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Bruton's tyrosine kinase is dispensable for the Toll-like receptor-mediated activation of mast cells

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ABSTRACT

Bruton's tyrosine kinase (Btk) represents an important signaling element downstream of ITAM-containing receptors, e.g. FcεR1 and BCR. Btk is part of the calcium signalosome and thus, critically involved in intracellular calcium mobilization. Loss of Btk or expression of mutant forms results in severe disease phenotypes, X-linked agammaglobulinemia (XLA) and Xid in humans and mice, respectively. Previously, roles for Btk in TLR-mediated signal transduction have been found in monocytes/macrophages. In the present study we show that Btk deficiency moderately enhances or has no influence on the LPS- or lipopeptide-induced secretion of IL-6 and TNF-α from murine bone marrow-derived mast cells (BMMCs). Furthermore, activation of p38 kinase, which is required for cytokine production, is comparable in WT and Btk−/− BMMCs. Moreover, stability of the adaptor protein Mal as well as LPS-induced H_2O_2 production does not vary between WT and Btk−/− cells. Interestingly, PKC-β deficiency, which results in a Xid-like phenotype as well, has also no negative effect on LPS-induced cytokine secretion, suggesting that proteins of the calcium signalosome are not involved in TLR-mediated BMMC activation. In conclusion, the study reveals that Btk is dispensable for TLR signaling and function in murine BMMCs.

1. Introduction

Btk is a cytoplasmic tyrosine kinase expressed in certain hemopoietic cells including B-lymphocytes, macrophages, mast cells, and platelets [1]. The importance of Btk is particularly obvious during B cell development and activation [2,3]. Btk deficiency manifests as X-linked agammaglobulinemia (XLA) and Xid in humans and mice, respectively [1]. Not only complete absence of Btk, but also defects in Btk, like point mutations in the PH and SH3 domains, can cause this severe disease [4]. Due to its role in B cell development, Btk-deficient patients/mice lack mature B cells and express dramatically reduced amounts of immunoglobulins [2]. Btk is part of the so-called calcium signalosome downstream of the BCR. It participates in the activation of PLC-γ2 and thus regulates the release of intracellular calcium from the endoplasmic reticulum [3]. The same function for Btk has been reported in mast cells (MCs) activated via the FcεR1 [5]. Both FcεR1 and BCR are ITAM-containing receptors [6] and share elements and mechanisms of signal transduction [5].

Btk has also been reported to be involved in the signal transduction of ITAM-independent receptor systems, e.g. TLR. However, the current literature provides in part conflicting data. Btk has been found to interact with components of the TLR4 and TLR2 signaling machinery, i.e. the adapter proteins, MyD88 and MyD88 adapter-like protein (Mal), as well as the interleukin-1 receptor-associated kinase-1 in HEK293 transfectants [7], and to be involved in transactivation of NFκB [8]. Furthermore, it was reported that Mal is subject to Btk-mediated tyrosine phosphorylation in response to TLR4 or TLR2 engagement [9] and this phosphotyrosine serves as an acceptor site for the suppressor of cytokine signaling 1 (SOCS1) protein. Binding of SOCS1 to Mal conveys this adaptor protein for degradation by the 26S proteasome [10]. Thus, Btk seems to be acting as part of a negative regulatory mechanism limiting primary innate immune responses. In accordance, degradation of Mal was absent in LPS-stimulated mouse Xid splenocytes [10]. From the involvement of Btk in degradation of Mal one would expect that cells lacking Btk function exhibit enhanced reactivity to LPS. However, the

Abbreviations: BMMC, bone marrow-derived mast cell; BMMΦ, bone marrow-derived macrophage; Btk, Bruton's tyrosine kinase; LP, lipopeptide; Mal, MyD88 adapter-like protein; MC, mast cell; SOCS1, suppressor of cytokine signaling 1; XLA, X-linked agammaglobulinemia.

