

Mutations in Bruton's tyrosine kinase impair IgA responses

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Received: 14 December 2014 / Revised: 5 January 2015 / Accepted: 5 January 2015 / Published online: 15 January 2015
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Abstract X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by mutations in Bruton's tyrosine kinase (BTK), and is characterized by markedly decreased numbers of blood B cells and an absence of all immunoglobulin isotypes. We performed whole exome sequencing in a male pediatric patient with dysgammaglobulinemia with IgA deficiency. Genetic analysis revealed a *BTK* missense mutation (Thr316Ala). To investigate whether a *BTK* mutation underlay this antibody deficiency with marked decrease of IgA in this patient, we performed functional analyses of B cells and phagocytes, and molecular analyses of somatic hypermutation and class switch recombination. The *BTK* missense mutation resulted in B cells with reduced BTK and high IgM expression. Equal proportions of CD19^{low} and CD19^{normal} fractions were observed, and both included naïve and memory B cells. Calcium influx and phospholipase C γ 2 phosphorylation upon IgM stimulation were marginally impaired in CD19^{low}, but

not in CD19⁺ B cells. Similar to XLA patients, IgA transcripts showed low SHM levels, whereas IgG transcripts were hardly affected. Our analyses suggest that the *BTK* mutation likely underlies the disease in this case, and that hypomorphic *BTK* mutations can result in normal circulating B cell numbers, but specifically impair IgA responses.

Keywords Bruton's tyrosine kinase · X-linked agammaglobulinemia · IgA deficiency · Class switch recombination · Somatic hypermutation

Abbreviations

AID	Activation-induced cytidine deaminase
APRIL	A proliferation-inducing ligand
BAFF	B cell-activating factor
BTK	Bruton's tyrosine kinase
CDR	Complementarity-determining region
CSR	Class switch recombination
CVID	Common variable immunodeficiency
KRECs	Kappa-deleting recombination excision circles

Electronic supplementary material The online version of this article (doi:10.1007/s12185-015-1732-1) contains supplementary material, which is available to authorized users.

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NF- κ B	Nuclear factor kappa B
PLC γ 2	Phospholipase C γ 2
ROS	Reactive oxygen species
RSS-KDE	Recombination signal sequence-kappa-deleting element
SHM	Somatic hypermutation
TNFRSF13B	Tumor necrosis factor receptor superfamily, member 13B
TACI	Transmembrane activator and calcium-modulator and cyclophilin ligand interactor
TRECs	T cell receptor excision circles
XLA	X-linked agammaglobulinemia

Introduction

X-linked agammaglobulinemia (XLA) is a human immunodeficiency disease caused by mutations of the *Bruton's tyrosine kinase* (*BTK*) gene. XLA is characterized by a profound deficiency of B lymphocytes and a decrease of all classes of immunoglobulin (Ig), because BTK mutations impair the differentiation of pro-B cells beyond the earlier pre-B cell stage [1–3].

BTK is a key molecule in pre-B cell activation and differentiation, and has multiple roles in the signaling, survival, and proliferation of B cells [4]. BTK is involved in the signaling pathways of various cell types through its association with the B cell antigen receptor (BCR) and Toll-like receptors (TLRs). After BCR ligation by antigen, phosphorylated B cell linker protein binds to BTK and phospholipase C (PLC) γ 2 via the SH2 domains of BTK and PLC γ 2. Activated BTK directly phosphorylates PLC γ 2 at tyrosine residue 759, after which phosphorylated PLC γ 2 causes calcium release and activation of canonical nuclear factor κ B (NF- κ B) signaling [4–7]. BTK is also a key signaling molecule of phagocytes; and recent studies have demonstrated the excessive production of reactive oxygen species (ROS) and increased apoptosis in human BTK-deficient neutrophils when stimulated via TLRs [8].

Over 600 different mutations in *BTK* have been reported in patients with XLA [9], but the genotype/phenotype correlation is unclear, and some missense mutations are associated with milder or atypical disease [4, 10]. The pathogenicity of a mutation in the *BTK* gene is less clear when BTK variants are found in antibody-deficient patients who have peripheral B cell percentages that are higher than expected in XLA (>2 % of lymphocytes) [11]. The few residual B cells from XLA patients can produce IgE upon in vitro stimulation with anti-CD40 and interleukin (IL)-4 [12]. Moreover, BTK-deficient B cells can generate low, but detectable levels of specific antibodies upon in vivo immunization and often produce self-reactive and poly-reactive antibodies [12–14]. It is still unknown, however,

whether XLA B cells can undergo class switch recombination (CSR) to IgA, undergo somatic hypermutation (SHM), or have normal immunoglobulin receptor diversity.

