

# Monozygous twins with a microdeletion syndrome involving *BTK*, *DDP1*, and two other genes; evidence of intact dendritic cell development and TLR responses

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Received: 18 January 2007 / Revised: 28 March 2007 / Accepted: 29 March 2007  
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**Abstract** We report for the first time monozygous twins with a microdeletion syndrome involving genes coding for Bruton's tyrosine kinase (Btk) and deafness-dystonia peptide 1 (*DDP1*), and two other genes. Apart from its essential role in B cell development, Btk is indicated to affect signaling mediated by toll like receptors (TLRs) and development of dendritic cells (DCs) but results are conflictive. The twins revealed normal numbers of plasmacytoid and myeloid DCs (pDCs and mDCs). Moreover, *BTK* null cells from these patients exhibited robust responses to TLR agonists, normal natural killer (NK) cell activity, and normal pDC functions.

**Conclusion:** Our results do not indicate the essential role of Btk in TLR signaling and DC development.

**Keywords** *BTK* · *DDP1* · Microdeletion · TLR · XLA

## Abbreviations

*BTK* Bruton's tyrosine kinase gene  
DCs dendritic cells  
*DDP1* deafness, dystonia peptide 1 gene

IFN interferon  
Ig immunoglobulin  
IL interleukin  
IV intravenous  
mDCs myeloid DCs  
MTS Mohr-Tranebjaerg syndrome  
NK natural killer  
PBMCs peripheral blood mononuclear cells  
pDC plasmacytoid DCs  
TLR toll like receptor  
TNF tumor necrosis factor  
XLA X-linked agammaglobulinemia

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## Introduction

X-linked agammaglobulinemia (XLA, OMIM #300300) is caused by arrest of B cell development due to mutations in the *BTK* gene coding the Btk. Mutations in *BTK* are heterogeneous and some mutations are associated with more severe disease manifested as the absence of circulatory B cells, undetectable level of IgM, and early onset of severe microbial infection [3, 12]. Btk, a member of Tec family of kinases, is expressed on most hematopoietic lineage cells (except T cells and plasma cells) and activated by multiple extra-cellular signals [25]. In *xid* mice, a murine model of *BTK* deficiency, the role for Btk in TLR signaling has been indicated [15]. Impaired DC development is also implicated with *BTK* deficiency, which was attributed to lack of natural antibodies [1]. However, the role of Btk in human innate immunity is unclear secondary to conflicting results obtained with the use of human XLA cells most likely reflecting marked heterogeneity in genotypes of *BTK* deficiency [3].

One of the most severe forms of *BTK* deficiency is gross deletion, which can occur as a part of microdeletion syndrome. Contiguous deletion syndrome of XLA and sensorineural deafness has been reported in four subjects secondary to gross deletion of 3' end of *BTK* and deletion of *DDP1* gene (*DDP1*, OMIM #300356) located adjacent the 3' end of *BTK* [17]. Mutations in *DDP1* cause a rare X-linked neurodegenerative disease termed as Mohr-Tranebjaerg syndrome (MTS), which is characterized by progressive neurological deficits including early-onset of deafness [2].

Herein, we present for the first time monozygous twins with a microdeletion syndrome involving *BTK*, *DDP1*, and two other genes. Despite presence of *BTK* null cells, the twins revealed intact responses to the TLR agonists tested and normal DC development.

### Case report

Six-year-old, monozygotic Caucasian twin brothers from Ukraine were presented in the Pediatric Allergy/Immunology Clinic, New Jersey Medical School, University of Medicine and Dentistry of New Jersey in July 2006 for further immune work-up. They were born at full term in the Ukraine and their birth weights were 2.5 kg. They were adopted shortly after birth; no family history is available. They developed their first bouts of bacterial pneumonia at 5–6 months of age, requiring intravenous (IV) antibiotics. Since then, they have suffered numerous sino-pulmonary infections and chronic diarrhea requiring extensive oral and IV antibiotics. In 2004, following the finding of their agammaglobulinemia status, treatment of IV immunoglobulin (IVIG) (0.4 g/kg/dose every 4 weeks) was initiated in the Ukraine, which helped attenuate the microbial infection. Severe speech delay was noted at 2–3 years of age and they were taught sign language by their adoptive mother.

