

Bruton's Tyrosine Kinase Is Involved in p65-mediated Transactivation and Phosphorylation of p65 on Serine 536 during NF κ B Activation by Lipopolysaccharide*

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Bruton's tyrosine kinase (Btk) has recently been shown to participate in the induction of nuclear factor κ B (NF κ B)-dependent gene expression by the lipopolysaccharide (LPS) receptor Toll-like receptor-4 (TLR4). In this study we have examined the mechanism whereby Btk participates in this response. Treatment of the murine monocytic cell line Raw264.7 with LFM-A13, a specific Btk inhibitor, blocked LPS-induced NF κ B-dependent reporter gene expression but not I κ B α degradation. Transient transfection of HEK293 cells with Btk had no effect on NF κ B-dependent reporter gene expression but strongly promoted transactivation of a reporter gene by a p65-Gal4 fusion protein. I κ B α degradation activated by LPS was intact in macrophages from X-linked immunodeficiency (Xid) mice, which contain inactive Btk. Transfection of cells with a dominant negative form of Btk (BtkK430R) inhibited LPS-driven p65 mediated transactivation. Additionally LFM-A13 impaired phosphorylation of serine 536 on p65 induced by LPS in HEK293-TLR4 cells, and in Xid macrophages this response was impaired. This study therefore reveals a novel function for Btk. It is required for the signaling pathway activated by TLR4, which culminates in phosphorylation of p65 on serine 536 promoting transactivation by NF κ B.

Bruton's tyrosine kinase (Btk)¹ is a member of the Tec family of non-receptor tyrosine kinases and is found in all cells of the hematopoietic lineage except plasma cells and T-cells (1). In B-cells, activation of the B-cell receptor leads to Btk being rapidly recruited to the plasma membrane where it becomes phosphorylated and activated. Btk is required for normal B-cell development (2) and the gene encoding Btk was first identified

as the mutated gene in X-linked agammaglobulinemia (XLA) in humans, an immune disease characterized by a lack of circulating B lymphocytes and an absence of Igs of all classes (3). Mutations in the Btk gene also result in the less severe X-linked immunodeficiency (Xid) in mice. Various mutations have been identified as being responsible for the XLA phenotype, whereas the mutation in Xid mice has been mapped to arginine 28 (R28C) in the PH domain (4). This prevents Btk associating with the membrane, thus inactivating it. The PH domain is important in the process of Btk activation, since it localizes Btk to the membrane through its interaction with phosphatidylinositol triphosphate (PIP₃), which is generated by phosphatidylinositol 3-kinase (PI3K).

Due to the major phenotype of Btk deficiency being impaired B-cell development and function, the main focus of interest in Btk has centered around the B-cell. However, several studies have provided a more general role for Btk in immune regulation. Studies in Xid peritoneal macrophages have shown reduced responses to lipopolysaccharide (LPS) stimulation, with tumor necrosis factor α (TNF α) and IL-1 β production decreased and macrophage effector functions impaired (5). We have shown that Btk is recruited to the LPS receptor Toll-like receptor-4 (TLR4) where it is activated and is required for NF κ B activation (6). In addition, peripheral blood mononuclear cells (PBMCs) isolated from XLA patients have been shown to have impaired TNF α production induced by LPS (7).

The mechanism whereby Btk participates in TLR4 signaling to NF κ B is not known. The prototypical form of NF κ B is a heterodimer consisting of a 50-kDa DNA-binding subunit (p50) and a 65-kDa transactivation subunit (RelA/p65). NF κ B is ubiquitously expressed and in uninduced cells is retained in the cytoplasm in an inactive form by the I κ B family of proteins. Upon stimulation of cells with LPS four adapter proteins are engaged, MyD88, MyD88 adapter-like (Mal), Toll/IL-1 receptor domain-containing adaptor-inducing interferon β (TRIF), and TRIF-related adapter molecule (reviewed in Ref. 8). MyD88 and Mal engage with IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1 leading to activation of the I κ B kinase (IKK) complex via Traf-6 (9, 10). In this pathway I κ B α becomes phosphorylated by the IKK complex and is subsequently targeted for ubiquitination and degradation (11, 12). This results in the release of NF κ B, which rapidly translocates into the nucleus, binds to its target DNA triggering the transcription of various target genes (13). In addition, LPS signaling promotes phosphorylation of the p65 subunit of the NF κ B complex, which is required for transactivation of gene expression. To date, five distinct serine residues have been identified on p65 that are inducibly phosphorylated in response to TNF, IL-1, and/or LPS. Serine 276 on p65 is phosphorylated by protein kinase A. LPS induces serine 536 phosphorylation, which may involve IKK2 (14). Other candidate kinases include IKK ϵ and TBK1, and an

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¹ The abbreviations used are: Btk, Bruton's tyrosine kinase; NF κ B, nuclear factor κ B; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; I κ B α , inhibitory protein κ B; HEK, human embryonic kidney; XLA, X-linked agammaglobulinemia; Xid, X-linked immunodeficiency; PH, pleckstrin homology; TNF, tumor necrosis factor; IL, interleukin; PBMC, peripheral blood mononuclear cells; Mal, MyD88 adapter-like; TRIF, Toll/IL-1 receptor domain containing adaptor-inducing interferon β ; IRAK, IL-1 receptor associated kinase; IKK, I κ B kinase; TBK1, TRAF family member associated (TANK)-binding kinase 1; BMDM, bone marrow-derived macrophages; PIP₃, phosphatidylinositol triphosphate; PI3K, phosphatidylinositol 3-kinase; CRE, cAMP response element; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

as yet unknown kinase identified by chromatic fractionation of cell extracts (15) prepared from cells treated with IL-1, which signals in a similar manner to LPS (16). The details of the pathway activated by TLR4 leading to phosphorylation of p65 on serine 536 are, however, not fully understood.

