Defective Toll-like receptor 9-mediated cytokine production in B cells from Bruton's tyrosine kinase-deficient mice

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Summary

Bruton's tyrosine kinase (Btk), a member of the Tec family of tyrosine kinases, plays an important role in the differentiation and activation of B cells. Mutations affecting Btk cause immunodeficiency in both humans and mice. In this study we set out to investigate the potential role of Btk in Toll-like receptor 9 (TLR9) activation and the production of pro-inflammatory cytokines such as interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-12p40. Our data show that Btk-deficient B cells respond more efficiently to CpG-DNA stimulation, producing significantly higher levels of pro-inflammatory cytokines but lower levels of the inhibitory cytokine IL-10. The quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis presented in this work shows that mRNA production of one of the important new members of the IL-12 family, IL-27, was significantly increased in Btk-deficient B cells after CpG-DNA stimulation. In this study, we demonstrate significant differences in CpG responsiveness between transitional 1 (T1) and T2 B cells for survival and maturation. Furthermore, TLR9 expression, measured both as protein and as mRNA, was increased in Btk-defective cells, especially after TLR9 stimulation. Collectively, these data provide evidence in support of the theory that Btk regulates both TLR9 activation and expression in mouse splenic B cells.

Keywords: Bruton's tyrosine kinase; B cells; Toll-like receptor 9; cytokines; inflammation

Introduction

Bruton's tyrosine kinase (Btk) is a cytoplasmic protein tyrosine kinase that is essential for B-cell development.[1–3] Btk belongs to the Tec kinase subfamily of Src-like enzymes together with the homologues Tec, Itk, Bmx and Txk.[3] These kinases are characterized by the presence of an N-terminal pleckstrin homology (PH) domain followed by a Tec homology, a Src-homology 3 (SH3), an SH2 and a C-terminal kinase (SH1) domain. Mutations in the human *BTK* gene lead to X-linked agammaglobulinaemia (XLA), a primary immunodeficiency characterized by the absence of B lymphocytes with subsequent susceptibility to pyogenic bacterial infections and enteroviral disease.[4–7] A PH domain missense mutation in the corresponding mouse gene is responsible for X-linked immunodeficiency (*Xid*) with a milder phenotype.[8,9] Btk is predominantly expressed in B lymphocytes, myeloid cells and platelets, but not in T cells or terminally differentiated plasma cells.[10,11] Recent studies have described the involvement of Btk in Toll-like receptor (TLR) signalling.[12–15] TLRs recognize microbial products and initiate a complex immune response designed to eliminate invading pathogens.[16] So far, 11 different murine TLRs have been described, which are variably expressed in many different cell types such as macrophages, neutrophils, dendritic cells (DCs), lymphocytes, and endothelial cells.[17] Stimulation of these receptors by pathogen-associated molecular pattern antigens leads to the activation of common and specific intracellular signalling pathways resulting in nuclear factor-κB (NF-κB) translocation, cell activation and differentiation, and secretion of cytokines and chemokines.[18,19]

TLR9 is specialized for the recognition of microbial nucleic acids. One factor enabling TLR9 to respond to prokaryotic DNA and discriminate between self and

foreign DNA is the presence of unmethylated CpG dinucleotides.[20,21] The immune stimulation mediated by bacterial DNA can be mimicked, at least in part, by short single-stranded synthetic oligodeoxynucleotides (ODNs).[22–24] Recently it has been reported that TLR9 delivers an important signal for activation of human naïve B cells inducing proliferation, isotype switching and differentiation to immunoglobulin-secreting cells.[25]

Whether Btk participates in TLR9 signalling is not known. In the present study, we found that Btk-deficient B cells expressed higher levels of TLR9 when compared with normal cells. Activation of TLR9 signalling induced a strong pro-inflammatory cytokine response in Btk-deficient cells as well as decreased secretion of the immunoregulatory cytokine IL-10. Interestingly, CpG-DNA stimulation induced a time-dependent accumulation of transitional 2 (T2) B cells in both Btk-deficient and normal B cells. These results suggest that Btk is a critical molecule in regulating TLR9 activation in splenic B cells. In addition, CpG also regulates B-cell differentiation independently of Btk.

Materials and methods

ODN and lipopolysaccharide (LPS)

Synthetic, endotoxin-free, completely phosphorothioate-modified oligonucleotides (S-ODN) were supplied by DNA Technology (Aarhus C, Denmark) and used at a final concentration of 5 μg/ml. The sequences of S-ODN used were 5′-GACGTTTTGACGTT TTGACGTTGTTG GTGGTGGTG-3′ (CpG-DNA) and 5′-GAAGTTTTGAGG TTTTGAAGTT GTTGGTGGTGGTG-3′ (non-CpG-DNA). *Escherichia coli* LPS (O111:B4) was purchased from Sigma-Aldrich (St Louis, MO) and the concentration used was 1 μg/ml.

Mouse strains and preparation of splenic mature B cells

Xid/CBA mice (6–8 weeks old) were obtained from Charles River Laboratories (Kungsbacka, Sweden). $Btk^{-/-}$/CBA mice were created by back-crossing $Btk^{-/-}$/SW129 mice as described previously.[26] $Btk^{-/-}$ (C57BL/6 background) mice were provided by Johan Forssell (Department of Cell and Molecular Biology, Division of Tumour Biology, Umeå University, Umeå, Sweden). Wild-type (WT) CBA and C57BL/6 mice were used as controls. Splenic B cells from all strains were enriched essentially as described previously.[27] Briefly, mouse splenic B cells were enriched using a high-gradient magnetic separation column (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The spleen cells, which had been stained with anti-B220-Ab coupled micromagnetic particles (Miltenyi Biotec), were retained on the separation column under a high-gradient magnetic field; these cells were subsequently eluted from the column. The purity of the cells was examined by flow cytometric analysis following staining with phycoerythrin (PE)-conjugated rat anti-mouse anti-CD19 antibodies. The purity of the cells was > 95%.