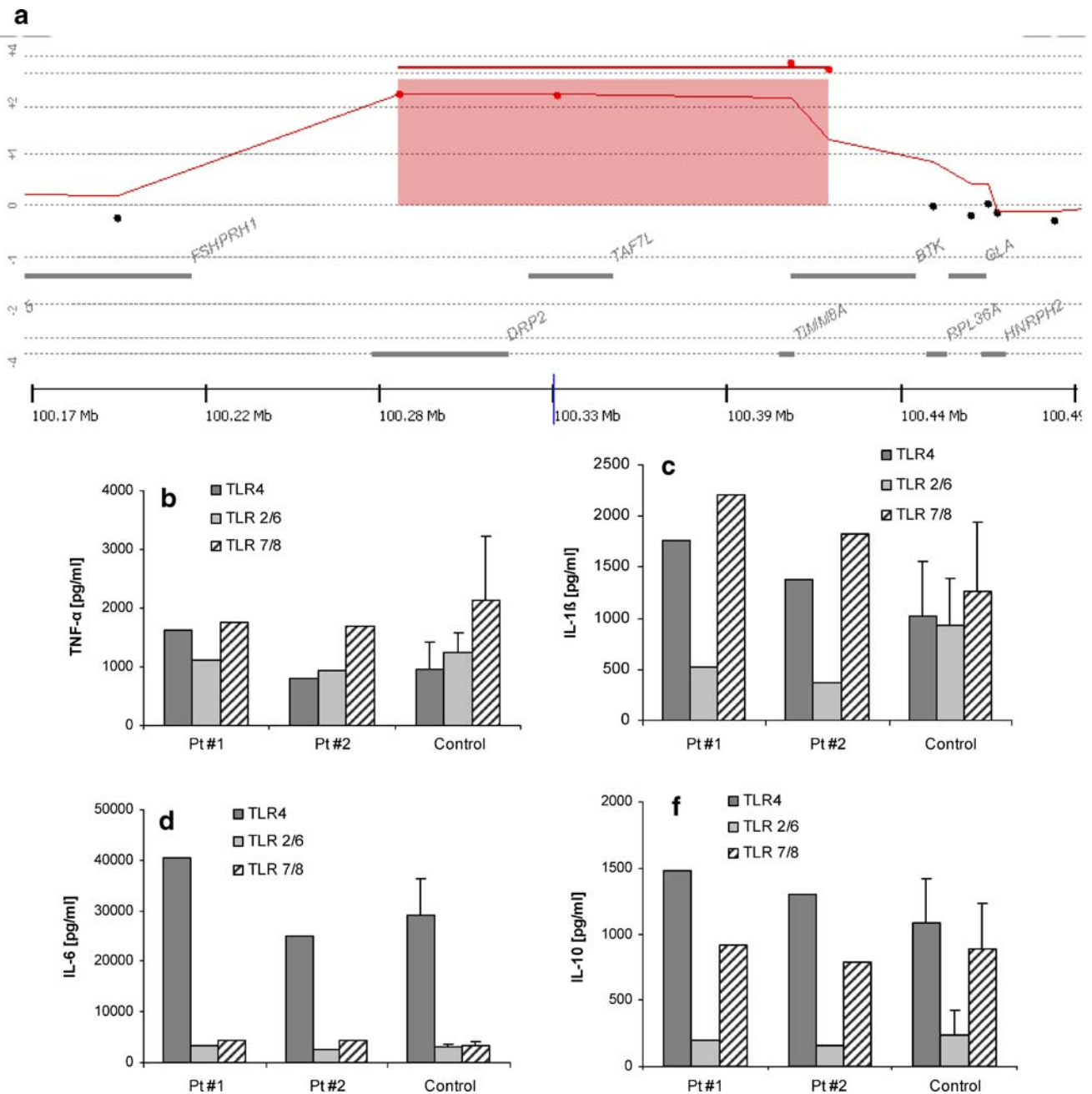
In their initial presentation, physical findings were significant for presence of scattered furuncles on trunk and extremities and multiple untreated dental caries; one brother revealed more severe furuncles and dental caries. They were also malnourished with body mass index being 12.93 and 13.89 (3–5 percentile). However, they did not reveal noticeable dysmorphisms or neurological deficits except severe hearing loss and speech delay. Normal lung function by spirometry was also revealed in these twins despite previous history of recurrent sinopulmonary infection. Initial work-up revealed absence of circulating CD19<sup>+</sup> B cells, along with undetectable levels of IgA, IgM, and IgE (<0.1 mg/ml for IgA/IgM and <0.1 IU/ml for IgE). Their IgG levels 3 months after the last dose of IVIG were 2.61 and 2.89 mg/ml. Antibody titers against tetanus and diphtheria were undetectable. Their neutrophil counts (6,200 and 4,000/m<sup>2</sup>), monocyte counts (1,200 and 800/m<sup>2</sup>), platelet