Having previously shown that LPS activated Btk and that inactive mutants of Btk inhibited LPS signaling to NF κ B-linked gene expression, we have investigated the function of Btk in NF κ B regulation. In the present study we have found that Btk is involved in the pathway leading to phosphorylation of p65 on serine 536 and is not involved in the process leading to I κ B α degradation. Our study therefore elucidates the role of Btk in TLR4 signaling and implicates Btk for the first time in the transactivation pathway of NF κ B.

MATERIALS AND METHODS

Cell Culture—HEK293, U373, and HEK293-TLR4 cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 μ g/ml gentamicin and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. CBA/CaOlaHsd and CBA/HNOlaHsd-Xid mice were obtained from P. Fallon (Trinity College, Dublin, Ireland). Peritoneal macrophages were prepared from CBA/CaOlaHsd and CBA/HNOlaHsd-Xid mice in the usual manner. Bone marrow-derived macrophages were prepared from CBA/CaOlaHsd and CBA/HNOlaHsd-Xid mice and cultured for 1 week in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 100 μ g/ml gentamicin and 2 mM L-glutamine, and driven to proliferate using granulocyte macrophage-colony-stimulating factor (10 ng/ml) (Calbiochem).

Plasmids and Reagents—The plasmid encoding the chimeric CD4-TLR4 was a kind gift from Dr. R. Medzhitov (Yale University, New Haven, CT) and has been described previously (17). The NF κ B-luciferase plasmid was a kind gift from Dr. R. Hofmeister (Universitat Regensburg, Regensburg, Germany) and contains 5 κ B sites upstream of the luciferase gene. The plasmid encoding wild-type Btk was obtained from R. Hendriks (Rotterdam, the Netherlands), and the dominant negative Btk (K430R) has been described previously (6). Gal4-p65⁽¹⁻⁵⁵¹⁾ plasmid encoding a full-length p65 subunit of NF κ B fused to the Gal4 DNA-binding domain was a kind gift from Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany) and has been described elsewhere (18). The Gal-luciferase reporter gene was obtained from Stratagene. The monoclonal p65 antibody was obtained from Santa Cruz Biotechnology, Inc., the polyclonal phospho-NF κ B p65 (Ser⁵³⁶) antibody was obtained from Cell Signaling Technology Inc., the monoclonal anti- β -actin antibody was obtained from Sigma (Poole, UK), and the I κ B antibody was a kind gift from Prof. R. Hay (University of St. Andrews, St. Andrews, UK). The Btk-specific inhibitor, LFM-A13, was obtained from Calbiochem (Nottingham, UK). All other reagents were obtained from Sigma unless otherwise stated.

Site-directed Mutagenesis—The Gal4-p65(S536A) plasmid was generated from the Gal4-p65⁽¹⁻⁵⁵¹⁾ plasmid using the QuikChange XL site-directed mutagenesis kit (Stratagene). The primers used were as follows: caggagatgaagactctccgccattcgccgacatggacttc (forward) and gaagtcacatgctcgcaatggcgagaaagcttctcatctctg (reverse).

Transient Transfections and Reporter Gene Assays—HEK293 or HEK293-TLR4 cells were seeded (1×10^5 ml⁻¹) onto 96-well plates 24 h prior to transfection. For the NF κ B-luciferase assay 80 ng of κ B-luciferase, 40 ng of *Renilla* luciferase, and the indicated amount of Btk-expressing plasmid (220 ng total) were transfected using Genejuice (Novagen, Madison, WI) according to the manufacturer's recommendations. For the p65 luciferase assay, 5 ng of Gal4-p65, 5 ng of Gal-luciferase, 40 ng of *Renilla* luciferase, and the indicated amount of Btk-expressing plasmid or CD4-TLR4-expressing plasmid (220 ng total) were transfected using Genejuice. In all cases the amount of DNA transfected was kept constant by the addition of various amounts of appropriate empty vector plasmid. Cells were either left untreated, pretreated with LFM-A13 (10 or 100 μ M, 45 min), or stimulated with LPS (1 μ g/ml) as indicated following a period of recovery (16–18 h). Firefly luciferase activity was assayed by the addition of 40 μ l of luciferase assay mix (20 mM Tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 MgSO₄, 0.1 M EDTA, 33.3 mM dithiothreitol, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to the sample, and *Renilla* luciferase was read by the addition of 40 μ l of a 1:500 dilution of Coelentrax in phosphate-buffered saline. Luminescence was read using the Reporter microplate luminometer (Turner Designs). The *Renilla* luciferase plas-

mid was used to normalize for transfection efficiency in all experiments.

