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Defects in acute responses to TLR4 in *Btk*-deficient mice result in impaired dendritic cell-induced IFN-γ production by natural killer cells

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Abstract This study defines a critical role for Btk in regulating TLR4-induced crosstalk between antigen presenting cells (APCs) and natural killer (NK) cells. Reduced levels of IL-12, IL-18 and IFN-γ were observed in *Btk*-deficient mice and ex vivo generated macrophages and dendritic cells (DCs) following acute LPS administration, whilst enhanced IL-10 production was observed. In addition, upregulation of activation markers and antigen presentation molecules on APCs was also impaired in the absence of Btk. APCs, by virtue of their ability to produce IL-12 and IL-18, are strong inducers of NK-derived IFN-γ. Co-culture experiments demonstrate that *Btk*-deficient DCs were unable to drive wild-type or *Btk*-deficient NK cells to induce IFN-γ production, whereas these responses could be restored by exogenous administration of IL-12 and IL-18. Thus Btk is a critical regulator of APC-induced NK cell activation by virtue of its ability to regulate IL-12 and IL-18 production in response to acute LPS administration.

1. Introduction

During infection, myeloid cells play a key role not only as our first line of defense but also as key cells that contribute to the activation of other innate immune cells such as natural killer (NK) cells. In this respect Toll-like receptor (TLR) stimulation of antigen presenting cells (APCs) such as macrophages and dendritic cells, drives the production of the key NK cell activating cytokines, IL-12 and IL-18, in addition to enhancing cell–cell contact between APCs and NK which is also known to be important in NK cell activation, interferon (IFN)-γ production and acquisition of effector function [1-5]. Given the critical importance of IFN-γ in immune response to pathogens, cross-talk between cells of the innate immune system is critical for the effective clearance of pathogens,

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particularly intracellular bacterial and viral infections. The pathways and proteins involved in the activation of TLRs and subsequent production of inflammatory cytokines and type I IFNs have been studied in great detail, however there have been conflicting reports regarding the role of Bruton's tyrosine kinase (Btk) in promoting Toll-like receptor (TLR)-4 mediated immune responses in both cells of the innate and adaptive immune response.

Btk is a non-receptor tyrosine kinase belonging to the Tec family of kinases (TFKs), which includes Tec, Itk, Txk and Bmx [6]. Btk has several structural domains, including N-terminus, PH, Tec homology (TH), SH2, SH3, and kinase (SH1) domains, that are important in interacting with a range of proteins critical for intracellular signaling [6]. Btk inactivation or deficiency gives rise to an immune-deficient condition known as X-linked agammaglobulinemia (XLA), where B-cells fail to progress from pro-B cell to pre-B cell in the bone marrow and subsequently these cells are unable to produce immunoglobulins (Ig) or mature into antibody secreting plasma cells [7]. Thus individuals with XLA are unable to mount a humoral immune response and suffer from recurrent bacterial and enteroviral infections [8], with reports strongly indicating that defects in other cell types may contribute to the poor immunity in XLA patients. In this regard, Btk has been shown to play an important role in signaling downstream of the LPS receptor, TLR4. A number of studies have independently shown that Btk is phosphorylated and thus activated following acute LPS stimulation of cells [9-11]. Btk activation in turn positively regulates the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. Regarding the molecular mechanism behind these effects, Btk has been demonstrated to interact with the TIR domains of TLR2, 4, 6, and 9, in addition to key signaling molecules such as MyD88, Mal, and IRAK-1 [9], leading to enhanced transactivation of the p65 subunit of NFκB (via serine 536 phosphorylation) [10,13,14]. Btk in addition has been shown to stabilize TNF-α mRNA via a p38-dependent pathway in both X-linked immunodeficiency (*Xid*) mice and in XLA patients, indicating additional roles for Btk in regulating cytokine production in cells [10,15].

However the role for Btk in TLR4 signaling remains somewhat unclear, with evidence indicating that Btk positively regulates the production of the immunosuppressive cytokine IL-10 in murine macrophages, and a concomitant reduction IL-6 production [11]. In murine bone marrow derived mast cells the absence of Btk has been shown to result in similar or marginally enhanced levels of TNF-α, IL-6, p38 kinase activity and H_2O_2 production when compared with wild type cells following TLR2 or TLR4 treatment [12]. In addition, Btk is known to phosphorylate the adaptor protein Mal following TLR2 and TLR4 stimulation, which has been suggested to act as a negative feedback mechanism on the TLR-4 pathway, with phosphorylated Mal being targeted for SOCS1-mediated polyubiquitination and subsequent degradation [16]. These findings are potentially consistent with a role for Btk in turning-off and limiting TLR4 responses, and it is conceivable that Btk is having a dual role in regulating LPS responses, positively regulating acute responses whereas acting as a negative regulator at later time points thus enabling or facilitating LPS tolerance.

Studies using immune cells from XLA patients have also been conflicting. Specifically, whilst one study suggests that Btk is not required for LPS induced MAPK activation and inflammatory cytokine production in human monocytes following LPS stimulation [17], a number of separate studies demonstrate defects in both phagocytosis and inflammatory cytokine production following TLR4 stimulation in monocytes isolated from XLA patients, in keeping with a positive role for Btk downstream of TLR4 [10,15,18].