We performed whole exome sequencing of a 3-year-old boy with dysgammaglobulinemia with a deficiency of IgA and identified a BTK missense mutation. This mutation has already been reported in a 31-year-old male with a reduced number of peripheral B cells (8 % at 12 years of age, and from 1.98 to 2.8 % at 28 years of age), dysgammaglobulinemia (normal serum IgG, low IgA and IgM), a history of recurrent infections, and an atypical clinical phenotype characterized by sclerosing cholangitis and chronic obstructive pancreatitis [15]. However, it is still unclear whether the mutation is causative of disease [16, 17], since expression of BTK and BTK phosphorylation was normal in this previous individual and no other biochemical analyses were performed.

In the present study, we showed the *BTK* (T316A) mutation underlies the decrease in the patient with dysgammaglobulinemia and examined B cell and phagocyte functions, as well as analyses of complementarity-determining region (CDR) length and SHM frequency in IgA and IgG transcripts to investigate the role of this mutation in IgA deficiency.

Materials and methods

Exome and sequencing analysis of *BTK*

All human exons were captured by SeqCap EZ Exome SR Version 3.0 (Roche NimbleGen, Inc., Madison, US), and paired-end sequencing of captured fragments was performed on an Illumina HiSeq 1000 deep sequencing instrument. DNA/RNA extraction, cDNA synthesis, cell isolation, and validation study information are described in detail in the supplementary methods section. This study design was approved by the local scientific ethics committee, and informed consent was obtained from all subjects.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were stained with fluorescein-conjugated antibodies and analyzed by Fortessa flow cytometry (Beckton Dickinson, Franklin Lakes, US) or FACS Calibur (Beckton Dickinson, Franklin Lakes, US) and FlowJo software (TreeStar Inc, Ashland, US). Additional antibody information is described in detail in the supplementary methods section.

Phosphorylation assay

A total of 1×10^6 cells/100 μ l PBMCs were incubated in RPMI 1640 and left untreated or stimulated with human

CD40 Ligand (2 μ g; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min or goat F(ab')₂ anti-human IgM UNLB (10 μ g; Southern Biotech, Birmingham, US) for 5 min at 37 °C [18]. Cells were then fixed and permeabilized using the BD Phosflow kit and stained with anti-NF- κ B p65 (pS529)-Alexa Fluor 488 and anti-PLC γ 2 (pY759)-Alexa Fluor 647 (all from BD Pharmingen Inc., San Diego, US).

Calcium flux

PBMCs were loaded with 2 μ M Fluo4AM (Life Technologies, Carlsbad, CA, US) in RPMI 1640 supplemented with 5 % heat-inactivated fetal bovine serum, incubated for 45 min at 37 °C, and stimulated with goat F(ab')₂ anti-human IgM (5.0 μ g/ml; Southern Biotech, Bergisch Gladbach, Germany) for 8 min and ionomycin (8 μ g/ml; Life technologies, Carlsbad, US) for the last minute, as previously described [19]. The results were analyzed by kinetic plots using FlowJo software (Tree Star Inc., Ashland, US).

Mutation analysis in IgA and IgG transcripts

IgA and IgG transcripts were amplified from PBMCs cDNA using primers in the leader sequence of the immunoglobulin heavy chain variable region (*IGHV*) in combination with an immunoglobulin heavy alpha (*IGHA*) or an immunoglobulin heavy gamma (*IGHG*) consensus reverse primer. All PCR products were subcloned and then sequenced, as previously described [20, 21]. The mutation frequencies within *IGHV* and the immunoglobulin heavy chain (IGH)-CDR3 sizes were determined from each unique clone. Statistical analyses were performed with the Mann–Whitney test. *P* values <0.05 were considered to be statistically significant.

Results

Patient and laboratory data

The patient is a 3-year-old male born to the first child of non-consanguineous parents, who suffered from perianal abscesses, recurrent acute otitis media, recurrent respiratory tract infections, and a skin eruption with fever, which was diagnosed pathologically as leukocytoclastic vasculitis. He did not present with lymphadenopathy, autoimmune disease, or gastrointestinal infection.

Diagnostic workup was started at 10 months of age, and the patient had normal serum IgG and IgG subclass (IgG 1335, IgG1 963, IgG2 337, IgG3 151, IgG4 < 3.0 mg/dl) and low IgM (from 14 to 49 mg/dl), but undetectable serum IgA levels <4 mg/dl (Supplementary Table 1), and

occasional neutropenia induced by infection. Specific IgG levels against previous vaccinations (measles and rubella) were normal, as were T cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) numbers. Since recurrent bacterial infections were not controlled by prophylactic use of antibiotics, we provisionally put him on intravenous immunoglobulin therapy at 2 years of age. This decreased the frequency of bacterial infections.