Immunoblot Analysis—Cells were seeded in 6-well plates at 1×10^6 ml⁻¹ for experiments and left untreated or pretreated with LFM-A13 or Me₂SO prior to stimulation with LPS as indicated in the figure legends. Treatment was terminated by the addition of 5 ml of ice-cold phosphate-buffered saline. Cells were lysed on ice (30 min) in ice-cold radioimmune precipitation buffer (phosphate-buffered saline buffer containing 1% Nonidet P40, 0.5% w/v sodium deoxycholate, 0.1% SDS, 10 μ g/ml phenylmethylsulfonyl fluoride, 30 μ l/ml aprotinin, and 10 μ g/ml sodium orthovanadate). Protein estimations of cell extracts were determined by the dye binding assay of Bradford (30). Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose, and after incubation with primary antibodies as indicated, blots were incubated with the appropriate peroxidase-conjugated secondary antibody. Visualization was by enhanced chemiluminescence according to manufacturer's recommendations (Amersham Biosciences).

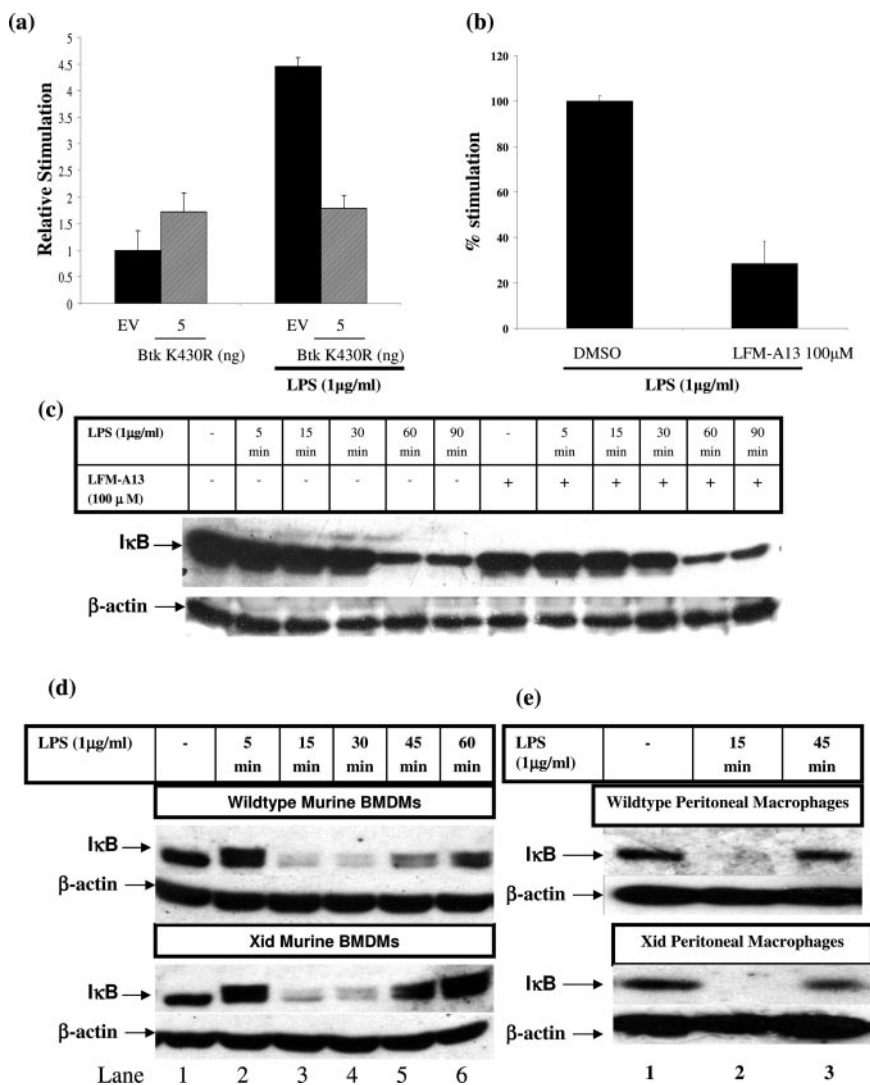
RESULTS

Btk Is Involved in the Activation of NF κ B-linked Reporter Gene Expression but Not I κ B α Degradation Activated by LPS—To gain insight into the function of Btk in NF κ B regulation by TLR4 we examined the effect of a kinase inactive form of Btk (Btk(K430R)) and the Btk-selective kinase inhibitor LFM-A13, on the induction of an NF κ B-linked reporter gene comprising five NF κ B sites linked to luciferase. We had previously shown that Btk(K430R) could block CD4-TLR4-driven NF κ B-linked luciferase in 293 cells (6); we now confirm that finding in the LPS-responsive, murine monocytic cell line RAW264.7. The ability of LPS to drive NF κ B-linked luciferase was abolished by transfecting cells with a plasmid encoding Btk(K430R) (Fig. 1a). We also tested the effect of the Btk inhibitor LFM-A13 on LPS-induced NF κ B-activation. Pretreatment of Raw264.7 cells with 100 μ M LFM-A13 reduced LPS-induced NF κ B activation by 70% (Fig. 1b). We next examined the activation of I κ B α degradation using the LPS-responsive cell line HEK293-TLR4. As shown in Fig. 1c, LPS induced I κ B α degradation in these cells from 30 min stimulation (*left-hand panel*). Pretreating the cells with LFM-A13 had no effect on this degradation (*right-hand panel*). To further test the possible role of Btk in LPS-induced I κ B α degradation, we isolated bone marrow-derived macrophages (BMDMs) and peritoneal macrophages from Xid and normal mice. Macrophages from Xid mice lack a functioning Btk (4). We confirmed the phenotype of the Xid mice, demonstrating a lack of Btk activation in response to LPS (not shown). As shown in Fig. 1d LPS-induced I κ B α degradation was identical in both normal and Xid BMDMs, with almost complete degradation occurring from 15 min stimulation (Fig. 1d, *lane 3*) in both cases, indicating no obvious impairment of the I κ B α