Bruton’s Tyrosine Kinase Is Required For Lipopolysaccharide-induced Tumor Necrosis Factor α Production

Nicole J. Horwood,¹ Tara Mahon,¹ John P. McDaid,¹ Jamie Campbell,¹ Hiroyuki Mano,² Fionula M. Brennan,¹ David Webster,³ and Brian M.J. Foxwell¹

¹Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, London W6 8LH, United Kingdom
²Jichi Medical School, Division of Functional Genomics, Tochigi 329-0498, Japan
³Royal Free Medical School, Department of Immunology, University College London, London NW3 2QG, United Kingdom

Abstract

Lipopolysaccharide (LPS), a product of Gram-negative bacteria, is potent mediator of tumor necrosis factor (TNFα) production by myeloid/macrophage cells. Inhibitors capable of blocking the signaling events that result in TNFα production could provide useful therapeutics for treating septic shock and other inflammatory diseases. Broad spectrum tyrosine inhibitors are known to inhibit TNFα production, however, no particular family of tyrosine kinases has been shown to be essential for this process. Here we show that the Bruton’s tyrosine kinase (Btk)-deficient mononuclear cells from X-linked agammaglobulinemia patients have impaired LPS-induced TNFα production and that LPS rapidly induces Btk kinase activity in normal monocytes. In addition, adenoviral overexpression of Btk in normal human monocytes enhanced TNFα production. We examined the role of Btk in TNFα production using luciferase reporter adenoviral constructs and have established that overexpression of Btk results in the stabilization of TNFα mRNA via the 3’ untranslated region. Stimulation with LPS also induced the activation of related tyrosine kinase, Tec, suggesting that the Tec family kinases are important components for LPS-induced TNFα production. This study provides the first clear evidence that tyrosine kinases of the Tec family, in particular Btk, are key elements of LPS-induced TNFα production and consequently may provide valuable therapeutic targets for intervention in inflammatory conditions.

Key words: tyrosine kinase • adenovirus • TNFα • macrophage • X-linked agammaglobulinemia

Introduction

TNFα is a proinflammatory cytokine pivotal to the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and Crohn’s disease (1), and in inflammatory responses such as leukocyte migration, tissue resorption, the acute-phase response, and fever (2). The major producers of TNFα are cells of the mononuclear phagocyte lineage including macrophages, microglia, osteoclasts, and myeloid dendritic cells. Despite considerable efforts, relatively few signaling molecules, e.g., nuclear factor (NF)κB (3, 4) and p38 MAPK/MK2 (5, 6), have been shown to be essential for TNFα expression in macrophages. It is well documented that tyrosine kinase inhibitors are potent suppressors of LPS-induced TNFα production (7, 8) and that

N.J. Horwood, T. Mahon, and J.P. McDaid contributed equally to this work.

Address correspondence to Brian M.J. Foxwell, Kennedy Institute of Rheumatology Division, Faculty of Medicine, Imperial College of Science, Technology and Medicine, Charing Cross Campus, ARC Building, 1 Aspenlea Road, London W6 8LH, United Kingdom. Phone: 44-208-383-4444; Fax: 44-208-383-4499; E-mail: b.foxwell@ic.ac.uk

*Abbreviations used in this paper: AdBtk, adenovirus-encoding human Bruton’s tyrosine kinase; ARE, AU-rich element; Btk, Bruton’s tyrosine kinase; IkBα, inhibitory protein κBα; MAPK, mitogen-activated protein kinase; m.o.i., multiplicity of infection; NF, nuclear factor; UTR, untranslated region; xid, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.
the activation of tyrosine kinase activity is one of the earli-
est detectable events after LPS stimulation of monocyte
cells (9–11). However, defining a causal relationship be-
tween a particular tyrosine kinase and TNFα production
has been difficult. LPS can activate the Src family kinases
p58hck, p53src, and p59shp, as well as p72vk (12–14), yet mac-
rophages from mice deficient in these kinases do not show
any impairment of LPS-induced TNFα production (15,
16). LPS has also been shown to activate the tyrosine kinase
Pyk-2 (17), but its role, if any, in TNFα production has yet
to be elucidated.