We carried out detailed immunophenotyping of the patient's B lymphocytes. The percentage of B cells within the lymphocyte gate at 1 year of age was 14.2 %; however, this gradually decreased with age to 1.4–5.7 % at 3 years of age (Fig. 1a and Supplementary Fig. 1). CD24⁺⁺ CD38⁺⁺ transitional B cells were increased at the age of 1 year; however, these were normal at 3 years of age compared to age-matched controls (Fig. 1b and Supplementary Fig. 1) [22]. The frequencies of IgD-CD27⁺ memory B cells at 1 year of age were reduced. Intriguingly, however, the frequencies of CD27⁺ memory B cells and IgG⁺ B cells were normal. In contrast, surface IgA⁺ B cells were not detected (Fig. 1c and Supplementary Fig. 1). CD19⁺ B cells consisted of two fractions: CD19^{low} and CD19^{normal} (Fig. 1a). Because previous observations found that decreased CD19 expression was associated with impaired BCR signaling or immature B cells [23, 24], we compared the expression of other surface markers between the two fractions. Expression of CD19 was low in the majority of CD21^{low} B cells (Fig. 1a). Transitional B cells were hardly detected in CD19^{low} B cells (Fig. 1b). Surface IgM expression was high on both CD19^{low} and CD19^{normal} cells compared to that in healthy donors (HD) (Fig. 1d).

Whole exome sequencing reveals a missense mutation in *BTK* (Thr316Ala)

To identify the causative gene defect in the patient with dysgammaglobulinemia with marked decrease of IgA, we performed whole exome sequencing. We excluded mutations in dbSNP database version 137 and selected coding, splice site, and non-synonymous mutations (Supplementary Fig. 2). This analysis identified possible mutations in *BTK*. Sanger sequencing of *BTK* confirmed the presence of a missense substitution in exon 11 (NM_000061.2:c.946A >G) of the patient, causing a change from threonine to alanine in position 316 of the SH2 domain (NP_000052.1:p. Thr316Ala). This missense substitution was located in a highly conserved site and was classified as a “deleterious mutation” by the effect predictor SIFT and as “probably damaging with a score of 1.000” by Polyphen 2 [25]. The same mutation was not found in exome sequencing on 26 patients with common variable immunodeficiency at our institute. This *BTK* (T316A) mutation has previously been

