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Brief Report

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Dysregulation of Toll-Like Receptor Signaling-Associated Gene Expression in X-Linked Agammaglobulinemia: Implications for Correlations Genotype-Phenotype and Disease Expression

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Keywords
Bruton's tyrosine kinase · B-lymphocytes · Gene expression profiling · X-linked agammaglobulinemia · Toll-like receptor · NF-kappaB

Abstract
Introduction: In X-linked agammaglobulinemia (XLA), the diversity of *BTK* variants complicates the study of genotype-phenotype correlations. Since BTK negatively regulates toll-like receptors (TLRs), we investigated if distinct *BTK* mutation types selectively modulate TLR pathways, affecting disease expression. ***Methods:*** Using reverse transcription-quantitative polymerase chain reaction, we quantified ten TLR signaling-related genes in XLA patients with missense (n = 3) and nonsense (n = 5) *BTK* mutations and healthy controls (n = 17). ***Results:*** *BTK*, *IRAK2*, *PIK3R4*, *REL*, *TFRC*, and *UBE2N* were predominantly downregulated, while *RIPK2*, *TLR3*, *TLR10*, and *TLR6* showed variable regulation. The missense XLA group exhibited significant downregulation of *IRAK2*, *PIK3R4*, *REL*, and *TFRC* and upregulation of *TLR3* and/or *TLR6*. ***Conclusion:*** Hypo-expression of *TLR3*, *TLR6*, and *TLR10* may increase susceptibility to infections, while hyper-expression might contribute to chronic inflammatory conditions like arthritis or inflammatory bowel disease. Our findings shed light on the important inflammatory component characteristic of some XLA patients, even under optimal therapeutic conditions.

Introduction

X-linked agammaglobulinemia (XLA) results from pathogenic genetic variants in Bruton's tyrosine kinase (BTK), a key regulator in B-cell development [1]. This primary antibody immune deficiency is characterized by the failure to produce mature B-lymphocytes and unsuccessful Ig heavy chain rearrangement. Genetic variants in all BTK domains can cause XLA, with missense mutations being most common, and numerous variants

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of uncertain significance and polymorphisms have been identified. Most mutations result in enzyme truncation, with CpG dinucleotide mutations being the most common events in XLA [2]. Two comprehensive cohort studies of XLA patients have revealed a wide spectrum of *BTK* mutation types, including missense, nonsense, indels, splicing, and deletions [3, 4]. However, no significant genotype-phenotype correlations were observed between patients' clinical manifestations and mutation types, either at diagnosis or during follow-up, underscoring the necessity for further research to fully elucidate this complex relationship [3, 4].

Toll-like receptors (TLRs) are an evolutionarily conserved receptor family involved in innate immunity by sensing structurally conserved molecules from microorganisms. TLRs are type I integral membrane glycoproteins with homology to interleukin (IL)-1R in the cytoplasmic region, an N-terminal extracellular domain with leucine-rich repeats, and a transmembrane domain activating a cytosolic signaling domain called TIR domain-containing adapter protein (TIRAP) [5]. This allows recognition of pathogen-associated molecular patterns, such as lipids, lipoproteins, and nucleic acids. TLRs are expressed in various cell types, including dendritic cells, phagocytes, B-lymphocytes, and endothelial cells [6, 7]. The mammalian TLR family has 13 members, with 10 identified in humans and 12 in mice [8]. Activation of pattern recognition receptors (PRRs) triggers intracellular pathways, inducing inflammatory cytokines, chemokines, and type I interferons, promoting the recruitment of leukocytes and other antigen-presenting cells [9].

BTK interacts with TIR domains of specific TLRs (4, 6, 8, and 9) and associates with signaling complex components like MYD88, TIRAP, and IRAK1 to activate nuclear factor kappa B (NF-kappaB), generating inflammation through proinflammatory cytokine transcription [10–13]. In XLA patients, BTK impairment affects lipopolysaccharide (LPS)-induced cytokine production (TNF, IL-1 beta, IL-6, and IL-10) by TLRs [14–17]. XLA patients are susceptible to encapsulated bacterial infections but typically not viral infections, except in cases such as echovirus-induced meningoencephalitis, which is often associated with a dermatomyositis-like syndrome and can be life-threatening [18]. Some patients develop autoimmune and/or inflammatory conditions like arthritis and inflammatory bowel disease (IBD) [19]. Low TLR activity may contribute to infection susceptibility, while hyperactivity is associated with chronic inflammatory diseases, including cardiovascular inflammation and arthritis [12, 20]. BTK inhibitors have been linked to rheumatoid arthritis prophylaxis, as BTK activates downstream of B-cell receptor, and Fc receptors in immunoglobulins [21]. However, BTK inhibitors do not fully mimic the XLA phenotype [22].

XLA-associated inflammatory conditions have been reported [19, 23, 24], with IBD/enteritis diagnosed in up to 10% of patients [23]. The risk of these complications is low, possibly due to immunoglobulin treatment's immunomodulation. A recent Chinese study of 98 XLA patients found 45.9% had arthritis, correlating with a higher incidence of *BTK* missense mutations, suggesting a genotype-phenotype relationship [25]. Our pilot molecular study aimed to evaluate TLR signaling pathway changes, shedding light on the impact of different *BTK* mutations on XLA clinical manifestations, diversity, and severity.

Methods

Subjects

Eight XLA patients were diagnosed based on the International Union of Immunology Societies criteria and the World Health Organization Scientific Group for primary immunodeficiency diseases [26, 27]: CD19+ B-lymphocytes below 2%, reduced or absent immunoglobulin subtypes in serum, and recurrent bacterial infection history. All patients received intravenous human immunoglobulin therapy. Demographic, laboratorial, and clinical characteristics are presented in Tables 1 and 2. Healthy male age-matched controls were used in all experiments. Total blood count and inflammatory markers (erythrocyte sedimentation rate, ferritin, and C-reactive protein) were tested for controls and patients at the Division of Clinical Pathology of the "Hospital de Clínicas da Unicamp."

Experiments

All reverse transcription-quantitative polymerase chain reaction (RT-qPCR) reagents and kits were acquired from Thermo Fisher Scientific Inc., Waltham, MA 02451, USA, and used according to manufacturers' standards. Ten TLR signaling-related target genes were quantified by RT-qPCR in XLA patients with missense (n = 3) and nonsense (n = 5) *BTK* mutations, compared to healthy controls (n = 17). Table 3 lists selected genes and their main functions. Quantifications were performed using a StepOnePlus™ Real-Time PCR System.

