaptor protein
TLR	Toll-like receptor

XLA X-linked agammaglobulinemia

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Clinical implications

TLR activation might enhance inflammatory disorders. Because BTK is involved in TLR signaling, inhibitors are being developed as therapeutic options; however, our data suggest that BTK might negatively regulate the inflammatory response.

FIG 1.

A and **B**, IκB-α and MAPK p38 phosphorylation in PBMCs treated with increasing concentrations of LPS (Fig 1, *A*) or CL097 (Fig 1, *B*) for 5 minutes. **C** and **D**, Phosphorylation after either no treatment (*NT*), LPS (1 μg/mL), or CL097 (2.5 μg/mL) was quantified and represented graphically as a percentage of β-actin in control cells (*solid bars*, *n* = 5) and cells from patients with XLA (*open bars*, *n* = 8). **E**, Phosphorylation of the MAPKs ERK, p38, and JNK in PBMCs treated as above with TLR ligands for 0 to 60 minutes.

FIG 2.

Cytokines secreted by control monocytes (*solid circles*) and those from patients with XLA (*open circles*) treated as above for 6 hours (**A** and **B**) or with LPS (1 μ g/mL) or CL097 (2.5 μ g/mL) or for 48 hours (**C**, **D**, and **E**). *ns*, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

FIG 3.

A–C, Cytokine production by control mDCs (*solid circles*) and those from patients with XLA (*open circles*) treated with LPS (1 μ g/mL) or CL097 (2.5 μ g/mL) for 24 hours. **D**, IFN- α production by pDCs from control subjects (*solid circles*) and patients with XLA (*open circles*) treated with CL097 (2.5 μ g/mL) or loxoribine (500 μ mol/L) for 24 hours. *ns*, Not significant. * P < .05, ** P < .01, and *** P < .001.

FIG 4.

A, Degradation of TIRAP in PBMCs from control subjects (*NL*; *solid bars*, $n = 10$) and patients with XLA (*open bars*, $n = 13$) treated with LPS ($1.0 \mu\text{g/mL}$) for 30 or 60 minutes. **B**, Representative Western blot of PBMCs from control subjects (*NL*) and 1 patient with XLA treated with LPS ($1 \mu\text{g/mL}$) for up to 240 minutes. *NT*, No treatment.