degradation pathway. LPS caused a classical response in the degradation of I κ B α in both wild-type and Xid BMDMs, with a doublet appearing after 5-min stimulation indicative of phosphorylation, degradation occurring from 15 min, and I κ B α induction occurring from 45 min. Similarly there was no impairment of I κ B α degradation in Xid peritoneal macrophages when compared with normal peritoneal macrophages, with complete degradation occurring from 15 min LPS stimulation (Fig. 1e, *lane 3*).

Btk Is Involved in LPS-induced p65-mediated Transactivation—The ability of dominant negative Btk and LFM-A13 to inhibit NF κ B-linked luciferase expression and yet the lack of a role for Btk in the degradation of I κ B α in response to LPS pointed toward a role for Btk on the p65-mediated transactivation pathway of NF κ B. To test this hypothesis we utilized the Gal4-p65⁽¹⁻⁵⁵¹⁾ trans-reporting system described previously (19, 20). Briefly, this assay employs an expression plasmid encoding the transactivation p65 subunit of NF κ B fused to the DNA-binding domain of Gal4 and a Gal4-responsive reporter plasmid, Gal-luciferase. The advantage of this system is that

FIG. 1. Lack of a role for Btk in LPS-activated I κ B degradation.

a, Raw264.7 cells (2.5×10^4) were transfected with plasmids containing κ B-luciferase (80 ng) and *Renilla* luciferase (40 ng) and a plasmid expressing dominant negative Btk(K430R) as indicated. 18 h after transfection, the cells were stimulated with LPS (1 μ g/ml, 6 h) or left untreated as shown, cells were then harvested, and luciferase activity was subsequently measured. Results (mean $\pm$ S.D. for triplicate determinations) are represented as fold increase compared with unstimulated controls and are representative of three separate experiments. **b**, Raw264.7 cells (2.5×10^4) were transfected with κ B-luciferase (80 ng) and *Renilla* luciferase (40 ng). 18 h after transfection, the cells were pretreated with Me₂SO (DMSO) as a control or the Btk specific inhibitor LFM-A13 as indicated for 45 min. After pretreatment, cells were stimulated with LPS (1 μ g/ml, 6 h) as shown. Results (mean $\pm$ S.D. for triplicate determinations) are represented as percentage LPS stimulation. **c**, HEK293-TLR4 cells (6×10^5) were pretreated for 45 min with Me₂SO as a control or the Btk specific inhibitor LFM-A13 as indicated. After pretreatment, cells were stimulated with LPS (1 μ g/ml) for the time points indicated. Cells were lysed and immunoblotted for I κ B α and β -actin. Results are representative of three separate experiments. **d** and **e**, control (top panel) and Xid (bottom panel) murine BMDMs (**d**) or peritoneal macrophages (**e**) were stimulated with LPS (1 μ g/ml) for the time points indicated. Cells were lysed and immunoblotted for I κ B α and β -actin. Results are representative of three separate experiments.