Another family of nonreceptor tyrosine kinases ex-
pressed by monocytic cells is the Tec family, and in par-
cular, Bruton’s tyrosine kinase (Btk). Recent studies
have shown that the Tec family kinases are important
components of both antigen receptor signaling and other
exertion surface receptors, resulting in the activation of nu-
merous signal transduction pathways, regulation of the
actin cytoskeleton, adhesion, migration, and transcrip-
tional activation (18). Btk is found in all cells of the
hematopoietic lineage except plasma and T cells (19), and
is required for normal B cell development and signal trans-
duction through cell surface molecules (20). Mutations in
the btk gene result in aberrant B cell development, lead-
ing to the X-linked agammaglobulinemia (XLA) pheno-
type in humans and to the less severe X-linked immu-
nodeficiency (xid) in mice. xid B cells show some
hyporesponsiveness to LPS stimulation, although the
precise cause has not been established (21, 22). Because
the major phenotype of Btk deficiency is impaired B cell
development and function, this cell type has been the
major focus of interest to date. Earlier studies performed
on xid mononuclear cells showed no obvious impairment
of TNFα production in response to a series of inflamma-
tory stimuli such as LPS and formalin-killed
Staphylococcus aureus (23). However, bone marrow–derived cultured
mast cells from xid mice have been shown to produce less
TNFα in passive cutaneous anaphylactic reactions (24)
and there is a recent report that xid peritoneal macro-
phages express reduced levels of TNFα and IL-1β in re-
response to LPS (25). Additionally, xid mice are resistant to
models of autoimmune diseases, e.g., collagen-induced
arthritis (26, 27).

In humans, XLA is characterized by the absence of ma-
ture B cells in the periphery with a marked reduction in
serum levels of all Ig isotypes resulting in a susceptibility
to recurrent and severe bacterial infections (20, 28). Yet
despite lacking Btk, XLA monocytes appear to develop
normally and are present in expected numbers in the cir-
culation. The importance of Btk in human monocyte/
macrophages remains to be fully elucidated. As the XLA
phenotype differs from that of the xid mice, we have ex-
amined the responses of XLA monocytes and macro-
phages to LPS stimulation. In addition, we have used an
adenovirus expressing Btk in conjunction with TNFα lu-
ciferase reporter adenoviruses to analyze the contribution
of Btk–dependent signaling events to the regulation of
TNFα production.

Materials and Methods

Isolation and Culture of Cells

Human blood samples were collected into lithium heparin vacu-
tainers. Each blood sample was mixed with an equal volume of
HBSS. PBMCs were prepared by ficoll-hypaque centrifugation
on a lymphoprep gradient. PBMCs were cultured in RPMI con-
taining 100 units/ml penicillin/streptomycin and 10% heat-inac-
tivated FCS at 37°C in a humidified atmosphere containing 5% CO2.
Monocytes were isolated from the PBMCs by adherence to
plastic for 1 h at 37°C in RPMI containing 100 units/ml penicil-
lin/streptomycin and 10% FCS. Nonadherent cells were then
washed off and the adherent monocytes were rested overnight
and stimulated with LPS. B cells were depleted from PBMC using
Dynabeads® coated with anti-CD19 antibodies. Cells were
incubated with the beads for 1 h with constant agitation at 4°C.
The beads were then magnetically removed and the remaining
cells were subjected to FACS® analysis with FITC–conjugated
anti-CD19 resulting in >90% B cell depletion.

