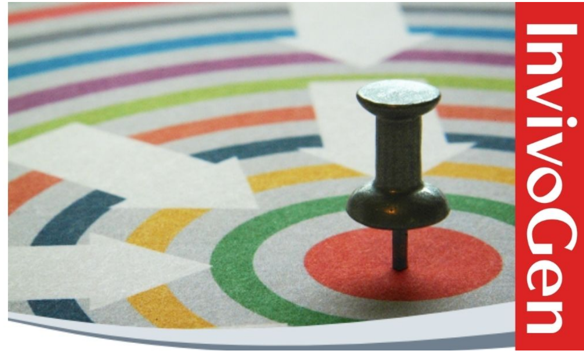
Custom Screening
& Profiling Services
for immune-modulating compounds

TLR - NOD 1/NOD2 - RIG-I/MDA5 - STING
DECTIN-1 - MINCLE



The Journal of Immunology

RESEARCH ARTICLE | MARCH 15 2006

Bruton's Tyrosine Kinase Is Required for TLR2 and TLR4-Induced TNF, but Not IL-6, Production[1]

Nicole J. Horwood; ... et. al

J Immunol (2006) 176 (6): 3635–3641.
https://doi.org/10.4049/jimmunol.176.6.3635

Related Content

Expression of Bruton's agammaglobulinemia tyrosine kinase gene, BTK, is selectively down-regulated in T lymphocytes and plasma cells.

J Immunol (January,1994)

Characterization of Mutations, Including a Novel Regulatory Defect in the First Intron, in Bruton's Tyrosine Kinase Gene from Seven Korean X-Linked Agammaglobulinemia Families

J Immunol (October,2001)

Six X-Linked Agammaglobulinemia-Causing Missense Mutations in the Src Homology 2 Domain of Bruton's Tyrosine Kinase: Phosphotyrosine-Binding and Circular Dichroism Analysis

J Immunol (April,2000)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

Bruton's Tyrosine Kinase Is Required for TLR2 and TLR4-Induced TNF, but Not IL-6, Production[1]

Nicole J. Horwood, Theresa H. Page, John P. McDaid,[2] Christine D. Palmer, Jamie Campbell,[3] Tara Mahon,[4] Fionula M. Brennan, David Webster,[5] and Brian M. J. Foxwell[6]

Bruton's tyrosine kinase (Btk), the gene mutated in the human immunodeficiency X-linked agammaglobulinemia, is activated by LPS and is required for LPS-induced TNF production. In this study, we have investigated the role of Btk both in signaling via another TLR (TLR2) and in the production of other proinflammatory cytokines such as IL-1β, IL-6, and IL-8. Our data show that in X-linked agammaglobulinemia PBMCs, stimulation with TLR4 (LPS) or TLR2 (*N*-palmitoyl-*S*-[2, 3-bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteine) ligands produces significantly less TNF and IL-1β than in normal controls. In contrast, a lack of Btk has no impact on the production of IL-6, IL-8, or the anti-inflammatory cytokine, IL-10. Our previous data suggested that Btk lies within a p38-dependent pathway that stabilizes TNF mRNA. Accordingly, TaqMan quantitative PCR analysis of actinomycin D time courses presented in this work shows that overexpression of Btk is able to stabilize TNF, but not IL-6 mRNA. Furthermore, using the p38 inhibitor SB203580, we show that the TLR4-induced production of TNF, but not IL-6, requires the activity of p38 MAPK. These data provide evidence for a common requirement for Btk in TLR2- and TLR4-mediated induction of two important proinflammatory cytokines, TNF and IL-1β, and reveal important differences in the TLR-mediated signals required for the production of IL-6, IL-8, and IL-10. *The Journal of Immunology,* **2006, 176: 3635–3641.**

The TLRs recognize conserved microbial pathogen-associated molecular patterns (PAMPs)[7] and have essential functions in both the innate and acquired immune systems (1–3). Stimulation through the TLRs by PAMPs triggers the production of various cytokines, resulting in an inflammatory response essential for the eradication of many infectious microorganisms. However, excessive and prolonged activation may contribute to sepsis: the most common cause of death in intensive care units. To date, 10 human TLRs have been identified that are variably expressed on many different cell types, including macrophages, neutrophils, dendritic cells, lymphocytes, and endothelial cells (4), each recognizing a distinct set of ligands. Enterobacterial LPS, the most commonly studied of the PAMPs, is recognized by TLR4, while LPS derived from *Leptospira interrogans* or *Porphyromonasgingivalis*, lipoarabinomannan, and bacterial lipoproteins are all recognized by TLR2 (1, 2). TLR2 can also function as a heterodimeric complex with other TLRs. Thus, the synthetic lipopeptide, macrophage-activating lipopeptide-2 (MALP2), and zymosan act via a TLR2/6 heterodimer, while *N*-palmitoyl-*S*-[2, 3-bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteine (PAM_3Cys) acts via a TLR2/1 heterodimer (4, 5). Zymosan has also been shown to act via TLR2 in synergy with Dectin-1, a yeast-binding C type lectin (6).

Signaling by TLRs requires the recruitment of various intracellular molecules, and all TLRs share a common sequence motif (known as the TOLL IL-1R or Toll-IL-1 region (TIR) homology domain) that mediates homotypic interactions with a group of intracellular TIR-containing adapter molecules: MyD88, MyD88 adaptor-like, TIR domain-containing adaptor inducing IFN-β, and TRIF-related adaptor molecules. Various other adapter molecules such as the IL-1R-associated kinases, TNFR-associated factor 6, and TNFR-associated factor family-associated NFκB activator-binding kinase 1 can then associate with the receptor complex, leading to the activation of IFN regulatory factor 3, NF-κB, p38, and other MAPK pathways. The activation of these pathways then regulates the expression of cytokines and other effector genes. However, the full nature of the signaling pathways used by TLRs to stimulate the release of cytokines and chemokines is still not fully understood, and also how many of the pathways described for the LPS/TLR4 system are applicable to other TLRs.