Cytokine detection

A total of 3×10^6 purified B cells/well (in 1 ml of medium) were stimulated for 24 hr with 5 μg/ml CpG-DNA, 5 μg/ml non-CpG-DNA or 1 μg/ml LPS. For all assays, cells were cultured in RPMI-1640 supplemented with 10% (volume/volume) heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES and 50 μM 2-mercaptoethanol (2-ME). Amounts of cytokines in the supernatants were assessed according to the manufacturer's recommendations using commercially available enzyme-linked immunosorbent assay (ELISA) kits [for interleukin (IL)-6 and tumour necrosis factor (TNF)-α] and sets (for IL-12p40 and IL-10) from BD Biosciences (San Diego, CA).

Fluorescence-activated cell sorter (FACS) analysis

Intracellular expression of TLR9 was detected by flow cytometry using fluorescein isothiocyanate (FITC) anti-mouse TLR9 (clone M9.D6 from eBioscience, San Diego, CA), gated on B cells using R-phychoerythrin (R-PE) anti-CD19 (BD Biosciences). Cells were fixed and permeabilized according to the manufacturer's procedure using Fix & Perm reagents (Caltag Laboratories, Vienna, Austria). Rat immunoglobulin G2a (IgG2a)-FITC and rat IgG2b-PE were used as isotype controls (BD Biosciences).

Identification of T1, T2 and mature B-cell subpopulations was based on a previous report.[28] Briefly, 1×10^6 cells were stained with anti-B220 PECy5 (BD Pharmingen, Palo Alto, CA), anti-CD21 (eBioscience) and anti-CD24 PE (BD Pharmingen). Cells were incubated for 15 min with these antibodies and washed with phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS); anti-rat IgG allophyocyanin (APC) (Caltag Laboratories) was used as a secondary antibody for anti-CD21. Finally, cells were fixed with PBS containing 1% formalin. Cell acquisition was performed in a FACS DIVa (BD Biosciences). For all samples, 100 000 events were computed and analysed for T1, T2 and mature B-cell subpopulations in WinMdi 2·8 software (Scripps Research Institute, http://facs.scripps.edu/software.html).

Cell proliferation

Cell proliferation was determined using the WST-1 assay (Roche, Mannheim, Germany), which measures the metabolic activity of the cells. Briefly, 2×10^5 cells/well were incubated with CpG-DNA (5 μg/ml), non-CpG-DNA

(5 μg/ml) or LPS (1 μg/ml) for 0, 3, 6, 24, 48, 72, or 96 hr. The cell proliferation reagent WST-1 (20 μl/well) was added to the cells which were cultured in 200 μl of medium.

RNA isolation, quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis and oligonucleotide array hybridization

Total RNA from splenic B cells was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA (100 ng) was reverse-transcribed into cDNA with avian myeloblastosis virus (AMV) reverse transcriptase using random hexamer primers (Roche). 18S rRNA was used as an endogenous control. Primers and probes for mouse TLR9, IL-23 and IL-27 were purchased as predeveloped TaqMan assays (Assays-on-Demand™; Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed as previously described.[26] The Affymetrix small sample protocol (Affymetrix UK Ltd, High Wycombe, UK), consisting of two rounds of amplification, was used to obtain a larger amount of cRNA. cRNA (100 ng) was hybridized to MOE430 2·0 GeneChips® (Affymetrix UK Ltd); one array per sample was run. The cRNA synthesis and hybridizations were performed in the BEA core facility at the Department of Biosciences and Nutrition (www.ki.se/bea), Karolinska Institute, Novum, Huddinge, Sweden.

Results

CpG-DNA induces an augmented pro-inflammatory cytokine response in Btk-deficient splenic B cells

We investigated the pro-inflammatory cytokine response in WT and Btk$^{-/-}$/C57BL6 mice by stimulating purified splenic B cells with CpG-DNA. We measured the concentrations of IL-6, IL-12p40 and TNF-α in the cultured supernatants by ELISA. B cells from Btk$^{-/-}$ mice revealed a significantly increased production of cytokines with an inflammatory profile when compared with normal mice (Figs 1a–c). For further confirmation,