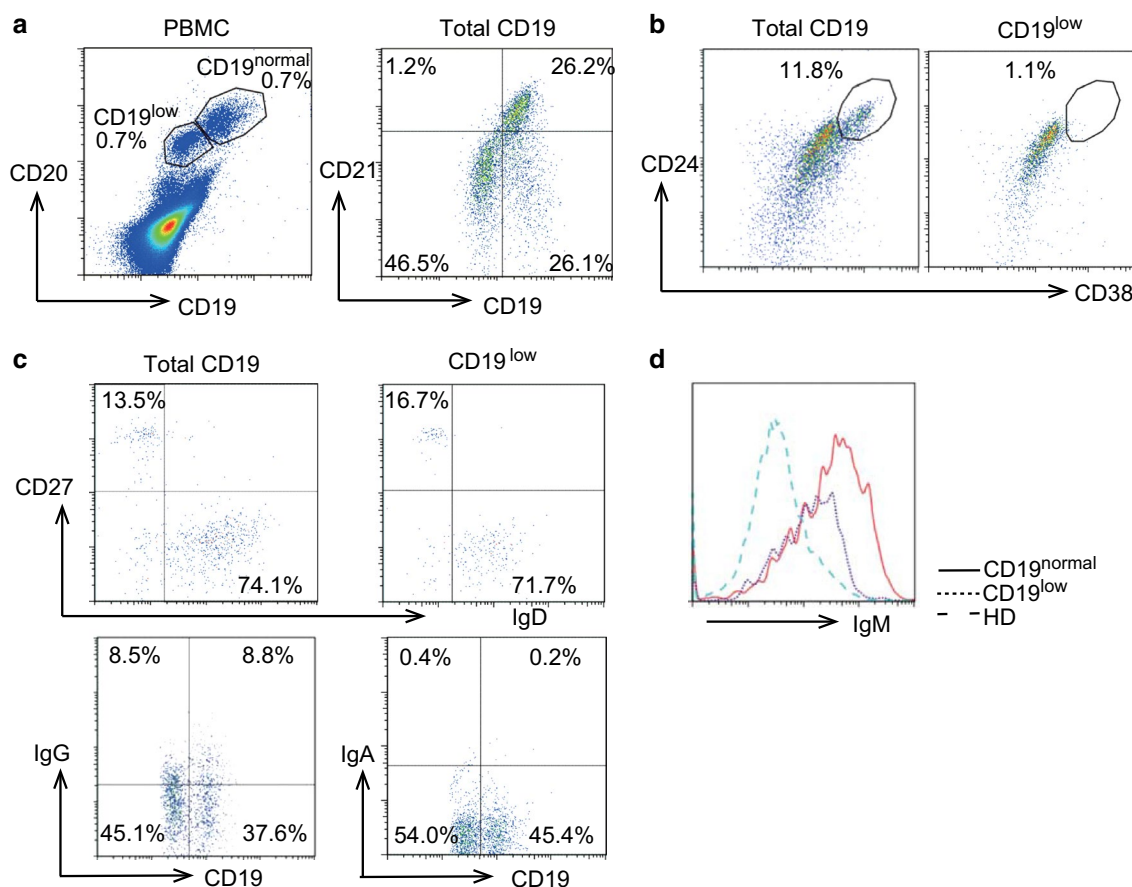


Fig. 1 Patient (3-year-old) peripheral B cell subpopulation. **a** Lymphocyte CD19, CD20, and CD21 expression. **b** Total CD19⁺ and CD19^{low} cells CD24 and CD38 expression. **c** Total CD19⁺ and

CD19^{low} cells CD27 and IgD expression, and total CD19⁺ cells IgG and IgA expression. **d** IgM expression in healthy donor (dotted line) and patient CD19⁺ cells (CD19^{normal}, solid; CD19^{low}, dotted lines)

reported in a 31-year-old male with atypical XLA that exhibited low IgA and normal IgG [15]. We observed no B cells with spontaneous reversion to normal BTK in the purified B cell fraction of the present patient (data not shown). The patient's parents did not harbor the same mutation, suggesting that it was de novo (Fig. 2a). BTK expression in monocytes was reduced in the patient when compared with that of control subjects (difference of mean fluorescence intensity (Δ MFI):HD, 1.985; patient, 0.996) (Fig. 2b).

In addition to the *BTK* mutation, the patient and his mother carried a heterozygous missense substitution in *TNFRSF13B* (tumor necrosis factor receptor superfamily, member 13B (TNFRSF13B), encoding transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)); NM_012452.2:c.637G > A, NP_036584.1:p.Ala213Thr (Supplementary Fig. 3a). This mutation is listed in dbSNP (rs140914723) and does not appear to contribute to dysgammaglobulinemia because the immunoglobulin levels of the patient's mother were normal (serum IgG, 927 mg/dl; IgM, 132 mg/dl; IgA, 112 mg/dl), and TACI expression on memory B cells of the patient was

normal (Supplementary Fig. 3b). We found no mutations in other genes than *BTK* and *TNFRSF13B* that could explain the dysgammaglobulinemia (data not shown).

Normal BCR signaling in CD19⁺ B cells carrying the T316A BTK mutation

BTK signals to downstream pathways of the BCR. Therefore, we determined whether signaling in B cells harboring the mutated *BTK* was affected. To examine BCR signaling, we measured calcium flux and phosphorylation of PLC γ 2 upon BCR cross-linking. The calcium influx in CD19⁺ cells from the patient was normal even upon suboptimal stimulation with anti-IgM F(ab')₂ (Fig. 3a). However, the calcium influx in CD19^{low} cells was slightly low and delayed upon suboptimal stimulation (Fig. 3b). The BCR-mediated PLC γ 2 phosphorylation of CD19⁺ cells was normal compared to B cells from controls [Δ MFI: controls, 5.2 ± 2.2 ; patient, 6.0 ± 0.1 (mean \pm SD)] (Fig. 3c). PLC γ 2 activation in CD19^{low} cells was marginally low compared to that in CD19^{normal} cells (Supplementary Fig. 4a).