Whilst studies in cells derived from XLA patients have been controversial the increased incidence of mycoplasma and enteroviral infections in XLA patients is suggestive of defective TLR-mediated innate immune responses and possibly IFN-γ production, in addition to a lack of humoral immunity [19,20]. To date little is known regarding the contribution of Btk to the regulation of cross-talk between antigen presenting cells and other cells of the innate immune system, for example NK cells. Interestingly, a recent study examining DCs from XLA patients found that stimulation with a range of TLR ligands induced less maturation and reduced cytokine production from XLA patient derived cells compared to healthy control cells [21]. A role for Btk in regulating antigen presentation and maturation of antigen presenting cells is also indicated from the inability of MHC class II and CD40 to form a complex in the absence of Btk, resulting in impaired TLR-driven activation of APCs [22].

As such the current study sought to determine the precise role of Btk in TLR4-mediated immediate early responses and thus the role of Btk in shaping acute rather than chronic innate immune responses. We found that following 4 h administration of a lethal dose of LPS systemic levels of TNF-α, IL-1β, IL-12, IL-18 and IFN-γ were reduced or absent in Btk-deficient mice compared with wild-type mice, whereas IL-6 and IL-8 levels were unchanged. Importantly, APCs from Btk-deficient mice failed to mature and induce the production (both at the mRNA and protein level) of the NK cell activating cytokine IL-12 and IL-18. This consequently resulted in diminished activation of NK cells and an almost complete absence of IFN-γ production in Btk-deficient mice. Our findings suggest that in the absence of Btk, the ability of APCs to interface with other innate immune cells such as NK cells is lost, resulting in an almost complete absence of systemic IFN-γ production.

2. Materials and methods

2.1. Reagents

LPS was purchased from Cayla Invivogen (*Escherichia coli* 0111:B4). L929 conditioned medium (L929 CM) was used as a source of macrophage colony stimulating factor (M-CSF). Briefly L929 cells (ATCC) were incubated in RPMI-1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) for 5–7 days. Cell free supernatant was then harvested from confluent monolayers, passed through a 0.22 μm membrane filter and kept at −20 °C until use. CHO FLT3L cells were a kind gift from Dr. Kate Fitzgerald (UMASS Medical School, Worcester, MA) and were maintained in DMEM Hams F12 containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml). Recombinant murine

IFN-γ was purchased from ImmunoTools. Recombinant murine IL-12 and IL-18 were purchased from Sigma.

2.2. Mice

Btk deficient mice were housed and bred in the Biomedical Research Facility at Queens University Belfast and at the Royal College of Surgeons in Ireland under specific pathogen-free conditions and used at 6–10 weeks. The animals were used in accordance with ethical approval and license conditions.

2.3. Intraperitoneal (i.p) LPS injection

Age-matched (6 to 10 weeks) wild type (WT) C57BL/6 mice (Harlan Laboratories) and Btk-deficient mice were administered an intraperitoneal injection of 8 mg/kg LPS dissolved in sterile saline solution. Control mice were given an intraperitoneal injection of the equivalent volume of saline. Blood was obtained via tail vein bleed at various times prior to LPS injection and during treatment period to monitor serum cytokine levels. Following LPS treatment mice were culled by CO_2 asphyxiation.

2.4. Preparation of BMDMs and BMDCs

Bone marrow cells were flushed from hind legs of C57BL/6 mice or Btk-deficient mice and bone marrow derived macrophages (BMDMs) were derived by differentiating bone marrow cells in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% L929 conditioned medium, 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% L-glutamine for 7 days. Bone marrow derived dendritic cells (BMDCs) were derived by differentiating bone marrow cells in RPMI containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 1% L-glutamine, supplemented with 10% FLT3L supernatant for 7 days. Purity of cell populations was determined by flow cytometry.

2.5. Primary mouse splenocyte preparation and NK cell purification

Spleens were harvested from C57BL/6 or Btk-deficient mice, and splenocytes were obtained by mechanical cell dissociation of the spleens using a 70-μm cell strainer (BD Falcon, Fisher, Pittsburgh, PA). Red blood cells were lysed by treatment of the cell suspension with red blood cell lysis buffer (RBC lysis). Natural killer (NK) cells were purified from mouse splenocytes by negative selection using NK cell enrichment kit (Miltenyi). Purity of cell populations was determined by flow cytometry. Sample purities were approximately 90% $CD3^-DX5^+$ following negative selection.

2.6. Cytokine multiplex assay

Levels of mouse cytokines in serum and supernatant samples were simultaneously measured using a multiplex 7-spot electrochemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD) and read by an Imager 2400 plate reader (Meso Scale Discovery, Gaithersburg, MD). The cytokines analyzed included TNF-α, IL-1β, IL-6, IL-8, IL-12p70, IL-10, and IFN-γ.

2.7. ELISA

The IL-18 ELISA kit components were purchased from R&D Systems and used a rat anti-mouse IL-18 mAb (74) as the capture antibody, a biotin labeled rat anti-mouse IL-18 monoclonal antibody (93-10C) as the detection antibody, and recombinant mouse IL-18 as a standard. IFN-γ ELISA (R&D Systems) was performed according to manufacturer's instructions. TMB (3, 3, 5, 5-tetramethylbenzidine) HRP substrate solution was used for colorimetric detection (Sigma).