Gal4-p65⁽¹⁻⁵⁵¹⁾ is regulated independently of I κ B α , allowing the effect of LPS on the p65-transactivation pathway to be assessed. Transient transfection of HEK293 cells with increasing amounts of plasmid expressing wild-type Btk (Fig. 2a, left-hand panel) resulted in a dose-dependent increase in activation of Gal4-p65⁽¹⁻⁵⁵¹⁾, with 100 ng of plasmid encoding Btk resulting in a 9-fold Gal4-p65⁽¹⁻⁵⁵¹⁾ activation. However this Gal4-p65⁽¹⁻⁵⁵¹⁾ activation was substantially reduced when Btk was cotransfected with a mutant form of the Gal4-p65⁽¹⁻⁵⁵¹⁾ construct (Gal4-p65(S536A)), in which the serine at position 536 is mutated to an alanine (Fig. 2a, right-hand panel). The response of Btk in the Gal4-p65⁽¹⁻⁵⁵¹⁾ trans-reporting system contrasted with the lack of effect of overexpressed Btk on the NF κ B-linked luciferase reporter plasmid (Fig. 2b), which requires I κ B α degradation to release NF κ B, further indicating a lack of a role for Btk in this process. Furthermore, although Btk failed to induce the NF κ B-linked luciferase reporter plasmid, LPS-stimulated HEK293-TLR4 cells induced a response (Fig. 2b, right-hand panel), and overexpression of CD4-TLR4, which is constitutively active, induced both responses (Fig. 2, a and b, left-hand panels, last histogram). CD4-TLR4-induced Gal4-p65⁽¹⁻⁵⁵¹⁾ activation was also reduced when cotransfected with the mutant Gal4-p65S536A, compared with wild-type Gal4-p65⁽¹⁻⁵⁵¹⁾ (Fig. 2a, right-hand panel). We next examined the effect of Btk(K430R) on the Gal4-p65⁽¹⁻⁵⁵¹⁾ trans-reporting system using HEK293 cells. As shown in Fig. 2c HEK293-TLR4 cells stimulated with LPS could drive the Gal4-p65⁽¹⁻⁵⁵¹⁾ trans-

reporting system in a dose-dependent manner. This ability of LPS to activate Gal4-p65⁽¹⁻⁵⁵¹⁾ was inhibited in a dose-dependent manner when cells were transfected with a plasmid encoding Btk(K430R) (Fig. 2d). Btk(K430R) was, however, unable to inhibit TNF α -induced Gal4-p65⁽¹⁻⁵⁵¹⁾ activation indicating specificity in its effect (21) (Fig. 2d). We also assessed the effect of Btk(K430R) on CD4-TLR4. Btk(K430R) abolished the effect of CD4-TLR4 on Gal4-p65⁽¹⁻⁵⁵¹⁾ activity (Fig. 2e). We also investigated the effect of the Btk inhibitor LFM-A13 on LPS induced Gal4-p65⁽¹⁻⁵⁵¹⁾ activation in HEK293-TLR4 cells. Pretreatment of the cells with 100 μ M LFM-A13 reduced LPS-induced Gal4-p65⁽¹⁻⁵⁵¹⁾ activation by 70% compared with control cells (Fig. 2f).

LPS-induced Phosphorylation of Serine 536 on p65 Is Inhibited by the Btk-specific Inhibitor LFM-A13 and Abolished in Cells from Xid Mice—Overall, the data suggested that there was a role for Btk in LPS-induced p65-mediated transactivation of NF κ B. As Btk did not drive the mutant Gal4-p65(S536A) trans-reporting system (Fig. 2a, right-hand panel), and as it has been reported that LPS induces the phosphorylation of the p65 transactivation domain on serine 536 in monocytes/macrophages, and furthermore, that the phosphorylation of serine 536 increases p65 transcriptional activity (14), we next investigated this response by employing a specific phospho-p65 antibody targeted against serine 536. LPS induced a strong increase in p65 phosphorylation on serine 536 in multiple cell types tested, including HEK293-TLR4 and U373 cells