Total RNA Extraction from Peripheral Blood Mononuclear Cells and Complementary DNA Synthesis

Peripheral blood samples (6 mL) were collected in EDTA-containing tubes, centrifuged, and buffy coats were added to 1,200 µL RNAlater™ Stabilization Solution. PBMC total RNA was extracted using RiboPure™ Blood RNA Purification Kit and reverse transcribed with High-Capacity complementary DNA (cDNA) Reverse Transcription Kit with RNase Inhibitor. RNA quantification and purity were assessed by fluorometry (Qubit® 2.0 Fluorometer) and spectrophotometry (NanoDrop 2000). RNA integrity numbers were determined by Bioanalyzer 2100 Expert (B.02.08. SI648) (Agilent Technologies, Santa Clara, CA, USA).

Table 1. Mutational and clinical features of the studied X-linked agammaglobulinemia (XLA) patients

Patient	Mutations			Clinical manifestation	Reference (PMID)
	cDNA change (exon)	first published as	protein (domain)		
XLA01	c.83G>T (2)	c.215G>T (MS)	p.(Arg28Leu) (PH)	Atopic dermatitis, urticaria/obesity and dyslipidemia/UTI/eye infections/chronic cough with phlegm/IBD and diarrhea/testicular cancer	12204007
XLA02	c.1838G>A (18)	c.1970G>A (MS)	p.(Gly613Asp) (TK)	Chronic nonspecific inflammation/celiac disease linked to malabsorption	12204007
XLA03	c.119A>G (2)	c.251A>G (MS)	p.(Tyr40Cys) (PH)	Vaccine-associated paralytic poliomyelitis/erosive esophagitis/hyperplastic polyps of rectum/ nonspecific chronic gastritis (*Helicobacter pylori* positivity)/recently osteopenia	12204007
XLA04	c.586C>T (7)	c.718C>T (NS)	p.0 (TH)	UTI	20721470
XLA05	c.1041del (12)	1204delT (NS)	p.0 (SH2)	Left knee arthritis, chronic lung disease/asthma and left hearing loss/sore throat with mucus	20721470
XLA06	c.970_971insT (11)	c.1135insT (NS)	p.0 (SH2)	Pneumonia, bacterial infection, recurrent sinusitis and diarrhea/bronchiectasis and neutropenia	20721470
XLA07	IVS5+1G>A (5)	IVS5+1G>A (NS)	Exon 5 skipping (PH)	CMV, infectious mononucleosis, hepatitis A and B/ anal ulcer requiring surgery	26417435
XLA09	IVS5+1G>A (5)	IVS5+1G>A (NS)	Exon 5 skipping (PH)	Chronic lung disease/bacterial rhinosinusitis/severe diarrhea (large amount of liquid excreta), fever (38.5°C), diffuse abdominal pain/parietal wall pain and sigmoid associated with inflammatory process	

Family history is positive for all patients. XLA07 and XLA09 are maternal half-brothers. Three patients, XLA01–03, harbor missense mutations. In XLA01 and XLA02, a point mutation leads to an amino acid change, respectively in exons 2 (PH domain) and 18 (TK domain). In XLA03, the point mutation (exon 2; PH domain) leads to an amino acid change that causes an aberrant splicing and deletion of 23 nucleotides at the 3-end of exon 2. The variants shown are described using the *BTK* NM_000061.3 transcript reference sequence. For mutations: MS, missense; NS, nonsense or mutations which lead to a premature stop codon. For clinical manifestation: UTI, urinary tract infection; CMV, cytomegalovirus. For Reference: PMID, PubMed Identifier (also known as the PubMed reference number).

Qualified RNA samples were converted into cDNA (online suppl. Table 1; for all online suppl. material, see https://doi.org/10.1159/000540082). Absolute quantifications confirmed 10 ng cDNA was adequate for relative quantifications (online suppl. Table 2).

Gene Expression by RT-qPCR and Data Analyses

Relative quantification reactions were run in triplicates using TaqMan™ Universal Master Mix II, with UNG, respective primers, and purified deionized H_2O with 10 ng cDNA samples. Data were generated and analyzed by StepOne Software version 2.3. Quantifications with >0.3 CT value differences were excluded. GraphPad Prism 5 software (GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, (http://www.graphpad.com) was used for statistical analysis, with p values <0.05 considered significant. Wilcoxon matched-pairs signed rank test allowed comparison of relative quantification data to controls, calibrated to 1.0. For patients XLA04 and XLA05, five pairs were created; for others, 12 controls were considered. Spearman nonparametric test correlated all relative quantification data, including patients' ages.

Results and Discussion

The *BTK*, *IRAK2*, *PIK3R4*, *REL*, *TFRC*, and *UBE2N* targets were predominantly hypo-expressed, whereas *RIPK2*, *TLR3*, and *TLR10* did not show a specific predominance, with hypo- or hyper-expression. *TLR6* was the only gene that was predominantly hyper-expressed. Patients XLA01-03, who harbor a *BTK* missense mutation, had a marked downregulation for *IRAK2*, *PIK3R4*, *REL*, and *TFRC*, accompanied by a marked hyper-expression of *TLR3* and/or *TLR6* (Fig. 1). With the exception of *REL*, XLA09 followed a similar pattern. In contrast to the nonsense group (the other 5 patients in our cohort), the gene expression results were quite similar for the patients in the missense group, providing initial evidence of a gene expression signature.

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Table 2. Demographic and laboratorial features of the studied XLA patients

Patient	Age at collection	Age at onset	Age at diagnosis	BTK, %	Ig levels before diagnosis, mg/dL			ESR	CRP	Ferritin	WBC
				(>95%)	IgM	IgG	IgA	(≤10 mm)	(≤3.0 mg/L)	(30–400 ng/mL)	(4.0–10.0 × 10^3)
XLA01	25 years	4 years	6 years	26.0	6 (28–212)	298 (750–1,780)	20 (750–1,780)	13.00	18.50	214.90	18.61
XLA02	28 years	6 years	6 years	26.0	18 (28–212)	180 (750–1,780)	22 (90–450)	ND	5.03	ND	4.45
XLA03	27 years	6 years	6 years	9.0	UD (28–212)	UD (750–1,780)	3 (90–450)	ND	ND	ND	ND
XLA04	10 years	5 months	11 months	ND	31 (40–156)	126 (282–1,115)	18 (12–104)	2.00	0.38	44.13	5.13
XLA05	13 years	3 months	2 years 8 months	39.0	22 (29–195)	149 (610–1,610)	1 (40–289)	3.00	12.30	39.38	15.59
XLA06	18 years	4 months	2 years 8 months	4.5	26 (29–195)	86 (610–1,610)	23 (40–289)	7.00	2.09	64.96	6.15[a]
XLA07	19 years	ND	1 year 8 months	3.0	ND	ND	ND	48.00	90.00	16.54	15.60
XLA09	28 years	ND	4 years	2.5	51 (52–180)	44 (660–2,120)	<40 (68–500)	ND	ND	ND	ND

Numbers between brackets are laboratory reference values. The unspecific inflammatory markers ferritin, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) showed higher levels in patients with a high leukocyte number. ND, not determined; UD, undetectable; WBC, white blood cell. [a]Documented severe neutropenia. Part of these data has been previously published in Teocchi et al. [28].