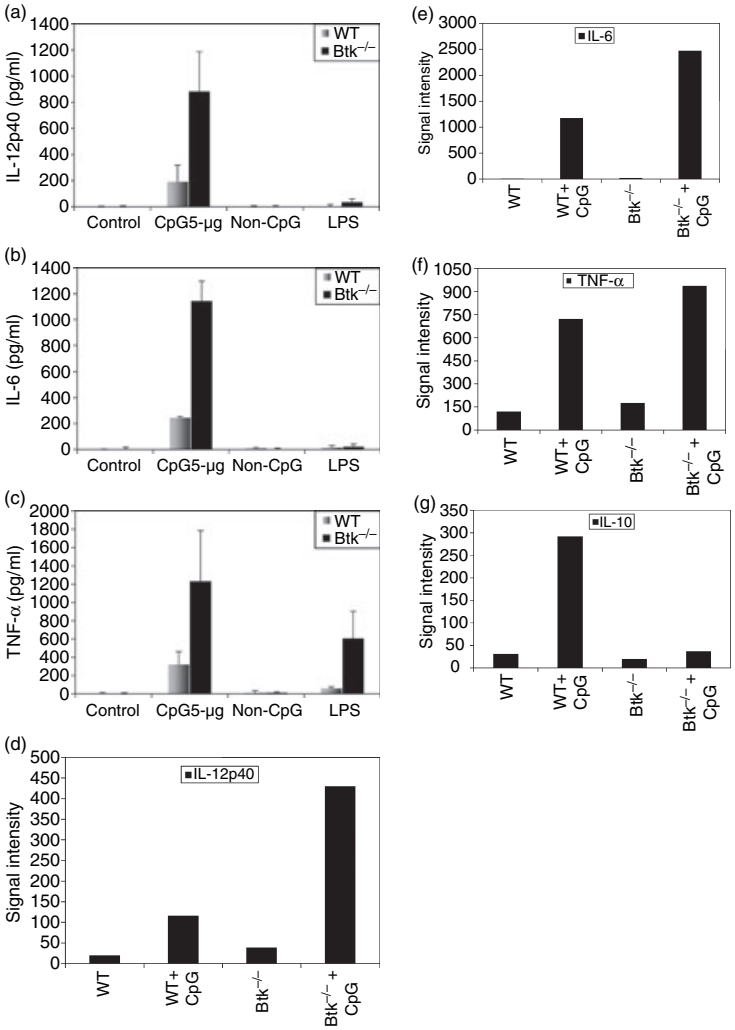

Figure 1. Pro-inflammatory cytokine production and microarray analysis for C57BL/6 wild-type (WT) and Bruton's tyrosine kinase-deficient (Btk$^{-/-}$) splenic B cells. (a) Interleukin (IL)-12p40, (b) IL-6 and (c) tumour necrosis factor (TNF)-α production. Purified B220^{+} cells were stimulated with CpG-DNA, non-CpG-DNA or lipopolysaccharide (LPS) as described in the 'Materials and methods'. Supernatants were collected after 24 hr of stimulation and assayed for cytokines using an enzyme-linked immunosorbent assay (ELISA). Data represent the average of three experiments including standard deviations. (d–g) Microarray analysis of CpG-DNA-stimulated and unstimulated splenic B cells. After 6 hr of stimulation, the pro-inflammatory cytokine gene products IL-12p40 (d), IL-6 (e) and TNF-α (f) were significantly up-regulated. Gene expression of the inhibitory cytokine IL-10 (g) was down-regulated in CpG-DNA-stimulated Btk$^{-/-}$ B cells.

these experiments were also carried out using *Xid* and Btk$^{-/-}$/CBA mice. Comparisons between Btk$^{-/-}$/CBA and WT/CBA mice showed a similar pattern to that found in C57BL/6 mice with CpG-DNA stimulation (data not shown). Taken together, these data indicate that Btk-deficient cells are more prone to produce inflammatory cytokines following TLR9 activation than WT cells.

Increased IL-27 mRNA in Btk$^{-/-}$ B cells following TLR9 activation

The IL-12 family is composed of three heterodimeric cytokines, IL-12 (p40p35), IL-23 (p40p19) and IL-27 (EBI3p28), and of monomeric and homodimeric p40.[29] Free p40 can form homodimers or be present as free monomers in mouse but not human cells.[30] It was previously reported that IL-23 plays an important role in autoimmune inflammation, inducing IL-17 production in the activated/effector CD4$^+$ T-cell population. In contrast, IL-27 has a role in limiting the intensity and duration of the adaptive immune responses.[31] Additionally, it has been reported that IL-27 modulates human B-cell differentiation and proliferation depending on the mode of cell activation.[32] We investigated the possibility that CpG-DNA might have an effect on the new members of the IL-12 family. Using quantitative RT-PCR analysis, we found that IL-27p28 mRNA production was significantly increased in Btk-deficient cells after CpG-DNA stimulation (Fig. 2). In contrast, IL-23p19 as well as IL-12p35 mRNA production was not affected, which was also confirmed by ELISA (data not shown).

Functional Btk is required for IL-10 production in B cells after CpG-DNA stimulation

IL-10 is an immunosuppressive cytokine that limits and terminates inflammatory responses. In addition to these activities, IL-10 regulates growth and/or differentiation of B cells, natural killer (NK) cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells (DCs), keratinocytes, and endothelial cells.[33] The importance of Btk in IL-10 production by DCs and bone marrow-derived macrophages upon LPS stimulation has recently been demonstrated.[34] We sought to investigate the effect of functional Btk on IL-10 production by B cells upon TLR9 activation. Experiments were performed in *Xid* and Btk$^{-/-}$/CBA murine splenic B cells. Importantly, we found that IL-10 production was dramatically reduced in Btk$^{-/-}$ B cells but moderately reduced in *Xid* B cells upon TLR9 activation when compared with normal cells (Fig. 3a). Similar results were obtained using Btk$^{-/-}$/C57BL/6 mice, further confirming our results (data not shown).

The reduction in IL-10 levels in the CpG-stimulated Btk$^{-/-}$ cells might be one of the mechanisms inducing increased pro-inflammatory cytokine production. We were interested in evaluating IL-12p40 production in IL-10-pretreated cells. Interestingly, we observed an abrogative effect of IL-12p40 production in the Btk$^{-/-}$ cells (Fig. 3b).

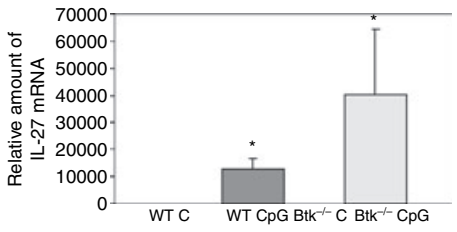

Figure 2. CpG-DNA-mediated induction of interleukin (IL)-27p28 mRNA in wild-type (WT) and Bruton's tyrosine kinase-deficient (Btk$^{-/-}$) splenic B cells. Purified B220$^+$ splenic cells were cultured with (CpG) or without (C, control) CpG-DNA. After 24 hr of stimulation, total mRNA was extracted and assayed for IL-27 transcripts by quantitative reverse transcription–polymerase chain reaction (RT-PCR). 18S rRNA was used as an endogenous control. Unstimulated WT or Btk$^{-/-}$ cells produced undetectable levels of IL-27. Data represent the average of three experiments including standard deviations. *P*-values were calculated using Student's *t*-test. $^*P < 0{\cdot}05$.

