REGULAR ARTICLE

blood advances

In BTK, phosphorylated Y223 in the SH3 domain mirrors catalytic activity, but does not influence biological function

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Key Points

- In publications on BTK and its inhibitors, phosphorylation of tyrosine 223 is often considered necessary for complete catalytic activity.
- We gene-edited mice substituting tyrosine 223 for nonphosphorylatable phenylalanine and found no difference from wild-type animals.

Bruton's tyrosine kinase (BTK) is an enzyme needed for B-cell survival, and its inhibitors have become potent targeted medicines for the treatment of B-cell malignancies. The initial activation event of cytoplasmic protein-tyrosine kinases is the phosphorylation of a conserved regulatory tyrosine in the catalytic domain, which in BTK is represented by tyrosine 551. In addition, the tyrosine 223 (Y223) residue in the SRC homology 3 (SH3) domain has, for more than 2 decades, generally been considered necessary for full enzymatic activity. The initial recognition of its potential importance stems from transformation assays using nonlymphoid cells. To determine the biological significance of this residue, we generated CRISPR-Cas–mediated knockin mice carrying a tyrosine to phenylalanine substitution (Y223F), maintaining aromaticity and bulkiness while prohibiting phosphorylation. Using a battery of assays to study leukocyte subsets and the morphology of lymphoid organs, as well as the humoral immune responses, we were unable to detect any difference between wild-type mice and the Y223F mutant. Mice resistant to irreversible BTK inhibitors, through a cysteine 481 to serine substitution (C481S), served as an additional immunization control and mounted similar humoral immune responses as Y223F and wild-type animals. Collectively, our findings suggest that phosphorylation of Y223 serves as a useful proxy for phosphorylation of phospholipase Cγ2 (PLCG2), the endogenous substrate of BTK. However, in contrast to a frequently held conception, this posttranslational modification is dispensable for the function of BTK.

Introduction

The cytoplasmic protein-tyrosine kinase BTK is essential for the development of B lymphocytes in humans[1,2] because inherited variations cause X-linked agammaglobulinemia (XLA). This disease is characterized by an essential absence of mature B-cell lineage cells and a lack of humoral immune responses. Moreover, inhibitors of BTK represent a new and highly successful paradigm for targeted

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Original data and the gene-edited knockin mouse model are available on request from the corresponding authors, Robert Månsson (robert.mansson@ki.se) and C. I. Edvard Smith (edvard.smith@ki.se).

The full-text version of this article contains a data supplement.

therapies for leukemias and lymphomas.[3-5] This makes the understanding of BTK's signaling properties a very important topic in hematology.

BTK is involved in transferring signals from the B-cell receptor (BCR) to activate development in the bone marrow and effector functions of B cells in the perifery.[1,2] In humans, this process is already crucial at the pro-B to pre-B transition.[6,7] In mice, the importance of BTK was first realized when the cause of the x-linked immunodeficiency (xid) phenotype was identified as a missense variation in the N-terminal, pleckstrin homology (PH) domain of BTK.[2,8] This replacement results in the exchange of an arginine residue for a cysteine impairing membrane translocation. The corresponding variation in humans causes XLA,[9] demonstrating that there are species differences, with the xid phenotype being mild compared with XLA.

The signal transduction activity of BTK is complex, but phosphorylation of phospholipase Cγ2 (PLCG2) is an essential ingredient, with PLCG2 being considered the key substrate of BTK.[10-12] Like all other cytoplasmic protein-tyrosine kinases, BTK is activated by transphosphorylation of the tyrosine 551 residue (Y551) in the kinase domain, altering the structure and promoting enzymatic activity. A key question has been whether additional tyrosine (Y) phosphorylations of BTK contribute to its activity. In addition, serine/threonine phosphorylations of BTK are also involved in its metabolism, including degradation.[11,13-15]

In 1996, Owen Witte's laboratory reported that Y223 represents an autophosphorylation site in BTK. Exchanging Y223 for the aromatic residue phenylalanine (F), which cannot be phosphorylated, in the SH3 domain of BTK and combining this alteration with the substitution of glutamic acid 41 for lysine in the PH domain potently enhanced BTK's transforming activity in NIH 3T3 fibroblasts.[16] This study was followed by the analysis of pY551 and pY223 in stimulated B lymphocytes.[17] Based on the finding that upon activation some BTK molecules were either phosphorylated on Y551 or on Y223 or on both sites, it was proposed that there is a sequential phosphorylation of Y551 followed by Y223 and subsequent dephosphorylation of pY551 followed by pY223. Functionally, pY223 did not enhance kinase activity but was hypothesized to have a regulatory function related to the conformation of BTK.[17] Although the effects of pY223 in NIH 3T3 cells and in B lymphocytes were dramatically different, the 2 publications have become highly influential with regard to the concept of how BTK is regulated.[18] Thus, in spite of no evidence for a 2-step potentiation of BTK's catalytic activity, a very large number of subsequent scientific reports anyway state that for BTK to become fully activated, BTK first needs to be phosphorylated on Y551 (pY551) in the kinase domain and subsequently on Y223 (pY223) in the SH3 domain.

Although pY551 is caused by phosphorylation exerted by SRC-family kinases, especially LYN in B lymphocytes,[19-21] pY223 is achieved through transautophosphorylation, in which 1 molecule of BTK phosphorylates another. Although the functional importance of SH3 domain phosphorylation remains elusive, similar autophosphorylations have also been identified among other kinases belonging to the BTK family.[22] To date, only this family of cytoplasmic tyrosine kinases has been shown to carry a tyrosine that can be phosphorylated in the SH3 domain.