Table 3. TLR signaling genes quantified by RT-qPCR in XLA patients and controls

Approved symbol and TaqMan® Identification	Approved name	Alias symbols	Gene groups	Function (UniProt)
BTK (Hs00975865_m1)	Bruton's tyrosine kinase	AGMX1, IMD1, XLA	Non-receptor tyrosine kinases	BTK is a non-receptor tyrosine kinase essential for B-cell development, differentiation, and signaling. It plays a critical role in B-cell receptor signaling, activating transcription factors such as nuclear factor kappa B (NF-kappaB), AP-1, and NFAT. Additionally, BTK is involved in cytokine receptor signaling and integrin-mediated adhesion and migration of B cells. Pathogenic variants in *BTK* result in XLA, a primary immunodeficiency characterized by the absence of mature B cells and low immunoglobulin levels
IRAK2 (Hs00176394_m1)	Interleukin 1 receptor-associated kinase 2		IRAK family	IRAK2, a serine/threonine kinase, plays a critical role in initiating innate immune responses by activating NF-kappaB and MAPK signaling pathways. It is involved in the signaling of various pattern recognition receptors (PRRs), including TLRs and IL-1 receptors. IRAK2 forms a complex with other signaling molecules, such as MYD88, IRAK1, and TRAF6, to propagate the immune response
PIK3R4 (Hs00300795_m1)	Phosphoinositide-3 kinase regulatory subunit 4	VPS15	Class III phosphatidylinositol 3 kinases (PI3Ks), phosphoinositide-3 kinase family	PIK3R4, also known as VPS15, is a regulatory subunit of the class III PI3K complex, playing a crucial role in autophagy, endosome trafficking, and lysosome biogenesis. It regulates autophagosome formation, maturation, and fusion with lysosomes, as well as endosome-to-Golgi retrograde transport
REL (Hs00968440_m1)	REL proto-oncogene, NF-kB subunit	c-Rel	NF-kappaB family	REL is a subunit of the NF-kappaB transcription factor complex, central to regulating immune and inflammatory responses, cell survival, and proliferation. It participates in B-cell development and function, as well as the regulation of various genes involved in immune responses, inflammation, and cell survival. Dysregulation of REL has been implicated in diseases such as B-cell lymphomas and autoimmune disorders
RIPK2 (Hs01572686_m1)	Receptor-interacting serine/threonine kinase 2	CARDIAK, RICK, RIP2	RIP homotypic interaction motif (RHIM)-containing kinases	RIPK2 is a serine/threonine kinase that plays a critical role in innate immune responses by activating NF-kappaB and MAPK signaling pathways. It is involved in the signaling of intracellular PRR, such as NOD1 and NOD2, which recognize bacterial peptidoglycan components. RIPK2 forms a complex with other signaling molecules, including XIAP, TRAF6, and IKK, to propagate the immune response and induce the production of proinflammatory cytokines and chemokines
TFRC (Hs00951083_m1)	Transferrin receptor	CD71, T9, TR, p90	CD molecules, transferrin family	TFRC, also known as CD71, is a cell surface receptor-mediating cellular uptake of iron-bound transferrin, crucial for iron homeostasis, cell growth, and differentiation. Highly expressed on rapidly dividing cells, including activated lymphocytes and malignant cells, dysregulation of TFRC is implicated in anemia, iron overload, and cancer. It is essential for erythropoiesis, neurologic development, and significantly affects thymocyte maturation and B-cell development

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Table 3 (continued)

Approved symbol and TaqMan® Identification	Approved name	Alias symbols	Gene groups	Function (UniProt)
TLR3 (Hs01551078_m1)	Toll-like receptor 3	CD283	TLR family, CD molecules	TLR3 is a PRR crucial for innate immune responses to viral infections. It recognizes double-stranded RNA (dsRNA), a molecular pattern associated with viral replication. TLR3 activation induces type I interferons and proinflammatory cytokines production through NF-kappaB, IRF3, and AP-1 transcription factors. TLR3 signaling is essential for host defense against various viruses, including herpes simplex virus, West Nile virus, and respiratory syncytial virus
TLR6 (Hs01039989_s1)	Toll-like receptor 6	CD286	TLR family, CD molecules	TLR6 is a PRR crucial for innate immune responses to microbial pathogens, particularly Gram-positive bacteria and fungi. It recognizes diacylated and triacylated lipopeptides, components of bacterial cell walls. TLR6 forms heterodimers with TLR2 to initiate signaling pathways, activating NF-kappaB and leading to the production of proinflammatory cytokines and chemokines. TLR6 signaling is essential for host defense against various bacterial and fungal pathogens, including *Staphylococcus aureus*, *Mycobacterium tuberculosis*, and *Candida albicans*
TLR10 (Hs01935337_s1)	Toll-like receptor 10	CD290	TLR family, CD molecules	TLR10 is a PRR with mainly inhibitory properties. Its specific ligand and signaling pathway are not well understood, but it is believed to modulate immune responses by interacting with other TLRs, such as TLR1, TLR2, and TLR6. Blocking TLR10 with antagonistic antibodies enhances the production of proinflammatory cytokines, including IL-1 beta, specifically after exposure to TLR2 ligands. TLR10 may play a role in the regulation of immune responses to various pathogens and in the development of chronic inflammatory conditions
UBE2N (Hs00854751_s1)	Ubiquitin-conjugating enzyme E2 N	UBC13, HEL-S-71	Ubiquitin-conjugating enzymes E2, ubiquitin-conjugating enzyme E2 N subfamily	UBE2N, also known as UBC13, is an E2 ubiquitin-conjugating enzyme crucial for regulating cellular processes such as DNA repair, immune signaling, and protein degradation. It is involved in activating NF-kappaB and MAPK signaling pathways in response to various stimuli, including TLR activation, DNA damage, and genotoxic stress. Dysregulation of UBE2N has been implicated in diseases like cancer, autoimmune disorders, and neurodegenerative diseases

Gene approved symbols, names, alias symbols, groups, and functions were accessed on the official websites of the HUGO Gene Nomenclature Committee (HGNC; genenames.org) and the Universal Protein Resource (UniProt; uniprot.org) on March 10, 2024. Codes between brackets under gene symbols are TaqMan® identification numbers for reverse transcription-quantitative PCR (RT-qPCR).