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opposite effect of Btk deficiency, an impaired TNF-α response to LPS, was observed in mononuclear cells from XLA patients [11]. XLA mononuclear cells showed attenuated LPS-induced phosphorylation of p38 kinase compared to cells from control donors and this seemed to cause decreased stability of TNF-α mRNA in XLA cells [11]. In another study [12] TLR2-mediated stimulation of human XLA mononuclear cells resulted in impaired production of TNF-α and IL-1β, while production of IL-6, IL-8, and IL-10 remained unimpaired [12]. Finally, in a very thorough study involving seven XLA patients expressing no Btk protein, no effect of Btk deficiency on LPS-induced TNF-α and IL-6 production as well as activation of p38 kinase in monocytes was found [13]. Contrary, in a murine study Btk-deficient macrophages stimulated with LPS or other TLR ligands secreted enhanced levels of IL-6, as a result of suppressed IL-10 production [14].

So far reported, investigations on the role of Btk in TLR signaling were carried out in monocytes/macrophages, but not in MCs. Unlike macrophages MCs do not express membrane (m)CD14 and differ from mCD14-positive macrophages in their LPS recognition properties [15]. MCs, in the absence of soluble (s)CD14, recognize and react to rough (R)-chemotypes of LPS, while the reactivity to smooth (S)-chemotypes is practically absent. In this respect, they behave like macrophages deficient for CD14 or those expressing a mutant variant of CD14 ('*heedless*') [16]. In contrast, wild-type macrophages are also able to recognize S-chemotypes of LPS in an LPS-binding protein/mCD14-dependent fashion [15,16].

In this work studying the requirement of Btk for LPS- as well as lipopeptide (LP) -induced MC activation, we demonstrate that Btk moderately inhibits or has no influence on the activation of bone marrow-derived MCs (BMMCs) mediated by TLR4 or TLR2. Induction of TNF-α and IL-6 by LPS or LP, and activation of p38 kinase, and H_2O_2 production by LPS in Btk-deficient MCs is in comparison to WT MCs moderately enhanced or comparable. Interestingly, deficiency of PKC-β, which also results in a Xid-like phenotype in mice [17], does not influence LPS-induced cytokine production in BMMCs, suggesting that proteins involved in the organization of the calcium signalosome do not, or only marginally participate in TLR4-mediated signal transduction in MCs. Finally, Btk plays no obvious role in activation of murine bone marrow-derived macrophages (BMMΦs) by LPS or LP.

2. Materials and methods

2.1. Reagents

R-form LPS from *S. minnesota* mutant R595 and S-form LPS from *S. abortus equi* were extracted and purified as described [18–20]. Synthetic lipopetides (Pam_3CSK_4, FSL-1) were obtained from Echaz Microcollections (Tübingen, Germany). Polyclonal anti-Mal (T-16) antibody was obtained from Santa Cruz (Heidelberg, Germany) and polyclonal anti-p85 antibody (#06195) from Upstate/Biomol (Hamburg, Germany). Polyclonal anti-P-p38 (T180/Y182) antibody was purchased from Cell Signaling Technology (Frankfurt a. M., Germany). DNP-HSA containing 30–40 mol DNP per mole albumin and monoclonal IgE with specificity for DNP (SPE-7) were purchased from SIGMA (Deisenhofen, Germany) and DMSO from J.T. Baker (Griesheim, Germany). Btk inhibitor, LFM A13 [alpha-cyano-beta-hydroxy-beta-methyl-*N*-(2, 5-dibromophenyl) propenamide], and cPKC-specific inhibitor, Gö6976, were obtained from Calbiochem (Schwalbach, Germany). p38 inhibitor, BIRB0796, was purchased from the Division of Signal Transduction Therapy, College Of Life Sciences, University of Dundee, Dundee, Scotland, U.K.

2.2. Animals

Breeding of mice and all experiments were done in accordance with national guidelines and were approved by a local ethics committee.