In particular, the role of tyrosine kinases in TLR signaling is unclear, which is surprising, as tyrosine phosphorylation is one of the earliest detectable events following LPS stimulation (7, 8). Some studies have suggested a role for both the Src and JAK family tyrosine kinases in TLR signaling (9, 10); however, the relationship between the tyrosine kinases and the different TLRs remains both largely unexplored and inconclusive.

Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Charing Cross Campus, London, United Kingdom

Received for publication July 27, 2005. Accepted for publication December 29, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by a Howard Florey Fellowship (to N.J.H.) and grants from the Arthritis and Rheumatism Council (to J.P.M. and T.H.P.), Wyeth (to J.C.), and the Medical Research Council (to N.J.H.).

[2] Current address: Renal Medicine, Division of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0HS, U.K.

[3] Current address: Department of Disease Biology, Rheumatology and Inflammation, GlaxoSmithKline Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts SG1 2NY, U.K.

[4] Current address: Avidex Limited, 57-59 Milton Park, Abingdon, Oxon OX14 4RX, U.K.

[5] Current address: Royal Free Medical School, Department of Immunology, University College, London, U.K.

[6] Address correspondence and reprint requests to Prof. Brian M. J. Foxwell, Kennedy Institute of Rheumatology Imperial College London, 1 Aspenlea Road, London, W6 8LH, U.K.

[7] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; Btk, Bruton's tyrosine kinase; C_t, threshold cycle; MALP2, macrophage-activating lipopeptide-2; moi, multiplicity of infection; PAM_3Cys, *N*-palmitoyl-*S*-[2, 3-bis(palmitoyloxy)-(2*R*)-propyl]-(*R*)-cysteine; PSI, proteasome inhibitor I; TIR, Toll-IL-1 region; XLA, X-linked agammaglobulinemia.

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$02.00

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

Recently, studies from our laboratory and others have demonstrated a role for Bruton's tyrosine kinase (Btk) in TLR signaling (11). Specifically, we have shown that LPS stimulation induces phosphorylation of Btk in human monocytes and macrophages. In humans, a lack of functional Btk results in X-linked agammaglobulinemia (XLA), and we have shown significantly reduced TNF production in response to LPS in PBMCs derived from XLA patients (11). *Xid* mice, which carry a defective Btk gene, also show reduced responses to LPS stimulation, with decreased NO production, and impaired cytokine production and macrophage effector functions (12, 13). Our studies also showed that the ability of Btk to modulate LPS-induced TNF production relied upon stabilizing TNF mRNA via the 3′ untranslated region. This stabilization was found to correlate with increased phosphorylation of p38 MAPK (11).

Although these data have shown that Btk is required for TLR4 signaling and the production of TNF, the role of Btk in signaling through other TLRs is unknown. Thus, because TLR4 and TLR2 are able to initiate a similar cascade of cytokine responses, we investigated the potential role of Btk in signaling via TLR2. In the studies described in this work, we have examined the responses of XLA PBMCs to the TLR2 ligand PAM_3Cys by measuring cytokine production compared with normal controls. Furthermore, we have now extended our previous work to examine the production of other important proinflammatory cytokines such as IL-1β and IL-6, as well as IL-8 and IL-10. The data clearly show that Btk also has an important role in signaling via TLR2, but reveal an unexpected complexity in the TLR2- and TLR4-mediated signaling pathways leading to the production of TNF and IL-1β vs those required for IL-6, IL-8, and IL-10.

Materials and Methods

Reagents

LPS was obtained from Sigma-Aldrich. PAM_3Cys was obtained from ECM Microcollections. MALP2 was acquired from Alexis Biochemicals. SB203580 and proteasome inhibitor I (PSI) were purchased from Calbiochem. All reagents (other than LPS) were tested for the presence of endotoxin using a *Limulus* amebocyte assay (BioWhittaker). Levels of contaminating LPS were below the limits of detection.

Isolation and culture of PBMCs from XLA patients and control donors

Human blood samples were collected into lithium heparin vacutainers. Each blood sample was mixed with an equal volume of HBSS, and PBMCs were prepared by Ficoll-Hypaque centrifugation on a Lymphoprep gradient. PBMCs were cultured at a concentration of 1×10^6 cells/ml in RPMI 1640 containing 100 U/ml penicillin/streptomycin and 10% heat-inactivated FCS at 37°C in a humidified atmosphere containing 5% CO_2. The diagnosis in the XLA patients depended on demonstrating mutations in the coding gene for Btk and failure to express the protein. Ethical permission for the study was obtained from the local Royal Free Ethics committee.

Isolation of monocytes by elutriation

PBMCs were prepared from buffy coat fractions of a unit of blood from a single donor using Ficoll-Hypaque. The monocytes were then isolated by centrifugal elutriation, as previously described (14). Monocyte fractions of >85% purity were routinely collected in this manner. Monocytes were cultured in RPMI 1640 containing 100 U/ml penicillin/streptomycin and 10% heat-inactivated FCS at 37°C in a humidified atmosphere containing 5% CO_2. For adenoviral infection, monocytes were treated with M-CSF (100 ng/ml) (gift from Wyeth-Ayerst Pharmaceuticals) for 72 h before viral infection.

Generation of adenoviral vectors and cell infection

Recombinant, replication-deficient adenoviral constructs encoding wild-type human Btk, Pyk2, and PykM were prepared using the AdEasy system previously described (14). M-CSF-derived monocytes were plated in 96-well plates at 1.5×10^5 cells/well and allowed to settle for at least 4 h. The cells were washed in serum-free RPMI 1640 medium and then exposed to virus at different multiplicity of infection (moi) for 2 h in serum-free RPMI 1640 at 37°C, following which cells were washed in RPMI 1640 and cultured in complete medium for 24 h before stimulation with LPS, as previously described (11). Inhibitors (SB203580 or PSI) were added at the concentrations indicated for 1 h before stimulation.

ELISA

Human macrophages and PBMCs were cultured at 1.5×10^5 and 2×10^5 cells/well, respectively, in 200 μl of medium. At 4 or 18 h poststimulation with TLR ligands, supernatants were harvested and stored at −20°C. The concentrations of TNF-α, IL-1β, IL-6, IL-8, and IL-10 were determined by ELISA (BD Pharmingen), according to the manufacturer's instructions. Absorbance at 450 nm was measured on a spectrophotometric ELISA plate reader (Labsystems Multiskan Biochromic), and data were analyzed using the Ascent software program.