As determined by 3-dimensional nuclear magnetic resonance (NMR) spectroscopy, Y223 was found to be located on the surface of the SH3 domain and therefore readily accessible for posttranslational modifications.[23] However, the question remains whether the pY223 transautophosphorylation is accidental and unimportant or essential for what is often referred to as full activation of BTK. Although many publications state that this is the case, there are 2 observations that argue against this possibility. In 1997, it was reported[24] that the BCR-induced, impaired calcium mobilization in the BTK-defective DT40 chicken B lymphoma cell line was restored upon transfer of a construct in which Y223 was replaced by the unphosphorylatable aromatic residue phenylalanine (Y223F). In contrast, a construct with the same substitution at position 551 in BTK did not restore calcium mobilization, demonstrating the essential role of the later tyrosine for BCR-mediated signaling.[24] Furthermore, in 2003, Rudolf Hendriks' laboratory reported on Y223F transgenic mice, in which BTK was expressed from a CD19 promoter.[18] In their study, it was shown that almost all features of BTK-deficient mice were corrected using this transgene, except for a slightly reduced number of λ light-chain–expressing B lymphocytes. However, despite the strong evidence above suggesting that phosphorylation of Y223 has no functional consequence, many researchers still consider both Y551 and Y223 phosphorylations to be essential for BTK to become fully activated. To finally resolve this issue, we have generated BTK Y223F knockin mice, which do not suffer from the criticism that could apply for transgene systems owing to copy number variations and insertions into new chromosomal sites.

Materials and methods

Animal studies

The Y223F mutation was introduced into exon 8 of the mouse *Btk* gene (Ensembl gene ID: ENSMUSG00000031264 and NCBI gene ID: 12229) using CRISPR/Cas9-mediated gene editing (via zygote injection) with a specific single guide RNA and an oligonucleotide (DNA template) carrying the modifications to be introduced. The targeting strategy was based on NCBI transcript NM_013482.2. The sgRNA was designed to be unique in GRCm38/mm10 (all potential off-target sequences had ≥2 mismatches). Mice were generated and maintained on C57BL/6 background. Analyzed Y223F mice and wild-type controls were sex- and age-matched. Experiments were performed on 7- and 22-week-old mice. Auto-antibody analysis was performed on 14- to 16-month-old animals. All experiments were approved by the local ethics committee.

Flow cytometry

Filtered (70 μm nylon filter) cell suspensions from dissected and crushed organs (bones, spleen, and thymus) were prepared in phosphate-buffered saline (PBS) with 2% fetal calf serum (FCS). Peripheral blood cells were obtained after red blood cells (RBC) were removed and lysed by using 1% dextran and 0.8% NH_4Cl, respectively.

Prepared cells were Fc-blocked using anti-CD16/32 (BD Biosciences) and stained with fluorochrome-conjugated antibodies as previously described.[25] Staining procedure and antibody panels were used as previously described.[26] Dead cell discrimination was performed using propidium iodide. Data were acquired on a

fluorescence-activated cell sorting FACSAria IIu (BD Biosciences) and analyzed using Flowjo 9.9.6 (FlowJo).

Immunofluorescence

Spleens and lymph nodes were frozen in cryostat medium (Bio-Optica, Milan, Italy) and cut into 8-mm-thick sections by using a cryostat microtome. Slides were acetone-fixed and blocked with 5% goat serum (Dako Cytomation, Fort Collins, CO) in PBS. Staining was performed overnight at 4°C with fluorophore-conjugated and biotinylated antibodies, and sections were washed and incubated with the fluorophore-conjugated streptavidin for 1 hour at room temperature. ProLong Diamond Antifade Mountant (Thermo Fisher Scientific) was used for mounting the slides. Data were acquired with the confocal microscope (Zeiss LSM880) and recorded with LSM Image software, as previously described.[25]

Histology

Spleen, liver, kidney, and lung were harvested, fixed in 4% neutral buffered formalin, and routinely processed for histology. Four µm thick paraffin sections were obtained and stained with hematoxylin-eosin (HE) for histopathological examination.

Enzyme-linked immunosorbent assay

For detection of IgM, IgG, IgG1, IgG2b, and IgG3, NP_{30}-BSA plates were incubated with diluted serum, then AP (alkaline phosphatase)-conjugated goat antibody to mouse IgM (1021-04; SouthernBiotech), IgG (1030-04, SouthernBiotech), IgG1 (1071-04, SouthernBiotech), IgG2b (1091-04, SouthernBiotech), and IgG3 (1100-04; SouthernBiotech) were incubated separately for 1 hour at room temperature and developed with phosphatase substrate (S0942-200TAB, Sigma). Results were measured at 405 nm and 620 nm with a Bio-Rad microplate reader.

Immunizations

For immunization with a T-cell independent (TI) antigen, mice were injected intravenously with 50 µg NP-Ficoll (F-1420-100; Biosearch Technologies) in a volume of 100 µL in PBS. Seven days after immunization, blood was collected from the ventral tail artery.

For immunization with a T-cell–dependent (TD) antigen, mice received an intraperitoneal injection with 100 µg NP-CGG (N-5055D-5; Biosearch Technologies) mixed with 100 µL Inject alum (77161, Thermo Scientific). Three weeks after the primary immunization, mice were boosted with 50 µg NP-CGG intravenously. Seven days after the first and booster immunization, blood was collected from the ventral tail artery.

Western blot analysis

Isolated splenocytes were starved in serum-free Iscove's modified Dulbecco's medium (IMDM) with 50 µM β-mercaptoethanol (Gibco, Life Technologies) for 1 hour. Subsequently, B lymphocytes were activated for 5 minutes at room temperature with H_2O_2 (4 mM) and anti-mouse IgM (10 µg/mL, 1022-01, Southern Biotech). Generation of whole-cell lysates and immunoblotting analysis were performed as previously.[25,27] The antibodies used for western blotting were anti-actin (A5441, Sigma-Aldrich), anti-BTK (270-284, Sigma-Aldrich), anti-BTK pY551 (24a/BTK, BD Biosciences), anti-BTK pY223 (EP420Y, Abcam), anti-PLCG2 (Rabbit polyclonal, Biotech), and anti-PLCG2 pY753 (polyclonal, Abcam). The secondary antibodies, goat anti-mouse 800CW, goat anti-rabbit 800CW, goat anti-mouse 680LT, and goat anti-rabbit 680, were purchased from LI-COR Biosciences GmbH, Lincoln, NE, USA. Odyssey Imager from LI-COR Biosciences GmbH was used for membrane scanning, and the signals of total and phosphorylated proteins were quantified by NIH ImageJ 1.52a.