2.3. Bone marrow-derived mast cells and macrophages

To obtain BMMCs, bone marrow cells (1×10^6/ml) from 6 to 8 week old male mice (129/Sv) were cultured (37 °C, 5% CO_2) as a single cell suspension in RPMI 1640 medium containing 15% FCS, 1% X63Ag8-653-conditioned medium as a source of IL-3 [21], 2 mM L-glutamine, 1×10^{-5} M 2-mercaptoethanol, 50 units/ml penicillin, and 50 mg/ml streptomycin. At weekly intervals, the non-adherent cells were reseeded at 5×10^5 cells/ml in fresh medium. By 4–6 weeks in culture, greater than 99% of the cells were c-kit and FcεR1 positive as assessed by phycoerythrin-labeled anti-c-kit antibodies (Pharmingen, Mississauga, Canada) and FITC-labeled rat anti-mouse IgE antibodies (Southern Biotechnology, Birmingham, AL, USA), respectively. Btk+/+ and −/− MCs were *in vitro* differentiated using the same protocol but starting from BM cells of 6 to 8 week old Btk+/+ and −/− littermates (C57BL/6). Wildtype and Xid MCs were *in vitro* differentiated from BM cells of 6 to 8 week old CBA/CaHN-Btk^{xid}/J and CBA/J mice (The Jackson Laboratory, Bar Harbor, Maine, USA). PKC-α+/+, PKC-α−/−, PKC-β +/+, and PKC-β −/− BMMC were *in vitro* differentiated from BM cells of 6 to 8 week old littermates (129/Sv).

To obtain BMMΦs, BM cells from Btk+/+ and −/− mice were grown in the presence of L-cell-conditioned medium in teflon bags as described previously [22].

2.4. MC stimulation and Western blotting

IgE-preloaded cells were washed twice in RPMI/0.1% BSA and resuspended in RPMI/0.1% BSA. Cells were adapted to 37 °C for 30 min and stimulated with the indicated concentrations of DNP-HSA, LPS, or LP. After stimulation for different length of time, cells were pelleted and solubilized with 0.5% NP-40 and 0.5% deoxycholate in 4 °C phosphorylation solubilization buffer [23]. After normalizing for protein content, the postnuclear supernatants were subjected directly to SDS-PAGE and Western blot analysis as described previously [24]. For measurement of extracellular cytokines, cell-free supernatants for cytokine measurements were stored in aliquots at −80 °C until use.

2.5. Macrophage stimulation

For induction of cytokines, macrophages were resuspended in serum-free DMEM (10^5 cells/0.2 ml/well), placed in 96-well plates (Nunc, Roskilde, Denmark) and cultured for 24 h at 37 °C in a humidified atmosphere containing 8% CO_2. Thereafter macrophages were washed and stimulated in triplicates with different amounts of LPS or LP in 0.2 ml of DMEM. The culture supernatants for TNF-α and IL-6 measurements were collected 4 h and 24 h later, respectively, and stored in aliquots at −80 °C until used for cytokine measurements.

2.6. Cytokine measurement

Mouse IL-6 and TNF-α ELISA's (BD Pharmingen, Heidelberg, Germany) were performed according to the manufacturer's instructions. The levels of cytokines in culture supernatants varied between experiments due to genetic background or age of the cells. Qualitative differences or similarities between WT and mutant cells, however, were consistent throughout the study.

2.7. Flow cytometric analysis of H_2O_2 production

IgE-sensitized BMMCs were washed twice with PBS, resuspended in 500 µl PBS, and stained with H2DCFDA (final concentration: 10 µM) for 30 min at 37 °C in the dark. Incubation in the dark was continued after the addition of the stimuli (Ag or LPS) for 5 min and flow cytometric analysis of cell samples was carried out subsequently using a FACScan cell analyzer (Beckton Dickinson, San Jose, USA).