Immunoprecipitation and in vitro kinase assay

Monocytes were prepared by elutriation and were cultured in the presence of 100 ng/ml M-CSF for 4 days. Cells were then replated into 10-cm cell culture dishes at a density of 6×10^6 cells/dish. The following day, cells were washed and rested in serum-free medium for 1.5–2 h. After stimulation with TLR ligands, cells were lysed in ice-cold lysis buffer (25 mM HEPES (pH 7.0), 150 mM NaCl, and 1% Nonidet P-40), containing 1 mM DTT, 1 mM PMSF, 10 mM NaF, aprotinin, and 100 μM sodium orthovanadate. Nuclei and cell debris were removed by centrifugation, and supernatants were precleared with protein G-Sepharose. Btk was then precipitated with 3 μl of polyclonal rabbit anti-Btk antisera (a gift from M. Tomlinson, University of Birmingham, Edgbaston, Birmingham, U.K.) and protein G-Sepharose for 1.5 h. Immunoprecipitated complexes were washed in radioimmunoprecipitation assay buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM DTT, and 100 μM vanadate) and once in kinase buffer (20 mM HEPES (pH 7.0), 5 mM $MnCl_2$, 5 mM $MgCl_2$, 100 μM vanadate, and 1 mM DTT). In vitro kinase assays were performed in kinase buffer in the presence of 10 mM ATP with 10 μCi of γ-ATP for 15 min at room temperature. The reaction was terminated by the addition of 5× gel sample buffer, and samples were resolved on 7% SDS-PAGE. Radiolabeled species were visualized by autoradiography using Hyperfilm-MP (Amersham Biosciences).

TaqMan RT-PCR

M-CSF-treated monocytes were plated at 5×10^5 cells/well in 24-well plates and infected, as described above. Cells were treated with LPS for 4 h before the addition of actinomycin D (2 μg/ml) and were harvested at 0, 15, 30, 60, 90, and 120 min. Total RNA was extracted using RNeasy Kit (Qiagen), according to the manufacturer's instructions. All semiquantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System; TaqMan One Step RT-PCR reagent; and TNF-α, IL-6, and GAPDH predeveloped assay reagents (PerkinElmer Applied Biosystems). All samples were run in triplicate. ABI PRISM 7700 Sequence detector was programmed for the reverse-transcription step of 30 min at 48°C, followed by a 5 min at 95°C deactivation step. Subsequent PCR amplification consisted of 40 cycles of denaturation at 94°C for 15 s and annealing/extension at 60°C for 60 s. All quantitations were normalized to an endogenous control, the housekeeping gene GAPDH, to account for variability in the initial concentration of RNA and in the conversion efficiency of the reverse-transcription reaction. The analysis of the relative quantitation required calculations based on the C_t; the cycle number at which the amplification plot crosses a fixed threshold above baseline is defined as threshold cycle (C_t). Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Results

PAM_3Cys, a TLR2 ligand, induces the activation of Btk in human macrophages

Primary human macrophages were stimulated with PAM_3Cys or MALP2 for various lengths of time, and endogenous Btk was immunoprecipitated and assessed for changes in intrinsic autokinase activity. Fig. 1 shows that Btk from stimulated cells exhibits increased autokinase activity compared with unstimulated cells or with immunoprecipitates using control Abs. Increased Btk kinase activity was observed using PAM_3Cys (Fig. 1*A*) and MALP2 (Fig. 1*B*) within 10 min of stimulation. These findings support a role for Btk in signaling via the TLR2 molecule.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

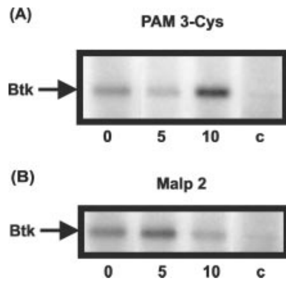


FIGURE 1. Increased phosphorylation of Btk in response to TLR2 ligands. Five-day M-CSF-treated monocytes (~6 × 100^{6}0/track) were either untreated or treated with either 10 ng/ml PAM3Cys (*A*) or 10 ng/ml MALP2 (*B*) for the indicated times (in minutes). Cells were lysed, and Btk was immunoprecipitated from lysates, as described in *Materials and Methods*. Immunoprecipitated Btk was subjected to in vitro kinase assay before resolution on 7% SDS-PAGE. c, Immunoprecipitation with an irrelevant rabbit sera. The kinase assays shown are representative of at least three separate experiments.

Btk deficiency results in impaired TLR2-induced TNF production

Previous data from our laboratory, using Btk-deficient XLA PBMCs, have shown that LPS (TLR4)-induced TNF production requires Btk (11). We therefore used the same approach to investigate the role of Btk in PAM_3Cys (TLR2)-driven cytokine production. In common with our previous findings for LPS, we also found that XLA PBMCs stimulated with PAM_3Cys over a variety of concentrations produced significantly lower amounts of TNF when compared with age- and sex-matched controls (Fig. 2*A*). This resulted in 5-fold lower expression of TNF from XLA PBMCs. It is important to note that due to the inherent variability of different donors, mean values were used to establish the statistical significance. Similar results were seen after stimulation with MALP2 over a range of concentrations (data not shown). In the absence of stimuli, there was no detectable expression of TNF in the culture supernatants.

Btk deficiency also results in impaired TLR-induced IL-1β production

IL-1β is also strongly implicated in inflammatory responses; however, our previous studies only addressed the role of Btk in TNF production. We therefore extended our studies to examine the requirement for Btk in the expression of this cytokine. Fig. 2*B* shows that XLA PBMCs stimulated with PAM_3Cys over a range of doses also have impaired IL-1β production when compared with age- and sex-matched controls. This was statistically significant at a dose of 10 ng/ml and was on average 2-fold lower in XLA than normal donors. This deficiency in IL-1β production was also seen in XLA PBMCs stimulated with LPS in which a 4-fold decrease in cytokine production was observed (Fig. 2*C*). In the absence of stimuli, there was no detectable expression of IL-1β in the culture supernatants. Taken together, these data indicate a common requirement for Btk in the production of both TNF and IL-1β in response to signaling via TLR4 and TLR2.