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 cen-
trifugal elutriation as previously described (4). Monocyte
fractions of >85% purity were routinely collected in this manner.
Monocytes were cultured in RPMI containing 100 units/ml
penicillin/streptomycin and 10% heat-inactivated FCS at 37°C
in a humidified atmosphere containing 5% CO2. For adenoviral
infection, monocytes were treated with 100 ng/ml M-CSF (pro-
vided by G. Larsen, Genetics Institute, Boston, MA) for 72 h be-
fore viral infection.

Generation of Adenoviral Vectors and Cell Infection. Recombi-
nant, replication-deficient adenoviral constructs encoding wild-
type human Btk (AdBtk; cDNA provided by C. Kinnon, Insti-
tute of Child Health, London, UK) were prepared using the AdEasy system as previously described (29). In short,
recombinant viral DNA was transfected into 293 cells in 6-well
tissue culture plates using lipofectamine (GIBCO BRL) accord-
ing to the manufacturer’s instructions. Cells were overlaid with
2% agarose/DMEM 24 h after transfection and viral plaques
were picked after 9–10 d. Viral clones were propagated in 293
cells and screened for transgene expression by Western blotting.
Clonal viruses were then prepared from 20 175 cm2 tissue cul-
ture flasks of 293 cells by ultracentrifugation through two cae-
sium chloride gradients. Titres of viral stocks were determined
by plaque assay and viral aliquots were stored at −70°C.
The AdGFP adenovirus was prepared according to this protocol us-
ing AdTrackGFP with no insert. AdTrack0 (Ad0), pAdTrack-
TNF 5′ promoter–Luc–3′ untranslated region (UTR; Ad5′Luc),
and pAdTrack–TNF 5′ promoter–Luc (Ad5′Luc) were generated
as previously described (30). The NF-κB luciferase adenovirus
(AdNF-κB–Luc) contains four tandem copies of the κ enhancer
element located upstream of the firefly luciferase gene (31). This
adenovirus was provided by P.B. McCray Jr. (University of
Iowa, Iowa City, IA) and is a modification of the pNF-κB re-
porter vector (BD Clontech). Recombinant adenoviral vectors
encoding Escherichia coli β-galactosidase (Adβgal) were provided
by M. Woods and A. Byrne (Oxford University, Oxford, UK).

M-CSF–derived monocytes were plated in 96-well plates at
1.5 × 105 cells/well and allowed to settle for at least 4 h. The
cells were washed in serum-free RPMI medium and then ex-
posed to virus at different multiplicity of infection (m.o.i.) for 2 h
in serum-free RPMI at 37°C, after which cells were washed in RPMI and cultured in complete medium for 24 h before stimulation with LPS as previously described (4).

**Immunoprecipitation and In Vitro Kinase Assay.** Btk autokinase activity was measured in response to LPS in primary human monocytes. Cells were stimulated with LPS for various times as detailed. Cells were pelleted by centrifugation and lysed for 20 min on ice with 1% NP-40 lysis buffer containing 20 mM Tris, pH 8, 130 mM NaCl, 10 mM NaF, 1 mM DTT, 20 μM leupeptin, 100 μM sodium orthovanadate, 1 mM PMSF, 10 μM E64, and 2 mg/ml aprotinin. Lysates were microfuged at 10 min at 13,000 rpm at 4°C and supernatants were removed and pre-cleared for 30 min at 4°C in 20 μl protein A–sepharose (previously washed in lysis buffer). After centrifuging the samples for 10 min at 1,300 rpm, the supernatants were removed and incubated with 2 μl rabbit polyclonal anti–Btk (provided C. Kinnon, Institute of Child Health, London, United Kingdom) for 1 h at 4°C. 30 μl protein A–sepharose was then added to each sample and incubated for an additional 1.5 h at 4°C. The beads were then washed three times in lysis buffer and once in kinase buffer (10 mM MgCl₂; 10 mM MnCl₂). Beads were mixed with kinase buffer containing γ³²P[ATP] and incubated for 15 min at room temperature. The reaction was stopped by the addition of 4× Laemmli buffer and the samples were boiled for 10 min. Samples were electrophoresed on an 8% SDS polyacrylamide gel. The gel was stained, fixed, dried, and autoradiographed. Tec autokinase activity was measured as previously described (32). Tec protein was immunoprecipitated, either with rabbit anti–Tec serum (32) or goat polyclonal anti–Tec (Santa Cruz Biotechnology, Inc.).