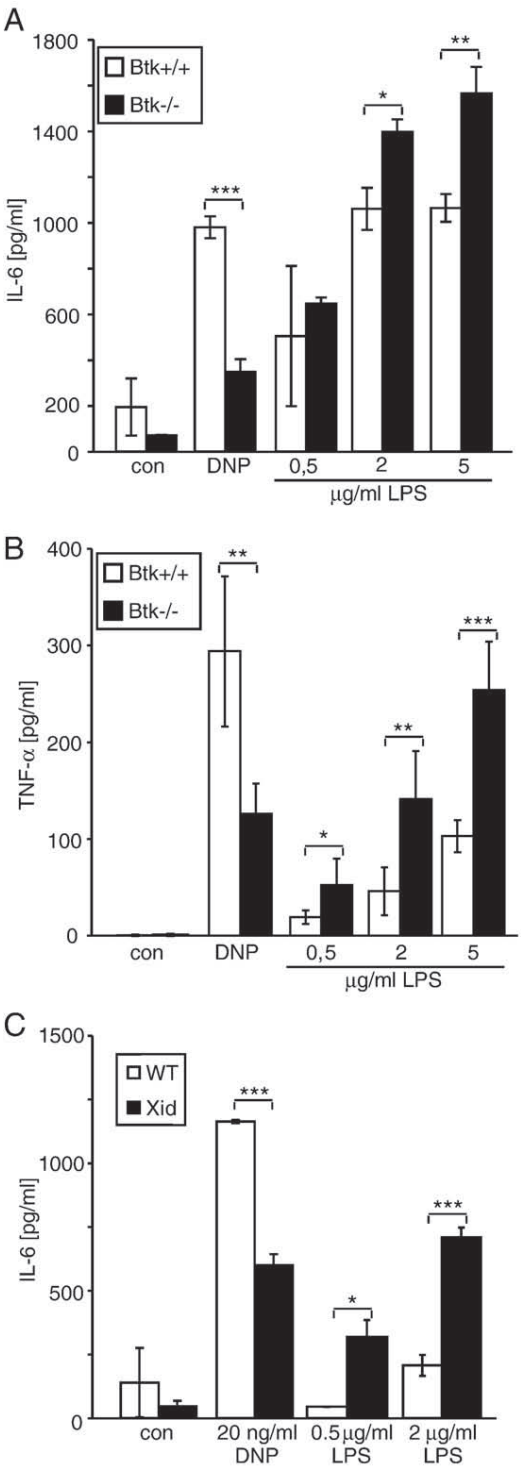

Fig. 1. Btk is not involved in the positive regulation of LPS-induced cytokine secretion in BMMCs. (A) IgE-loaded Btk+/+ and Btk−/− BMMCs were stimulated for 3 h with Ag (DNP), increasing concentrations of R-LPS, or left unstimulated (con). Secreted IL-6 was measured by ELISA. Each point is the mean of triplicates ± SD. Comparable results were obtained with independent BMMC cultures. (B) Supernatants from Btk+/+ and Btk−/− BMMCs treated as described in (A) were analyzed by ELISA for the presence of TNF-α. Each point is the mean of triplicates ± SD. Comparable results were obtained with independent BMMC cultures. (C) IgE-loaded WT and Xid BMMCs were treated for 3 h with Ag (DNP), increasing concentrations of R-LPS, or left unstimulated (con). IL-6 in the supernatants was measured by ELISA. Each point is the mean of triplicates ± SD. Comparable results were obtained with independent BMMC cultures.

Fluorescence intensity data (channel FL1) were collected from 25,000 cells per measurement.

2.8. RNA extraction and RT-PCR analysis

Total RNA was isolated from 5×10^6 cells by a guanidinium isothiocyanate-phenol-chloroform-isoamyl alcohol procedure [25], as described previously [26]. Total RNA (1 µg) was reverse-transcribed with Moloney Murine Leucaemia Virus reverse transcriptase and oligo-p(dT) primers (Expand reverse transcriptase kit; Roche) according to the manufacturer's recommendations. PCR was performed using HotStart *Taq* DNA polymerase (Genaxxon) according to the manufacturer's instructions. The primer pairs for amplification of murine S0CS1, S0CS3, CISH, and actin were: S0CS1: sense, CCCTGGCGACACTCACTT; antisense, GAGCGCGAAGAAGCAGTT (199-bp product size); S0CS3: sense, AGCGTCAAGACCCAGTCG; antisense, ACTTCGGACGAGGGTTCC (194-bp product size); CISH: sense, TCGGGAATCTGGGTGGTA; antisense, TCCAGCCGGAAGCTAGAA (192-bp product size), and actin: sense, TGG AAT CCT GTG GCA TCC ATG AAA; antisense, TAA AAC GCA GCT CAG TAA CAG TCC (348-bp product size). An annealing temperature of 56 °C was used for actin primer pairs and 61 °C was used for S0CS1, S0CS3, and CISH primer pairs. An annealing time of 25 s was used for S0CS1 as well as actin and of 45 s for S0CS3 and CISH. All PCR products were resolved by 2% agarose gel electrophoresis and were visualized by ethidium bromide staining.