TLR signaling via Btk does not influence IL-6, IL-8, or IL-10 production

However, in contrast to our findings with TNF and IL-1β production, stimulation of XLA PBMCs with either TLR4 (LPS) or TLR2 (PAM_3Cys) ligands showed that the lack of Btk in these cells has no influence on the production of IL-6: although there appears to be a minor trend toward a reduced level of IL-6 in the XLA PBMCs with PAM_3Cys, this was not statistically significantly different between XLA patients and normal age- and sex-matched controls (Fig. 3, *A* and *B*).

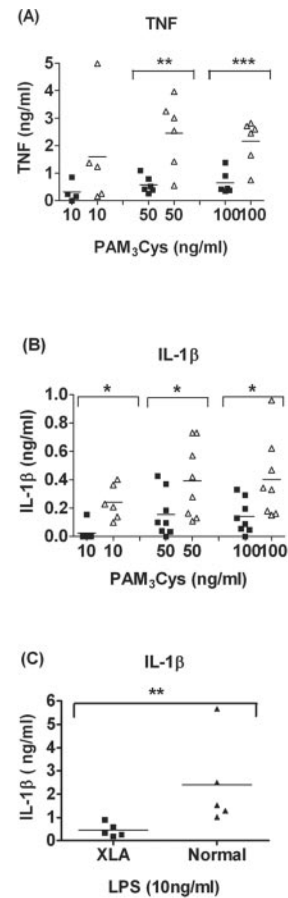


FIGURE 2. TLR2-induced TNF-α and IL-1β expression in PBMCs from XLA and normal donors. PBMCs were prepared from XLA (■) and normal (△) male donors (age range 17–46 years). Cells were stimulated with PAM_3Cys (100 ng/ml) for 18 h, and supernatants were assayed for TNF (*A*) and IL-1β (*B*) production by ELISA. Alternatively, cells were stimulated with LPS (10 ng/ml) and analyzed for IL-1β expression by ELISA (*C*). Each data point shown represents a single donor. Results from Student's *t* test *p* values are: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

Experiments performed with PBMCs from XLA patients stimulated with LPS also indicate that a lack of Btk has no influence on the production of IL-8 or IL-10 in these cells (Fig. 3, *C* and *D*). Preliminary data also indicate that production of IL-8 and IL-10 in response to TLR2 ligands (PAM_3Cys and MALP2) is also unimpaired in XLA patients (data not shown).

Overexpression of Btk enhances TLR2-induced TNF production, but not IL-6, IL-8, or IL-10

We have previously shown that infection of 3-day M-CSF-treated monocytes with an adenovirus encoding wild-type Btk results in the overexpression of TNF. This in turn results in enhanced LPS-induced TNF production, thus supporting a role for Btk in TLR4-induced TNF production (11). To further test a role for Btk in TLR2 signaling, similar experiments were performed with PAM_3Cys stimulation. Using an adenoviral delivery system, Btk (AdBtk) was overexpressed in primary human macrophages. Stimulation of these cells via either TLR2 (PAM_3Cys) or TLR4 (LPS) resulted in increased expression of TNF (Fig. 4, *A* and *B*). In contrast, overexpression of another LPS-activated tyrosine kinase, Pyk2, or a kinase-dead version of Pyk2 (PykM) had no effect on TNF production, indicating a specific role for Btk. Unfortunately,