**Western Blot Analysis.** Cells were lysed in lysis buffer and debris pelleted as described above. Lysate protein concentration was assessed by Bradford assay and equivalent amounts of lysate protein were electrophoresed on 8% SDS polyacrylamide gels. Membranes were blocked in 10% Marvel/PBS/Tween (0.05%) and probed with either rabbit polyclonal anti–Btk (BD Biosciences) at 1 μg/ml in 5% Marvel/PBS/Tween (0.05%), anti–inhibitory protein kβα (Iβκα; Santa Cruz Biotechnology, Inc.), anti-p54/JNK (Santa Cruz Biotechnology, Inc.), or rabbit anti–Tec for 2 h. After washing, membranes were incubated with anti–rabbit horseradish peroxidase at 1:5,000 in 5% Marvel/PBS/Tween (0.05%). After washing in PBS/Tween (0.05%), the membranes were developed using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences).

**ELISA.** Supernatants were harvested 18 h after stimulation of human macrophages or PBMCs. The concentration of TNFα (BD Biosciences) was determined by ELISA according to the manufacturer’s instruction. Absorbance was read and analyzed at 450 nM on a spectrophotometric ELISA plate reader (Labsystems Multiskan Biochromic) using the Ascent 2.4.2 software program. Results are expressed as the mean concentration of triplicate cultures ±SD.

**Luciferase Assays.** After LPS stimulation, cells were washed once in PBS and lysed with 100 μl CAT lysis buffer (0.65% [vol/vol] of NP-40, 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, pH 8, 150 mM NaCl). 50 μl cell lysate were transferred into the well of a luminometer cuvette strip containing 120 μl luciferase assay buffer. Luciferase activity was measured with a Luminostat Luminometer by dispensing 30 μl luciferin (Bright-Glo luciferase assay system; Promega) per assay point. Cell lysates were assayed for protein concentration by Bradford assay and luciferase activity was adjusted accordingly.

**Results**

**Btk Deficiency Results in Impaired TNFα Production.** PBMCs from XLA blood were used to see if Btk deficiency had any effect on LPS-induced TNFα production. When compared with PBMC from normal age and sex-matched controls, the LPS response of XLA cells was impaired. At the lowest dose of LPS (0.1 ng/ml), XLA and normal PBMCs produced similar levels of TNFα, but increasing concentrations of LPS failed to generate the increased response seen in the normal cells. At 10 ng/ml, LPS-induced TNFα production by XLA PBMCs was only 15% of the control cells, whereas at 100 ng/ml LPS the response was only 10% of control (Fig. 1 A). Although B cells are not known to produce TNFα in response to LPS, the involvement of B cells from the normal PBMCs in LPS-induced TNFα production was investigated to negate any secondary effects due to the absence of B cells in XLA PBMCs. Depleting B cells from normal PBMC showed no effect on LPS-induced TNFα production (Fig. 1 B). Furthermore, we found that the T cells, the major component of PBMCs, did not produce TNFα in response to LPS (unpublished data) and these cells do not express Btk (33). Although the amount of blood available made purifying the monocytes from XLA blood difficult, we managed to separate the monocytic cells using adherence. LPS-induced TNFα production was again significantly reduced, although at 50% of normal controls, the impairment was not as great as seen in PBMCs (Fig. 1 C).

