

Composition of Precursor B-Cell Compartment in Bone Marrow from Patients with X-Linked Agammaglobulinemia Compared with Healthy Children

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ABSTRACT

X-linked agammaglobulinemia (XLA) is characterized by a severe B-cell deficiency, resulting from a differentiation arrest in the bone marrow (BM). Because XLA is clinically and immunologically heterogeneous, we investigated whether the B-cell differentiation arrest in BM of XLA patients is heterogeneous as well. First, we analyzed BM samples from 19 healthy children by flow cytometry. This resulted in a normal B-cell differentiation model with eight consecutive stages. Subsequently, we analyzed BM samples from nine XLA patients. Eight patients had amino acid substitutions in the Bruton's tyrosine kinase (BTK) domain or premature stop codons, resulting in the absence of functional BTK proteins. In seven of these eight patients a major differentiation arrest was observed at the transition between cytoplasmic $Ig\mu^-$ pre-B-I cells and cytoplasmic $Ig\mu^+$ pre-B-II cells, consistent with a role for BTK in pre-B-cell receptor signaling. However, one patient exhibited a very early arrest at the transition between pro-B cells and pre-B-I cells, which could not be explained by a different nature of the *BTK* mutation. We conclude that the absence of functional BTK proteins generally leads to an almost complete arrest of B-cell development at the pre-B-I to pre-B-II transition. The ninth XLA patient had a splice site mutation associated with the presence of low levels of wild-type *BTK* mRNA. His BM showed an almost normal composition of

the precursor B-cell compartment, suggesting that low levels of BTK can rescue the pre-B-cell receptor signaling defect, but do not lead to sufficient numbers of mature B lymphocytes in the peripheral blood. (*Pediatr Res* 51: 159–168, 2002)

Abbreviations

XLA, X-linked agammaglobulinemia
PB, peripheral blood
BTK, Bruton's tyrosine kinase
aa, amino acids
BM, bone marrow
Cy, cytoplasmic
Sm, surface membrane
wt, wild type
LWBM, lysed whole BM
MC, mononuclear cell
PE/Cy5, phycoerythrin-cyanine5
TdT, terminal deoxynucleotidyl transferase
NK, natural killer
RT, reverse transcriptase
LR, long range
BCR, B-cell receptor

XLA is a primary immunodeficiency, which affects approximately 1 in 100,000 boys and is characterized by agamma- or hypogammaglobulinemia and strongly reduced numbers of B lymphocytes in PB (1). The clinical picture of XLA generally develops during the first year of life, after maternal antibodies have disappeared from the serum, with frequent infections of

different organ systems, particularly upper and lower respiratory tract and gastrointestinal infections, but also bacterial skin infections, meningitis, septicemia, or osteomyelitis (1, 2).

XLA is caused by mutations in the gene encoding BTK, located on Xq21.3 (3, 4). BTK is a cytoplasmic protein of 659 aa, composed of five different domains, the pleckstrin homology, Tec homology, Src homology 3, Src homology 2, and kinase domain (1). So far, 341 unique mutations have been reported, which are scattered throughout the gene (5). No correlation has been described between the type and position of

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the mutations and phenotypic variables, such as age or serum Ig levels at diagnosis or severity of infections (2, 6). XLA is a heterogeneous disease (7, 8), which could be the result of differences in mutations in the *BTK* gene, in combination with other genetic or environmental factors, infections, or age of the patients.

Early studies have shown that in XLA a B-cell differentiation arrest occurs in BM (9–11). However, controversy exists regarding the stage in B-cell development that is primarily affected by mutations in the *BTK* gene. Differentiation arrests have been described between $\text{CyIg}\mu^+$ precursor B cells and SmIgM^+ immature B cells as well as at the transition of $\text{CyIg}\mu^-$ into $\text{CyIg}\mu^+$ precursor B cells (10, 11). These findings could reflect heterogeneity in the stages at which B-cell development is arrested, in parallel with the clinical and immunologic heterogeneity of XLA (12–14). In a recent study, MAbs against the surrogate light chains were used to characterize the differentiation arrest (15). It was shown that the major blockade in XLA patients was located at the transition from large to small $\text{CyIg}\mu^{\text{low}}$ -surrogate light chain^{bright} pre-B cells (15). However, the authors did not discuss the severity of the blockade. The severity of this arrest might be variable, because XLA patients differ in the absolute numbers of mature B lymphocytes in their PB, which suggests that variable degrees of leakiness exist (16). The residual B cells can proliferate, undergo IgH isotype switching, and differentiate into specific antibody-producing cells (17). We hypothesized that an accurate characterization of the B-cell differentiation arrest in BM of XLA patients would help to explain the level of immunologic variation and provide information on the function of BTK in early B-cell development in man.