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

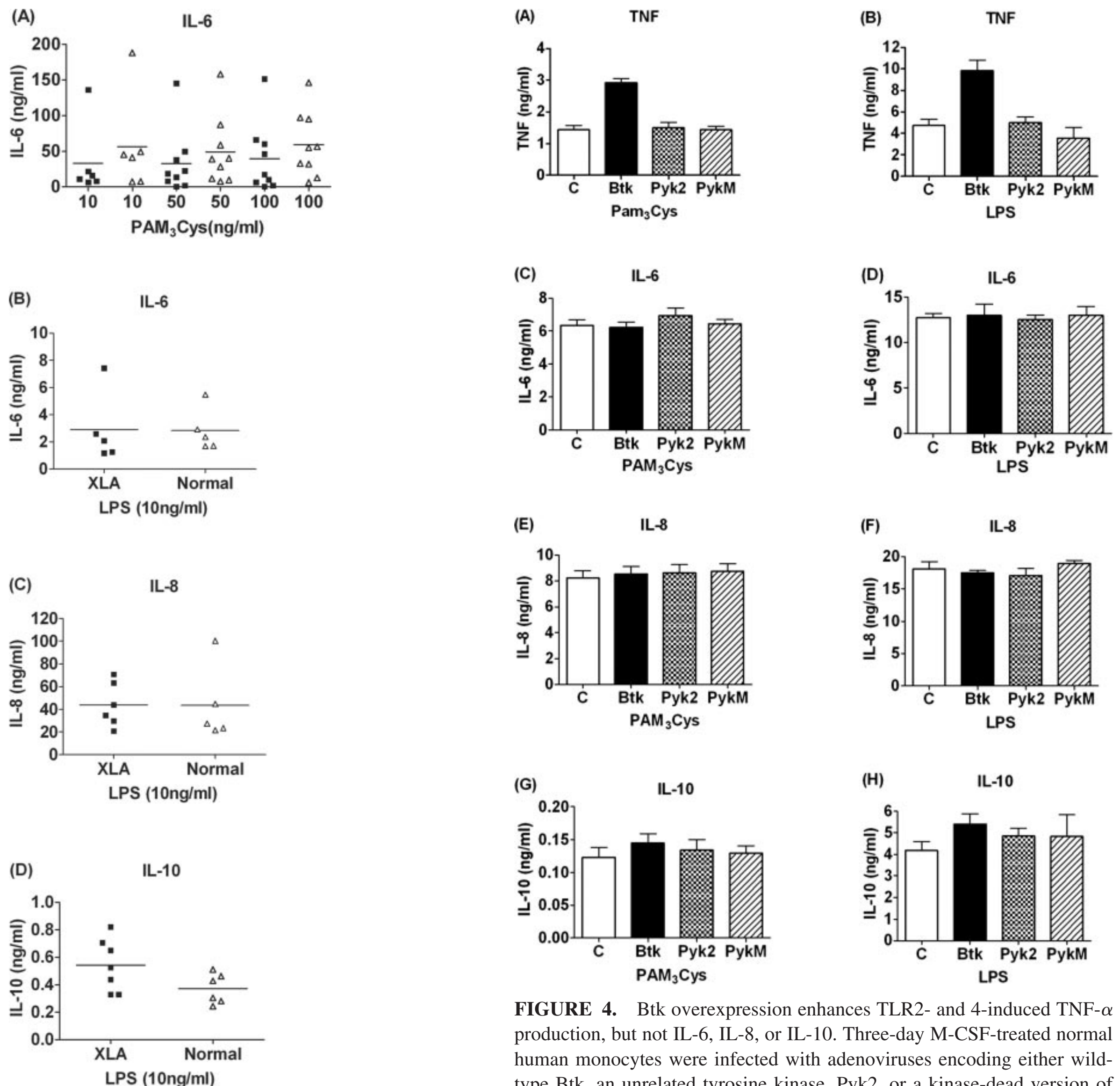


FIGURE 3. TLR-induced IL-6, IL-8, and IL-10 expression in PBMCs from XLA and normal donors. PBMCs were prepared from XLA (■) and normal (△) male donors (age range, 17–46 years). Cells were stimulated with PAM_3Cys (*A*; 100 ng/ml) or LPS (*B*; 10 ng/ml) for 18 h, and supernatants were assayed for IL-6 production by ELISA. Each data point shown represents a single donor. Supernatants from LPS-stimulated cells were also analyzed for IL-8 (*C*) and IL-10 (*D*) production. For *C* and *D*, each point also represents a single donor. The *p* value (Student's *t* test) for IL-10 is not statistically significant (0.187).

FIGURE 4. Btk overexpression enhances TLR2- and 4-induced TNF-α production, but not IL-6, IL-8, or IL-10. Three-day M-CSF-treated normal human monocytes were infected with adenoviruses encoding either wild-type Btk, an unrelated tyrosine kinase, Pyk2, or a kinase-dead version of Pyk2 (PykM) (moi 100:1). After infection for 24 h, cells were stimulated with either PAM_3Cys (100 ng/ml) or LPS (10 ng/ml) for 18 h. Supernatants were assayed for TNF-α (*A* and *B*), IL-6 (*C* and *D*), IL-8 (*E* and *F*), and IL-10 (*G* and *H*). Experiments were performed in triplicate, and the data are presented in ng/ml (± SD). Results from Student's *t* test *p* values: **, $p < 0.01$ compared with activated uninfected controls. Data are representative of at least four different experiments.

overexpression studies for IL-1β were not possible as, unlike PBMCs, the 3-day M-CSF-treated monocytes no longer produce detectable levels of IL-1β. However, we were able to measure IL-6, IL-8, and IL-10 production in these supernatants in response to the stimulation of TLR2 and TLR4. In direct contrast to the findings with TNF, overexpression of Btk had no effect on either the TLR2- or TLR4-induced expression of IL-6, IL-8, or IL-10 (Fig. 4, *C–H*).

Overexpression of Btk stabilizes TNF, but not IL-6 mRNA induced by PAM_3Cys

We have shown previously that Btk-regulated LPS-induced TNF expression is mediated by stabilization of TNF mRNA (11). To determine whether the same mechanism applied to TLR2-induced TNF, we examined mRNA stability by assessing the decay kinetics of mRNA following actinomycin D treatment using TaqMan PCR. Fig. 5*A* shows that overexpression of Btk increased the stability of TNF mRNA induced by PAM_3Cys in human primary macrophages, indicating that the same mechanism does indeed apply to TLR2 signaling. In contrast, overexpression of Btk was unable to stabilize TLR2-induced IL-6 mRNA (Fig. 5*B*). Similar data were obtained for IL-6 mRNA decay with LPS-activated cells (data not shown). Overexpression of the control kinase Pyk2 had no effect on either TNF or IL-6 mRNA stability.

Effect of inhibition of p38MAPK activity on TNF and IL-6 production

The regulation of TNF mRNA stability in response to LPS has been associated with the activation of p38 MAPK (15), and our previous studies have shown that Btk appears to be part of the

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

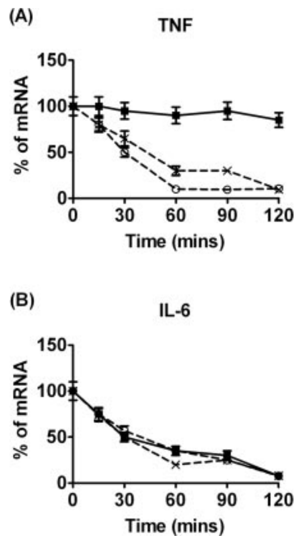


FIGURE 5. Btk overexpression does not stabilize IL-6 mRNA. Cells were either uninfected or infected with AdBtk or AdPyk2 (moi 100:1) and activated with PAM_3Cys for 4 h. Actinomycin D (2 μg/ml) was added to halt mRNA synthesis, and the cells were incubated for a further 0, 15, 30, 60, 90, or 120 min, after which time they were harvested in RNA lysis buffer and the supernatants reserved for ELISA. Total mRNA was prepared and TaqMan RT-PCR was used to access the quantity of TNF (*A*) and IL-6 (*B*) mRNA. The results were normalized to 100% at the 0-min time point. The lines are representative of cells infected with Btk (■), GFP control (○), and Pyk2 (×). Data are means of triplicate reactions ± SD expressed as a percentage of the control and are representative of three experiments performed using different donors.