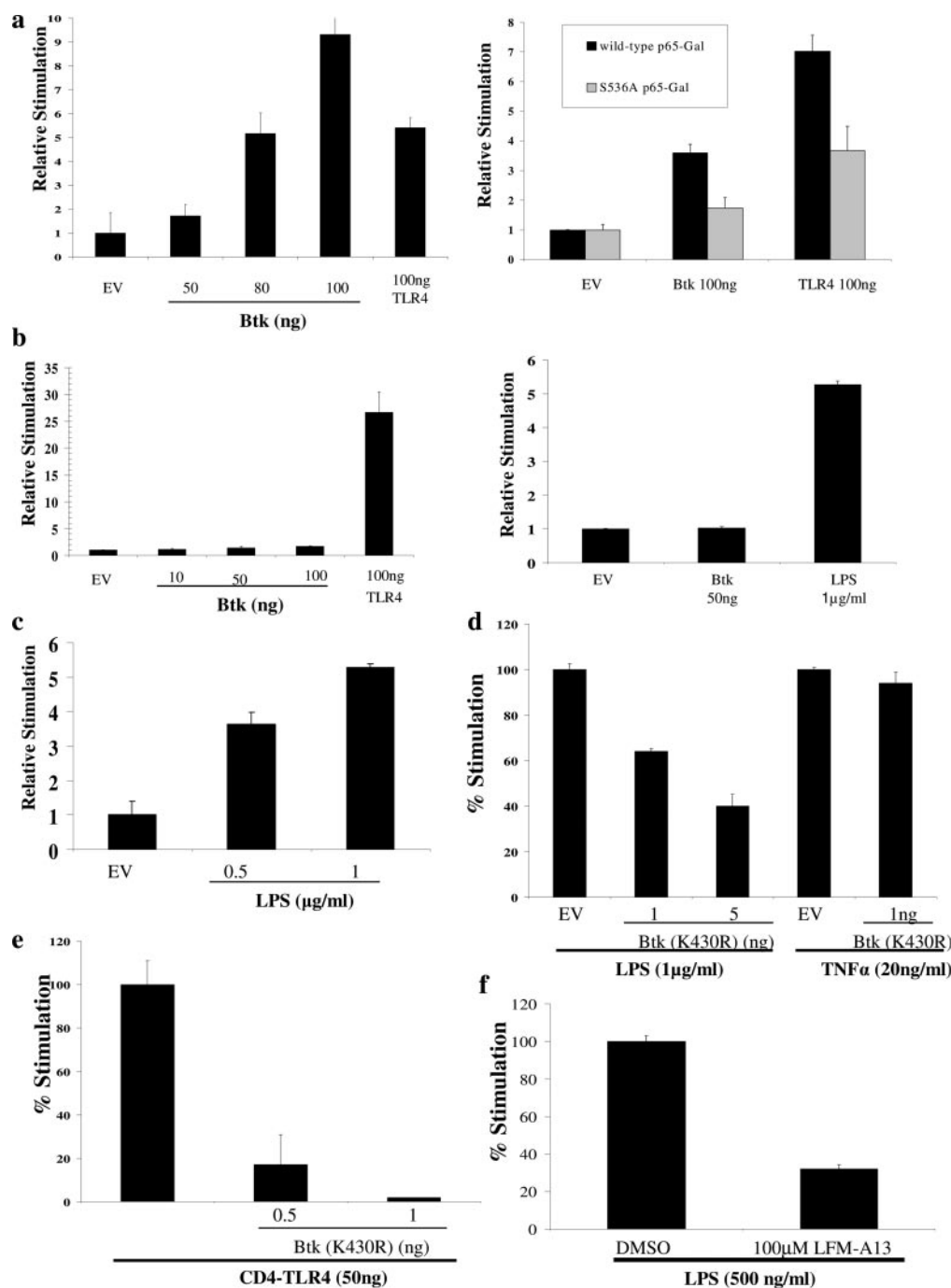


FIG. 2. Btk drives p65-mediated transactivation. *a*, HEK293 cells (2.5×10^4) were transfected with plasmids containing Gal-luciferase (5 ng), Renilla luciferase (40 ng), and plasmids expressing Gal4-p65 (5 ng) (left-hand panel) or Gal4-p65(S536A) (right-hand panel) and Btk or CD4-TLR4, as indicated. 18 h after transfection the cells were harvested, and luciferase activity was subsequently measured. Results (mean $\pm$ S.D. for triplicate determinations) are represented as fold increase compared with unstimulated controls. *b*, HEK293 cells (2.5×10^4) (left-hand panel) and HEK293-TLR4 cells (2.5×10^4) (right-hand panel) were transfected with plasmids containing κ B-luciferase (80 ng) and Renilla luciferase (40 ng) and a plasmid expressing wild-type Btk as indicated or CD4-TLR4 (100 ng). 18 h after transfection the cells were either left untreated or treated with LPS (1 μ g/ml, 6 h) harvested, and luciferase activity was subsequently measured. Results (mean $\pm$ S.D. for triplicate determinations) are represented as fold increase compared with unstimulated controls. *c-f*, HEK293-TLR4 cells (2.5×10^4) were transfected with plasmids containing Gal-luciferase (5 ng), Renilla luciferase (40 ng), and a plasmid expressing Gal4-p65 (5 ng). 18 h after transfection the cells were stimulated with LPS at the indicated doses for 6 h or left untreated as shown (*c*), transfected for 18 h with a plasmid expressing Btk(K430R) as indicated and treated with LPS (1 μ g/ml, 6 h) or TNF α (20 ng/ml, 3 h) (*d*), transfected with plasmids expressing CD4-TLR4 (50 ng) and Btk(K430R) as indicated for 18 h (*e*), or pretreated with Me₂SO (DMSO) as a control or the Btk-specific inhibitor LFM-A13 as indicated for 45 min, after which cells were stimulated with LPS (500 ng/ml, 6 h) as shown (*f*). Results shown are mean $\pm$ S.D. for triplicate determinations and are either fold increase over untreated cells (*a-c*) or are represented as percentage of LPS (*d-f*) or CD4-TLR4 stimulated cells (*e*). All results are representative of three separate experiments.

(Fig. 3a, left- and right-hand panels, respectively). A response in both cell types from 15 min was evident. We examined the effect of LFM-A13 in HEK293-TLR4 cells. As shown in Fig. 3b p65 phosphorylation was greatly reduced when the cells were

pretreated with LFM-A13; however, some residual phosphorylation was still evident (Fig. 3b, compare lanes 6, 8, and 10 with lanes 5, 7, and 9, respectively).