All experiments were approved by the local animal experimentation ethics committee, ID 1679. Mice were generated and maintained on C57BL/6 background. Analyzed Y223F mice and controls were sex- and age-matched. Experiments were performed on 7- and 22-week-old mice. Auto-antibody analysis was performed on 14- to 16-month-old animals.

Results

To determine the biological importance of Y223 for BTK's activity, we generated a CRISPR-Cas9 knockin mouse model in which Y223 was replaced by the nonphosphorylatable, aromatic, phenylalanine residue in the exon 8 of the mouse *Btk* gene (Figure 1). The tyrosine-ATA codon was exchanged for the phenylalanine-TTT codon to generate the Y223F substitution; additional silent mutations were introduced to avoid further Cas9 activity and to generate an AfeI restriction site for analytical purposes (Figure 1).

Mice lacking the Y223 phosphorylation site in BTK show normal B-cell development and maturation

We first aim to evaluate whether replacement of Y223 affects B-cell development. B-cell progenitors from bone marrow (BM) and peripheral B cells from the peritoneal cavity (PeC) and spleen (Sp) were analyzed by flow cytometry (Figure 2A-C). No significant differences were found in the distribution of the B-cell subsets from the different tissues, suggesting that phosphorylation at Y223 is

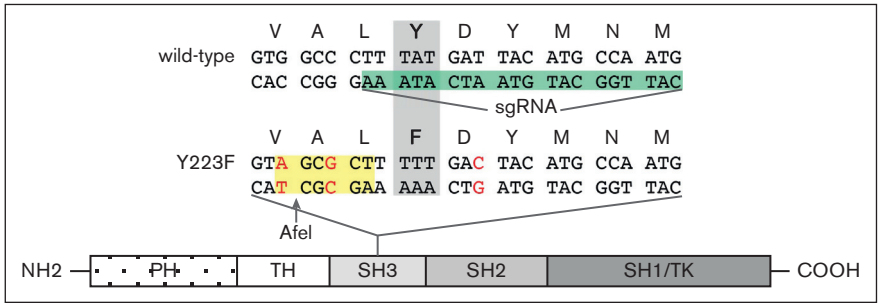

Figure 1. Overview of the wild-type and Y223F mouse *Btk* locus after CRISPR-Cas9–mediated gene editing. The Y223F substitution was introduced into exon 8 of the Btk gene using CRISPR-Cas9–mediated gene editing. Additional silent mutations were introduced to create an AfeI restriction site (AGCGCT) for analytical purposes and to avoid further editing of the locus. The sequence of the single guide RNA (sgRNA, green), silent point mutations (red), position of amino acid 223 (grey), and the introduced AfeI site (yellow) are indicated.