2.8. Realtime PCR

Total RNA was extracted from cell cultures using an RNeasy kit (Qiagen) and reverse transcribed to cDNA using Omniscript reverse transcriptase (Qiagen) according to manufacturer's recommendations. Quantitative realtime PCR was performed using SYBR Green Taq ReadyMix™ (Sigma) and the data was normalized to a *Gapdh* reference. The primer pairs for analysis are listed in Table 1. Realtime PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method [23].

2.9. Flow cytometric analysis

BMDMs, BMDCs and splenocytes were collected as detailed above from WT or Btk-deficient mice and either left unstimulated or stimulated for 24 h with 100 ng/ml LPS. Cells were stained for F4/80, CD11b, CD11c, CD86, IA/IE, (MHCII), CD107a, CD69 and DX5 using specific antibodies (BD Biosciences) and analyzed by flow cytometry using a FACSCantoII flow cytometer (BD Biosciences). Intracellular levels of IFN-γ were determined in splenocytes following 24 h treatment with 100 ng/ml LPS. For intracellular staining splenocytes were surface stained followed by fixing and permeabilizing using a Fix & Perm cell permeabilization Kit (Invitrogen) according to manufacturer's instructions and staining with a FITC conjugated anti-IFN-γ antibody (eBioscience). Quadrants in dot plot graphs and

Table 1 Primer pairs for gene induction analysis.

Gene	Forward	Reverse
IL-18	AAAGTGCCAGTGAACCC	TTTGATGTAAGTTAGTGAGAGTGA
IL10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
IL12p40	GGAAGCACGGCAGCAGAATA	AACTTGAGGGAGAAGTAGGAATGG
GAPDH	CATCAAGAAGGTGGTGAAGC	CCTGTTGCTGTAGCTGTATT

histograms were defined using fluorescently conjugated isotype controls.

2.10. NK cell stimulation with BMDCs

NK cells (1×10^5) were cultured with BMDCs (1×10^5) in 96-well flat bottomed plates in RPMI. LPS (100 ng/ml) was added and the cells cultured for 24 h. The level of IFN-γ in the culture supernatants was measured by ELISA (R&D Systems). Where indicated, co-culture experiments were performed in the presence of IL-12 (10 ng/ml) or IL-18 (10 ng/ml).

2.11. Statistical analyses

Two-tailed Students *t* tests were used to evaluate changes in cytokine production and gene expression. Data were deemed to be significantly different at *P* values less than 0.05.

3. Results

3.1. Btk enhances proinflammatory cytokine production following LPS administration

Although a role for Btk as a regulator of TLR4 responses in myeloid cells has been previously demonstrated there has been a certain degree of controversy within this field to date. For example, whilst we and others have shown that Btk is critically involved in NFκB activation and specifically p65 phosphorylation downstream of multiple TLRs including TLR4, TLR7 and TLR9 [13,14], other groups have reported that Btk is not essential for TLR4 driven inflammatory cytokine production [17]. To definitively characterize the role of BTK in TLR4-mediated immune responses, we challenged C57BL/6 (WT) or Btk-deficient mice with a lethal dose of the TLR4 ligand, LPS (8 mg/kg). Four hours post LPS challenge, systemic levels of inflammatory cytokines (IL-1β, IL-