We next tested the response in cells from wild-type and Xid

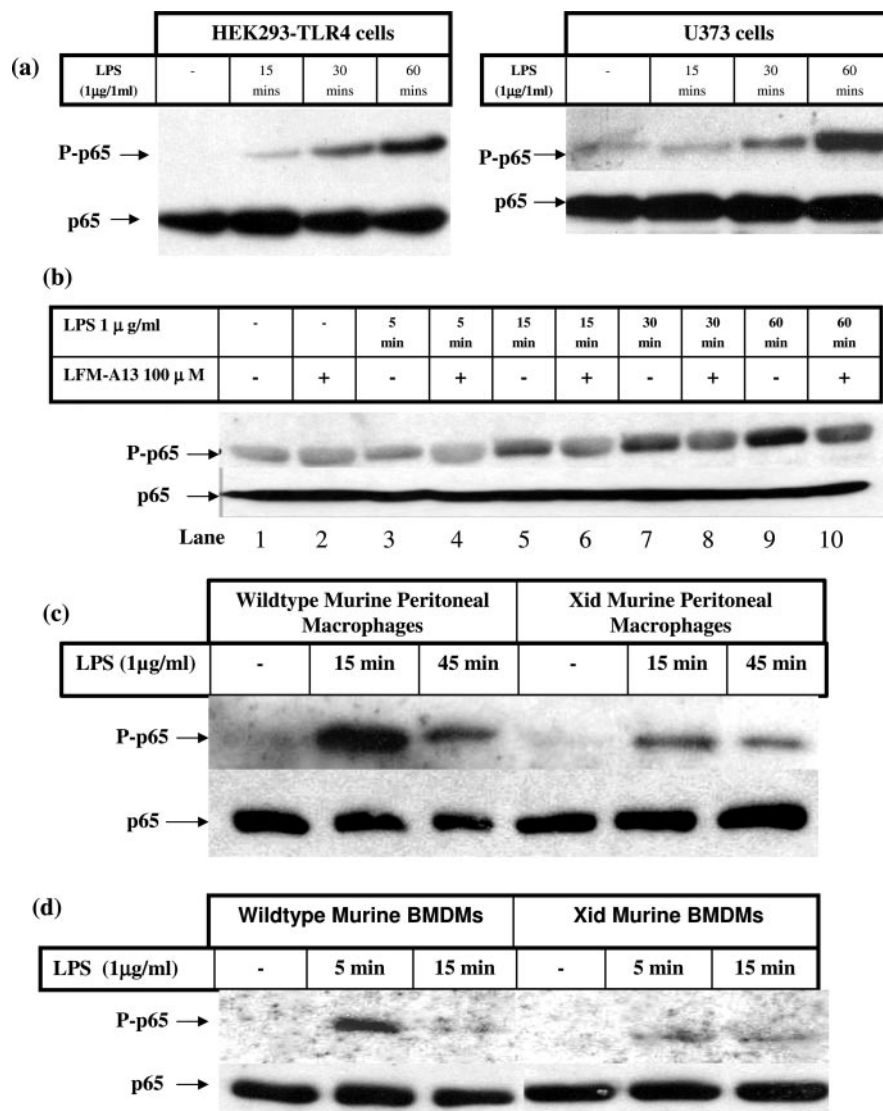


FIG. 3. LPS-induced phosphorylation of serine 536 on p65 involves Btk. *a*, HEK293-TLR4 cells (6×10^5) (left-hand panel) or U373 cells (6×10^5) (right-hand panel) were stimulated with LPS (1 µg/ml) for the time points indicated or left untreated as shown; cells were then lysed and immunoblotted for phosphoserine 536 on p65 and total p65. Results are representative of three separate experiments. *b*, HEK293-TLR4 cells were pretreated for 45 min with Me₂SO as a control or the Btk-specific inhibitor LFM-A13 as indicated. After pretreatment, cells were stimulated with LPS (1 µg/ml) for the time points indicated. Cells were lysed and immunoblotted for phosphoserine 536 on p65 and total p65. Results are representative of three separate experiments. *c*, control and Xid murine peritoneal macrophages were stimulated with LPS (1 µg/ml) for the time points indicated. Cells were lysed and immunoblotted for phosphoserine 536 on p65 and total p65. Results are representative of three separate experiments. *d*, control and Xid murine BMDMs were stimulated with LPS (1 µg/ml) for the time points indicated. Cells were lysed and immunoblotted for phosphoserine 536 on p65 and for total p65. Results are representative of three separate experiments.

mice. LPS caused a transient increase in p65 phosphorylation, which occurred at 15 min with the response decreasing at 45 min in wild-type peritoneal macrophages (Fig. 3c, left-hand panel). This response was much decreased in peritoneal macrophages from Xid mice (right-hand panel). In BMDMs, the increase in phosphorylation was also transient, occurring at 5 min and declining at 15 min (Fig. 3d, left-hand panel). This response was abolished in BMDMs from Xid mice (Fig. 3d, right-hand panel).

Taken together our results suggest a model whereby Btk is required for the TLR4-activated pathway leading to phosphorylation of p65 on serine 536, promoting transactivation of NFκB-dependent genes.

DISCUSSION

In this study we have found that Btk is involved in the LPS-induced pathway leading to phosphorylation of the p65 subunit of NFκB on serine 536, promoting transactivation of gene expression. We had previously discovered a role for Btk in TLR4 signaling to NFκB, demonstrating activation of Btk upon stimulation of cells with LPS, an interaction between Btk and TLR4, and an inhibitory effect of dominant negative Btk on NFκB-linked gene expression (6). Our study therefore pinpoints the process in the NFκB system which involves Btk, providing a novel function for the Btk enzyme.