Several recent flow cytometric studies have shown that in human BM, five to six B-cell differentiation stages can be distinguished (18–22). According to Ghia *et al.* (19, 21), pro-B, pre-B-I, large pre-B-II, small pre-B-II, immature B, and mature B cells can be recognized in man on the basis of Ig rearrangement and cytoplasmic or surface Ig expression. BTK functions as a signal-transducing element downstream of the BCR, thereby directing B-cell development (23).

To unravel the precise B-cell differentiation arrest in XLA patients and to obtain information on the degree of leakiness, we performed detailed flow cytometric studies of nine BM samples from XLA patients together with 19 BM samples from healthy children younger than 16 y of age as controls.

METHODS

Cell samples. We received BM samples from 19 healthy children (youngest age, 1 y 7 mo; oldest 16 y 1 mo; median, 8 y 2 mo; 10 boys, 9 girls), who were donors for BM transplantation of their siblings (Department of Pediatrics, Leiden University Medical Center; Drs. P.M. Hoogerbrugge and J.M.J.J. Vossen). These control BM samples were used for detailed flow cytometric studies, via the LWBM method (24) as well as after Ficoll-Paque density centrifugation (density, 1.077 g/mL; Pharmacia, Uppsala, Sweden).

We received BM and PB samples from nine XLA patients during the last 15 y. BM samples from four recent XLA

patients were used for flow cytometric analysis according to the LWBM method. The BM samples from the other five XLA patients were subjected to Ficoll density centrifugation. The recovered MC were frozen and stored in liquid nitrogen and thawed later for flow cytometric immunophenotyping studies.

Granulocytes and MC were isolated from PB by Ficoll density centrifugation and used for DNA or RNA extraction.

All cell samples were obtained according to the informed consent guidelines of the Medical Ethics Committees of the Leiden University Medical Center and the University Hospital Rotterdam.

Comparison of cell samples. In four healthy children, we compared the results of flow cytometric analysis of fresh BM via the LWBM method with the results obtained from thawed BMMC. Furthermore, in five other healthy children, we compared the results of flow cytometric analysis of fresh BM via the LWBM method with the results obtained from freshly isolated BMMC. All flow cytometric data concerning the precursor B-cell compartment were analyzed independently by two experienced technicians and did not show significant changes before and after Ficoll density centrifugation as well as before and after freezing and thawing of MC, implying that no major selective loss of particular precursor B-cell subsets occurred. Moreover, using BMMC from our cell bank (storage in liquid nitrogen) was the only way we could obtain BM samples from enough XLA patients to perform this study.

Flow cytometric analysis of BM from healthy children and XLA patients. BM samples were diluted with PBS (pH7.8) to a concentration of 15×10^6 cells/mL. Twenty-five microliter aliquots of whole BM or BMMC were incubated for 10 min at