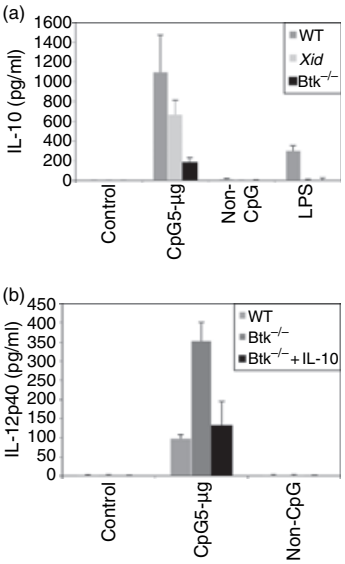

Figure 3. (a) Interleukin (IL)-10 production by wild-type (WT), X-linked immunodeficient (*Xid*) and Bruton's tyrosine kinase-deficient (Btk$^{-/-}$) splenic B cells after Toll-like receptor 9 (TLR9) activation. Purified B220$^+$ splenic cells were incubated with or without CpG-DNA, non-CpG-DNA or lipopolysaccharide (LPS). Twenty-four hours later, supernatants were collected and assayed for IL-10 production by enzyme-linked immunosorbent assay (ELISA). Data represent the average of three experiments including standard deviations. (b) Effect of recombinant IL-10 on production of the pro-inflammatory cytokine IL-2p40. Purified B cells ($1{\cdot}5 \times 10^6$) from C57BL/6 WT and Btk$^{-/-}$ mice were preincubated with IL-10 (2 ng/ml) for 1 hr. CpG or non-CpG-DNA was added, and supernatants were collected and assayed for IL-12p40 production by ELISA after 24 hr of incubation. Data represent the average of two experiments including standard deviations.

CpG-DNA induces up-regulation of TLR9 expression in WT and Btk$^{-/-}$ mouse splenic B cells

The TLR9 family (TLR7, 8 and 9) is predominantly confined to intracellular compartments.[35] We observed that CpG-DNA induced significant increases in TLR9 expression levels in both normal (36%) and Btk$^{-/-}$ (82%) splenic B cells (C57BL/6 background) (Fig. 4a), in comparison to corresponding unstimulated cells 3·1% (normal cells) versus 11% (Btk$^{-/-}$). The non-CpG-DNA control did not show changes in TLR9 expression levels. These data are consistent with recent studies showing an increase in TLR9 expression level in human B cells after B-cell receptor (BCR) or CpG-DNA stimulation.[36,37] All experiments were repeated in the Btk$^{-/-}$ and normal mice on the CBA background with similar results (data not shown). In contrast, it has been demonstrated previously that CpG-DNA reduces TLR9 mRNA expression levels in the human myeloma cell line RPMI 8226 and in human PBMC.[38,39]

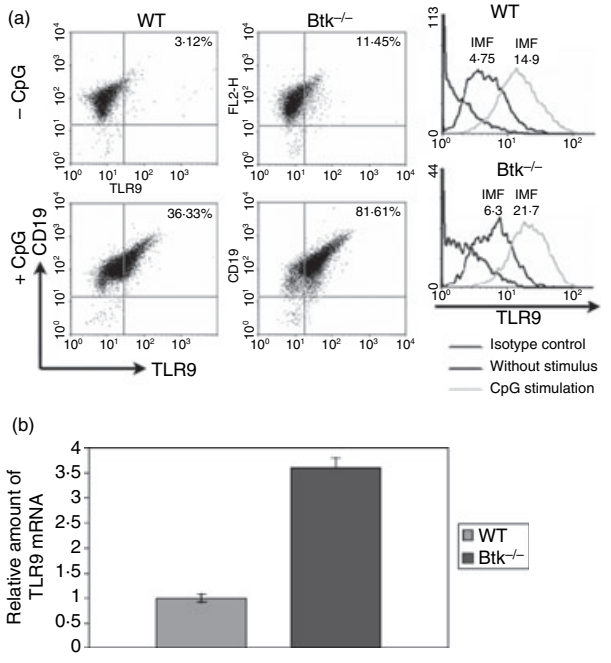

Figure 4. Toll-like receptor 9 (TLR9) expression in C57BL/6 wild-type (WT) and Bruton's tyrosine kinase-deficient (Btk$^{-/-}$) splenic B cells. Splenic cells from WT and Btk$^{-/-}$ mice were obtained and the B-cell fraction was magnetically purified using anti-B220 antibodies. (a) Fluorescence-activated cell sorter (FACS) analysis of splenic B220^{+} cells stained with anti-CD19 and anti-TLR9 antibodies showing the expression pattern under steady-state conditions (upper panel) and after 24 hr of CpG-DNA stimulation (lower panel). Histograms indicating the mean fluorescence intensity (MFI) with the respective isotype controls are shown in the right panel. (b) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of TLR9 mRNA expression in purified splenic B cells. Data represent the average of three experiments including standard deviations. RNA extraction and cell staining for FACS analysis were performed as described in the 'Materials and methods'.