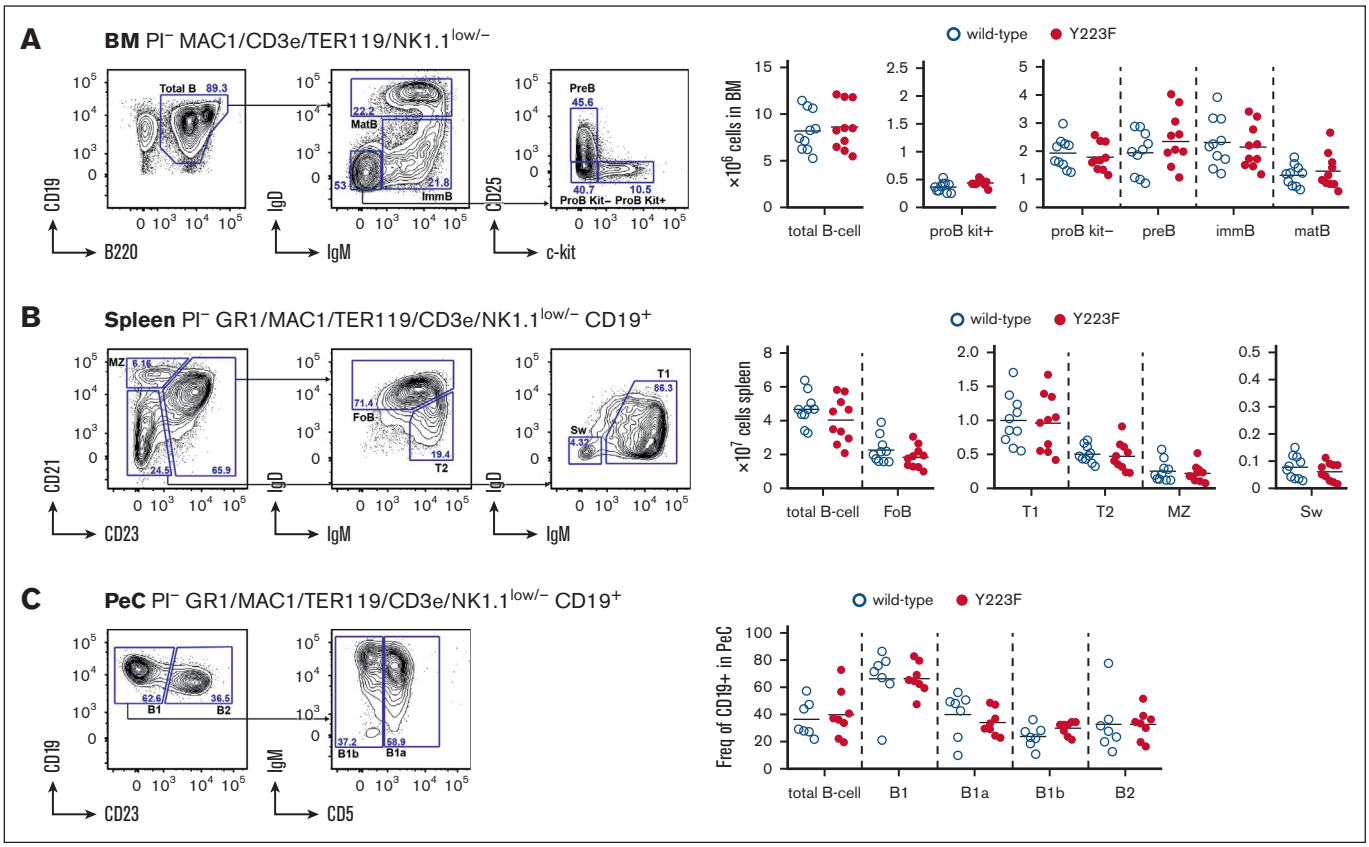

Figure 2. Tyrosine 223 to phenylalanine substitution in BTK does not affect B-cell development in bone marrow or peripheral B-cell subsets in spleen and peritoneal cavity. (A-C) (left) Gating strategy for identification of indicated B-cell subsets. Prior gating was performed as indicated above the fluorescence-activated cell sorting plots. Propidium iodide (PI) was used for dead cell discrimination. (A-B) (right) Absolute numbers of total B cells and B-cell subsets in bone marrow (BM) and spleen. (C) (right) Frequency of subsets within B cells in the peritoneal cavity (PeC). In (A-C) (right), each dot represents data from individual animals, vertical bars indicate mean and 2-tailed Mann-Whitney test was used to calculate significance. Immature B cell (immB); mature B cell (matB); transitional 1 B cell (T1); transitional 2 B cell (T2); marginal zone B cell (MZ); follicular B cell (FoB); switched B cell (Sw). None of the comparisons yielded any significant difference.

dispensable for BM B-cell development and peripheral B-cell maturation (Figure 2A-C).

The distribution of κ- and λ-light chain fractions was also assessed in different B-cell subsets from BM and Sp. A tendency of enriched κ-chain fraction was observed in immature and mature B-cell populations from BM; however, after applying multiple test comparison with Sidak-Bonferroni correction (alpha: 0.05), we confirmed the difference only in immature B cells (Figure 3A). This finding was not observed in the analyzed B cells from Sp, suggesting that if there really is a difference, normalization of the κ- and λ-light chain frequencies occurs during development (Figure 3B).

To extend these findings, peripheral B-cell and T-cell spatial distribution was evaluated by immunofluorescence in Sp and lymph nodes (LN). Well-defined B-cell and T-cell areas were found within the follicular structure, and no noticeable changes in white and red pulp were observed in the Sp when comparing Y223F knockin and wild-type control mice (Figure 4A, right). Normal structure and spatial distribution of B-cell and T-cell areas were also found in LN from both genotypes (Figure 4A, left). Taken together, this shows that Y223F substitution has no detectable effect on B-cell development, including their distribution in peripheral lymphoid organs.

Lack of Y223 phosphorylation does not affect kinase activity upon stimulation of the B-cell receptor

Each B cell expresses a unique BCR,[28,29] which, upon stimulation, initiates a cascade of secondary events that activates LYN, a SRC-family kinase that transphosphorylates the Y551 residue in BTK, leading to enhanced catalytic activity.[30,31] This is followed by transautophosphorylation of the Y223 residue, in which 1 BTK molecule phosphorylates another. This scenario was proposed as crucial for BTK activation and, consequently, for its biological effect.[17] Activated BTK phosphorylates phospholipase C-γ2 (PLCG2), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate and diacylglycerol at the cell membrane. This leads to the activation of protein kinase Cβ (PKCβ) and downstream transcription factors involved in the regulation of B-cell proliferation, differentiation, migration, and survival.[32-34]

Here, we evaluated endogenous BTK's catalytic activity by western blotting after ex vivo anti-IgM stimulation. We measured the phosphorylation of Y551 (pY551) as an indicator of BTK's initial activation and pY223 and pY753-PLCG2 as measures of BTK's catalytic activity. As expected from the amino acid substitution,