**Stimulation with LPS Induces Btk Kinase Activity In Vitro.** To further establish a role for Btk in LPS-induced TNFα production, we attempted to determine whether LPS was able to regulate Btk activity. Autokinase assays of enzyme immunoprecipitated from human primary monocytes demonstrated that LPS could rapidly activate Btk (Fig. 2 A). Btk autokinase activity was also observed in the murine macrophage cell line RAW 264.7 after LPS stimulation (unpublished data). Additionally, the effect of overexpression of Btk was examined by infecting 48-h M-CSF–treated monocytes (4) with increasing amounts of adenovirus-encoding human Btk (AdBtk). Infection with AdBtk
alone did not induce TNFα production (unpublished data). However, infection with increasing amounts of virus synergized with LPS to increase TNFα expression, resulting in a two- to threefold increase in TNFα at the highest m.o.i. of 100 (Fig. 2 B). This effect correlated with the increased amounts of Btk protein seen in the infected cells (Fig. 2 C). No effect on TNFα production was seen with control viruses encoding either β-galactosidase or Pyk2, another LPS-activated tyrosine kinase (17), at the highest m.o.i. of 100 (Fig. 2 B).

**LPS-induced IkBα Degradation Is Intact in XLA PBMCs.**

Overall, the data strongly suggested that there was a role for Btk in LPS-induced TNFα production. However, there was the possibility that this was the result of some development defect, rather than a direct effect of an absent kinase. As others have reported (34), FACS® analysis showed no impairment of CD14 expression, a component of the LPS binding on XLA monocytes (unpublished data). We also used the LPS-induced degradation of IkBα as a measure of signaling function, as this event does not appear to require tyrosine kinase activity (35, 36) and therefore should be intact. As shown in Fig. 3 A, there was a similar degradation of IkBα in XLA and normal monocytes in response to LPS, indicating no obvious impairment of the NF-κB activation pathway. We further investigated the involvement of the NF-κB signaling pathway by coinfecting M-CSF–treated monocytes with AdBtk and an NF-κB reporter virus that encodes luciferase under the control of a series of NF-κB binding sites (31). M-CSF monocytes were simultaneously infected with these viruses and controls, and the luciferase activity and TNFα production were measured after 18 h of LPS stimulation. Although we still observed an increase in TNFα production in these cultures (Fig. 3 C), there was no corresponding increase in luciferase activity (Fig. 3 B), indicating that Btk was able to promote TNFα production independently of the NF-κB activation.

**Overexpression of Btk Acts via the 3′ UTR of TNF mRNA.**

To establish the mechanism of Btk action on TNFα production, the activity of the human TNF promoter in stimulated human macrophages was studied using 5′ promoter-luciferase and 5′ promoter–luciferase–3′ UTR adenoviral TNF gene reporter constructs (30). Primary human macrophages were simultaneously infected with reporter viruses at an m.o.i. of 40, whereas the Ad0 or AdBtk were used at an m.o.i. of 100. 24 h after adenoviral infection, macrophages were treated with LPS or left unstimulated for 18 h before assay of luciferase activity and ELISA for TNFα pro-
duction. There was a threefold increase in luciferase activity in the cells containing the TNF5/H11032/3-H11032/UTR reporter construct compared with the Ad0 control cells (Fig. 4 A). However, there was no enhancement of luciferase activity from the TNF5/H11032 promoter construct (Fig. 4 C). Overexpression of Btk enhanced LPS-stimulated TNF5/H9251 production by threefold in the presence of either TNF reporter construct (Fig. 4, B and D). There was no significant difference in TNF production and luciferase activity when comparing Ad0-infected macrophages with controls infected with LPS for 18 h before assay of (B) luciferase activity and (C) TNF5 production. Data are means of triplicate cultures ± SD and are expressed as a percentage of the control level. This graph is representative of five experiments performed using different donors.