Purified splenic B cells from both Btk$^{-/-}$ mice (C57BL/6 background) and WT mice were also examined to determine TLR9 mRNA levels under steady-state conditions. Quantitative RT-PCR analysis (Fig. 4b) showed that the TLR9 mRNA level was up-regulated in Btk$^{-/-}$ cells, which is comparable to our FACS data.

CpG-DNA stimulation favours the T2 B-cell population

The immature, or transitional, splenic B cells are subdivided into two subsets, T1 and T2 cells. These subsets of cells express distinct surface markers and are located in different microenvironments in the spleen. Based on the expression of surface markers T1 (CD21^{low} CD24^{high}) and T2 (CD21^{high} CD24^{high}), cells can be distinguished from mature B cells (CD21^{low} CD24^{low}).[28] In this study, we evaluated the TLR9 signalling capacities of the T1 and T2 B-cell subsets. In response to CpG-DNA, T2 cells accumulated and proliferated during the first 72 hr, becoming mature B cells after 96 hr of stimulation (Fig. 5a). In contrast, T1 cells did not proliferate and died after CpG-DNA stimulation. Similarly, it was noted that T2 cells survived and proliferated whereas T1 cells died upon BCR stimulation in normal WT cells.[40] To determine whether the T1 population was differentiating into T2 cells following TLR9 activation, we isolated B cells by FACS from newborn mice (7 days old) lacking both T2 and mature B cells. Re-analysis of the sorted samples revealed a sorting purity of 95%. Cells were cultured with CpG-DNA and evaluated every 24 hr for T1, T2 and mature phenotypes using CD21 and CD24 markers. In this setting also, immature T1 cells did not proliferate and died after 24 hr (data not shown).

We also sought to determine whether T1, T2 and mature cells expressed different levels of TLR9 in both normal and Btk$^{-/-}$ B cells. We observed that TLR9 expression differed between T1 and T2 cells (Fig. 5b) in both mice in steady-state conditions. Furthermore, TLR9 expression was considerably augmented in the T2 cell population upon CpG-DNA stimulation (Fig. 5c).

TLR9 activation induces Btk$^{-/-}$ B-cell survival and proliferation

Btk-deficient B cells die rapidly *ex vivo*, and their survival is contingent upon rescue from programmed cell death by signals from the environment. It has been shown that CpG-DNA prolongs survival of different cell types such as eosinophils and neutrophils.[41,42] Here we investigated the survival and proliferation of splenic