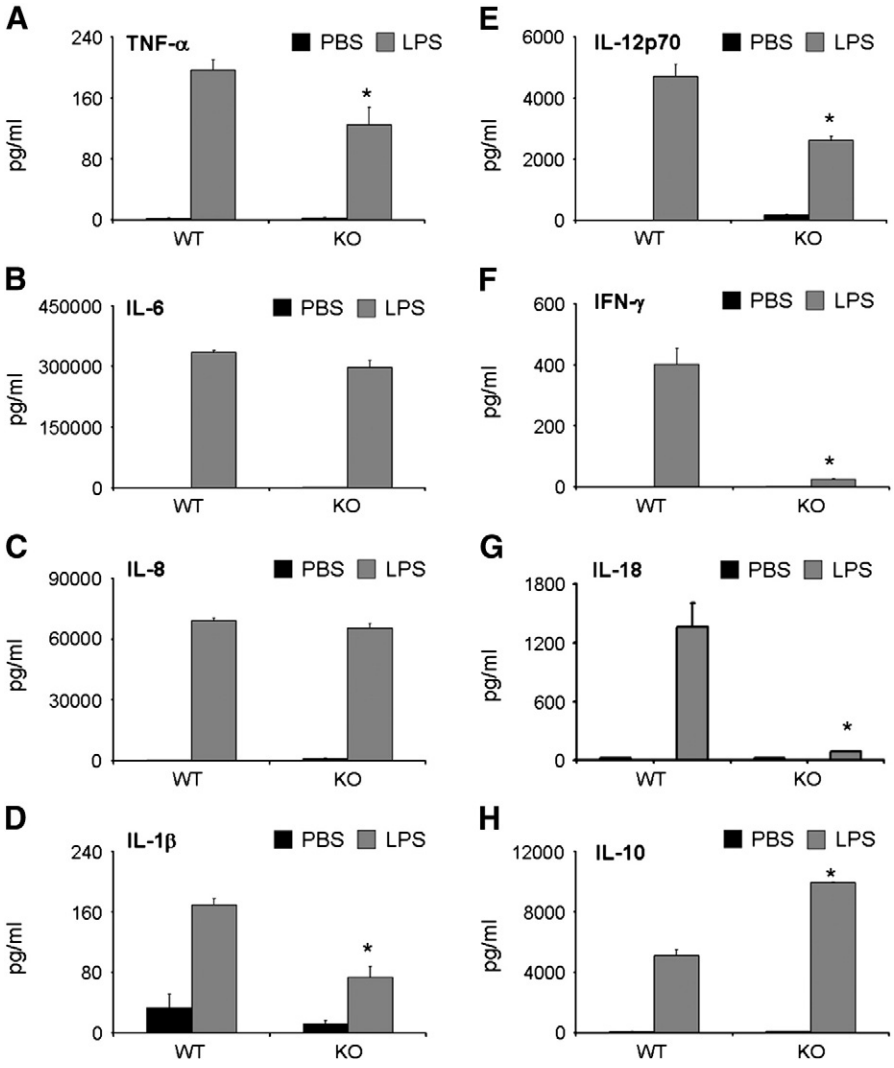

Figure 1 Proinflammatory cytokine production in mice lacking Btk. Wild-type (WT) and Btk-deficient mice were injected i.p. with 8 mg/kg LPS or saline as control and 4 h later serum was collected by tail-vein bleed and assayed for (A) TNF-α, (B) IL-6, (C) IL-8, (D) IL-1β, (E) IL-12p40, (F) IFN-γ, (H) IL-10 using Meso Scale Discovery multiplex ELISA. (G) IL-18 was assayed by standard sandwich ELISA (R&D). (n=4 for each treatment group). Bars represent mean ± SD. *$p<0.05$.

6, IL-8, IL-10, IL-12p40, IL-18, TNF-α and IFN-γ) were measured (Fig. 1). Consistent with a role for Btk as a positive regulator of TLR4 responses, LPS treatment of Btk-deficient mice resulted in reduced production of the pro-inflammatory cytokines TNF-α, and IL-1β compared to WT controls (Figs. 1A and B), whereas IL-6 and IL-8 levels were unaffected between the two groups (Figs. 1C and D). Strikingly, the ability of Btk-deficient mice to produce IL-12, IL-18, and IFN-γ in response to LPS was also severely impaired indicating that the mechanisms that control and regulate IFN-γ production may be defective in the Btk deficient mice (Figs. 1E–G). Interestingly we observed enhanced production of the key immunomodulatory cytokine IL-10 in Btk-deficient mice in response to LPS (Fig. 1H), suggesting that a skewing of cytokine responses from immunostimulatory to immunosuppressive occurs in response to LPS in the absence of Btk.

As myeloid cells are a key source of IL-12 and IL-18 and promote the production of IFN-γ from natural killer (NK) cells, we next sought to investigate the ability of APCs