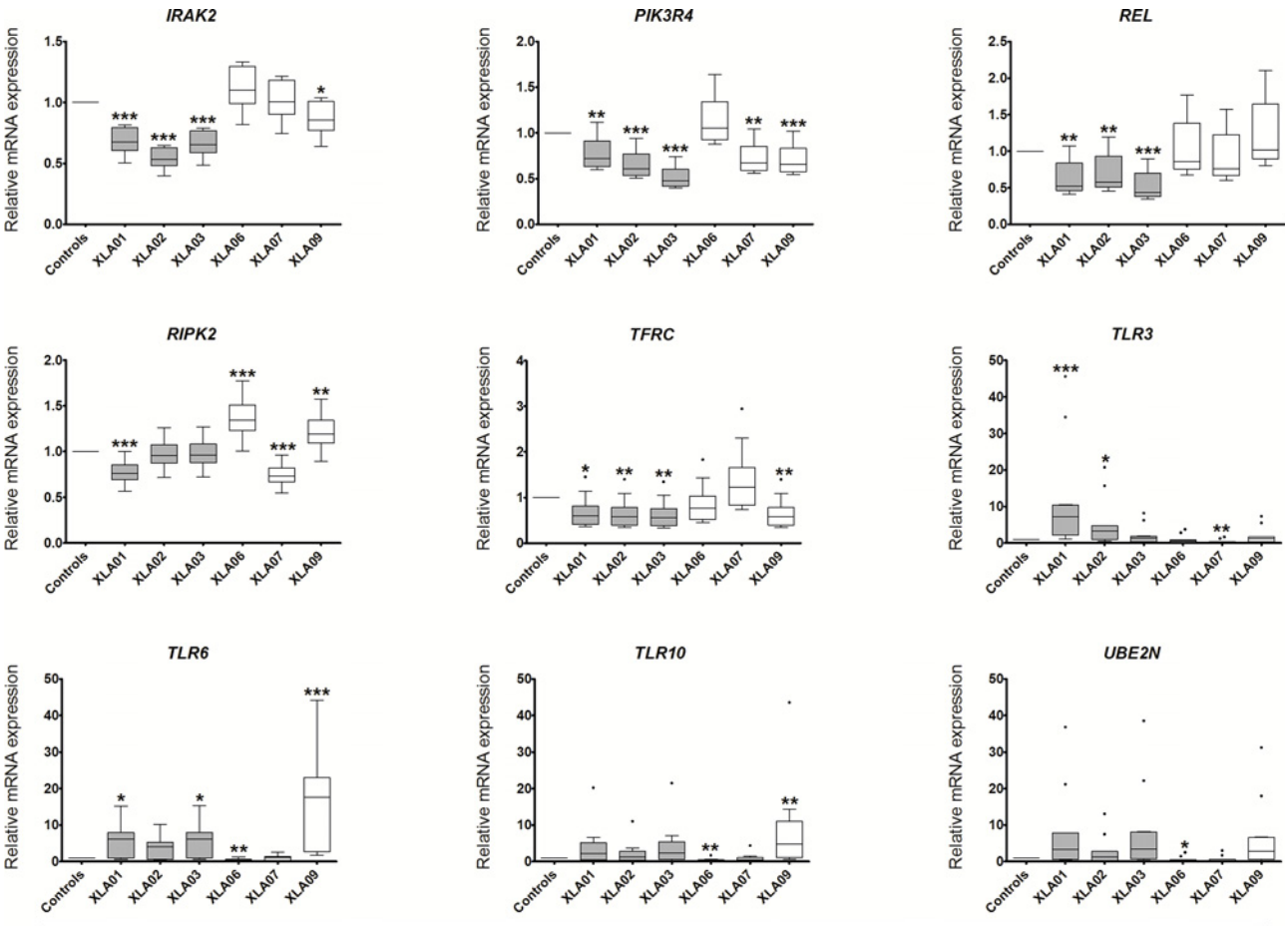
BTK mutation consequences analyzed in the XLA scenario revealed a complexity through numerous signaling pathways affecting the expression of hundreds of genes, proteins, and molecular regulators [29]. BTK functions in the immune system analyzed as a whole – which may differ in specific cell lines and subgroups [30–32] – have not been completely elucidated notwithstanding its well-known role as a key regulator of B-cell development [33]. Accordingly, correlations between the XLA clinical manifestation diversity/severity and the genetic defect are inconclusive [3, 34]. Mutations in *BTK* are very miscellaneous with no single mutation accounting for more than 3% of patients. Missense mutations seem to impact more severely on XLA [23, 34]. Autoimmunity and/or chronic inflammation in XLA may be directly related to TLR signaling malfunction, leading to complications that are consistent with a diagnosis of arthritis, IBD, or other inflammatory conditions [19, 23]. Large studies described the high morbidity and mortality of XLA patients despite progress in diagnosis and treatment [4]. Regional differences were shown in the frequency of IBD and enteroviral meningoencephalitis complications that were found to be more prevalent in South American patients [24].

As anticipated, *BTK* downregulation was observed in all patients, except for XLA03 (Fig. 1, 2), who exhibited significant *BTK* hyper-expression ($p = 0.001$) despite known protein-level downregulation [28]. The mutation type may influence *BTK* expression, with nonsense mutations leading to RNA decay and *BTK* hypo-expression [28]. However, XLA03 (missense mutation) represents the first instance of *BTK* hyper-expression, possibly due to a compensatory mutational mechanism. Although inefficient since the protein remains defective, further studies on this patient could elucidate *BTK* expression regulators, as the molecular mechanisms behind this upregulation are unclear. It is crucial to note that the youngest patients, XLA04 and XLA05, were compared to only five control individuals instead of 12, potentially affecting the significance of our results. Consequently, we adjusted the threshold of significance ($0.1 > p > 0.05$) for some data (Fig. 1, 2).

TLR signaling-associated gene expression variability in XLA patients may influence clinical manifestations (Fig. 3). Despite individual differences and epigenetic factors, we assume that for each patient, dysregulation of TLR signaling gene expression results from mutations causing absent or defective BTK. BTK is involved in sensing microbes through multiple TLRs, including TLR2, TLR3, TLR4, TLR7/8, and TLR9 in human and mouse macrophages and dendritic cells [33]. However, it has been shown that the absence of BTK in XLA neutrophils does not impair functional responses to TLR signals (LPS for TLR4; imidazoquinoline compound for TLR7/8) [30]. Lee et al. [35] demonstrated that BTK directly phosphorylates TLR3, playing a critical role in initiating its signaling. Without BTK, TLR3-induced PI3K, AKT, and MAPK signaling, as well as activation of NF-kappaB, interferon regulatory factor 3, and AP-1 transcription factors, are defective, compromising the formation of the downstream TICAM1-RIPK1-TBK1 complex. Additionally, BTK-deficient macrophages fail to secrete inflammatory cytokines and interferon beta 1 (IFNB1) upon TLR3 stimulation [35].