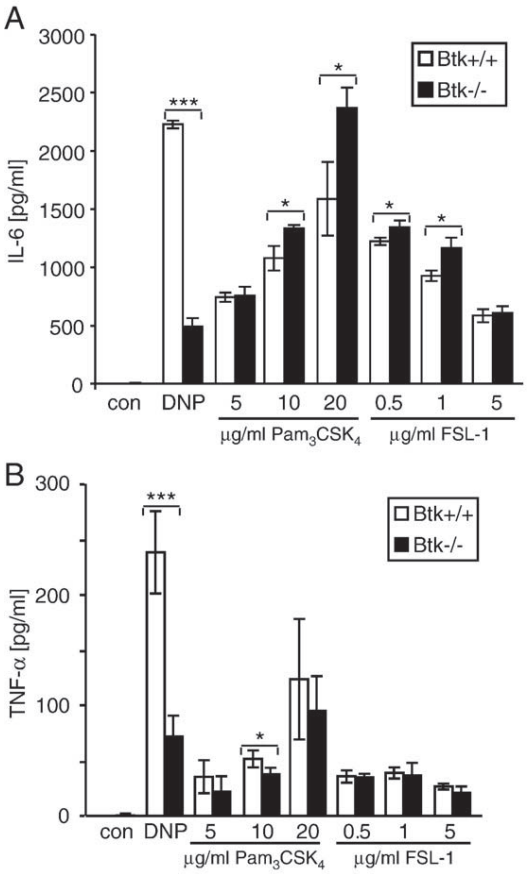

Fig. 2. No attenuation of TLR2-mediated cytokine secretion in Btk-deficient BMMCs. (A) IgE-loaded Btk+/+ and Btk−/− BMMCs were stimulated for 3 h with Ag (DNP, 20 ng/ml), indicated concentrations of Pam_3CSK_4 (TLR2/TLR1 ligand) or of FSL-1 (TLR2/TLR6 ligand), or left unstimulated (con). Secreted IL-6 was measured by ELISA. Each point is the mean of triplicates ± SD. Comparable results were obtained with independent BMMC cultures. (B) Supernatants from Btk+/+ and Btk−/− BMMCs treated as described under (A) were analyzed by ELISA for TNF-α expression. Each point is the mean of triplicates ± SD. Comparable results were obtained with independent BMMC cultures.

2.9. Statistical analysis

All values in the figures (concerning bar graphs) are expressed as mean of SD of *n* observations (with *n* indicated in the respective figure legends). The relevant data sets were compared by unpaired two tailed Student's *t* test using Graph Pad Prism3 evaluation software (Graph Pad, San Diego, CA). *P* values of * <0.05, ** <0.005, and *** <0.0005 were considered statistically significant. Densitometric