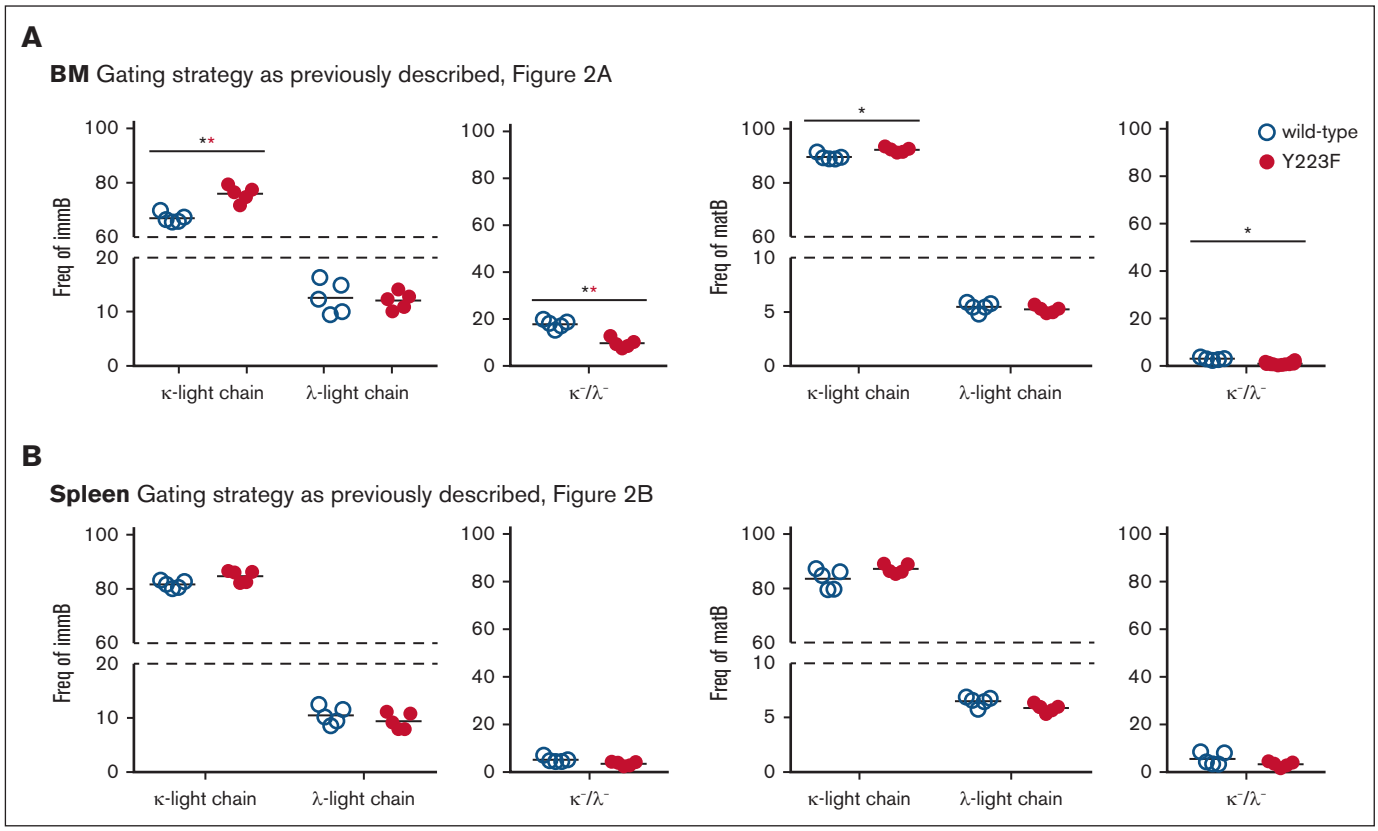

Figure 3. Distribution of κ- and λ-light chain fractions in the B-cell lineage from bone marrow and spleen. (A-B) Gating strategy as indicated for B-cell subsets in Figure 2. Left, absolute numbers of total B-cell subsets in bone marrow (BM) and spleen. Right, frequencies as indicated for each B-cell subset. Each dot represents data from individual animal. Gated cells lacking light-chain expression are shown as κ^-/λ^-. Mann-Whitney U test was used to calculate significance when 2 groups were compared (*$P < .05$ and **$P < .01$). (*) Indicates multiple comparison t test using Sidak-Bonferroni correction method (alpha: 0.05), which confirmed significant values only for κ-light chain in the immature B-cell (immB) population from BM.

pY223 was not found in Y223F B cells (Figure 4B). In contrast, phosphorylation of Y551 was similar in both Y223F and wild-type mice (Figure 4B). No significant differences were obtained from the comparison of the Y753-PLCG2 phosphorylation status between the genotypes (Figure 4B). Together, this indicates that absent Y223-BTK phosphorylation does not interfere with BTK's catalytic activity.

Y223F mice display no alterations in hematopoietic progenitors or non–B-lineage leukocytes

After finding that BTK function, B-cell maturation and distribution appear to be normal in Y223F mice, we set out to determine by flow cytometry whether the substitution affected the development of early lymphoid progenitors, T-cell subsets, and non–B-cell BTK-expressing cell populations. We found no differences in the number of hematopoietic stem cells, lymphoid-primed multipotent progenitors, or common lymphoid progenitors (supplemental Figure 1A) in BM of Y223F mice. Moreover, we found no significant differences in thymic T-cell progenitors (supplemental Figure 1B) nor in the $CD4^+$ and $CD8^+$ T-cell subsets from Sp and peripheral blood (PB) (supplemental Figure 2A-B). Similarly, no significant changes were identified in macrophages, granulocytes, dendritic cells, and natural killer cell numbers in Sp and PB when comparing Y223F and wild-type animals (supplemental Figure 2A-B).

Y223F and C481S knockin and wild-type mice show comparable levels of serum antibodies upon immunization with T-cell–dependent and –independent antigens

To assess B-cell function and ability to efficiently mount in vivo antigen-specific T-cell–dependent and –independent responses, we measured levels of serum immunoglobulins upon NP-CGG and NP-Ficoll immunization (Figure 5) in wild-type and Y223F mice. As an additional control group, we included mice carrying an edited BTK gene (C481S) resistant to irreversible BTK inhibitors.[26] In line with the results obtained for the analyzed B-cell populations, similar IgG1 and IgG3 subclass levels were observed in all 3 genotypes at baseline and after either NP-Ficoll or NP-CGG immunization (Figure 5A-B). We observed a significantly higher IgM response upon immunization and higher IgG2b levels at baseline (Figure 5A-B). Furthermore, upon immunization with either T-cell–independent type II NP-Ficoll or T-cell–dependent NP-CGG antigens, no differences in other antibody responses were found in C481S or Y223F mice when compared with wild-type animals (Figure 5A-B). Similar to Y223F animals, a tendency toward higher IgG2b levels