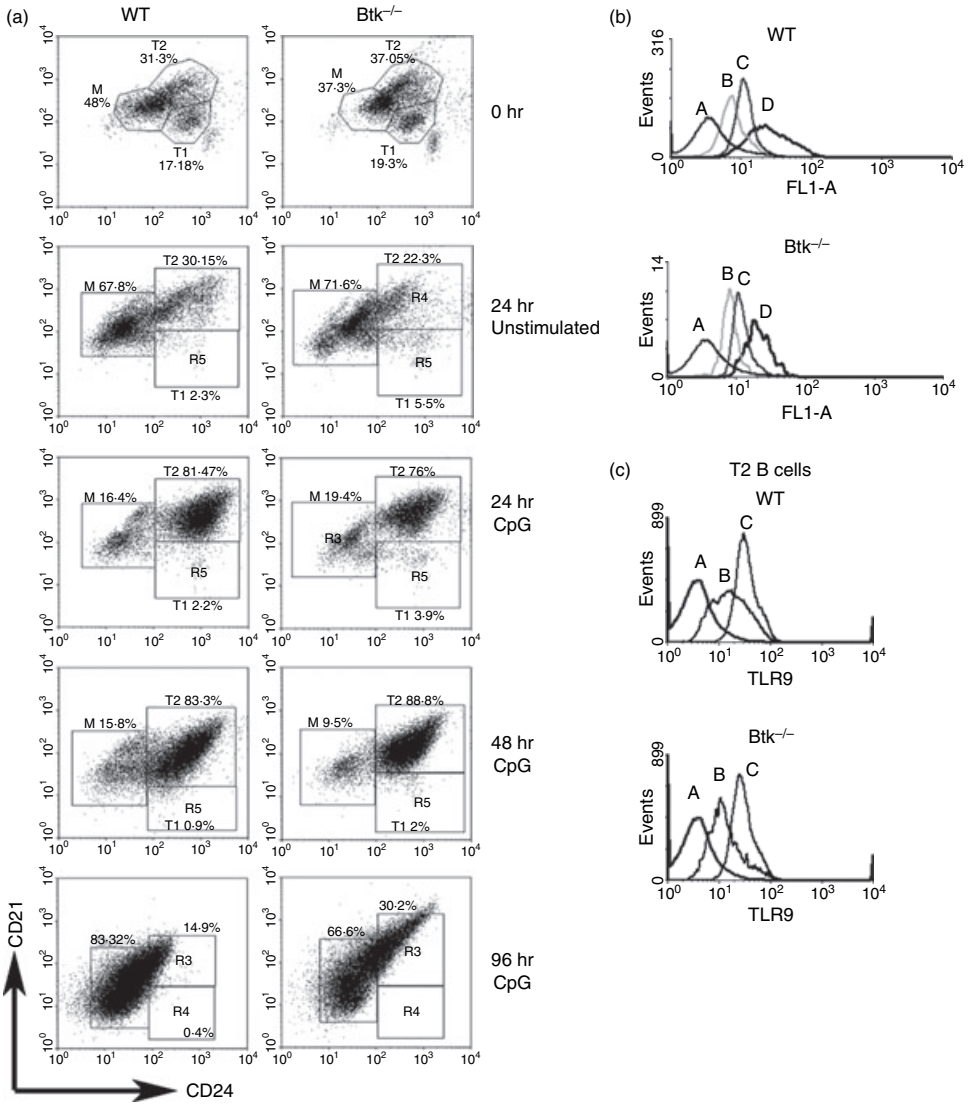

Figure 5. (a) Effect of CpG-DNA on different subsets of splenic B cells and their differential Toll-like receptor 9 (TLR9) expression in wild-type (WT) and Bruton's tyrosine kinase-deficient ($Btk^{-/-}$) B cells. WT and $Btk^{-/-}$ splenic B cells were cultured with or without CpG-DNA for different times. Transitional 1 (T1), T2 and mature B cells were identified and analysed by flow cytometry. One representative experiment of three is shown. (b) T1 TLR9 expression levels differ from those of the T2 cell population under steady-state conditions. A, isotype; B, mature B cells; C, T1 B cells; D, T2 B cells. (c) TLR9 expression levels in the T2 B-cell population after 24 hr of CpG-DNA stimulation. A, isotype control; B, control 24 hr; C, CpG 24 hr.

$Btk^{-/-}$ and WT B cells upon stimulation by CpG-DNA and LPS. Our results showed that $Btk^{-/-}$ B cells continued to proliferate until 72 hr after TLR9 activation (Figs 6a and b). Survival of $Btk^{-/-}$ B cells was found to be increased from 20% (untreated) to 80% when cells were treated with CpG-DNA. The same pattern was also observed in the WT cells, but it was less dramatic (an increase from 55 to 90%) (Fig. 7). All these results are consistent with our RT-PCR data, where we saw up-regulation of the antiapoptotic gene B-cell leukemia/lymphoma 2 gene (*Bcl-2*) and the gene encoding the cell cycle regulator cyclin D2 after TLR9 activation (Figs 8a and c).

Gene expression profile in CpG-DNA-stimulated splenic B cells

To extend and confirm our previous results, we used Affymetrix oligonucleotide arrays complementary to around 39 000 transcripts. A selective gene list with differentially regulated transcripts between the $Btk^{-/-}$ and control mouse groups (both unstimulated and CpG-DNA-stimulated) was generated using the dChip software (http://biosun1.harvard.edu/complab/dchip/, manuscript in preparation). There was a good correlation between the cytokine production and gene expression data for all genes analysed (*IL-12p40*, *IL-6*, *TNF-α* and *IL-10*)

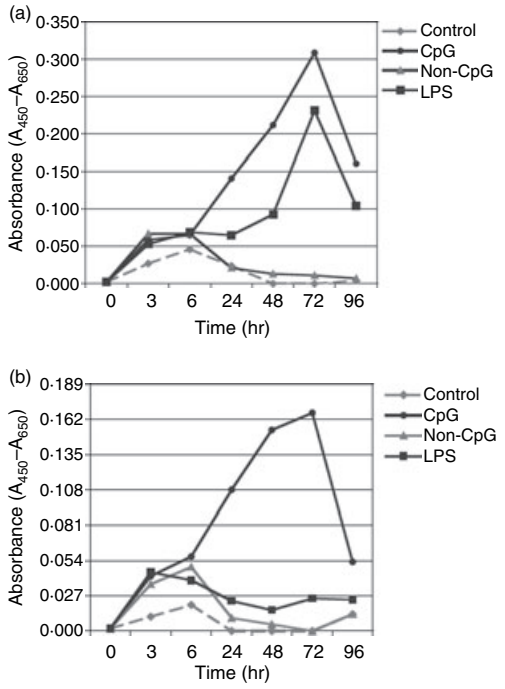

Figure 6. CpG-DNA-dependent proliferation in wild-type (WT) and Bruton's tyrosine kinase-deficient ($Btk^{-/-}$) splenic B cells. Purified $B220^{+}$ cells (2×10^5) were cultured with CpG-DNA, lipopolysaccharide (LPS) or non-CpG-DNA and assayed at different time-points. Proliferation in WT (a) and $Btk^{-/-}$ (b) purified B cells was determined using the WST-1 assay kit.

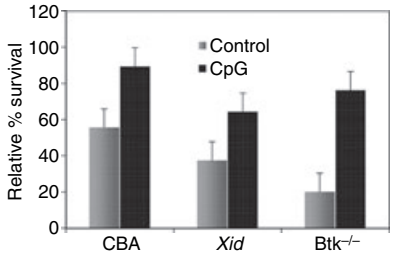

Figure 7. Splenic B-cell survival after CpG-DNA stimulation in wild-type (WT), X-linked immunodeficient (*Xid*) and Bruton's tyrosine kinase-deficient ($Btk^{-/-}$) cells. Purified B cells (1×10^6) were cultured and propidium iodide incorporation was measured after 24 hr. Results show the relative survival of B cells in culture with CpG-DNA (black bars) or without CpG-DNA (grey bars). Data represent the average of three experiments including standard deviations.

(Figs 1d–g). Furthermore, analysis of other genes such as the B-cell regulator gene *Bcl-2* and the cell cycle regulator gene cyclin D2 (Figs 8b and d, respectively) strongly indicates that there is a significant CpG-DNA-dependent increase in both WT and $Btk^{-/-}$ B cells. These results are consistent with the effect of TLR9 activation on B-cell proliferation, survival and/or differentiation.

Discussion

Numerous studies have shown that the innate immune system has a high degree of specificity and that it is highly developed in its ability to discriminate between self and foreign pathogens.[43] This recognition relies to a great extent on a family of evolutionarily conserved germline-encoded receptors known as TLRs. The innate immune system stimulated via TLRs activates the adaptive immune system through the production of pro-inflammatory cytokines such as IL-6, TNF-α and IL-12.[16] Cytokines, however, induce pronounced positive feedback in the immune system, which, if left uncontrolled, can cause severe immunopathologies. The mechanism by which innate immunity is held in check is largely unknown.