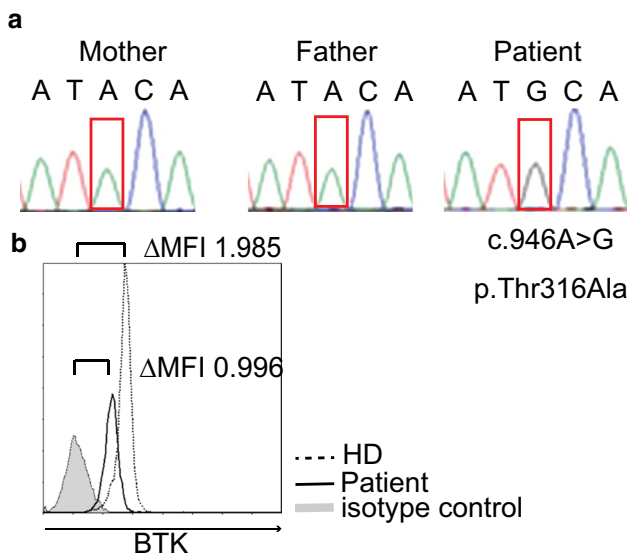


Fig. 2 Genetic analysis of *BTK*. **a** Sanger sequencing of *BTK* exon 11 in the patient and his parents. **b** Flow cytometric analysis of BTK expression in the patient. Dotted line, healthy donor; solid line, patient; gray, isotype control. Values represent Δ MFI. One representative dataset out of two independent experiments is shown

Defective IgA production upon in vitro CD40 ligand stimulation

To evaluate CD40 signaling, we analyzed the phosphorylation of NF- κ B p65 at S529 in patient B cells stimulated with recombinant CD40 ligand and found it to be normal [Δ MFI: controls, 7.1 ± 5.1 ; patient, 4.1 ± 1.2 (mean \pm SD)] (Fig. 3d). To study whether this signal induced IgG and IgA production, we purified B cells (patient) and naïve B cells (healthy donors), cultured them in the presence of anti-CD40 and IL-21 for 12 days, and measured supernatant IgG and IgA levels by ELISA. B cells harboring the *BTK* (T316A) mutation produced comparable levels of IgG to control subjects; however, the production of IgA was significantly impaired in mutant B cells (Supplementary Fig. 4b). Thus, despite seemingly normal CD40 signaling, *BTK* mutant B cells were unable to induce class switching and production of IgA.

Strongly reduced SHM levels in IgA transcripts of the patient and classical XLA patients

B cells from the patient produced IgG both in vitro and in vivo, and the patient showed a normal IgG response to vaccination against measles and rubella. To further investigate the quality of the patient's antibodies, we analyzed SHM frequencies in *IGHV* genes and the IgH-CDR3 sizes of IgG and IgA transcripts. SHM frequencies of IgG transcripts in B cells with *BTK* (T316A) were slightly, but

significantly, reduced as compared to those in age-matched healthy volunteers and IgA-deficient patients ($P < 0.01$). A similar decrease was observed in classical XLA patients; however, this was much less severe than what was previously observed for CD19-deficient patients (Fig. 4a). SHM frequencies in IgA transcripts of the patient were lower than in controls and IgA-deficient patients ($P < 0.001$), and were similar to classical XLA patients and CD19-deficient patients. (Figure 4a). The median IgH-CDR3 sizes in patient IgA transcripts were significantly longer than in controls ($P = 0.003$) (Fig. 4b); however, there were no significant differences in patient and control CDR3 sizes in IgG transcripts ($P = 0.36$) (Fig. 4b).

Thus, both in our patient and in classical XLA patients, the mutation levels and the selection mechanism for short IGH-CDR3 regions are specifically impaired in IgA transcripts. This indicates that the observed anomaly in class switched transcripts is not peculiar to the *BTK* (T316A) mutation, but is generally observed in classical *BTK* mutations.

Neutrophils with BTK (T316A) exhibit excessive ROS production

To further investigate whether the *BTK* mutation leads to a functional impairment in innate immune cells [8, 26, 27], we analyzed the function of neutrophils. In a luminol assay, neutrophils with *BTK* (T316A) produced excessive ROS compared to normal neutrophils (Supplementary Fig. 5).

Discussion

Whole exome analysis of a 3-year-old male with dysgammaglobulinemia with IgA deficiency unexpectedly revealed a *BTK* missense mutation (Thr316Ala). The numbers of peripheral blood B cells, total and specific serum IgG levels, and KRECs levels of the patient at diagnosis were normal. We nevertheless confirmed that this mutation was disease causing, because this de novo mutation was located in a highly conserved site; no other mutation found in whole exome analysis could explain the antibody deficiency; the percentage of peripheral B cells was gradually decreased to around 1 %; *BTK* expression in B cells was low; and neutrophils exhibited augmented ROS production. Our patient was similar to the previously reported patient with T316A in clinical phenotype and immunological data. Both patients exhibited recurrent bacterial infections; the frequency of B cells gradually decreased with age; and they showed normal IgG and low IgA. Although the previous reports did not show the abnormalities in the expression of *BTK*, we detected a low expression of *BTK*. Moreover, similar to classical XLA patients, the B cells had high