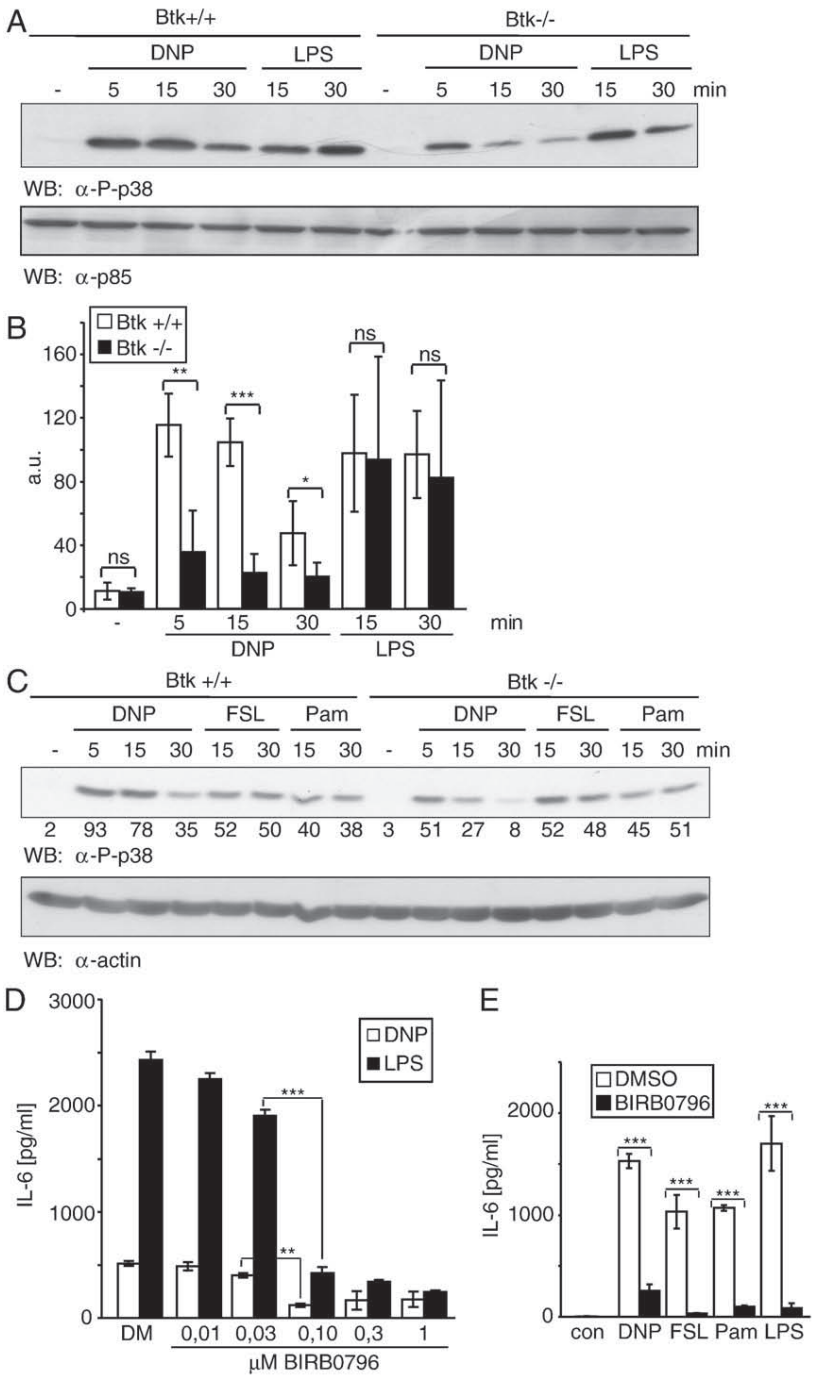

Fig. 3. p38 activation is important for LPS-induced IL-6 secretion, but is not dependent on Btk activity. (A) Btk+/+ and Btk−/− BMMCs were pre-loaded with IgE and stimulated with Ag (DNP, 20 ng/ml) or R-LPS (5 μg/ml) for the indicated time points, or were left untreated (−). Subsequently, postnuclear supernatants were analyzed by anti-P-p38 (upper panel) and anti-p85 (lower panel; loading control) immunoblotting. (B) Anti-P-p38 Western Blot shown in (A) together with two further experiments performed with independently cultured BMMCs were statistically evaluated with respect to changes between Btk+/+ and Btk−/− BMMCs. (C) IgE-preloaded Btk+/+ and Btk−/− BMMCs were stimulated with Ag (DNP, 20 ng/ml), FSL-1 (1 μg/ml), or Pam_3CSK_4 (Pam; 10 μg/ml) for the indicated time points, or were left untreated (−). Subsequently, postnuclear supernatants were analyzed by anti-P-p38 (upper panel) and anti-actin (lower panel; loading control) immunoblotting. Densitometry was performed, and relative expression levels are indicated under each band. (D) IgE-loaded BMMCs were stimulated with Ag (DNP, 20 ng/ml) or R-LPS (5 μg/ml) for 3 h in the presence of the indicated concentrations of p38-specific inhibitor, BIRB0796, or vehicle (DMS0, DM). Inhibitor treatment was started 30 min before the addition of stimulus. IL-6 in the supernatants was measured by ELISA. Each point is the mean of triplicates±SD. Comparable results were obtained with independent BMMC cultures. (E) IgE-loaded BMMCs were left untreated (con) or stimulated with Ag (DNP, 20 ng/ml), FSL-1 (FSL, 1 μg/ml), Pam_3CSK_4 (Pam, 10 μg/ml), or R-LPS (5 μg/ml) for 3 h in the presence of 0.1 μM BIRB0796 or vehicle (DMS0). Inhibitor treatment was started 30 min before the addition of the stimuli. IL-6 in the supernatants was measured by ELISA. Each point is the mean of triplicates±SD. Comparable results were obtained with independent BMMC cultures.

analysis of Western blots was performed using publicly available ImageJ software [27].