It has been reported that Btk is a component of TLR signalling and therefore might play an important role in the function of immunocompetent cells of innate as well as adaptive immunity.[12–15,44] In particular, it has been shown that Btk interacts with the conserved cytosolic domain termed the Toll/interleukin-1 receptor (TIR) of TLRs 4, 6, 8 and 9, and was also found to specifically associate with MyD88, Mal and IRAK1.[15,45] There is growing evidence that TLRs play a crucial role in B-cell physiology. Importantly, TLR4 favours B-lymphocyte maturation, whereas TLR2 arrests or retards that process.[46] Furthermore, TLR9 stimulation plays an essential role in induction of isotypic switch and B-cell terminal differentiation.[25] Stimulation of the human monocytic cell line THP-1 with LPS resulted in an increase in tyrosine phosphorylation of Btk.[15] Recently, it has been reported that Btk induces the phosphorylation of p65 on serine 536, thereby promoting transactivation by NF-κB in response to LPS.[44] Involvement of Btk in TLR2 signalling, where a TLR2 agonist (PAM3-Cys) induces increased phosphorylation of Btk, has also been shown.[12] Taken together, these data support the notion that Btk provides a common signalling mechanism for TLR2 and TLR4. The exact relationship between Btk and the different TLRs remains largely unexplored and uncertain. The molecular mechanisms leading to the activation of Btk via TLR triggering as well as its downstream signal transduction pathways remain to be determined.

Little is known about the effect of Btk on B-cell physiology after TLR9 activation. In the present study we have shown that Btk has a critical role in TLR9 signalling in murine splenic B cells. Consistent with this function, purified B cells from $Btk^{-/-}$ mice showed increased pro-inflammatory cytokine production when stimulated with CpG-DNA. This exacerbated response of $Btk^{-/-}$ cells is likely to result from enhanced TLR signalling. It has been reported that TLR9 activation by CpG-DNA either up-regulates or down-regulates TLR9 mRNA.[37–39,47] This is still a matter of controversy, and the discrepancy may be species dependent. It has been shown that there are

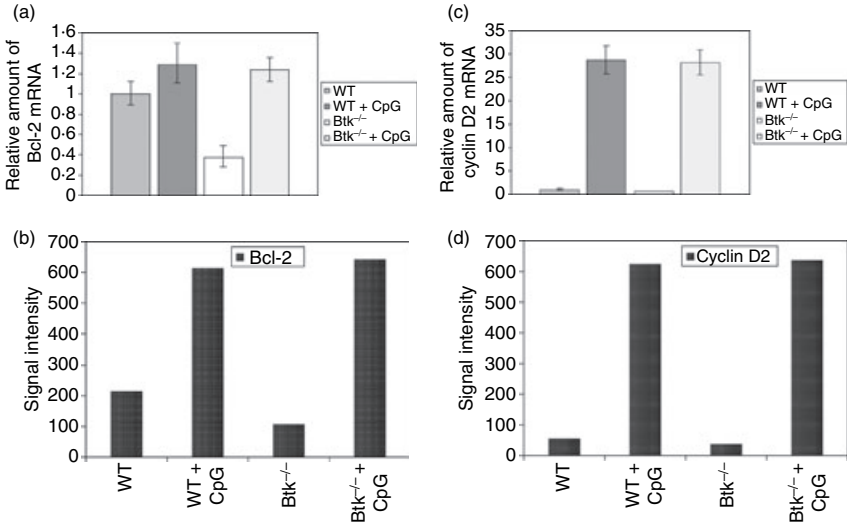

Figure 8. B-cell leukemia/lymphoma 2 gene (*Bcl-2*) and cyclin D2 gene expression analysis. After 24 hr of stimulation, total mRNA was extracted and assayed for (a) *Bcl-2* and (c) cyclin D2 transcripts by quantitative reverse transcription–polymerase chain reaction (RT-PCR). 18S rRNA was used as an endogenous control. Data represent the average of three experiments including standard deviations. Microarray analysis was performed after 6 hr of stimulation. (b) *Bcl-2* and (d) cyclin D2 transcripts were increased in both wild-type (WT) and Bruton's tyrosine kinase-deficient ($Btk^{-/-}$) B cells after CpG-DNA stimulation.

species-specific differences in the optimal CpG motif, with GACGTT being optimal for mice and GTCGTT for humans.[23,48,49] Our results showed that the TLR9 expression level was higher in $Btk^{-/-}$ murine splenic B cells than in WT, and in both cell types CpG-DNA up-regulated TLR9 expression. In agreement with this result, we found in the microarray analysis that stimulation of TLR9 up-regulated CCAAT/enhancer binding protein (C/EBP), a transcription factor that has been reported previously to interact with the TLR9 promoter.[37–39,47] It has also been reported that IL-10 does appear to increase the expression of human B-cell TLR9.[50] This might not be the underlying mechanism under our assay conditions. Thus, $Btk^{-/-}$ B cells showed a substantial decrease in the production of this immunosuppressive cytokine, suggesting the involvement of environmental or other cellular factors, such as pro-inflammatory cytokines. While this work was in progress, an article on common variable immunodeficiency (CVID) was published reporting TLR9 activation defects in this immune deficiency disorder.[51] This study and ours suggest that TLR9 dysregulation may play an important role in primary immunodeficiencies involving B cells.