Figure 3. LPS-induced IkBα degradation in XLA and normal PBMCs. (A) PBMCs stimulated with 10 ng/ml LPS for 20 min were lysed and sequentially immunoblotted for the indicated proteins. This result is representative of three different experiments. (B and C) M-CSF monocytes were simultaneously infected with NF-κB luciferase reporter construct (m.o.i. of 100) alone, or coinjected with either AdBtk or Ad0 at various m.o.i. 24 h after adenoviral infection, cells were treated with LPS for 18 h before assay of (B) luciferase activity and (C) TNF5 production. Data are means of triplicate cultures ± SD and are expressed as a percentage of the control level. This graph is representative of five experiments performed using different donors.

Figure 4. Btk overexpression enhances TNF5 via the 3′ UTR. (A) Schematic representation of the human TNF 5′ promoter-luciferase-3′ UTR and TNF 5′ promoter-luciferase adenoviral constructs. Activity of the human TNF 5′ UTR (B and C) or TNF 5′ promoter (D and E) in stimulated human macrophages was tested by simultaneously infecting primary human macrophages with reporter virus at an m.o.i. of 40 and either Ad0 or AdBtk at various m.o.i. 24 h after adenoviral infection, macrophages were treated with LPS for 18 h before assay of luciferase activity (B and D) and TNF5 production (C and E). Data are means of triplicate cultures ± SD and are expressed as a percentage of the control level. Each graph is representative of five experiments performed using different donors.

Overexpression of Btk Leads to Stabilization of TNF5 mRNA. The importance of the 3′ UTR in the stability of TNF5 mRNA has been studied extensively (37, 38). Because Btk appeared to be acting via the 3′ UTR, it seemed likely that it was also affecting the stability of TNF5 mRNA. To further analyze the mechanism by which Btk determines the levels of TNF5, we blocked transcription in LPS-stimulated M-CSF monocytes by using actinomycin D and analyzed half-life of TNF5 mRNA by Taqman RT-PCR analysis. As a control, GAPDH mRNA was detected and used to normalize the TNF5 mRNA levels. For each of the controls, uninfected Ad0 and PykM, the half-life of TNF5 mRNA was <45 min after actinomycin D treatment. However, in the presence of Btk, TNF5 mRNA levels were maintained at 70–100% of the time 0 control for the duration of the 120-min time course (Fig. 5 A). These data demonstrate the ability of Btk to stabilize TNF5 mRNA and consequently this may result in the elevated protein expression observed in these cultures. As the stability of TNF5 mRNA is known to be associated with p38 mitogen-activated protein kinase (MAPK) activation, we examined the ability of PBMCs from XLA patients to phosphorylate p38 MAPK in response to LPS treatment. We were able to observe consistently low levels of p38 MAPK phosphorylation in XLA PBMCs (Fig. 5 B), however there was considerable variability in the degree of p38 MAPK phosphorylation from person to person in the normal controls. There was no difference in p38 MAPK phosphorylation after LPS treatment of normal PBMCs in either the presence or absence of B cells (unpublished data). Due to the limitation of patient samples we were unable to
examine the kinetics of p38 MAPK activation in XLA patients compared with normal controls. To further establish the link between p38 MAPK and Btk, we used adenoviral overexpression of Btk to determine if there was any increase in p38 MAPK activity in normal M-CSF-treated monocytes. As shown in Fig. 5 C, we were able to observe a 1.5–2-fold increase in phosphorylated p38 MAPK.