XLA patients typically respond to various viral infections; however, their responses to enterovirus genus (e.g., echovirus), coxsackie, and live polio vaccine virus are compromised, potentially causing progressive or chronic enteroviral meningoencephalitis, arthritis, or enteritis [36, 37]. Recently, Hedin et al. [38] described a case of a 47-year-old man with XLA who developed severe tick-borne encephalitis (TBE) and was treated with plasma containing TBE virus (TBEV) IgG antibodies as rescue therapy. The patient carries a *BTK* splice-site mutation (c.1349+4A>G), which was predicted to cause skipping of exon 14 and a frameshift with a premature stop codon (p.V450Gfs*13). Despite the absence of B cells, the patient mounted a robust TBEV-specific CD8+ T cell response [38]. This finding suggests that XLA patients can clear acute viral infections and may benefit from future TBEV vaccination, highlighting the importance of cell-mediated immunity in controlling viral infections in patients with humoral immunodeficiencies [38].

BTK appears to positively regulate TLR3 signaling and antiviral responses through direct phosphorylation of TLR3, while other proteins like UBAC1 and TRIM8 provide negative regulation of the TLR3 pathway. The BTK-TLR3 interaction may have implications for antiviral immunity and potentially vaccine responses [33]. *TLR3* hypo-expression may be linked to higher susceptibility to viral infections, as observed in patient XLA07. Conversely, *TLR3* hyper-expression, found in patients XLA01 and XLA02, may contribute to their susceptibility to chronic inflammatory conditions. Our data on *TLR3* expression dysregulation supports Barmetller et al.'s [23] hypothesis that XLA patients with missense *BTK* mutations exhibit more clinical phenotypes than those with nonsense mutations.



XLA patients			Genes									
Samples	*BTK* Mutation	Age	*BTK*	*IRAK2*	*PIK3R4*	*REL*	*RIPK2*	*TFRC*	*TLR3*	*TLR6*	*TLR10*	*UBE2N*
XLA01	Missense	25	***0.0005	***0.0005	**0.0034	**0.0034	***0.0010	*0.0122	***0.0005	*0.0161	ns0.0771	ns0.0640
XLA02	Missense	28	**0.0034	***0.0005	***0.0005	**0.0068	ns0.3804	**0.0093	*0.0210	ns0.0640	ns0.4238	ns0.3394
XLA03	Missense	27	***0.0010	***0.0005	***0.0005	***0.0005	ns0.5693	**0.0068	ns0.4238	*0.0161	ns0.0640	ns0.0640
XLA04	Nonsense	10	0.0625	ns>0.9999	ns0.1875	ns0.625	ns0.3125	ns0.4375	*0.0313	0.1563	*0.0313	*0.0313
XLA05	Nonsense	13	0.0625	0.0625	ns0.8125	ns>0.9999	ns>0.9999	0.0625	*0.0313	*0.0313	0.0625	*0.0313
XLA06	Nonsense	18	***0.0005	ns0.0640	ns0.2334	ns0.6772	***0.0005	ns0.2036	ns0.2334	**0.0024	**0.0034	*0.0425
XLA07	Nonsense	19	***0.0005	ns0.9697	**0.0015	ns0.6221	***0.0005	ns0.0923	**0.0034	ns0.9697	ns0.1294	ns0.0923
XLA09	Nonsense	25	***0.0005	*0.0210	***0.0010	ns0.5693	**0.0049	**0.0093	ns0.9697	***0.0005	**0.0093	ns0.0640

Fig. 1. Peripheral blood mononuclear cell (PBMC) expression of *IRAK2*, *PIK3R4*, *REL*, *RIPK2*, *TFRC*, *TLR3*, *TLR6*, *TLR10*, and *UBE2N* in X-linked agammaglobulinemia (XLA) patients. *GAPDH* and *HPRT1* were used as reference genes. Control samples were calibrated as reference samples, and their relative quantification were always 1.0. All quantifications for patients were benchmarked to the reference samples. Filled boxes correspond to patients with missense *BTK* mutations (XLA01–03) while clear boxes correspond to those with mutations that lead to a premature stop codon (XLA06, XLA07, and XLA09). For statistical analyses, patients were separated in two groups: children (XLA04 and XLA05; $n_{controls} = 5$) and young XLA patients (XLA01–03, XLA06, XLA07, and XLA09; $n_{controls} = 12$). Pediatric patients are not represented by box plots. The Wilcoxon matched-pairs signed rank test was used for the comparison between pairs. All p values are described in the table below the figure. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, nonsignificant. Significant hyper-expressions were marked with hot colors, e.g., yellow (*), orange (**), and red (***). Cold colors were used for meaningful hypo-expressions: light green (*), green (**), and light blue (***). p values in the threshold of significance ($0.1 > p > 0.05$) were shown with red (upregulation) or blue (downregulation) fonts.

BTK deficiency and the resulting impairment of TLR3-induced PI3K and AKT activation likely affect the mechanistic target of rapamycin (mTOR) signaling pathway, a major downstream target of PI3K/AKT [39]. mTOR exists in two distinct multiprotein complexes, mTORC1 and mTORC2, which regulate