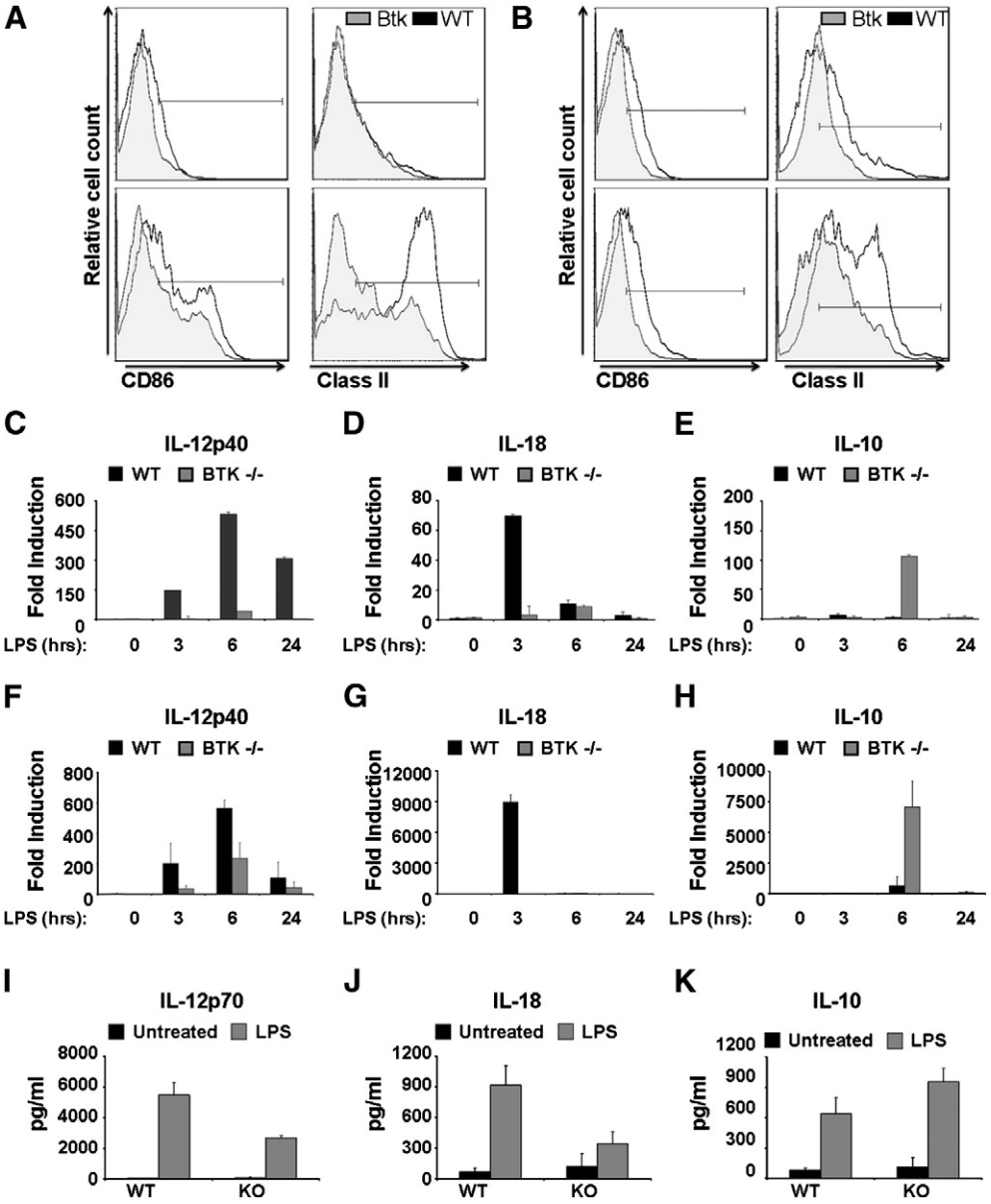

Figure 2 Impaired TLR4 responses in antigen presenting cells in the absence of Btk. (A-B) BMDMs (A) and BMDCs (B) were generated from WT and Btk-deficient mice and treated with 100 ng/ml LPS for 24 h. Activation was determined by examining expression of CD86 and MHC Class II by flow cytometry (FACSCanto; BD Biosciences, San Diego, CA) using an APC-conjugated and a FITC-conjugated antibody respectively. (C–H) WT or Btk-deficient BMDMs (C–E) or BMDCs (F–H) mice were treated with LPS (100 ng/ml) over the indicated time course. Induction of IL-12p40, IL-18 and IL-10 mRNA induction relative to baseline levels was measured over the indicated time course by real-time PCR following normalization with β-actin mRNA expression. In all cases, results shown are mean ± SD from three independent experiments. (I–K) WT or Btk-deficient BMDMs were treated with LPS (100 ng/ml) for 24 h. Supernatants were harvested and assayed for (I) IL-12p70 and (K) IL-10 using Meso Scale Discovery multiplex ELISA. (J) IL-18 was assayed by standard sandwich ELISA (R&D).

derived from Btk-deficient mice to respond to LPS and to drive IL-12 and IL-18 production (Fig. 2). Bone marrow derived macrophages (BMDM) and bone marrow derived dendritic cells (BMDC) derived from WT or Btk deficient mice were stimulated ex vivo with LPS (100 ng/ml) and the expression of activatory and co-stimulatory surface antigens was determined by flow cytometry (Figs. 2A–B). The absence of Btk resulted in impaired LPS mediated induction of the co-stimulatory molecule CD86 (37% v 53%) and reduced expression of MHC class II (49% v 74%) when compared to similarly treated WT BMDMs (Fig. 2A). Similarly we observed that BMDCs derived from Btk-deficient mice had reduced expression of CD86 (11% v 28.1%) and MHC class II (50.3% v 76.8%) following LPS exposure when compared to BMDCs derived from WT mice (Fig. 2B), indicating a global defect in APCs to become activated efficiently in the absence of Btk following TLR4 stimulation.

Having established impaired activation of Btk derived APC populations we next determined the ability of BMDMs and BMDCs to drive IL-12, IL-18 and IL-10 gene expression by real-time PCR (Figs. 2C–H). In all cases WT BMDMs and WT BMDCs (Figs. 2C–D and F–G, black bars) responded more efficiently to LPS by inducing significant increases in IL-12 and IL-18 gene expression. In contrast Btk-deficient BMDMs and BMDCs displayed virtually no increase in IL-12 or IL-18 mRNA (Figs. 2C–D and F–G, gray bars). Consistent with ELISA data from in vivo studies, induction of IL-10 mRNA was shown to be significantly increased in Btk-deficient macrophages and dendritic cells compared to WT cells (Figs. 2E and H, respectively), in keeping with our profile of an immunosuppressive cytokine milieu in the absence of Btk. This altered profile of gene induction observed in the absence of Btk was mirrored by impaired production of IL-12 and IL-18 protein and enhanced IL-10 protein production as measured by ELISA for TLR 4 treated BMDMs (Figs. 2I–K), indicating that the loss of IL-12 and IL-18 at the protein level is as a result of decrease in mRNA synthesis, rather than an inability to process and secrete the relevant cytokines

3.2. Btk-deficient APCs are unable to drive NK cell activation and IFN-γ production

The reduced ability of Btk-deficient macrophages to drive IL-12 and IL-18 production prompted us to investigate what effect loss of Btk would have on the ability of macrophages and dendritic cells to drive IFN-γ production from Natural Killer (NK) cells, a key early source of IFN-γ. Splenocytes were isolated from WT and Btk-deficient mice and were left untreated or stimulated with LPS (100 ng/ml) ex vivo for 24 h and the relative expression of the early activation marker CD69 was determined on NK cells by flow cytometry. Whereas LPS induced a 2 fold increase in CD69 expression on WT NK cells in a mixed culture experiment expression of CD69 was reduced on LPS treated NK cells from Btk-deficient mice in comparison to WT NK cells (Fig. 3A). In addition we also observed that production of IFN-γ was reduced in NK cells from Btk-deficient mice compared to WT controls (Fig. 3B). These results indicate either a possible defect in NK cell responses upon LPS exposure or that signals that drive NK cell activation by macrophages and dendritic