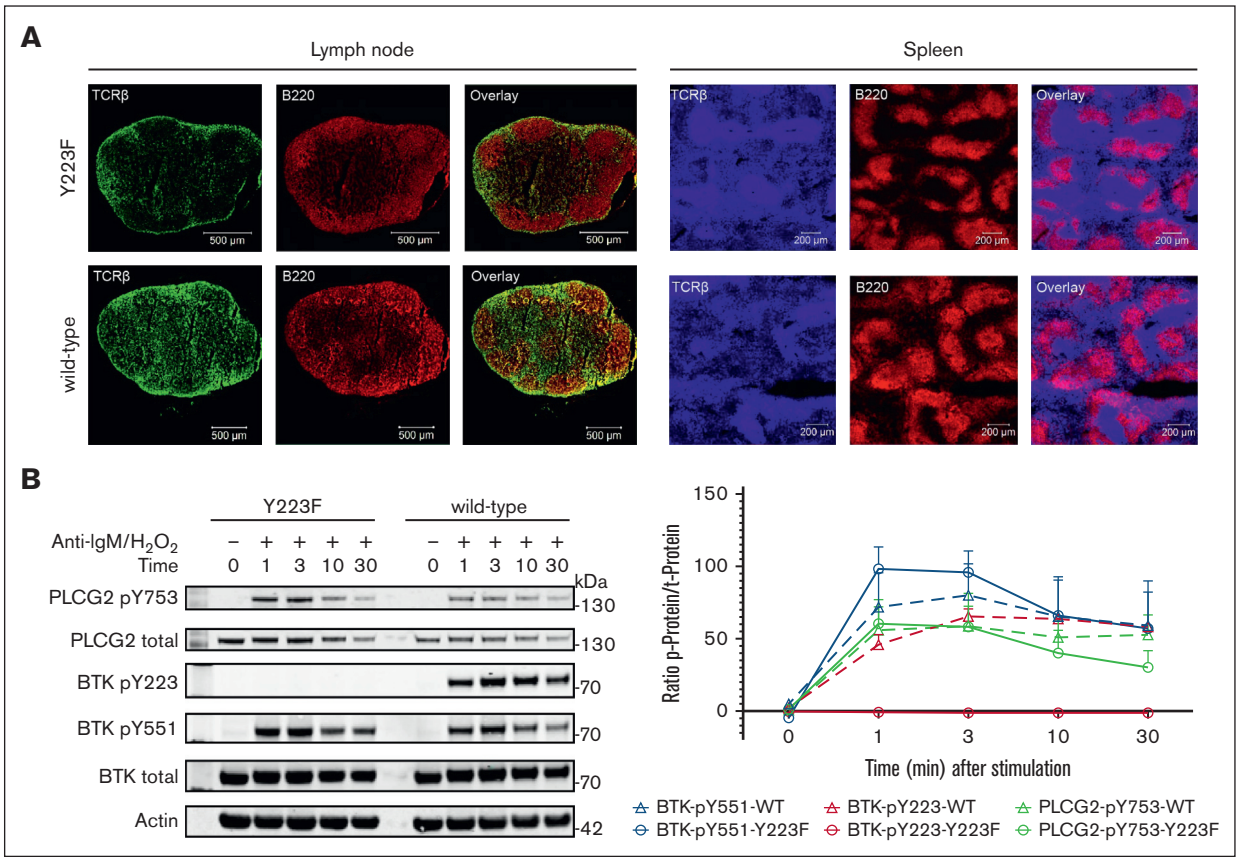

Figure 4. Y223F mice have unaltered structure of spleen and lymph nodes and functional BTK kinase activity upon stimulation of the BCR. (A) Immunofluorescence staining of spleen and lymph nodes sections. TCRβ (green) and B220 (red) staining in lymph nodes; TCRβ (blue) and B220 (red) staining in spleen. Scale bar 500 μm lymph nodes and 200 μm spleen. (B) Left, western blot on whole-cell lysates from stimulated WT and Y223F B cells. Right, ratio of phosphorylated protein over total protein (as quantified by densitometric analysis) from splenocytes. Splenocytes were stimulated with anti-IgM/H_2O_2 and whole-cell lysates were prepared after 1, 3, 10, and 30 minute after stimulation. None of the comparisons yielded any significant difference.

was observed in C481S mice before and after immunization, but there was no statistically significant difference when corrected for multiple testing (Figure 5A-B).

Old Y223F mice displayed no significant phenotypic changes when compared with wild-type mice

Dysregulation of B-cell activation could lead to the development of natural polyreactive antibodies with affinity for DNA and induce autoimmune disease caused by deficient B-cell tolerance checkpoints in aged mice.[35] We therefore quantified levels of anti-DNA reactivity in 14- to 16-month-old mice and did not find significant differences in Y223F mice when compared with wild-type controls (Figure 6A). Further studies that included histopathological analysis were also performed for the lung, spleen, kidney, and liver (Figure 6B; supplemental Figure 3-7; Table 1). Certain changes are known to be acquired over time, but no differences in the type or severity of the lesions were observed or associated in the presence of the Y223F substitution. Histological findings such as splenic and pulmonary hyperplasia, hepatic macrovesicular or microvesicular lipidosis, or renal tubule degeneration were considered age-related incidental lesions and found in both Y223F and wild-type mice (Figure 6B; supplemental Table 1). This indicates that there is no particular finding associated with the Y223F genotype that leads to differences in polyreactive antibody production or to the early onset of pathologies associated with aging.

Discussion

A detailed understanding of BTK's intracellular signaling is of particular importance in the field of hematology, given the success of inhibitors of BTK for the treatment of leukemia and lymphoma. Using a Y223F knockin mouse, we found no phenotypic differences among multiple parameters, including the usage of the κ- and λ-light chains in B-cell lineage from Sp, as compared with normal wild-type littermates. In contrast, phosphorylation of the Y223 residue, as noted by us and many other investigators, correlates well with BTK-induced phosphorylation of its major substrate, PLCG2, making it a useful readout for activated BTK.

The previous, comprehensive work on transgenic Y223F mice, support the idea that pY223 is not necessary for the activation of BTK. In these transgenic mice, a large number of phenotypes were completely normalized, with the exception of the mentioned, slightly reduced number of B lymphocytes using the λ light chain.[18] The reason for the discrepancy regarding the λ-light chain phenotype is unknown, but in contrast to the knockin mice, the transgenic strains express BTK from the CD19 promoter. It should also be noted that,