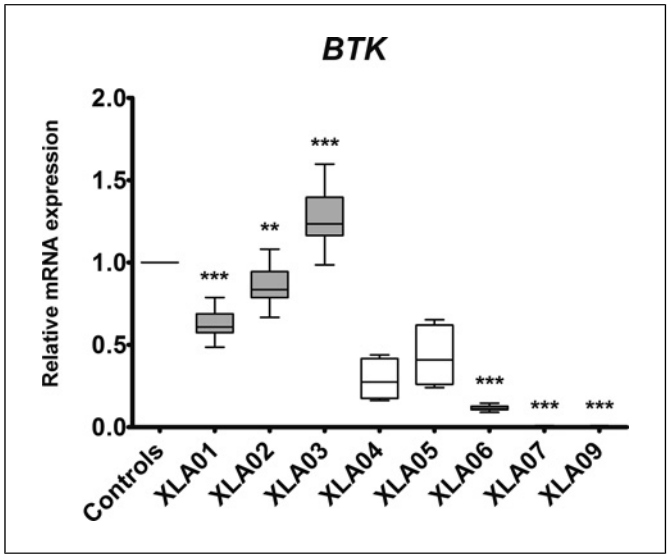


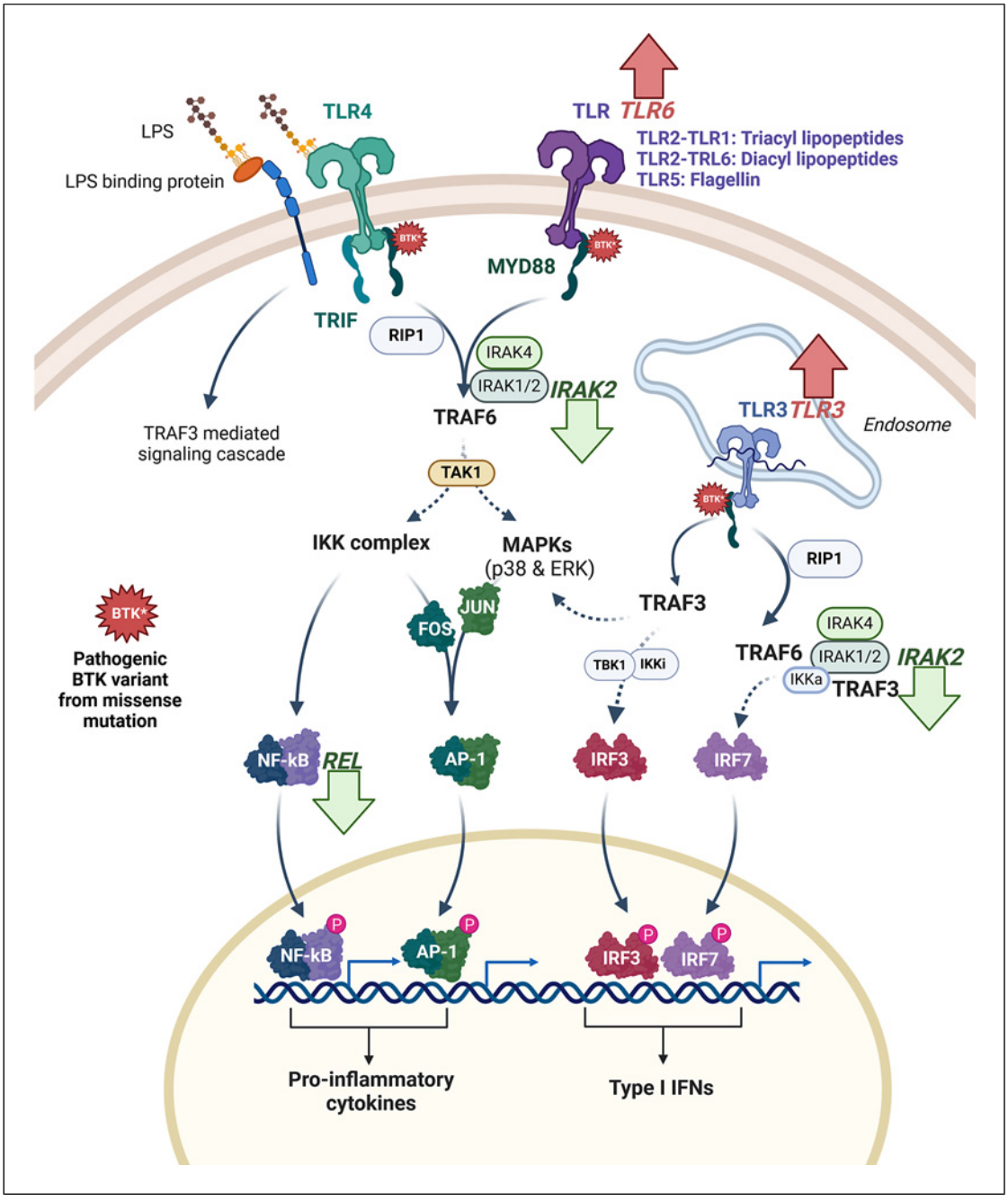
Fig. 2. Expression of *BTK* in PBMC from XLA patients and healthy controls. Relative expression of the *BTK* gene in XLA patients with mutations that lead to a premature stop codon (clear boxes) and patients with missense mutations (filled boxes). The *y*-axis represents the quantitative data of the relative mRNA expression of *BTK* (fold-change). Data from RT-qPCRs were generated from *GAPDH* and *HPRT1* as reference genes, in combination. Data are shown as median with interquartile range (whiskers: minimum to maximum). The Wilcoxon matched-pairs signed rank test was carried out for statistical analysis, in which all patients were compared to 12 healthy matched controls, except for XLA04 and XLA05 (young patients), who were compared to 5 healthy matched controls. For them, *BTK* was under-regulated with a p value of 0.0625, while for other patients the p value reached 0.0034 for XLA02 and 0.0005 for the remaining patients (**$p < 0.01$; ***$p < 0.001$). RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

protein and lipid syntheses, autophagy, and cell survival [39]. In XLA, impaired PI3K and AKT activation may reduce mTORC1 activity, as AKT phosphorylates and inhibits TSC1/2, a negative regulator of mTORC1 [39]. This could decrease phosphorylation of mTORC1 targets, RPS6KB1 (S6K1) and 4E-BPs, involved in protein synthesis and cell growth [39]. Additionally, mTORC2, regulated by PI3K, phosphorylates and activates AKT [39]. Thus, impaired PI3K activation due to BTK deficiency may affect mTORC2 activity, reducing AKT phosphorylation and potentially impacting cell survival and metabolism. This scenario may contribute to defective inflammatory cytokine and IFNB1 secretion in BTK-deficient macrophages upon TLR3 stimulation [35]. Further studies investigating the impact of BTK deficiency on mTORC1 and mTORC2 activity and their downstream targets in TLR3 signaling would provide more definitive insights into mTOR's role in this cascade.

TLR6 showed the highest upregulation in XLA patients, with significant downregulation in XLA06, characterized by pneumonias, bacterial infections, and recurrent sinusitis. XLA09 had the highest *TLR6* mRNA level ($p = 0.0005$) and shared some manifestations with XLA06, but experienced worsened symptoms due to a sigmoid inflammatory process (Table 1). TLR6 overregulation appears to promote inflammation, which becomes chronic over time. Conversely, *TLR10* hyper-expression in XLA patients might indicate anti-inflammatory signaling. Blocking TLR10 with antagonistic antibodies enhances proinflammatory cytokine production, including IL-1 beta, specifically after exposure to TLR2 ligands [40, 41]. This modulatory property seems to follow *TLR6* expression in our patients. Oosting et al. [40] emphasized that inhibitory effects of TLR10 are likely mediated through interaction with TLR2 and possibly TLR1 and TLR6. TLR6 and TLR10 expressions showed the highest correlation in our patients ($p = 0.0004$), with significant overregulation in XLA09 ($p = 0.0093$). TLR10 hyper-expression was observed in patients with chronic inflammatory conditions, while hypo-expression was seen in XLA06, prone to bacterial infections. Differently, patient XLA04 also had *TLR10* downregulation without *TLR6* upregulation. As the youngest patient in our cohort, his *TLR10* quantification was in accordance with the positive correlation with patient's age ($p = 0.0218$). His clinical manifestations were mild, mainly characterized by urinary tract infections.