Table 1. Triple labelings for analysis of B-cell differentiation

	FITC-conjugates	PE-conjugates	PE-Cy5-conjugates
MAB directed against membrane antigens			
1	CD34 (HPCA-2)*	CD22 (Leu-14)*	CD19 (HIB19)§
2	CD34 (HPCA-2)	CD10 (J5)	CD19 (HIB19)
3	CD34 (HPCA-2)	CD38 (Leu-17)*	CD19 (HIB19)
4	CD34 (HPCA-2)	CD20 (Leu-16)*	CD19 (HIB19)
5	CD10 (J5)†	CD20 (Leu-16)	CD19 (HIB19)
6	SmIgM	CD10 (J5)	CD19 (HIB19)
7	SmIgD	SmIgM (MB-11)‡	CD19 (HIB19)
MAB directed against membrane antigens and TdT via FACS Lysing Solution			
8	TdT (hTdT-6)¶	CD22 (Leu-14)	CD19 (HIB19)
9	TdT (hTdT-6)	CD34 (HPCA-2)	CD19 (HIB19)
10	TdT (hTdT-6)	CD10 (J5)	CD19 (HIB19)
MAB directed against membrane antigens and $\text{CyIg}\mu$ via IntraPrep			
11	$\text{CyIg}\mu$	CD34 (HPCA-2)	CD19 (HIB19)
12	$\text{CyIg}\mu$	CD10 (J5)	CD19 (HIB19)
13	$\text{CyIg}\mu$	CD38 (Leu-17)	CD19 (HIB19)
14	$\text{CyIg}\mu$	SmIgM (MB-11)	CD19 (HIB19)

MAB were derived from * Becton Dickinson (San Jose, CA, U.S.A.); † Beckman Coulter (Brea, CA, U.S.A.); ‡ Sigma Aldrich (St. Louis, MO, U.S.A.); § Serotec (Raleigh, NC, U.S.A.); || Kallestad/Sanofi-Synthelabo (Paris, France); ¶ Supertechs (Bethesda, MD, U.S.A.).

Table 2. Composition of the BM lymphogate

	CD22 ⁺ B cells (%)		CD3 ⁺ T lymphocytes		CD13 ⁺ /CD33 ⁺ myeloid cells	CD71 ⁺ /GpA ⁺ /CD45 ⁻ normoblasts (%)
	Precursor B cells*	Mature B cells†	(%)	CD16 ⁺ /CD56 ⁺ NK cells (%)	(%)	
Healthy children <5 y (n = 6)	36 ± 7	12 ± 9	22 ± 13	3 ± 1	6 ± 3	25 ± 16
Healthy children 5–10 y (n = 7)	27 ± 9	7 ± 3	28 ± 8	3 ± 2	5 ± 2	31 ± 7
Healthy children 10–16 y (n = 6)	26 ± 11	7 ± 4	25 ± 5	3 ± 1	5 ± 2	35 ± 13
XLA1 (LWBM)	15	0.2	55	6	5	20
XLA2 (thawed BMMC)	8	0.2	39	15	11	26
XLA3 (thawed BMMC)	40	<0.01	22	11	3	13
XLA4 (thawed BMMC)	21	0.04	38	6	6	22
XLA5 (thawed BMMC)	39	0.2	23	12	5	17
XLA6 (LWBM)	12	0.03	68	5	7	15
XLA7 (thawed BMMC)	20	0.03	52	16	2	6
XLA8 (LWBM)	9	0.1	56	6	3	25
XLA9 (LWBM)	26	2.2	29	7	5	34

* Percentage of precursor B cells was calculated by subtracting the percentage of mature B cells from the total percentage of CD22⁺ B cells.

† Percentage of mature B cells was based on the CD10⁻/SmIgM⁺ or SmIgM⁺/SmIgD⁺ populations (labelings 6 and 7 from Table 1).

room temperature with combinations of optimally titrated MAb: 25 μ L FITC-conjugated MAb, or 25 μ L phycoerythrin-conjugated MAb, or 25 μ L PE/Cy5-conjugated Mab, or combinations of preceding MAbs were used to detect membrane-bound antigens. After incubation, the cells were washed and further processed depending on the type of triple labeling. The 14 applied triple labelings are summarized in Table 1.

Triple labelings for three membrane-bound antigens (labelings 1–7 in Table 1) were directly analyzed by flow cytometry using FACScan (Becton Dickinson, San Jose, CA, U.S.A.) in case of BMMC, whereas whole BM samples were first subjected to lysis of the erythrocytes using FACS Lysing Solution (Becton Dickinson).