counts (270 and 352 × 10<sup>3</sup>/m<sup>2</sup>), and T cell numbers/functions were normal. They also revealed normal complement 3 and 4 levels and normal CD16<sup>+</sup>CD56<sup>+</sup> NK cell numbers (194 and 394/m<sup>2</sup>). Given these findings, IVIG treatment was promptly resumed and after reaching normal serum IgG levels, extensive dental procedures were performed without complications in both boys. Oral antibiotics and IVIG treatment effectively controlled furuncles without further intervention. Following a 5 weeks' stay in the USA, the twin brothers returned to the Ukraine in much improved physical condition.

Additional work-up during their stay in the USA revealed the following:

- *Audiology evaluation*: Profound bilateral sensorineural hearing loss was found in both twins.
- *Genetic work-up*: Initial attempts of sequencing *BTK* revealed normal sequences of exons 1 and 2 but failed to amplify exons 3 through 19. Array CGH with oligonucleotide microarrays (21) revealed a 155kb microdeletion (chr X: 100,288,859–100,453,630). It resulted in gross deletion of *BTK* (OMIM #300300, exons 3 through 19) and complete deletion of *DDP1* (OMIM #300356), *TAF7L* (OMIM #300314), and *DRP2* (OMIM #300052) (Fig. 1a). Common missense mutations of TLR2 (Arg753Gln) and TLR4 (Asp299Gly and Thr399Ile) were also studied secondary to the fact that the presence of heterozygous alleles of these mutations were implicated with increased susceptibility to certain microbes [22]. No mutated alleles were found.
- *Additional immune work-up*: We also assessed production of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, IL-12p40, and sTNFRII in response to TLR2/6 (zymosan; 50  $\mu$ g/ml), TLR3 (poly I:C; 0.1  $\mu$ g/ml), TLR4 (lipopolysaccharide, LPS; 0.1  $\mu$ g/ml), and TLR7/8 (imidazoquinoline, 20  $\mu$ M) agonists by their peripheral blood mononuclear cells (PBMCs), since the previous reports indicated impaired TLR signaling in XLA cells [7, 8, 20]. PBMCs (10<sup>6</sup> cells/ml) were stimulated overnight with these stimuli and concentrations of cytokines were measured by enzyme-linked immunosorbent assay as previously reported [10]. Figure 1b–f reveals equivalent production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 with TLR2/6, TLR4, and TLR 7/8 agonists in these twins as compared to age-matched controls ( $N=17$ , median 6.8 years). The production of IL-12p40 or sTNFRII with these TLR agonists was all within normal limits. Likewise, responses to TLR3 agonist were also within normal limits in terms of production of the above-described cytokines.