The role of IRAK2 in inflammatory signaling pathways has been extensively studied, revealing important differences between humans and mice. In humans, IRAK2 is a critical positive regulator of TLR/IL-1R signaling, essential for early NF-kappaB and MAPK activation, cytokine production, and post-transcriptional regulation of inflammatory mediators like TNF [42]. However, in mice, IRAK2 appears to play a more nuanced role, with studies in *Irak2*$^{-/-}$ cells showing intact early TLR responses but impaired late NF-kappaB activation and cytokine production [43, 44]. This discrepancy likely stems from the existence of inhibitory IRAK2 splice variants in mice, which are absent in humans [45].

Wang et al. [46] identified a functional IRAK2 variant in humans associated with increased colorectal cancer risk, potentially by impairing IRAK2-mediated TRAF6 ubiquitination and subsequent IL-6 transcription.



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(For legend see next page.)

Notably, patient XLA03 was diagnosed with hyperplastic rectum polyps. Considering IRAK2's crucial role in propagating inflammatory signaling in human cells, its reduced expression may contribute to an aberration in this signaling cascade in patients with XLA [42]. However, the overall impact on the immune dysregulation and clinical manifestations in XLA likely depends on the intricate interplay between various inflammatory and anti-inflammatory pathways regulated by IRAK2 and its interacting partners. Moreover, a significant positive correlation was found between IRAK2 and PIK3R4 expressions (p = 0.0218), which suggests a potential

crosstalk between IRAK2-mediated inflammatory pathways and PI3K signaling, which warrants further investigation in the context of XLA pathogenesis.

PIK3R4 plays a crucial role in autophagosome biogenesis and endosome trafficking [47]. In innate immunity, autophagy is responsible for eliminating intracellular microbes through autolysosomes, distributing cytosolic microbial antigens to PRRs, and acting as an antimicrobial effector for TLRs and other PRR signaling [48]. Deficient autophagy from aberrant variants, linked to inflammatory disorders like Crohn's disease [48], may contribute to the molecular pathogenesis of gastrointestinal illnesses in the missense group (Table 1) if hypoexpression correlates with protein levels. Moreover, a study associated autosomal recessive agammaglobulinemia with a homozygous mutation in *PIK3R1* [49], emphasizing the crucial role of class IA phosphoinositide-3 kinases in immune function and regulation.

All 3 patients harboring a *BTK* missense mutation exhibited significant downregulation of *REL*. Also, taking all 45 comparison pairs for all the 10 targets into account, the only meaningful negative correlation was between *BTK* and *REL* ($p = 0.0368$). Four other genes are members of the NF-kappaB complex subunits gene family: *RELA* (p65), *RELB*, *NFKB1* (p50/p105), and *NFKB2* (p52) [50]. Different dimer combinations act as transcriptional activators or repressors, with the RELA/REL heterodimer being an activator [51]. *REL* polymorphisms are associated with ulcerative colitis and RA, and variants in *RELB* and *NFKB1* have been linked to primary immunodeficiencies [52–55]. Low *REL* mRNA levels in patient XLA03, associated with rectum hyperplastic polyps, may be linked to increased susceptibility to disease observed in $Rel^{-/-}$ knockout mice with higher intestinal polyp formation and proliferation rates [56, 57].

The IKK complex, composed of CHUK, IKBKB, and IKBKG (also known as IKK-alpha, IKK-beta, and IKK-gamma or NEMO, respectively), is a key player in the NF-kappaB signaling pathway downstream of IRAK/TRAF/TRADD [58]. TRAF6 activates the IKK complex through ubiquitination events [58], and impaired TRAF6 ubiquitination due to IRAK2 dysfunction could reduce IKK complex activation, affecting NF-kappaB signaling and target gene expression, such as IL-6. The significant downregulation of *REL* in patients with BTK missense variants suggests altered IKK complex activity, as the IKK complex phosphorylates and degrades NF-kappaB inhibitor proteins, which normally sequester NF-kappaB dimers (including REL-containing dimers) in the cytoplasm [58]. Moreover, autophagy deficiency resulting from aberrant PIK3R4 variants and autosomal recessive agammaglobulinemia associated with a homozygous *PIK3R1* mutation may also be connected to altered IKK complex function, as autophagy regulates IKK activity and NF-kappaB signaling [59], and PI3K signaling can modulate IKK activity through AKT-mediated phosphorylation of CHUK [60].

Of note, BTK is a negative regulator of the NLRP3 inflammasome in human and murine myeloid cells [61]. BTK deficiency in XLA patients and mice or pharmacological BTK inhibition leads to enhanced NLRP3-mediated IL-1 beta processing and release, suggesting a

Fig. 3. TLR3/6 signaling dysregulation in XLA due to *BTK* missense mutations. Bruton's tyrosine kinase (BTK) directly interacts with the TIR domains of specific TLRs, including TLR4, TLR6, TLR8, and TLR9, and associates with key signaling components such as MYD88, TIRAP, and IRAK1. Also, BTK initiates the signaling of TLR3 by directly phosphorylating it, playing a pivotal role in the process. Upon ligand binding, TLRs dimerize and initiate downstream signaling cascades through the recruitment of adapter proteins like MYD88 and TRIF. The MYD88-dependent pathway involves the formation of the Myddosome complex, comprising MYD88, IRAK4, and IRAK1/2. IRAK1 activates the E3 ubiquitin ligase TRAF6, leading to the synthesis of K63-linked polyubiquitin chains. This recruits and activates the TAK1 complex, which phosphorylates and activates the canonical IKK complex, ultimately resulting in NF-kappaB activation. Additionally, TAK1 activates the MAPK pathways, including JNK, p38, and ERK1/2. In the TRIF-dependent pathway, TRIF recruits TRAF6 and TRAF3. TRAF6, a critical signaling molecule, could potentially convert TLR3 and TLR6 signals to activate MAPK and ERK pathways, leading to the induction of additional inflammatory mediators. TRIF also promotes TRAF3-dependent activation of TBK1 and IKKi, which phosphorylate and activate IRF3. The activation of transcription factors such as NF-kappaB, AP-1, CREB (through p38, not shown), and IRF3/7 cooperatively induces the expression of proinflammatory cytokines and type I interferons. In XLA patients, the impaired TLR signaling leads to dysregulated cytokine production, including reduced levels of TNF, IL-1 beta, IL-6, and IL-10 in response to LPS stimulation (TLR4). Conversely, in these patients, impaired TLR BTK-negative regulation could result in chronic inflammation driven by sustained activation of other transcription factors, such as AP-1 and CREB, downstream of MAPK pathways. This dysregulation may contribute to the clinical manifestations of XLA, such as recurrent infections and potential inflammatory complications like arthritis and inflammatory bowel disease, particularly in patients harboring *BTK* missense mutations. A different scenario may be involved in the pathophysiology of X-linked agammaglobulinemia (XLA) due to *BTK* nonsense mutations, supporting a possible genotype-phenotype correlation (Fig. 2). The green arrows indicate downregulated targets in the signaling pathway; the red ones, upregulated. Created using BioRender (biorender.com).