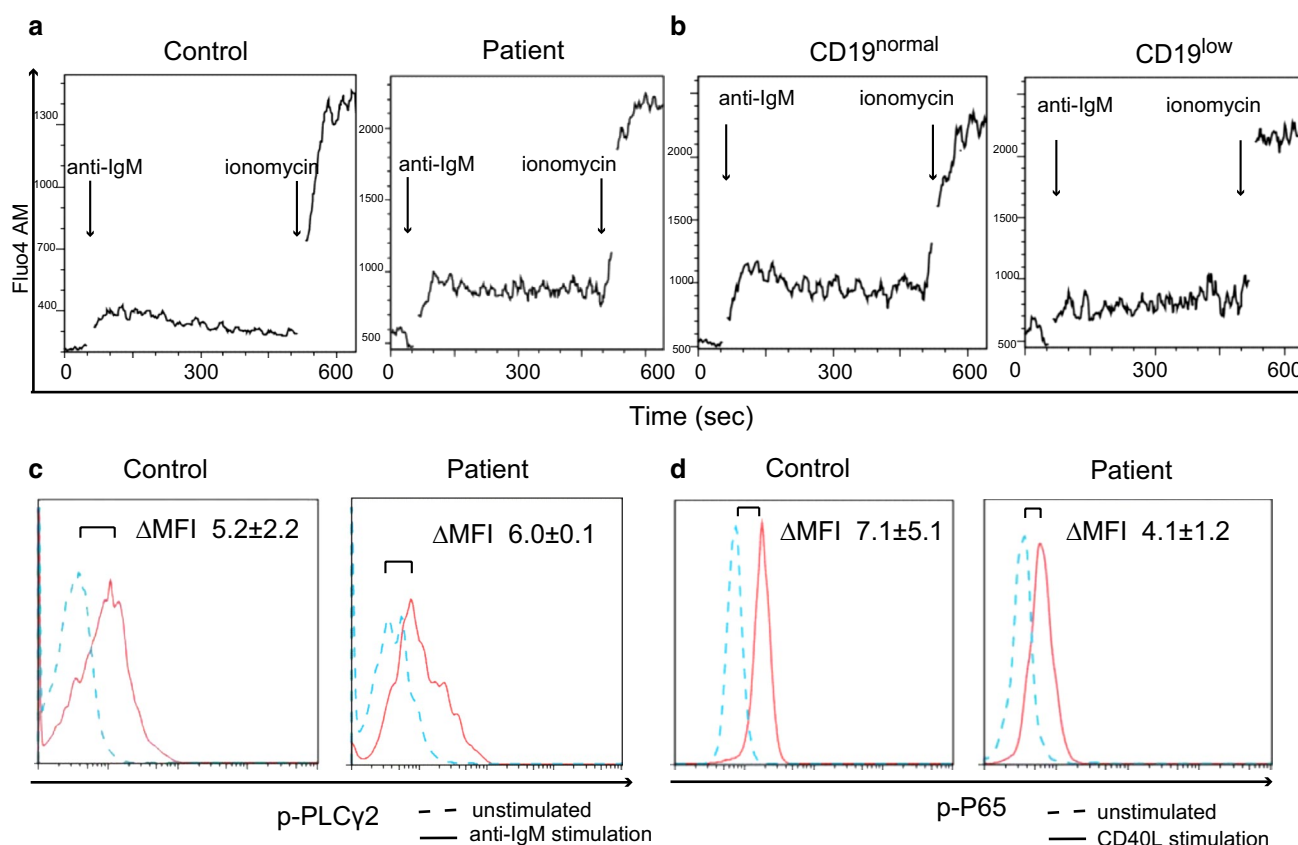


Fig. 3 B cell signaling. Calcium mobilization in patient and healthy donor CD19⁺ (a), and patient CD19^{normal} and CD19^{low} cells (b). Arrows indicate time points of stimulation. Flow cytometric analyses of PLC γ 2 phosphorylation in CD19⁺ (c) and NF- κ B p65 phosphorylation in CD19⁺ (d) stimulated with anti-IgM antibody or CD40

ligand. Solid lines, stimulated cells; dotted lines or gray lines, unstimulated cells. Values represent Δ MFI \pm SD. Healthy volunteers ($n = 4$ or 5) and patients with CVID ($n = 3$ or 4) and IgA deficiency ($n = 1$ or 2) were enrolled as controls

surface IgM levels and carried similarly low levels of SHM in IgA transcripts compared to IgG transcripts. Impairment in antibody maturation and diversity may have been involved in his susceptibility to infection which was controlled by intravenous immunoglobulin supplementation.