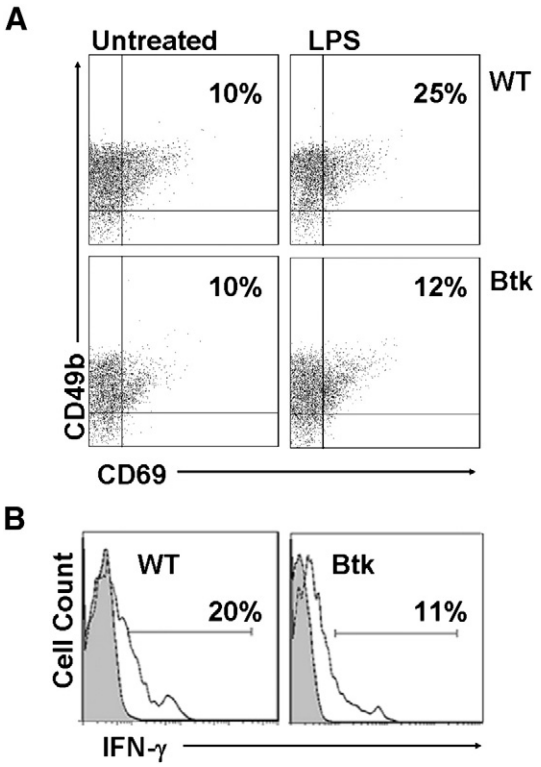

Figure 3 Absence of Btk results in impaired activation of NK cells. (A) Splenocytes were harvested from WT or Btk-deficient mice and were left untreated or were stimulated ex vivo with LPS (100 ng/ml) for 24 h. Expression of CD69 on NK cells was determined by co-staining with DX5. (B) Splenocytes were treated with LPS (100 ng/ml) for 24 h. Intracellular levels of IFN-γ were determined in NK cells by co-staining with DX5. Quadrants in dot plot graphs and histograms were defined using fluorescently conjugated isotype controls.

cells (IL-12/IL-18 and interaction with APCs) are impaired in Btk-deficient mice.

To investigate these responses in greater detail, we purified NK cells ($DX5^+$ cells) by negative selection (Miltenyi) from splenocytes and co-cultured them with bone marrow derived dendritic cells (BMDCs) from either WT or Btk-deficient mice and examined NK cell activity by examining surface expression of CD107a, which is associated with NK cell cytotoxic function [24] and by measuring IFN-γ production. Co-culture of BMDCs derived from WT mice with purified WT NK cells in the presence of LPS resulted in a fourfold increase in CD107a expression, indicating NK cells were activated (Fig. 4A). In contrast, Btk-deficient BMDCs failed to induce any significant increase in CD107a expression on NK cells derived from Btk-deficient mice (Fig. 4B). Importantly we found that whilst WT DCs had the capacity to activate Btk-deficient NK cells (Fig. 4C), leading to a comparable increase in CD107a expression observed in WT NK cells, Btk-deficient DCs failed to induce any increase in CD107a expression on WT NK cells (Fig. 4D). Similarly, WT DCs were capable of inducing IFN-γ production from WT and Btk-deficient NK cells with equal efficiency following LPS treatment (Fig. 4E), whilst Btk-deficient DCs failed to induce NK cell activation and hence IFN-γ production from either WT or Btk-deficient NK cells (Fig. 4E). Our results