3. Results

3.1. Btk deficiency enhances or does not affect LPS- or LP-triggered cytokine secretion in BMMCs, respectively

The role of Btk in TLR signaling in MCs was not studied yet. Such investigation appeared interesting since activation of MCs via TLR4 or TLR2, unlike a similar activation of macrophages, proceeds without help of mCD14 and may thus differ from the latter [15]. Here, mast cells generated from bone marrow precursors, BMMCs, of wild type and Btk-deficient mice were stimulated with LPS *in vitro* and cytokine responses were measured. As shown in Fig. 1A, the levels of IL-6 secreted by LPS-stimulated Btk−/− and WT BMMCs were either comparable or higher in the cultures of Btk−/− cells. Likewise, LPS-stimulated Btk−/− BMMCs secreted higher TNF-α levels than WT cells (Fig. 1B). However, as reported previously [28], marked reduction of secretion of IL-6 and TNF-α was observed in Btk-deficient BMMCs compared to WT cells after stimulation via the FcεR1 (Fig. 1A and B). Comparable results to those obtained with Btk-deficient BMMCs were obtained in studies of IL-6 responses to LPS or Ag of cells derived from Btk^{Xid} mice (Fig. 1C).

In this study we also investigated the role of Btk in the IL-6 and TNF-α responses of BMMCs to LPs. As shown in Fig. 2A and B, WT and Btk-deficient BMMCs stimulated with the synthetic analoga of bacterial lipopeptides, FSL-1 (TLR2/TLR6 ligand) and Pam_3CSK_4 (TLR2/TLR1 ligand), secreted moderately enhanced or comparable levels of IL-6 and TNF-α, indicating that neither TLR2/TLR6- nor TLR2/TLR1-mediated signaling depends on Btk activation in BMMCs.

3.2. TLR-mediated activation of p38 is independent of Btk in BMMCs

In addition to the impaired TNF-α response, impaired activation/phosphorylation of p38 kinase was observed earlier in Btk-deficient/-compromised monocytes stimulated with LPS [11]. Fitting with our data on LPS-induced cytokine secretion in BMMCs, no defect in p38 phosphorylation was observable in LPS-treated Btk−/− compared to WT BMMCs (Fig. 3A and B). Furthermore, also lipopeptide-induced p38 phosphorylation was independent of Btk (Fig. 3C). These data indicate that in MCs TLR-driven activation of p38 is independent of Btk. Ag-triggered p38 activation, however, was severely compromised in Btk-deficient BMMCs (Fig. 3A–C). To proof that LPS-induced IL-6 secretion in BMMCs indeed is dependent on p38 activation, we performed dose–response analyses with the highly specific p38 inhibitor, BIRB0769 [29]. This inhibitor, at the dose of 0.1 μM, blocked both Ag- and LPS-triggered IL-6 and TNF-α secretion in BMMCs (Fig. 3D and data not shown). Likewise, LP-induced IL-6 and TNF-α responses were abrogated by using this inhibitor (Fig. 3E and data not shown). These data indicate that TLR-mediated cytokine secretion in BMMCs is, like in monocytes, dependent on p38 activation. However, this activation is, unlike in monocytes [11], independent of Btk.

3.3. Btk deficiency in BMMCs does neither influence stability of Mal nor TLR4-mediated H_2O_2 production

In macrophages, Btk has been demonstrated to be responsible for Mal tyrosine phosphorylation and the subsequent S0CS1/proteasome-mediated degradation of this adapter protein [10]. Next, we aimed at studying this feedback mechanism in BMMCs. However, we did not observe degradation of Mal, neither in WT nor Btk−/− BMMCs, during the first 3 h of stimulation (Fig. 4A). This suggests that the respective pathway (e.g. S0CS1 expression) is not functional in BMMCs. Indeed, performing RT-PCR analysis we observed increased expression of the S0CS family member CISH in BMMCs, whereas S0CS1 expression was