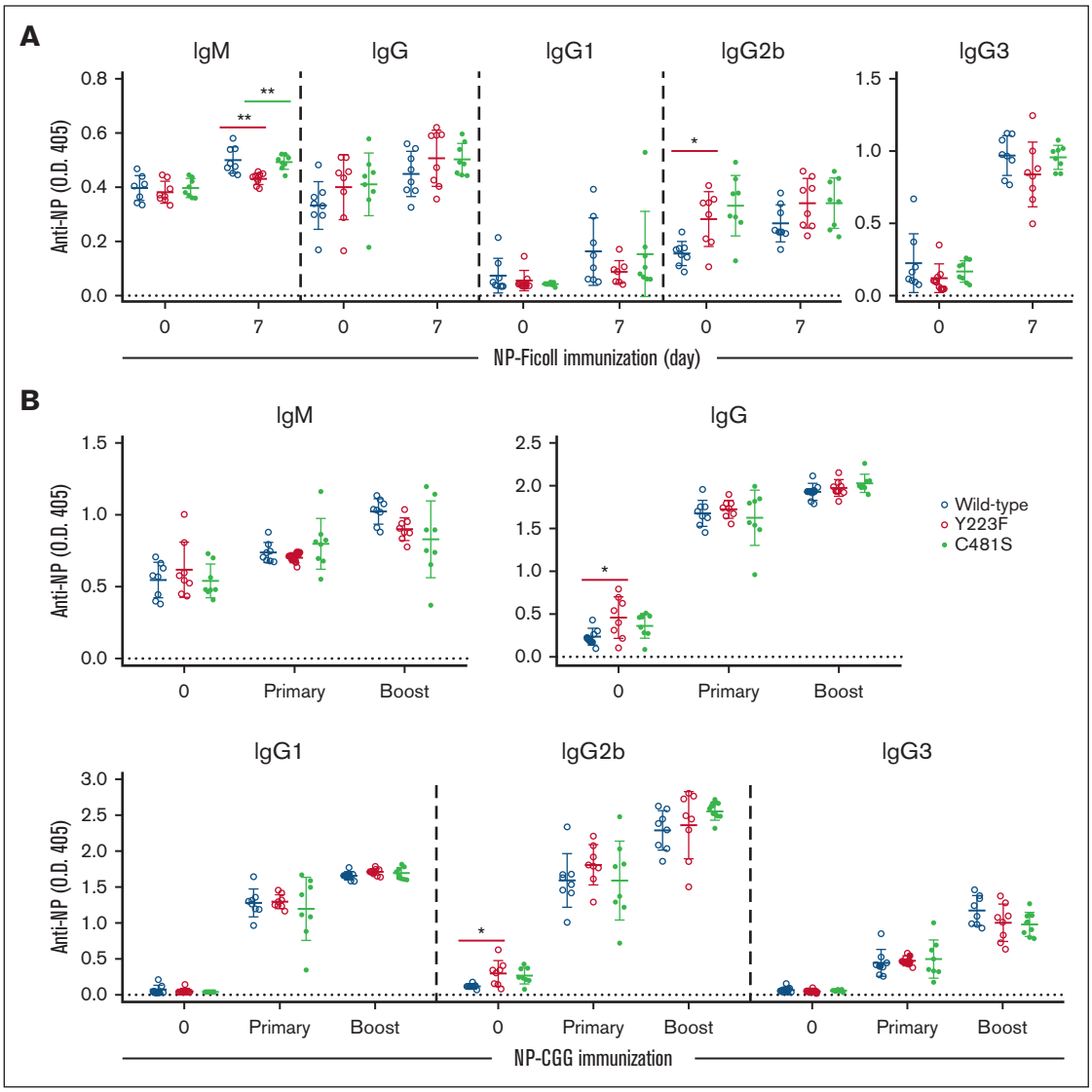

Figure 5. Levels of serum antibodies in response to immunization with both T-cell–dependent and –independent antigens. (A-B) Serum IgM, IgG, IgG1, IgG2b, and IgG3 anti-NP levels before and after NP-Ficoll or NP-CGG immunization. (A) Mice were injected intravenously with 50 μg NP-Ficoll in PBS and 7 days postimmunization blood was collected for the analysis. (B) Mice were intraperitoneally immunized with 100 μg NP-CGG, 100 μL inject alum and 3 weeks after mice were intravenously boosted with 50 μg NP-CGG. Seven days after first and boost immunization, blood was collected for quantification of serum antibodies levels. *$P < .05$ and **$P < .005$ (Kruskal-Wallis test). All significant values were corrected by multiple comparison Dunn test (alpha: 0.05).

as opposed to knockin mice, transgenic mice normally integrate various copy numbers of the transgene, and, moreover, the chromosomal location for the integration may affect the expression. Hence, in the reported transgenic Y223F strains,[18] 5 different lines were generated with the corresponding BTK expression levels in pro/pre-B, immature B, and mature cells varying considerably, making the readout complex.

Another observation of importance relates to patients with XLA. Since 1995, a mutation repository, BTKbase, has been collecting all mutations causing XLA, and the most recent update was published in 2023.[36] Substitutions in the SH3 domain are highly underrepresented as compared with all other domains,[36] and this may be related to the fact that SH3 domains in ABL and SRC kinases have a negative regulatory role,[37-39] with the possibility that this is also the case for BTK. In contrast, an exon deletion affecting the SH3 domain causes XLA.[40] Moreover, among the 1025 unique mutations reported to date, out of which 40.8% are missense mutations, none substitutes the Y223 residue in the SH3 domain for another amino acid. Although this does not disprove the idea that pY223 plays a role in the activation of B lymphocytes, it aligns with the idea that this residue is tolerant to replacements, although those causing instability of the protein are expected to cause XLA. In contrast, substitution of Y551 is a known variation that causes XLA with reported replacements to both asparagine (N) and histidine (H).[36,41,42]

A limitation of this project is that we are studying mice because it is known that in this species, variations in the *Btk* gene cause milder phenotypes as compared with humans. Thus, the final proof would