Circulating B cells in XLA patients in previous reports were largely CD38^{high} CD24^{high} transitional B cells and were IgM^{high}, CD19^{low}, and CD21^{low} [24, 28, 29]. Expression of IgM is usually down-regulated as B cells mature [30]. High IgM expression of B cells from XLA patients was reported to reflect the immature stage of B cell differentiation [31]. In contrast, all the B cells with *BTK* (T316A), including naïve and memory B cells, showed high IgM expression. Unusually high level of IgM expression in mature B cells was observed in CD19- or CD45-deficient mice, and it is speculated that this may be a reflection of impairment of constitutive BCR signaling [32, 33]. These cumulative data suggest that all B cells in our patient have some defects in in vivo BCR signaling.

Interestingly, B cells with the *BTK* (T316A) mutation consisted of CD19^{normal} and CD19^{low} subsets, in which

CD19^{low} B cells showed marginally reduced calcium influx and PLC γ 2 response. This observation was not peculiar to our *BTK* deficiency, since CD19^{low} B cells from other B cell deficiency patients also exhibited impaired BCR-mediated response (data not shown). Expression of CD19 is affected by CD21 and CD81 that form a signaling complex together with CD19 [23, 34]. Decreased expression of CD19 has also been seen in B cells from a patient with Ig β deficiency and with B cell linker protein deficiency; however, the factors that regulate CD19 expression are not well understood [24]. The reason for the presence of CD19^{normal}/CD21^{normal} and CD19^{low}/CD21^{low} populations in *BTK* (T316A) B cells is unknown.

CD40 signaling is critical in both CSR and SHM [4]. CD40-mediated NF- κ B p65 phosphorylation was normal; and induction of activation-induced cytidine deaminase (AID) was observed in B cells with *BTK* (T316A) after stimulation with an anti-CD40 antibody and IL-21 (data not shown). The involvement of *BTK* in CD40 signaling is still controversial [35]. Several reports suggested that *BTK* was activated upon CD40 stimulation and was required for