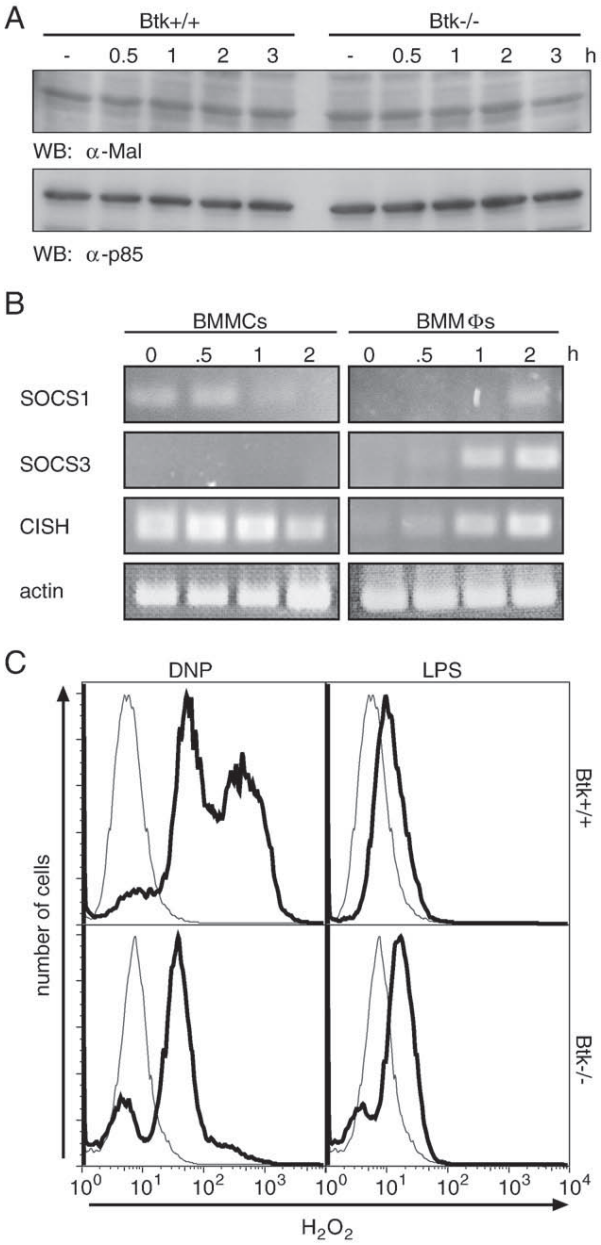

Fig. 4. Btk deficiency does interfere neither with Mal stability nor LPS-induced H_2O_2 production. (A) Btk+/+ and Btk−/− BMMCs were stimulated with R-LPS (5 μg/ml) for the indicated times or left unstimulated (−). Postnuclear supernatants were subjected to Western blot analysis with anti-Mal (upper panel) and anti-p85 (lower panel; loading control) antibodies. (B) BMMCs (left panels) and BMMΦs (right panels) were stimulated with 10 μg/ml and 1 μg/ml R-LPS, respectively, for the indicated time points. RT-PCR analyses were performed using primers for S0CS1, S0CS3, CISH, and actin (loading control). Comparable results were obtained with independent BMMC and BMMΦs cultures. (C) Btk+/+ (upper panels) and Btk−/− BMMCs (lower panels) were loaded with IgE overnight and subsequently incubated for 30 min with the dye H2DCFDA. Cells were then either left unstimulated (thin lines) or treated with Ag (DNP, 20 ng/ml; left panels) or R-LPS (5 μg/ml; right panels) (thick lines) and H_2O_2 production analyzed by FACS. Comparable results were obtained with independent BMMC cultures.

reduced in response to LPS (Fig. 4B). In contrast, in BMMΦs S0CS1, S0CS3, and CISH expression were induced in response to LPS stimulation (Fig. 4B).

Previously, Mangla et al. reported on the positive role of Btk in the generation of bursts of reactive oxygen intermediates in macrophages [30]. We and others described PI3K-dependent H_2O_2 production in Ag-triggered BMMCs [31,32]. Here we investigated whether LPS is able

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