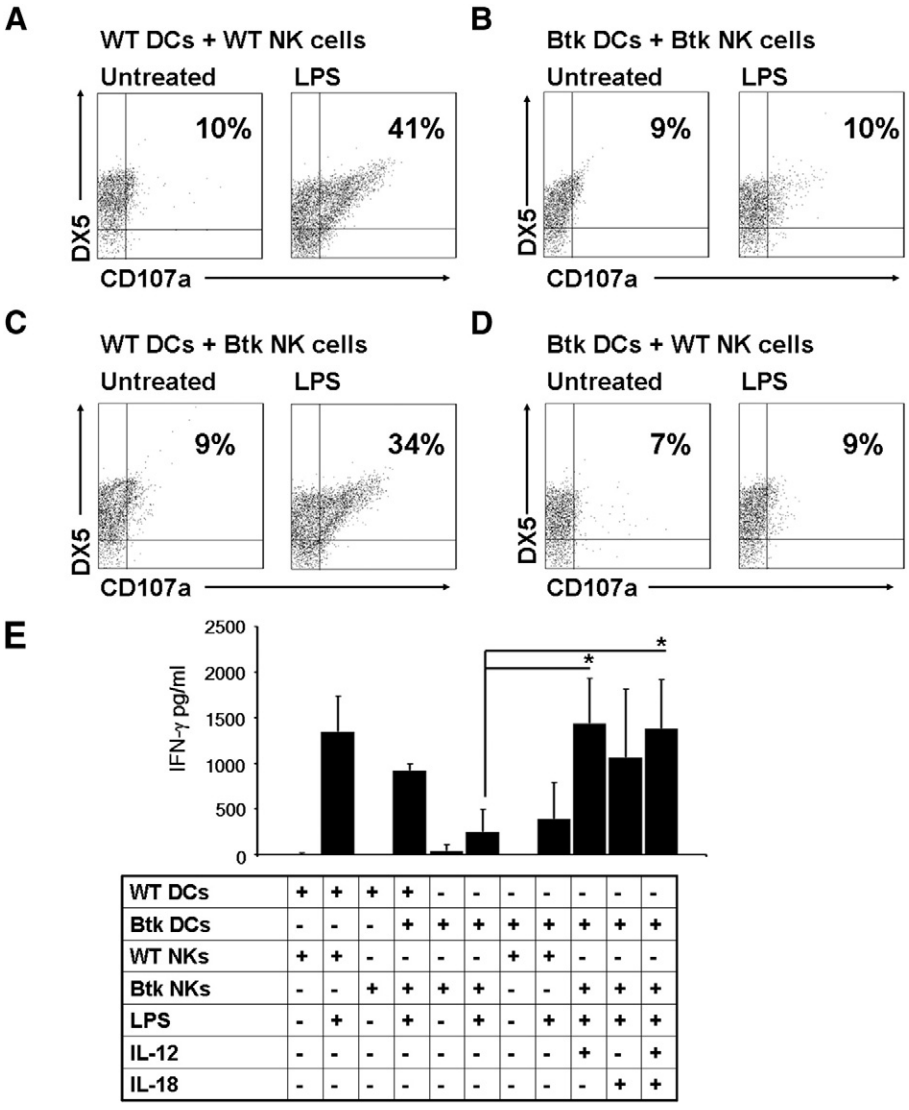

Figure 4 TLR4 mediated DC:NK cell crosstalk is impaired in the absence of Btk. (A–D) NK cells isolated from WT or Btk-deficient splenocytes were co-cultured with corresponding BMDCs (A–B) or with BMDCs from counterparts (C–D) and CD107a expression was determined by flow cytometry. Quadrants in dot plot graphs and histograms were defined using fluorescently conjugated isotype controls. (E) 1×10^5 NK cells derived from WT or Btk-deficient mice were co-cultured with 1×10^5 BMDCs derived from WT or Btk-deficient mice as indicated and left untreated or were treated with LPS (100 ng/ml). Where indicated co-culture experiments were performed in the presence of IL-12 (10 ng/ml) or IL-18 (10 ng/ml) alone or in combination. Supernatants were collected 24 h post-stimulation to determine IFN-γ production by ELISA. Results are expressed as mean ± SD for three independent experiments. $^*p<0.05$.

strongly point to the defect in IFN-γ production in response to LPS being due to a failure of Btk-deficient APCs to activate NK cells, rather than any inherent defect in the Btk-deficient NK cells to respond to activatory signals.

In keeping with the important role for DC derived IL-12 and IL-18 in the activation of NK cells, inclusion of the cytokines IL-12 or IL-18 alone or in combination rescued the ability of Btk-deficient DCs to activate Btk-deficient NKs following LPS treatment (Fig. 4E). These results support an important role for Btk in regulating APC function downstream of TLR4 and indicate that the NK cell defect observed in the absence of Btk is a result of impaired APC activation which can be restored with the exogenous addition of IL-12 or IL-18 alone or in combination. These results have major implications for our understanding of enteroviral susceptibility in the XLA patients and opens possible novel avenues for therapeutic intervention.

4. Discussion

Our current findings demonstrate that Btk is critically required by macrophages and dendritic cells to enable them to respond appropriately to TLR4 and induce expression of cytokines and cell surface receptors that contribute to NK cell activation. Our novel finding that TLR4-induced IL-12 and IL-18 production is completely defective in the absence of Btk and results in complete lack of NK cell activation and hence IFN-γ production, has important implications for our understanding of the role of Btk in anti-viral and anti-

tumor responses. Critically, although TLR-stimulated APCs are unable to induce activation of NK cells in the absence of Btk, the ability of NK cells to induce IFN-γ is restored via exogenous addition of IL-12 and IL-18 in the presence of Btk-deficient APCs, demonstrating that Btk-deficiency does not impair NK cell responses but that the defect lies at the level of APCs. This finding has important implications as IFN-γ is a key anti-viral cytokine which plays a central role in mediating innate and adaptive immune responses to pathogen.

Evidence from both in vitro and in vivo studies has clearly implicated a role for Btk downstream of TLRs and in particular downstream of the LPS receptor, TLR4. However, analysis of the role of Btk in regulating systemic responses to acute LPS administration is lacking. The results presented herein suggest that Btk is required in order to drive optimal cytokine responses to administration of the TLR4 ligand LPS. In keeping with previous studies, systemic levels of the pro-inflammatory cytokines TNF-α and IL-1β are significantly reduced compared with responses induced in wild-type (WT) mice, whereas IL-6 levels are unchanged [9,10,13-15]. Our findings also show that IL-8 levels are unchanged in response to LPS administration, supporting previous studies indicating that Btk is not required for induction of IL-8 downstream of TLR4 [15]. In light of the known role for Btk in regulating phosphorylation of the p65 subunit of the proinflammatory transcription factor NFκB downstream of TLR4 and TLR7 these results support previous findings suggesting Btk acts as a positive regulator of the early phase of TLR-driven cytokine responses [13,14]. Additionally we observe a significant increase in the production of the immunosuppressive cytokine IL-10 in Btk-deficient mice following ip LPS injection. Increased IL-10 production together with a significant reduction in IFN-γ production suggests that loss of Btk may result in altered Th_1/Th_2 balance although further studies are required to test this hypothesis.