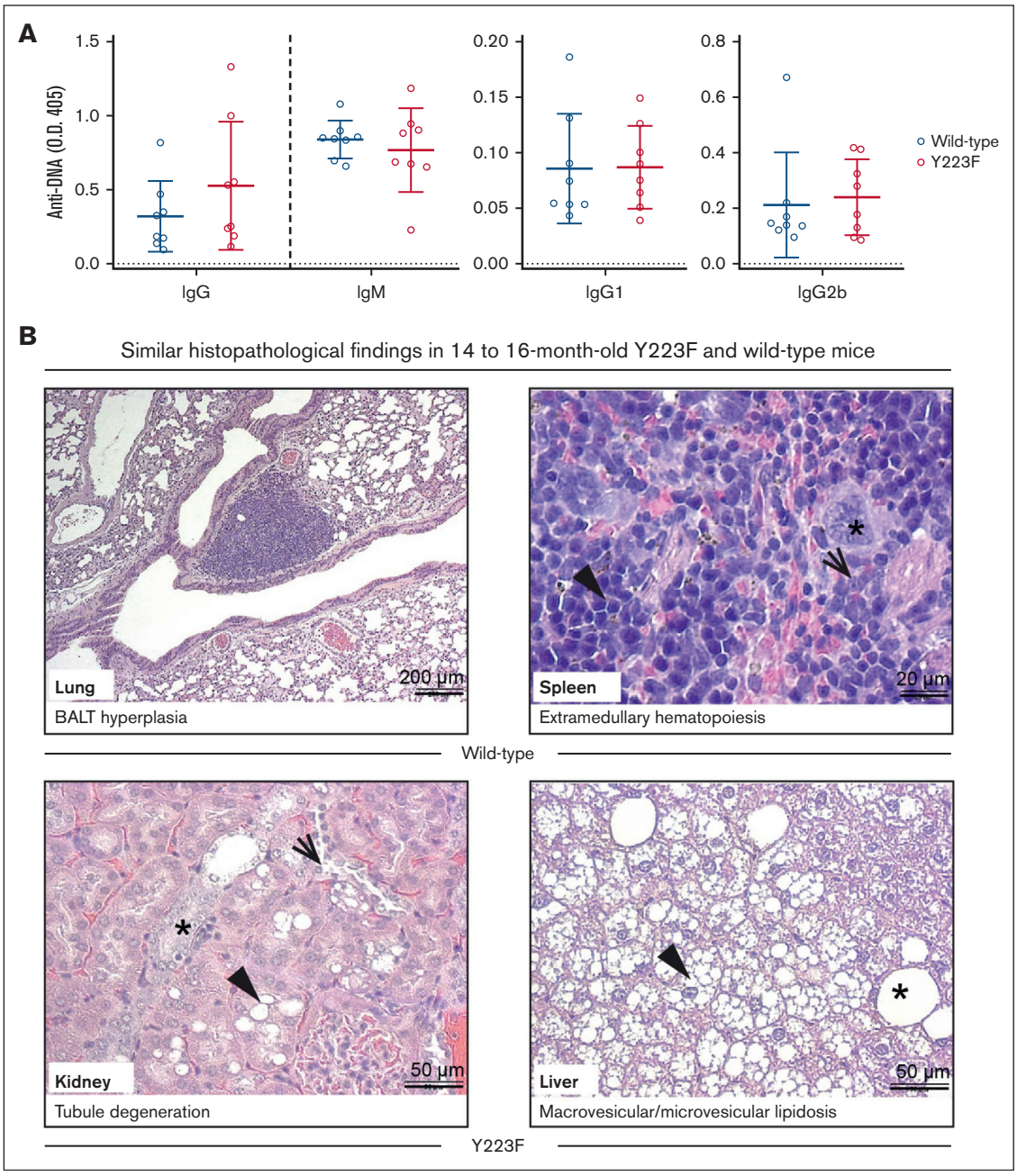

Figure 6. Similar serum anti-DNA antibody levels and histopathological findings were found in 14- to 16-month-old Y223F and wild-type mice. (A-B) Serum and organs were obtained from 14- to 16-month-old mice. (A) Serum IgM, IgG, IgG1, IgG2b anti-double strand DNA level at steady state. *$P < .05$, **$P < .01$, and ***$P < .001$ (Mann-Whitney test). None of the comparisons yielded any significant difference between Y223F and wild-type mice. (B) Representative data from histopathological analysis of formalin-fixed organs routinely processed with hematoxylin and eosin. Lung: prominent nodule of bronchus-associated lymphoid tissue (BALT); Spleen: filled arrow – erythroid precursor, arrow – myeloid precursor and (*) – megakaryocyte; Kidney: filled arrow – vacuolation, arrow – cellular sloughing and (*) – tubule basophilia; Liver: filled arrow – microvesicular and (*) – macrovesicular fatty changes.

be the detection of a Y223 substitution variation in humans and whether this causes any phenotype. To this end, in the recently released gnomAD mutation repository, in the v4 data set (GRCh38), which spans 730 947 exome sequences and 76 156 whole-genome sequences from unrelated individuals of diverse ancestries, there is a reported substitution of Y223 for cysteine (https://gnomad.broadinstitute.org/gene/ENSG00000010671?dataset=gnomad_r4).[43] This mutation was found in a female, and we cannot exclude that it could cause manifest disease in males. However, since this repository is not collecting patients with XLA, this finding is in line with our observations.

In conclusion, we believe that the concept of Y223 being of importance for the activation of BTK should be repudiated. The initial observation that Y223 plays a role in a heterologous fibroblast in vitro system and the idea that this would be of importance

also for BCR signaling is not supported by previous studies of chicken B cells or of primary B lymphocytes in transgenic mice. We have now taken this a step further by studying our knockin model with its introduced point mutation. Taken together with the fact that there are no reported replacements of Y223 in more than 1000 unrelated XLA families, this strongly argues against the concept that phosphorylation of Y223 promotes activation of BTK. In contrast, similar to all other cytoplasmic protein-tyrosine kinases, phosphorylation of Y551 in the kinase domain is essential for the activation of BTK. Given the great importance of BTK as a therapeutic target, we believe that it is crucial to understand the role of tyrosine phosphorylations for its enzymatic activity, and our findings strongly suggest that phosphorylation of Y223 is not essential for activity but remains a useful marker for BTK's catalytic activity.

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Authorship

Contribution: C.I.E.S. perceived the project and proposed the generation of the mouse model; R.M. and C.I.E.S. conceptualized the project, designed the work, and obtained the mouse model; H.Y.E., T.B., and R.M. performed phenotyping by flow cytometry; H.Y.E. performed western blot for kinase activity and in vivo experiments; H.Y.E., and N.F. performed κ- and λ-light chain analysis; A.C. performed histopathological analysis with hematoxylin and eosin; A.B. performed preparation and coordination of animal experiments; C.H. and M.C.I.K. carried out immunofluorescence staining, enzyme-linked immunosorbent assay, and autoantibodies assays; H.Y.E., T.B., A.B., C.H., M.C.I.K., and C.I.E.S. analyzed and interpreted results; R.Z. interpreted the results and assisted in obtaining the mouse model; T.B., C.H., and M.C.I.K. wrote selected parts of the manuscript; and H.Y.E., A.B., R.M., and C.I.E.S. wrote the manuscript.

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