Regulation of NFκB following stimulation with LPS occurs

via activation of two pathways. The best characterized of these regulates the release of NFκB from its inhibitory protein IκBα, subsequently allowing NFκB to translocate to the nucleus. However, this nuclear translocation of NFκB is not sufficient to activate NFκB-dependent gene transcription alone. The second pathway, which involves post-translational modifications, regulates the transactivating ability of the p65 subunit of NFκB once bound to its consensus sequence. Using the Btk-selective kinase inhibitor LFM-A13 and Xid macrophages, which lack a functioning Btk, our results clearly show no role for Btk in activation of IκBα degradation in response to LPS. Its role is in transactivation by p65, occurring most likely via phosphorylation of p65 on serine 536. Our results in relation to IκBα are consistent with a previous study on PBMCs isolated from control and XLA donors, who have an inactive Btk, where it was observed that LPS-induced IκBα degradation was intact in XLA PBMCs, but the induction of the NFκB-dependent gene TNF was impaired (7). In addition, the general protein-tyrosine kinase inhibitor genistein, which has been shown to affect Btk (22), was shown not to inhibit LPS-activated NFκB nuclear translocation as measured by DNA binding assays but blocked the induction of IL-1β, again consistent with a lack of a role for tyrosine kinases in activating IκBα degradation (23, 24).

The role of serine 536 phosphorylation in coupling p65 to coactivators, corepressors, or components of the basal tran-

scriptional machinery has recently been investigated. Buss *et al.* (15) reported that p65 phosphorylated on serine 536 binds to the promoter of IL-8 in response to IL-1 stimulation. They found that reconstitution of mutant p65 proteins in p65-deficient fibroblasts that either mimicked phosphorylation (S536D) or preserved a predicted hydrogen bond between Ser⁵³⁶ and Asp⁵³³ (S536N) revealed that phosphorylation of Ser⁵³⁶ favors IL-8 transcription mediated by TATA-binding protein-associated factor II31, a component of TFIID. They suggest that serine 536 phosphorylation disrupts a hydrogen bond between Ser⁵³⁶ and Asp⁵³³, and as a result of this, p65 gains a lower affinity for the corepressor amino-terminal enhancer of split and can therefore interact efficiently with TAFII31 (TATA-binding protein-associated factor II31), which induces transcription of IL-8, a known p65 target gene (15). This process is likely to be similarly activated by LPS.

The mechanism of NF κ B activation by LPS involves a pathway activated by the adapters MyD88 and Mal, which acting via the IRAks and TRAF6 activate the IKK complex. Regulation of phosphorylation of p65 on the other hand is not fully understood. There is clear evidence that the two pathways, although sharing some components, are distinct (25). Overexpression of MyD88 and Mal both promote transactivation by p65 (19, 26). The effect of Btk may involve these adapters, since it has been found in a complex with both proteins (6). The most detailed study to date of kinases that phosphorylate p65 identified four kinases possibly involved in this process, namely IKK1, IKK2, IKK ϵ , and TBK1. An as yet unknown kinase was also identified by chromatic fractionation of IL-1-stimulated cell extract (15). Of these IKK2 has been shown to be involved in phosphorylation of p65 on serine 536 in response to LPS in murine embryonic fibroblasts, although whether this occurs in macrophages was not examined (14). No role for IKK1 or IKK2 in the activation of this process by IL-1 was found in another study (15). Since IL-1 and LPS signaling to the IKK complex is broadly similar, this difference with LPS where IKK2 has a role (at least in murine embryonic fibroblasts) is somewhat unexpected. There is also evidence for NF κ B-inducing kinase activating IKK1, which phosphorylates p65 on serine 536 but does not phosphorylate I κ B α (27). This process may be involved in the Btk pathway in macrophages. No study has shown the direct phosphorylation of p65 on serine 536 by IKK2, and so the identity of the direct kinase downstream of Btk in this process remains undetermined. Btk might also phosphorylate p65 itself. It is a dual specificity kinase and as such phosphorylates both tyrosine and serine/threonine residues of exogenous substrates. It has been shown to activate another transcription factor cAMP response element (CRE)-binding protein (CREB) and subsequent CRE-mediated gene transcription by directly phosphorylating CREB at serine 133 (28). We are currently exploring the possibility that Btk directly phosphorylates p65.

Consistent with our data, it has been shown that PI3K activated by IL-1 is required for p65 phosphorylation rather than I κ B α phosphorylation and degradation (29). PI3K regulates Btk by generating PIP₃, which in turn binds the PH domain of

Btk localizing it to the membrane. Given our evidence for Btk involvement it is likely that the TLR4 pathway culminating in p65 phosphorylation involves both PI3K and Btk.

In conclusion our results demonstrate that in response to LPS stimulation Btk functions on the pathway leading to enhanced transactivation by p65 via phosphorylation of serine 536 but not I κ B α phosphorylation and degradation. These novel findings provide an additional function for Btk and identify a new process in LPS signal transduction leading to NF κ B activation.

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