IFN- $\gamma$ , IL-5, TNF- $\alpha$ , and IL-17 production with T cell mitogens (PHA and Con A) were also equivalent to normal



**Fig. 1** Panel **a** reveals the chromosomal segment, in which the deletion is present. The grey horizontal bars indicate the genes and the corresponding gene symbol is at the top right corner of each bar. The deleted segment is shown by the shaded areas which included four

genes: *BTK*, *DDP1* (*TIMMBA*), *TAF7L* and *DRP2*. Panels **b**, **c**, **d**, and **f**, respectively, show production of TNF-α, IL-1β, IL-6, and IL-10 by PBMCs from the twin brothers along with values obtained from age-matched controls (*N*=17, median 6.8 years)

controls. Due to chronic diarrhea and apparent clinical response to a dairy-free and wheat-free diet, we also assessed responses to milk and wheat proteins by measuring production of IFN-γ, TNF-α, IL-12, IL-17, and IL-10 by their PBMCs; however, no significant reactivity was found. The brothers subsequently tolerated dairy and wheat products without difficulty.

Since XLA patients are known to be susceptible to enterovirus infections [24], we tested NK cell activity and

number and the functions of pDCs in the twins given their pivotal roles in viral infection [5, 9, 14]. Their NK cell activity against herpes simplex virus (HSV) infected fibroblasts was normal (3.2 and 1.9 lytic units; control donor 1.2 lytic unit) [9]. When both twin brothers had active infection (furuncles), they revealed increased numbers of pDCs (CD123<sup>+</sup>, HLA-DR<sup>+</sup>, CD11c<sup>-</sup> cells) (34.4 and 23.6/μl, *N*=4-17/μl) that normalized following intensive IVIG treatment (15.7 and 11.0/μl) [4]. Their pDCs revealed

normal levels of production of both IFN- $\alpha$  and TNF- $\alpha$  in response to HSV which stimulates through the TLR9 pathway [11]. The numbers of mDCs (CD 123<sup>lo</sup>, HLA-DR<sup>+</sup>, CD11c<sup>+</sup> cells) were normal in both boys (18.7 and 12.8/ $\mu$ l) [4].

## Discussion

XLA can be caused by more than 500 mutations in *BTK*. There appears to be some genotype/phenotype correlations in XLA; mutations causing lack of Btk expression tend to reveal more severe phenotypes with undetectable levels of IgM/circulating B cells and early onset of severe infection [3, 12]. However, individual clinical phenotypes appear to vary considerably even among XLA patients lacking Btk expression, indicating the effects of other genetic and environmental factors.

One of the most severe forms of *BTK* mutations is gross deletion observed in up to 10% of XLA patients [3]. Such gross deletion of *BTK* can occur as a part of microdeletion syndrome and deletion of other genes can further affect XLA phenotypes. The currently known microdeletion syndrome involving *BTK* is a contiguous deletion syndrome of XLA and sensorineural deafness previously reported in four patients [17]. In addition to gross deletion of *BTK*, deletion of *DDP1* gene adjacent to the 3' end of *BTK* causes progressive sensorineural deafness. *DDP1* protein located in the inter-membrane space of mitochondria forms the complex with Tim13 and facilitates the import of nuclear-encoded precursor proteins into the mitochondrial inner membrane [2]. MTS patients with *DDP1* mutations suffer from progressive neurological deficits characterized by early onset of deafness, dystonia, cortical blindness, dementia, and mental retardation [2]. Neurological deficits are known to develop later in life except deafness. As with our cases, the previously reported four patients [17] had no neurological deficits except for sensorineural deafness, most likely because of their young age. However, in these four patients, the effects of *BTK* deficiency in innate immunity were not addressed.