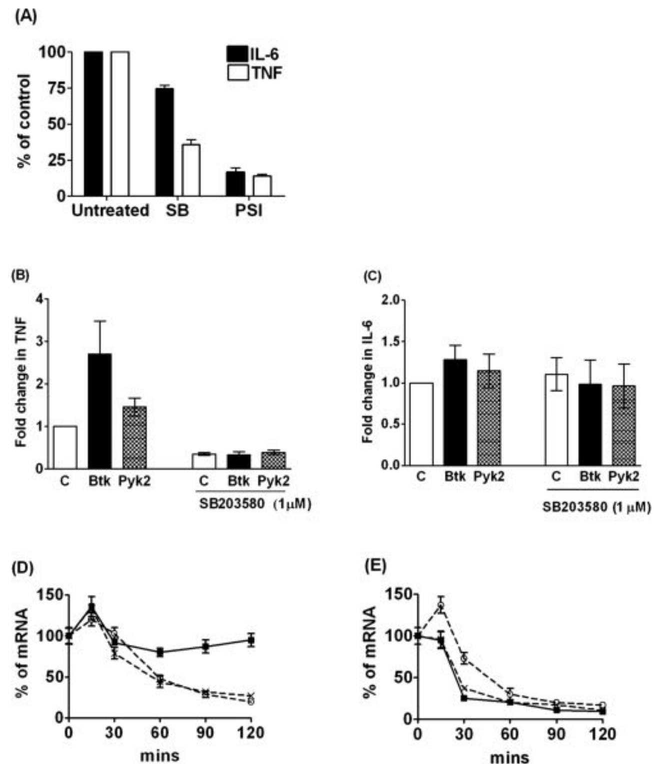


FIGURE 6. p38 MAPK activity influences TLR-induced TNF production. *A*, 3-day M-CSF-treated normal human monocytes were preincubated with either SB203580 (1 μM) or PSI (5 μM), or left untreated. After 30 min, cells were stimulated with LPS (10 ng/ml). Cell supernatants were collected after 4 h and assayed for the presence of IL-6 or TNF by ELISA. Data shown are means of triplicate experiments ± SD. *B* and *C*, 3-day M-CSF-treated normal human monocytes were left uninfected, or infected with AdBtk or AdPyk2 (moi 100:1) for 24 h. Cells were left untreated or preincubated with 1 μM SB203580 for 30 min before stimulation with 10 ng/ml LPS for 18 h. Supernatants were collected and assayed for TNF (*B*) and IL-6 (*C*) by ELISA. Student's *t* test was performed for each of the donors. *, $p < 0.05$. Statistical significance levels for groups treated with SB203580 are compared as follows: untreated vs treated for each virus and uninfected. Data are representative of five different experiments. *D* and *E*, Cells were either uninfected or infected with AdBtk or AdPyk2 (moi 100:1) for 24 h. Cells were either left as control (*D*) or pretreated with SB203580 (*E*) (1 μM) 30 min before stimulation with PAM_3Cys (100 ng/ml) for 4 h. Actinomycin D (2 μg/ml) was added, and the cells were incubated for 0, 15, 30, 60, 90, or 120 min before cell lysis for RNA preparation. TaqMan RT-PCR was used to access the quantity of TNF mRNA. The results were normalized to 100% at the 0-min time point. The lines are representative of cells infected with Btk (■), GFP control (○), and Pyk2 (×). Data are means of triplicate reactions ± SD expressed as a percentage of the control and are representative of three experiments performed using different donors.

TLR4-mediated signaling pathway leading to LPS-induced p38 MAPK activation (11). Given the apparent role of Btk in regulating TLR-induced TNF, but not IL-6 mRNA levels, we investigated whether the requirement for Btk correlated with a requirement for p38 MAPK activity in the production of these cytokines. Using SB203580, a selective inhibitor of p38 MAPK, we observed that LPS-induced expression of IL-6 in human monocytes/macrophages was significantly less sensitive to p38 MAPK inhibition than was TNF (a 20–25% reduction for IL-6 vs a 60–65% reduction for TNF) (Fig. 6*A*). In comparison, the proteosome inhibitor PSI, which blocks the activation of NF-κB, a transcription factor that is required for both the expression of TNF and IL-6 (16), inhibited both cytokines equally.

Furthermore, we examined the effect of SB203580 on the Btk enhancement of TNF expression. One would expect that if Btk was affecting TNF via p38 MAPK, inhibiting p38 MAPK would ablate this effect. Monocytes were infected with adenoviruses encoding either Btk or Pyk2, or cells were left uninfected. Pretreatment with SB203580 before LPS stimulation inhibited TNF by at least 80% compared with controls and completely ablated the ability of Btk to superinduce TNF production (Fig. 6*B*). We have previously shown that Btk overexpression was unable to superinduce IL-6 production (Fig. 4, *C* and *D*). Likewise, Btk was unable to increase IL-6 production, and in the presence of SB203580 there was no significant effect on IL-6 in all lanes (Fig. 6*C*).

To confirm that the ability of Btk to stabilize TNF mRNA was dependent on p38 MAPK activity, we performed actinomycin D experiments in the presence or absence of SB203580. As shown in Fig. 6*D*, overexpression of Btk was able to stabilize TNF mRNA induced by PAM_3Cys in primary human macrophages, whereas Pyk2-infected cells and uninfected controls were not. However, overexpression of Btk was no longer able to stabilize TNF mRNA in the presence of SB203580, and similar decay kinetics were observed for Btk-infected cells compared with the uninfected control and Pyk2-infected cells (Fig. 6*E*).

Overall, these data indicate that p38 MAPK is distal of Btk on the pathway regulating TNF mRNA stability. The lack of a significant role for p38 MAPK in IL-6 expression would also explain why this cytokine is not regulated by Btk.

Discussion

This study has produced two key observations. First, signaling via TLR2 activates Btk, and the activation of this kinase is required for the TLR2-induced expression of TNF and IL-1β. Second, we show that Btk has a selective role, as it is required for TNF and IL-1β

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

production, but not for IL-6. Together these data suggest that Btk provides a common signaling mechanism for TLR2 and TLR4, but only in some aspects of their biology.