**Tec Is Phosphorylated in Response to LPS.** Although we have demonstrated that Btk is able to modulate TNFα expression, XLA patients do not show a serious impairment in their innate immune function. Treatment of XLA monocytes for 2 d with M-CSF greatly enhanced their production of TNFα in response to LPS to levels similar to those obtained with normal monocytes (Fig. 6 A). Consequently, we examined the mechanism of the increased responsiveness of monocytes after M-CSF treatment. Because members of the Tec family of kinases can functionally complement each other (39), we tested whether M-CSF can regulate the expression of an alternative Tec family kinase to Btk. An obvious candidate was Tec kinase, as this enzyme is expressed in myeloid cells and does, to some degree, complement Btk activity in xid B cells (40). Western immunoblot analysis showed that Tec kinase was expressed at low levels in untreated monocytes from both normal donors and XLA patients (Fig. 6 B), however, after treatment with M-CSF for 48 h the expression of Tec protein was increased. Counter blots confirmed that the expression of Btk was restricted to the normal cells (Fig. 6 B). Next, the response of Tec kinase to LPS in M-CSF–treated monocytes was investigated using autokinase assays of immunoprecipitated enzyme. Like Btk, Tec kinase activity was increased in response to LPS treatment of human M-CSF–treated monocytes (Fig. 6 C).

**Discussion**

Our data has shown that stimulation of human monocytes with LPS triggers the activation of the nonreceptor tyrosine kinase, Btk. Moreover, in XLA monocytes that lack functional Btk, there is a failure to respond to LPS treatment as determined by their reduced TNFα production after stimulation. Our initial findings led us to further examine the control of Btk in controlling TNFα production. Adenoviral gene transfer has been used extensively to investigate the role of different transgenes in primary cells due to the ability of adenoviruses to infect both quiescent and dividing cells at high efficiency (4). Overexpression of Btk resulted in a consistent two- to threefold increase in TNFα production from M-CSF–treated monocytes. In B cells, activation of the NF-κB transcription complex by signals derived from the B cell antigen receptor act via Btk to control B cell development, survival, and antigenic responses. However, the actions of Btk in macrophages appear to be independent of NF-κB activation and there is no defect in IkBα degradation in response to LPS in XLA monocytes.

TNF biosynthesis is under the control of multiple and complex regulatory mechanisms. Because the Tec family is...
known to interact with a wide range of signal transduction
to interact with a wide range of signal transduction
molecules (18), we took the alternate approach of examining
the effect of Btk on the UTRs of TNFα. Using the 5′
reporter constructs, we were able to show that the
ability of Btk to enhance TNFα production required
the 3′ UTR. There is considerable evidence indicating that
some of these may occur at the translational level and be
mediated by AU-rich elements (AREs) in the 3′ UTR of
the mRNA. Certain inflammatory gene mRNAs, including
cyclooxygenase-2, IL-6, IL-8, and TNFα mRNAs, are
stabilized by activation of the p38 MAPK pathway by stimu-
uli such as IL-1 and LPS (37, 41–43). Studies with mRNA
reporter constructs have shown that the p38 MAPK medi-
ated stabilization directly involves AREs (38). Thus, AREs
confer instability on mRNAs. However, after activation of
the p38 MAPK pathway, they allow mRNA stabilization
and hence increased protein expression. There is also a
strong association between Btk and p38 MAPK in B cells
showing that the activation of p38 MAPK was completely
inhibited in cells deficient in Lyn and Btk, and the intro-
duction of wild-type Btk, but not kinase-inactive Btk, re-
stored the p38 MAPK activation in response to 280 nm
UV irradiation in chicken DT40 B cells (44). The precise
regions of the TNF 3′ UTR involved in this interaction
with Btk and the potential involvement of p38 MAPK and
other intermediary molecules is a field of ongoing research
in our laboratory.