The role of TLR signalling in B-cell development is poorly understood. In this report, we set out to determine whether T1, T2 and mature B cells express different levels of TLR9 in both normal and $Btk^{-/-}$ cells. Furthermore, we explored the ability of T1 and T2 cells to respond to CpG-DNA. Our results indicate that TLR9 expression was augmented in the T2 population after CpG-DNA stimulation. In response to CpG-DNA, T2 cells started to accumulate, probably as a result of cell proliferation, becoming mature after 3 days of stimulation, whereas T1 cells did not respond and died. Similar results have been previously reported in response to BCR engagement in normal T2 B cells, whereas T2 cells from *Xid* mice failed to generate any proliferative and survival response after BCR cross-linking.[40] In contrast, we found that T2 cells from Btk-deficient mice were able to generate proliferative and survival responses upon CpG-DNA stimulation, suggesting that TLR9-mediated signalling rescues the defect seen after BCR cross-linking.

We are aware of the fact that cells with high expression of both CD21 and CD24 markers include both T2 and marginal zone (MZ) B-cell subsets. In this study, we did not evaluate the CD23 marker to differentiate between T2 and MZ subpopulations. It has been shown that 88% of $CD21^{high}$ $CD24^{high}$ cells are CD23 positive, indicating a T2 B-cell phenotype, and for this reason we believe that the increased levels in the mature cell population are mostly attributable to T2 cells following CpG stimulation.[40]

Further experiments will be necessary to explain the molecular differences among immature transitional B-cell subsets, MZ cells and follicular (FO) cells. In addition, it will be very interesting to evaluate the costimulatory effect of BCR and TLR9 on B-cell development. It is possible that the reported synergistic effect of the two receptors on B-cell physiology could contribute to the Btk deficiency phenotype.[52]

Interestingly, there is evidence that both endogenous (self-antigen) and exogenous TLR ligands in the bone marrow may influence B-cell development.[53] This result supports the notion that TLR signalling may enhance

B-cell survival and may be involved in B-cell development, having a relevant effect during infectious episodes. In conclusion, TLR9 stimulation can result in the selection, expansion or differentiation of certain B-cell subsets, and we are currently investigating the direct mechanism of such effects. Furthermore, analysis of gene expression and quantitative RT-PCR analysis of the gene encoding the cell cycle regulator cyclin D2 and the antiapoptotic B-cell leukaemia/lymphoma 2 gene *Bcl-2* show that there is CpG-DNA-dependent production of these transcripts. Previous reports have shown that Btk regulates the antiapoptotic proteins Bcl-2 and Bcl-X_L.[54,55] In *Xid* mice, there is a decrease in the amount of endogenous Bcl-2 and this is correlated with an increase of B-cell apoptosis in culture.[54] Our results suggest the possibility that TLR9 activation might provide the means to circumvent the proliferative and/or survival defect caused by Btk mutations. However, LPS stimulation did not restore cell survival/proliferation to a similar extent in the Btk-deficient cells (Fig. 6b), as previously shown.[54,55]

Interestingly, the block in BCR signalling produced by the Btk mutations is reversed by CD40 engagement, although the underlying mechanism remains unknown.[56] In this context, more experiments will be required to explore the possibility that CpG treatment allows BCR signalling to successfully bypass Btk and propagate otherwise blocked downstream events.

Another alternative that might explain the substantial restoration of B-cell survival in $Btk^{-/-}$ cells after CpG stimulation observed in this study is that this phenomenon is a consequence of the effect of one or several cytokines produced via TLR9 signalling. We found that IL-27p28 mRNA production was significantly increased in Btk-deficient B cells after CpG-DNA stimulation. It has been shown that IL-27 modulates human B-cell differentiation and proliferation depending on the mode of cell activation.[32] These results are also consistent with the effect of TLR9 activation on B-cell proliferation, survival and differentiation, suggesting a molecular basis for the increased TLR9 expression observed in the $Btk^{-/-}$ B cells.

In the current work, we also evaluated the production of the immunosuppressive cytokine IL-10. This inhibitory cytokine regulates the growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, DCs, keratinocytes and endothelial cells.[33] Importantly, we found that IL-10 production was dramatically reduced in $Btk^{-/-}$ B cells but moderately reduced in *Xid* B cells upon TLR9 activation compared with normal cells. This result probably explains the altered pro-inflammatory cytokine profile (e.g. TNF-α, IL-12 and IL-6) observed in $Btk^{-/-}$ B cells after TLR9 activation. The differences in IL-10 reduction between *Xid* and $Btk^{-/-}$ (Fig. 3a) might be an effect of a residual activity of the mutant version of Btk in the *Xid* mice. In this context, it has also been reported that transfected cells with the mutated version of Itk-kinase inactive (another member of the Tec family) only had a minimal reduction in Erk activation in response to stromal cell derivated factor (SDF)-1a.[57] While our work was in progress, two other groups demonstrated that Btk is required for TLR4-induced IL-10 production in both macrophages and DCs.[34,58] These results suggest that Btk regulates the balance of pro- and anti-inflammatory cytokine production. Moreover, when we supplemented Btk-deficient B-cell cultures with IL-10, we noted that pro-inflammatory cytokine production decreased compared with normal B cells (Fig. 3b). Further experiments are necessary to determine the role of TLR9-induced IL-10 production in B cells. It is also plausible that Btk exerts a negative regulatory effect on TLR9 signalling, but the mechanism is still unknown.

In conclusion, functional Btk is necessary for inhibitory cytokine production, indicating that Btk is a key component of a feedback regulatory system of innate immunity. All our findings taken together suggest that the regulatory effect of Btk in both innate and adaptive immune signalling plays a crucial role in the maintenance of homeostasis of the immune system. Finally, elucidation of the physiological role of TLRs in B cells will provide important clues for understanding the molecular mechanisms underlying inflammation, infectious diseases, autoimmunity, immunodeficiencies, cancer and allergy/asthma. TLR9 could turn out to be a target for both agonists and antagonists having the therapeutic potential, depending on the clinical setting.

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