With the exception of the NF-κB and p38 MAPK pathways and those leading to the activation of the IFN regulatory factors, the remainder of the TLR signaling pathways is still largely uncharacterized. In particular, while LPS is known to activate a range of tyrosine kinases, including hck, lyn, fgr, syk, and pyk2, the role of these in LPS signaling is still unclear, and none appear to be essential for cytokine production (7, 8, 10). Using a combination of studies involving XLA (Btk deficient) and normal human monocytes and macrophages, we have shown that members of the Tec family of protein tyrosine kinases, namely Btk and Tec itself, are also activated by LPS. Furthermore, Btk was found to be required for LPS-induced TNF production (11). These findings correlate well with those from Btk defective *xid* mice (12), which show defects in the secretion of LPS-induced TNF and IL-1β by macrophages.

The question naturally arose whether Btk would provide a signaling mechanism for other TLRs. The data described in this manuscript show that signaling via PAM_3Cys (a TLR2/TLR1 ligand) also activates Btk, supporting the inference that this kinase is also a basic part of TLR2 signaling. This observation is underscored by the work of Jefferies et al. (17), who have shown that Btk can bind the TIR domain found in TLR4 as well as TLR6, TLR8, and TLR9, and TIR adapter molecules such as MyD88 and Mal. The importance of the Btk pathway to TLR2 and TLR4 function is clearly demonstrated by the reduced levels of both TNF and IL-1β produced by XLA PBMCs in response to LPS and PAM_3Cys when compared with normal human cells. We have observed similar lower responses by XLA PBMCs in preliminary studies with another TLR2 ligand, MALP2 (data not shown), further supporting a role for Btk in TLR2-induced TNF and IL-1β production.

Recently, Mangla et al. (18) showed that *xid* mice have multiple defects in the development and function of the myeloid lineages. In light of this observation and our data, it is surprising that XLA patients do not suffer a profound defect in their innate immune system given their impairment in TLR signaling. However, this may be explained by two separate observations. One was revealed in our previous study, in which we showed that while XLA monocytes had impaired TNF production, this was not the case when the cells were matured to macrophages. The most likely explanation for this is the increased expression of the Btk-related Tec kinase as the cells mature (11). Tec is also activated by LPS, and functional redundancy between Btk and Tec is well documented (18, 19). It is for this reason that simple Btk-reconstitution experiments in XLA monocytes are likely to be unhelpful. The other reason is the new observation made in this study that Btk is not essential to all TLR2- and 4-induced activities, as IL-6 production was not significantly impaired in XLA PBMCs. The slightly lower levels of IL-6 that are seen in XLA patients may be secondary to the lower levels of TNF or IL-1β induced in these cultures; both cytokines are potent stimuli of IL-6 production.

The failure of Btk to contribute to TLR2- and TLR4-induced IL-6 expression is supported by the fact that, in contrast to TNF, overexpression of Btk in primary human macrophages does not enhance IL-6 production in response to TLR2 or TLR4 stimulation. The failure of Btk to contribute to IL-6 production may also extend to other cytokines, as Btk overexpression in these cells also failed to enhance both IL-8 and IL-10 production in response to LPS, PAM_3Cys, and other TLR2 ligands. This finding is underscored by results from LPS-treated XLA PBMCs, which also suggest that IL-8 and IL-10 production in response to TLR4 stimulation is not significantly impaired. Preliminary data from XLA PBMCs treated with PAM_3Cys and MALP2 suggest that the same may also be true of TLR2 stimulation (data not shown). The role of Btk/Tec in aspects of TLR biology other than cytokine production is the subject of ongoing studies within this laboratory.

Although these findings reveal a previously unreported level of complexity in the TLR-mediated production of cytokines, they are not entirely unexpected. The IκB-like molecule IκBζ (also known as molecule possessing ankyrin repeats induced by LPS/IL-1-inducible nuclear ankyrin-repeat protein) is induced by signaling via a number of TLRs, including TLR2 and TLR4, and has recently been described to induce the production of IL-6, but not TNF, demonstrating clear differences in the way that these two cytokines are regulated (20). In addition, it has been shown recently that SOCS1 selectively inhibits LPS-induced IL-6 production, but not TNF, and that this effect is independent of the NF-κB pathway, demonstrating that these cytokines are not only differentially regulated, but also use multiple signal transduction pathways (21).

The studies described in this work have indicated that Btk lies on a TLR2-mediated pathway that regulates the stability of TNF mRNA, and these findings agree with our previous data showing that TLR4 signaling increases TNF levels by stabilizing TNF mRNA via its 3′ untranslated region. At present, the details of the mechanism that mediates this effect are unclear, but our previous data suggest that Btk may be on a pathway proximal to p38 MAPK. This is in agreement with data from Btk-defective DT40 cells, which are no longer able to activate p38 MAPK in response to UV light (22). p38 MAPK has been implicated in the stabilization of TNF mRNA. Accordingly, we have now shown that TLR4-induced TNF production is sensitive to p38MAPK inhibitors, while the production of IL-6 is markedly less affected. As the ability of Btk to stabilize TNF mRNA and superinduce its production is ablated in the presence of the p38 MAPK inhibitor, SB203580, we conclude that the p38 MAPK pathway is essential for the effects of Btk on TLR-induced TNF production.

In contrast to the selective requirement for p38 activity, TLR-driven expression of both TNF and IL-6 in human macrophages is known to be dependent on the NF-κB transcription factor, and recent data from transfected HEK cells have shown that Btk induces the phosphorylation of p65 on serine 536 in response to LPS, thereby promoting *trans* activation by NF-κB (23). Our own results imply that the TLR-induced NF-κB pathway in human monocytes/macrophages is not regulated by Btk, as it does not appear to be required for IL-6 expression: a highly NF-κB-dependent process (24). The reason for this discrepancy is unclear at present, but may simply reflect different signaling mechanisms used by transfected cell lines and primary cells.

In summary, our data provide support for Btk being a common signaling mechanism for TLR2- and TLR4-induced TNF and IL-1β production in primary human monocytes/macrophages. However, the role of Btk is selective, as it is not required for IL-6 and probably not for IL-8 and IL-10 production. Given the importance of TLRs to the defense of the host against bacterial and viral infection and their potential role in inflammatory diseases, the identification of Btk as a selective mediator of TLR signaling provides an important insight in the biology of these molecules.