The proposition that Btk is essential for LPS-induced
TNFα production is not easily reconciled with the XLA
phenotype that shows no obvious gross defect of the innate
immune system. However, there have been limited reports
that XLA patients have reduced inflammatory responses
(45). This is in contrast to patients with common variable
immunodeficiency who are prone to multiorgan granulo-
matous disease, although they too have a type of antibody
deficiency similar to the XLA patients (28, 46). In humans,
Btk is absolutely required for the progression of developing
B cells through the pro-B to pre-B stage. However, in xid
mice the absence of Btk alone does not result in the same
phenotype and it is only when Tec is also ablated that the
same B cell deficiency is observed (40). It would be of in-
terest to investigate LPS responsiveness in monocyte/mac-
rophages from these mice. This suggests that Tec might be
able to substitute for the absence of Btk in mouse pro-B
cells, but at this stage of development in man, Tec is either
unavailable or is incapable of substituting for Btk (47). It is
of interest to note that monocyte differentiation toward
macrophages ablates the differences observed between the
XLA patients and normal donors (Fig. 6 A) and that this is
accompanied by an increase in Tec levels (Fig. 6 B). The
observation that Tec kinase is expressed in resting mono-
cytes from XLA and normal individuals, although at a
lower level, may explain why there is some response to
LPS in the Btk-deficient cells. The data obtained here
would suggest that without an increase in Tec kinase ex-
pression induced by M-CSF, there is insufficient kinase ac-
tivity in the resting XLA monocytes to support a full LPS
response. This hypothesis is supported by the observation
that at the lowest LPS concentration used in this study (0.1
ng/ml), TNFα production by XLA and normal cells was
similar (Fig. 1 A). The majority of the studies in mice (23,
24), with the exception of the study by Mukhopadhyay et
al. (25), have been unable to find any differences in LPS
responsiveness between xid and normal macrophages. We
also examined the responsiveness of peritoneal macro-
phages from xid mice versus normal mice and were unable
to observe any differences in TNFα production and NF-κB
DNA binding activity in response to LPS (unpublished
data). Taken together, these data suggest that rather than a
difference between murine and human cells, that it is in
fact a difference between monocytes and macrophages. It is
therefore possible that the up-regulation of Tec, or other
Tec kinase family members, in human macrophages may
explain why there is no major impairment of innate immu-
nity in XLA patients although further work is required to
confirm this hypothesis.

In summary, this study has demonstrated that Btk is not
only another tyrosine kinase activated by LPS, but also that
deficiency in its expression is associated with impairment of

Figure 6. The effect of M-CSF treatment on LPS-stimulated TNFα
production and Tec expression. (A) Adherent monocytes from XLA and
normal donors were either stimulated with LPS for 2 h, or incubated with
M-CSF for 48 h before LPS stimulation for 2 h. Supernatants were as-
sayed for TNFα production. (B) Adherent monocytes from normal (lanes
1 and 2) and XLA donors (lanes 3 and 4) were either lysed immediately,
or after M-CSF treatment for 48 h. Western blot analysis was performed
using either α-TecSH3, α-Btk, or α-actin antibody. (C) Elutriated mono-
cytes from normal donors were stimulated with LPS for the indicated time
periods. Tec was immunoprecipitated and in vitro autokinase assay was per-
formed. Each study is representative of at least three separate experiments.
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May have in mediating TNFα expression. Furthermore, overexpression of Btk in macrophages synergizes with LPS to induce TNFα production by stabilizing TNFα mRNA. Although these data imply that Btk is the first described tyrosine kinase having a key role in LPS-induced production of TNFα by monocytes, this role is probably not restricted to Btk alone. The intriguing question remains as to what role Tec kinases may have in mediating TNFα production by myeloid cells to other stimuli, particularly those involved in inflammatory conditions like rheumatoid arthritis and Crohn’s disease, where TNFα is a validated therapeutic target.

The authors would like to thank Dr. V. Tybulewicz and Ms. L. Vanes (National Institute for Medical Research, Millhill, United Kingdom) for providing access to the xid mice. Many thanks to Prof. C. Kinnon and Dr. M. Tomlinson for providing access to the Btk and Tec family antibodies used in this study. The authors would also like to thank Dr. A. Clark for constructive criticism of the manuscript.

N.J. Horwood is a Howard Florey Research Fellow. T. Mahon is a Training and Mobility of Researchers Marie Curie Fellow. These studies were supported by a block grant from the Arthritis Research Campaign, UK.

Submitted: 22 October 2002
Revised: 11 February 2003
Accepted: 11 March 2003

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