In addition to *BTK* and *DDP1*, *TAF7L* and *DRP2* genes are also present in deleted genomic segment. Animal studies indicate *TAF7L* encodes RNA polymerase II TBP-associated factor II protein. This protein is expressed only in male spermatogonia and may have a role in pre-meiotic stages of mammalian spermatogenesis [23]. *DRP2* encodes a dystrophin 2 protein which is principally expressed in spinal chord and brain. Rodent studies indicate its role in regulation of myelination [19]. No human pathology has been reported due to a mutation of these two genes.

Apart from its essential role in B cell development, recent studies indicate a role of Btk in TLR signaling. LPS,

a TLR4 agonist, induces phosphorylation of Btk in human monocytes and phosphorylated Btk can interact with downstream signaling molecules in TLR pathways, including MyD88, Mal, IRAK (IL-1 receptor associated kinase), and TRAF6 (TNF-receptor-associated factor 6). In *xid* mice, a murine model of *BTK* deficiency is caused by missense mutation in the N-terminal portion of mouse *BTK*; others report impaired production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-10, defects in respiratory oxygen bursts, and attenuated inflammatory responses to LPS [13, 15, 18].

In human XLA monocytes lacking Btk expression, Horwood et al. reported decreased TNF- $\alpha$  but not IL-6 production in response to TLR2 and TLR4 agonists [7, 8], while others reported impaired production of IL-6 and TNF- $\alpha$  in response to TLR8 agonist but not to other TLR agonists [20]. Lack of sequence data of *BTK* genes in these XLA subjects makes it difficult to assess conflicting results. In seven XLA patients with *BTK* mutations causing absence of Btk expression, others report intact intracellular expression of TNF- $\alpha$  and IL-6 and oxidative burst in response to LPS [16]. Likewise, studies of DCs in XLA patients yielded conflicting results. Bayry et al. reported impaired differentiation and maturation of DCs in seven XLA patients which was attributed to lack of natural antibodies (IgM) [1]. However, Gagliardi et al. reported normal differentiation, maturation, and antigen-presenting function of mDCs in five XLA patients with absent Btk expression [6]. IgM levels of XLA patients were equivalent in both studies [1, 6].

The presented cases in this report likely represent the most severe form of XLA with gross deletion of *BTK* involving exons 3 to 19 as evidenced by undetectable levels of serum IgM, IgA, and IgE and the absence of circulating B cells. Since natural antibodies all belong to the IgM isotype, these twin brothers are thought to lack natural antibodies. Nevertheless, we found normal numbers of mDCs and pDCs in these twin brothers. We also observed normal signaling of pDCs by HSV-1 which is known to signal through TLR9 [11]. Likewise, NK cell activity against HSV-infected fibroblasts, known to be dependent upon DCs [9], was also normal in both patients. These results caution the essential role of natural antibodies for development of DCs.

These twin brothers also revealed normal production of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-10, and IL-12 in response to TLR2/6, 3, 4, 7/8 agonists. These results also caution an essential role of Btk in TLR signaling in human monocytes. The conflicting results observed in *BTK* null human monocytes can be partly attributed to marked heterogeneity of *BTK* mutations and may also be attributed to the effects of other genetic and environmental factors. For example, heterozygous alleles of missense mutations of TLR4 (Asp299Gly and Thr399Ile) are present in about 10% of the general population and their presence is implicated in impaired responses to LPS [21],

which might be aggravated in *BTK* null cells. We confirmed the absence of such mutations in the presented cases. In the presented cases, their immune systems may also be affected by the deletion of other genes including *DDP1* which appears to play a role in the mitochondrial functioning essential for normal cellular functions. The *DDP1* deficiency can potentially affect immune function later in their lives secondary to impaired mitochondrial functions. Careful follow-up will be necessary along with close monitoring of their neurological status.

In summary, we report the results of evaluation of innate immunity including DCs, NK cells, and TLR-mediated cytokine production in patients with contiguous deletion syndrome of XLA and sensorineural deafness caused by deletion of *BTK*, *DDP1*, and two other genes. Our results caution an essential role of Btk in TLR signaling pathways and DC development.

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