Acknowledgments

We thank Dr. M. Tomlinson for the gift of the anti-Btk polyclonal, and acknowledge the skilled technical assistance of Alison Davis. Also, thanks to Drs. J. Dean, K. Midwood, and A. Clark for critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025

References

1. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol.* 2: 675–680.
2. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406: 782–787.
3. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20: 197–216.
4. Akira, S., and H. Hemmi. 2003. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett.* 85: 85–95.
5. Brown, G. D., J. Herre, D. L. Williams, J. A. Willment, A. S. Marshall, and S. Gordon. 2003. Dectin-1 mediates the biological effects of β-glucans. *J. Exp. Med.* 197: 1119–1124.
6. Rogers, N. C., E. C. Slack, A. D. Edwards, M. A. Nolte, O. Schulz, E. Schweighoffer, D. L. Williams, S. Gordon, V. L. Tybulewicz, G. D. Brown, and E. S. C. Reis. 2005. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* 22: 507–517.
7. Han, J., J. D. Lee, P. S. Tobias, and R. J. Ulevitch. 1993. Endotoxin induces rapid protein tyrosine phosphorylation in 70Z/3 cells expressing CD14. *J. Biol. Chem.* 268: 25009–25014.
8. Weinstein, S. L., J. S. Sanghera, K. Lemke, A. L. DeFranco, and S. L. Pelech. 1992. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J. Biol. Chem.* 267: 14955–14962.
9. Hazeki, K., N. Masuda, K. Funami, N. Sukenobu, M. Matsumoto, S. Akira, K. Takeda, T. Seya, and O. Hazeki. 2003. Toll-like receptor-mediated tyrosine phosphorylation of paxillin via MyD88-dependent and -independent pathways. *Eur. J. Immunol.* 33: 740–747.
10. Karaghiosoff, M., R. Steinborn, P. Kovarik, G. Kriegshauser, M. Baccarini, B. Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, et al. 2003. Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. *Nat. Immunol.* 4: 471–477.
11. Horwood, N. J., T. Mahon, J. P. McDaid, J. Campbell, H. Mano, F. M. Brennan, D. Webster, and B. M. Foxwell. 2003. Bruton's tyrosine kinase is required for lipopolysaccharide-induced tumor necrosis factor α production. *J. Exp. Med.* 197: 1603–1611.
12. Mukhopadhyay, S., M. Mohanty, A. Mangla, A. George, V. Bal, S. Rath, and B. Ravindran. 2002. Macrophage effector functions controlled by Bruton's tyrosine kinase are more crucial than the cytokine balance of T cell responses for microfilarial clearance. *J. Immunol.* 168: 2914–2921.
13. Mukhopadhyay, S., A. George, V. Bal, B. Ravindran, and S. Rath. 1999. Bruton's tyrosine kinase deficiency in macrophages inhibits nitric oxide generation leading to enhancement of IL-12 induction. *J. Immunol.* 163: 1786–1792.
14. Foxwell, B., K. Browne, J. Bondeson, C. Clarke, R. de Martin, F. Brennan, and M. Feldmann. 1998. Efficient adenoviral infection with IκBα reveals that macrophage tumor necrosis factor α production in rheumatoid arthritis is NF-κB dependent. *Proc. Natl. Acad. Sci. USA* 95: 8211–8215.
15. Grzybowska, E. A., A. Wilczynska, and J. A. Siedlecki. 2001. Regulatory functions of 3′ UTRs. *Biochem. Biophys. Res. Commun.* 288: 291–295.
16. Bondeson, J., K. A. Browne, F. M. Brennan, B. M. Foxwell, and M. Feldmann. 1999. Selective regulation of cytokine induction by adenoviral gene transfer of IκBα into human macrophages: lipopolysaccharide-induced, but not zymosan-induced, proinflammatory cytokines are inhibited, but IL-10 is nuclear factor-κB independent. *J. Immunol.* 162: 2939–2945.
17. Jefferies, C. A., S. Doyle, C. Brunner, A. Dunne, E. Brint, C. Wietek, E. Walch, T. Wirth, and L. A. O'Neill. 2003. Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor κB activation by Toll-like receptor 4. *J. Biol. Chem.* 278: 26258–26264.
18. Mangla, A., A. Khare, V. Vineeth, N. N. Panday, A. Mukhopadhyay, B. Ravindran, V. Bal, A. George, and S. Rath. 2004. Pleiotropic consequences of Bruton tyrosine kinase deficiency in myeloid lineages lead to poor inflammatory responses. *Blood* 104: 1191–1197.
19. Humphries, L. A., C. Dangelmaier, K. Sommer, K. Kipp, R. M. Kato, N. Griffith, I. Bakman, C. W. Turk, J. L. Daniel, and D. J. Rawlings. 2004. Tec kinases mediate sustained calcium influx via site-specific tyrosine phosphorylation of the phospholipase Cγ Src homology 2-Src homology 3 linker. *J. Biol. Chem.* 279: 37651–37661.
20. Yamamoto, M., S. Yamazaki, S. Uematsu, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Kuwata, O. Takeuchi, K. Takeshige, et al. 2004. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ. *Nature* 430: 218–222.
21. Kimura, A., T. Naka, T. Muta, O. Takeuchi, S. Akira, I. Kawase, and T. Kishimoto. 2005. Suppressor of cytokine signaling-1 selectively inhibits LPS-induced IL-6 production by regulating JAK-STAT. *Proc. Natl. Acad. Sci. USA* 102: 17089–17094.
22. Kabuyama, Y., M. K. Homma, T. Kurosaki, and Y. Homma. 2002. Early signaling events induced by 280-nm UV irradiation. *Eur. J. Biochem.* 269: 664–670.
23. Doyle, S. L., C. A. Jefferies, and L. A. O'Neill. 2005. Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NFκB activation by lipopolysaccharide. *J. Biol. Chem.* 280: 23496–23501.
24. Libermann, T. A., M. Lenardo, and D. Baltimore. 1990. Involvement of a second lymphoid-specific enhancer element in the regulation of immunoglobulin heavy-chain gene expression. *Mol. Cell. Biol.* 10: 3155–3162.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/176/6/3635/1215554/3635.pdf by guest on 26 March 2025