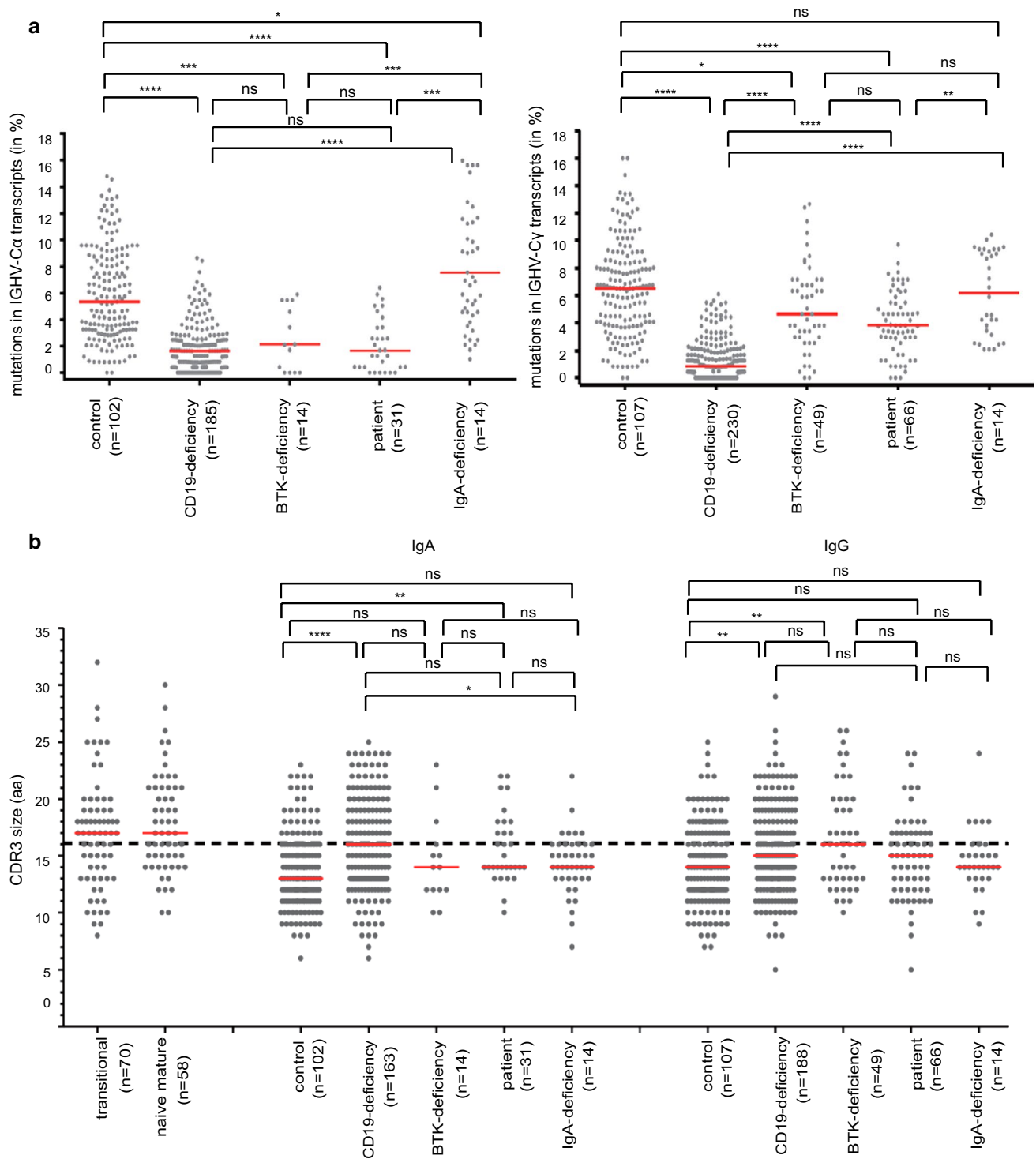


Fig. 4 Somatic hypermutation and BCR repertoire analyses. **a** *IGHV* mutation frequencies of IgA and IgG transcripts. **b** *IGH*-CDR3 sizes of IgA and IgG transcripts. *Dashed lines* represent centroblast median values as previously reported [23]. *Gray dots*, unique sequences; *red lines*, median values. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (Mann–Whitney test). The values in *parentheses*

indicate the total number of analyzed sequences in each subset. The total number of clones from IgA transcripts that we were able to generate from six classical BTK-deficient patients (<0.5 % B cells) was only fourteen. We also analyzed age-matched healthy volunteers ($n = 4$) as controls, IgA-deficient patients ($n = 2$), and CD19-deficient patients ($n = 7$)

the synergy between CD40 and BCR [36–38]. Although anti-CD40-induced NF- κ B phosphorylation was normal in the patient, we could not exclude the possibility that CD40 signaling was defective in the B cells, because B cells failed to produce IgA upon stimulation not only with an anti-CD40 antibody and IL-21, but also with an anti-CD40 antibody and IL-10 or IL-4 (data not shown).

CSR to IgA was severely affected, and SHM and CDR3 sizes of IgA transcripts were abnormal in the B cells with BTK (T316A). This agrees with a previous study that reported normal IgG but low IgA in a patient with the same T316A mutation [15], and reduced IgA levels in an XLA patient with a splice-site mutation in intron 10 of BTK [39]. Interestingly, we found that SHM frequencies of IGHV genes in C α transcripts were more severely affected than C γ transcripts in classical XLA patients as well, unlike in IgA-deficient patients. Cumulatively, these results indicate that BTK function is particularly important in isotype switch to IgA. What would be a potential explanation for the specific defects in IgA in BTK deficiency? Signaling through TACI or transforming growth factor- β 1 receptor is important for CSR to IgA [40]. BTK may be involved in these signaling pathways. One possibility is that BTK is involved in the TACI signaling pathway. TACI signaling is essential for AID expression and IgA secretion in response to a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) [41], but the mechanism by which TACI triggers CSR to IgA is unclear [40, 42]. Previous reports showed that PBMCs from patients with a TACI deficiency were able to produce IgG, but not IgA when stimulated with anti-CD40 and IL-4 or IL-10 [43, 44]. These data raised the possibility that TACI signaling was impaired in our patient. Further experiments are necessary to address this issue using functional assays such as stimulation with APRIL, BAFF, and TLRs agonists.

In conclusion, we identified a *BTK* (T316A) mutation in a young boy with dysgammaglobulinemia with marked decrease in IgA, despite normal numbers of circulating B cells and serum IgG levels. Thus, mildly affected BTK function is sufficient for early B cell differentiation, but cannot fully support IgA responses.

Acknowledgments The authors would like to thank Eri Kumaki, Miko Okamura, Shizuko Minegishi, Takashi Watanabe, Masaki Takazawa, and Atsushi Hijikata for technical assistance.

Conflict of interest The authors declare that they have no conflict of interest.

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