potential role for dysregulated NLRP3 inflammasome activation in the inflammatory phenotype of some XLA patients [61]. Negative regulatory mechanisms may have a crucial role in controlling inflammatory responses [62]. Low levels of REL and IRAK2 could indicate reduced activation of the NF-kappaB signaling pathway and potentially lead to abnormal inflammatory responses, contributing to immune dysregulation and the development of inflammatory disorders or autoimmune conditions (Fig. 3). Proper regulation of inflammatory pathways, such as NF-kappaB and the NLRP3 inflammasome, is essential to prevent excessive inflammation, tissue damage, and the development of chronic inflammatory diseases [62].

Sanaei et al. [63] found increased transcripts of *SOCS1* and *RNF216* in XLA PBMCs compared to healthy PBMCs, with downregulation observed after TLR inductions in XLA patients. *IRAK3* showed a significant increase after LPS treatment in healthy PBMCs but a decrease in XLA PBMCs, with lower transcripts in XLA PBMCs compared to healthy PBMCs after both LPS and CpG-A treatments. The authors mention conflicting results from studies on cells from XLA patients regarding impairments in TLR signaling and cytokine production. In PBMCs from XLA patients, phosphorylation and degradation of CHUK were normal, while phosphorylation levels of p38 (MAPK14) were lower. This suggests that BTK may activate the MAPK pathway rather than the canonical NF-kappaB pathway in these cells [14, 17, 63].

Furthermore, the X-box-binding protein 1 plays a pivotal role in the unfolded protein response (UPR) and is also activated by TLR signaling, linking the UPR to the inflammatory response [64]. This activation is distinct from its canonical role in the UPR, and it has been demonstrated to be implicated in the pathogenesis of rheumatoid arthritis [64]. Also, the crosstalk between the UPR and the NF-kappaB signaling pathway has been well-documented [65]. X-box-binding protein 1 upregulation in XLA patients may be associated with inadequate TLR activation and could potentially contribute to their chronic inflammatory disorders [28].

Together with *IRAK2*, *PIK3R4*, and *REL*, *TFRC* forms the tetrad of downregulated genes in our XLA missense group. Marked *TFRC* downregulation in XLA01-03 and XLA09 ($p \leq 0.0122$) suggests a proinflammatory response and chronic inflammation in XLA patients, as TFRC is associated with combined immunodeficiency and adaptive immunity [66]. Our patients with *TFRC* downregulation displayed conditions related to chronic inflammation. Inflammatory macrophages (M1), acting as the first line of defense against pathogen invasion, showed low TFRC expression and high ferritin. On the other hand, alternately activated macrophages (M2), responsible for recycling Fe++ in the inflammatory process, expressed high levels of these proteins [67].

TFRC and *RIPK2* were not correlated with any of the other genes. *RIPK2* expression varied among patients (Fig. 2), with hypo-expression in XLA01 ($p = 0.0010$) and XLA07 ($p = 0.0005$) and hyper-expression in XLA06 ($p = 0.0005$) and XLA09 ($p = 0.0049$). No significant correlation was found, but *RIPK2* upregulation in XLA06 and XLA09 may be linked to bacterial infection susceptibility (Table 1). Further studies on NOD2 expression in XLA could enrich results. Both wild-type *NOD2* and *NOD2* polymorphisms/mutations can independently cause aberrant RIPK2 signaling, leading to immune dysregulation and multiple inflammatory diseases [68]. The NOD2-RIPK2 complex's role in inflammatory disease is contradictory. Loss-of-function *NOD2* mutations cause Crohn's disease, while gain-of-function mutations cause early-onset sarcoidosis. Wild-type *NOD2* and *RIPK2* activity have been implicated in asthma, inflammatory arthritis, and multiple sclerosis [68].

UBE2N, associated with various cancers and infections [69], contributes to proinflammatory scenarios when overregulated. Detected in XLA01, XLA03, and XLA09 ($p = 0.0640$), *UBE2N* overregulation contrasts with marked under-regulation in XLA04-06, potentially relating to their TLR expression and signaling. TLR genes and *UBE2N* were mostly under-regulated for XLA04-07, the youngest patients in our cohort. *TLR3*, *TLR6*, *TLR10*, and *UBE2N* expressions positively correlated with patient age ($0.0218 \leq p \leq 0.0368$). Additionally, all pairs composed of *UBE2N* and the three TLR genes had a significant positive correlation ($0.0107 \leq p \leq 0.0279$). STAT3, a pivotal transcriptional repressor on UBE2N, modulates NF-kappaB activity and restrains inflammation [69], making UBE2N a promising therapeutic target. Investigating this mechanism in XLA patients may provide valuable insights.

In conclusion, our study provides preliminary evidence for a distinct TLR pathway-gene expression signature in XLA patients harboring missense *BTK* mutations. This strongly suggests that pathogenic BTK variants may significantly impair TLR signaling, with complex functional impact predictions due to the vast spectrum of *BTK* mutations. The interplay between distinct TLR signaling alterations and impaired B-cell function likely contributes to the more severe clinical manifestations observed in XLA patients with missense *BTK* mutations. Despite the limited cohort size and

absence of protein analyses, our findings have the potential to contribute to prognosis and guide treatment strategies for XLA patients. Elucidating the TLR-/NF-kappaB-negative regulatory mechanisms is crucial for developing therapeutic strategies to modulate inflammation in chronic inflammatory and autoimmune pathologies. Future functional studies on biological samples from XLA patients should consider the BTK mutation/variant type and the cell subpopulation to better understand the genotype-phenotype correlations and the cell-specific impact of BTK deficiency on TLR signaling. Further research is warranted to expand these results and elucidate the underlying molecular mechanisms in a larger patient cohort.

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Statement of Ethics

Written informed consent was obtained from all adult participants and all underaged participants' parent/legal guardian for both publication of the details of their medical case and participation in the study. The research project was authorized by the Research Ethics Committee of the University of Campinas Medical School (FCM-UNICAMP), Campinas, São Paulo, Brazil (CEP #1176/2011).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest in regard to the research presented in this paper.

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Author Contributions

Conception and design of the study: M.T. and M.M.S.V. Acquisition of data: M.T., T.A.E., L.M., and M.M.S.V. Data analysis and interpretation: M.T. and T.A.E. Drafting and/or revising the manuscript: M.T., I.Q., and M.M.S.V.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding author (M.T.).

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