It is evident from the available data that Btk is necessary for regulating the balance of pro-inflammatory and immunomodulatory cytokines depending on the stimulus and the cell type involved. A significant body of evidence points to a critical role for Btk in driving inflammatory responses downstream of multiple TLRs. However, several studies show no such dependence on Btk for initiating an inflammatory response [17,25,26], indicating that the role of Btk in this respect is complicated. In the present study, immediate early levels of cytokine production in response to a lethal dose of LPS have been assessed, whilst other studies reporting that Btk negatively regulates pro-inflammatory cytokines such as IL-6 [11], have administered a sub-lethal dose of LPS and examined its effects on cytokine production at later time points, potentially mimicking tolerance induction. The possibility therefore exists that Btk may have a dual function in mediating TLR signals, functioning initially as driver of inflammation whereas at later time points it may play a role in limiting the effects of TLRs, acting as a negative regulator. Interestingly studies by Kawkami et al. suggest that Btk plays a key role in autologous IL-10 production and subsequent STAT-3 activation which functions to negatively regulate APC maturation and function [27]. Additionally a dual role hypothesis is further supported by studies demonstrating that Btk phosphorylates the key TLR4 adaptor Mal, resulting in SOCS1-mediated ubiquitination and degradation of Mal and hence negative regulation of TLR4-dependent pathways [16].

Interestingly, evidence is mounting supporting a role for Btk in regulating antigen presenting cell function. Recent studies by Lui et al. have shown that Btk is required to form a signaling complex with CD40 and intracellular MHC Class II at the endosome in order to drive potent pro-inflammatory cytokine and type I IFN production downstream of TLRs [22], suggesting that Btk plays a key role at the interface between innate and adaptive immunity. Our findings that not only is macrophage and DC activation suppressed in Btk-deficient mice in response to LPS, but that their ability to produce the cytokines IL-12, and IL-18 is impaired at both the mRNA and protein level would support this hypothesis and suggests that the inability of Btk-deficient APCs to drive activation of other key cells of the innate immune response may underlie the striking inability of Btk-deficient mice to produce IFN-γ in response to LPS administration. Interestingly previous studies examining LPS treated rheumatoid fibroblast-like synoviocytes (RA FLS) have suggested that Btk may play a role in regulating IL-18 production [28]. These studies found that Btk expression was not observed in RA FLS and attributed this to the induction of miR346 which was found to repress Btk expression in these cells and thus result in impaired stability of IL-18 mRNA.

Clearance of mycobacterial and enteroviral infections relies heavily on NK cell activation and consequent IFN-γ production [29-31]. The essential role that DC-derived cytokines, in particular IL-12, IL-15 and IL-18, play in the induction of NK cell cytotoxicity and IFN-γ production is well established [1,4,32,33]. Additionally optimal NK cell activation requires NK:DC cell contact in the form of DC expressed NKp30 which promotes NK cell cytotoxic function [34]. Interestingly this relationship has been shown to be reciprocal with NK cells playing a role in DC maturation and subsequent activation of naïve T cells via production of TNF-α and IFN-γ [2,33]. Our study now helps explain the increased susceptibility to recurrent enteroviral infections observed in XLA patients [19-21].

Given that NK cell activation and IFN-γ production is severely impaired in Btk-deficient mice and that this defect is not at the level of the NK cells themselves but instead resides in an inability of Btk-deficient DCs to drive the activation of the NK cells, our work suggests that reduced NK cell activity as a result of defective APC function in XLA patients may underlie this enhanced susceptibility. The fact that activity of NK cells derived from Btk-deficient mice could be restored following co-culture of Btk-deficient NK cells with activation competent WT BMDCs or Btk-deficient BMDCs supplemented with exogenous IL-12 or IL-18 suggests that therapies aimed at augmenting NK cell activation in XLA patients, such as exogenous IL-12, IL-18 or indeed IFN-γ, may be important in treatment of such infections. In keeping with our findings, studies by Jyonouchi et al. have demonstrated that XLA patients have similar levels of NK cell activity against herpes simplex virus infected fibroblasts as healthy controls [26]. Importantly exogenous IL-12 has been shown to provide protection from enteroviral infection through increasing IFN-γ production from NK and NK T cells [28], indicating that a similar mechanism may rescue the IFN-γ defect in the Btk-deficient mice observed in this

study. Interestingly, induction of IL-10 during coxsackie virus infection is associated with delayed induction of innate and adaptive responses [35]. Given that we observed enhanced IL-10 production in APCs derived from Btk-deficient mice in response to LPS administration, one might speculate that the shift of balance towards an immunosuppressive environment in the absence of Btk may in part explain the enhanced susceptibility to recurrent bacterial and enteroviral infections [20] observed in the XLA patients.

Overall our findings clearly demonstrate that Btk is functioning at an important interface between innate immune cells such that NK cell activation is impaired in the absence of Btk, as a result of reduced APC derived IL-18 and IL-12. These findings have important therapeutic implications, as mechanisms that promote DC-NK cell crosstalk could potentially reduce the incidence and severity of enteroviral infection in these individuals.

Conflict of interest statement

The authors declare that there are no competing interests.

Acknowledgments

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