Permeabilization of the BM cells using FACS Lysing Solution (Becton Dickinson) was performed before intranuclear staining of TdT both on whole BM samples and BMMC (labelings 8–10 in Table 1) (24, 25).

Intracellular staining of Ig μ (CyIg μ) was performed after permeabilization of the BM cells using IntraPrep Permeabilization Reagent (Immunotech, Marseille, France) both on whole BM samples and BMMC (labelings 11–14 in Table 1) (24, 25).

DNA and RNA extraction and RT reaction. DNA was extracted from PBMC or granulocytes using the QIAamp Blood kit (Qiagen, Chatsworth, CA, U.S.A.) (26). Total RNA was isolated from PB granulocytes according to the method of Chomczynski and Sacchi (27) using RNAzol B (Tel-Test, Friendswood, TX, U.S.A.). cDNA was prepared from 1 μ g of mRNA as described before, using oligo(dT) and avian myeloblastosis virus (AMV) RT (Promega, Madison, WI, U.S.A.) (28).

PCR amplification of (c)DNA. PCR was performed as described previously (29). Exons 1–13 and exon 19 of the *BTK* gene were amplified separately, whereas exons 14–18 were amplified in one LR-PCR reaction. In each 100 μ L of PCR reaction, 0.1 μ g of (c)DNA, 20 pmol of 5' and 3' oligonucleotides, and 1 U of AmpliTaq gold polymerase (Applied Biosystems, Foster City, CA, U.S.A.) were used. PCR conditions were 2–10 min at 94°C, followed by 60 s at 92°C, 60 s at 57–60°C, and 2 min at 72°C for 40 cycles, followed by a final extension step (7 min at 72°C).

In each 100 μ L of LR-PCR reaction, 0.1 μ g of DNA sample, 5.25 U of Expand enzyme mix (Boehringer Mannheim, Mannheim, Germany), and 30 pmol of 5' and 3' oligonucleotides were used. LR-PCR conditions were 2 min at 94°C, followed by 15 s at 94°C, 30 s at 57°C, and 4 min at 68°C for 40 cycles using 10 s autoextension from cycle 21 onward. After the last cycle an additional step of 10 min at 72°C was performed for final extension.

The sequences of the oligonucleotides used for PCR amplification of *BTK* were based on Oeltjen *et al.* (30) (Genbank accession number U78027) and were designed with the OLIGO 6 program (Dr. W. Rychlik, Molecular Biology Insights, Cascade, CO, U.S.A.) (31). Primer sequence data will be made available on request to interested readers.

Fluorescent sequencing reaction and analysis. LR-PCR products of *BTK* exons 14–18 were first purified with the QIAquick PCR purification kit (Qiagen), whereas short PCR products (exons 1–13 and 19) were used directly for sequencing with 5 μ L of dRhodamine dye terminator mix (Applied Biosystems), using 3.3 pmol of sequencing primers. The sequencing primers were positioned in the *BTK* introns just upstream and downstream of the exon-intron borders, so that

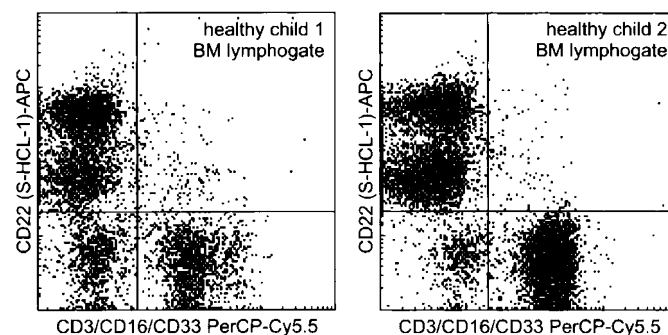


Figure 1. Flow cytometric analysis of BM samples from two healthy children using a mix of PerCP-Cy5.5-labeled MAbs to confirm the reliability of CD22 as a pan-B-cell marker. Flow cytometric analyses within a BM lymphogate showed that CD22 is rarely expressed on CD3⁺ T cells, CD16⁺ NK cells, or CD33⁺ myeloid cells, thereby confirming the reliability of CD22 as a pan-B-cell marker.

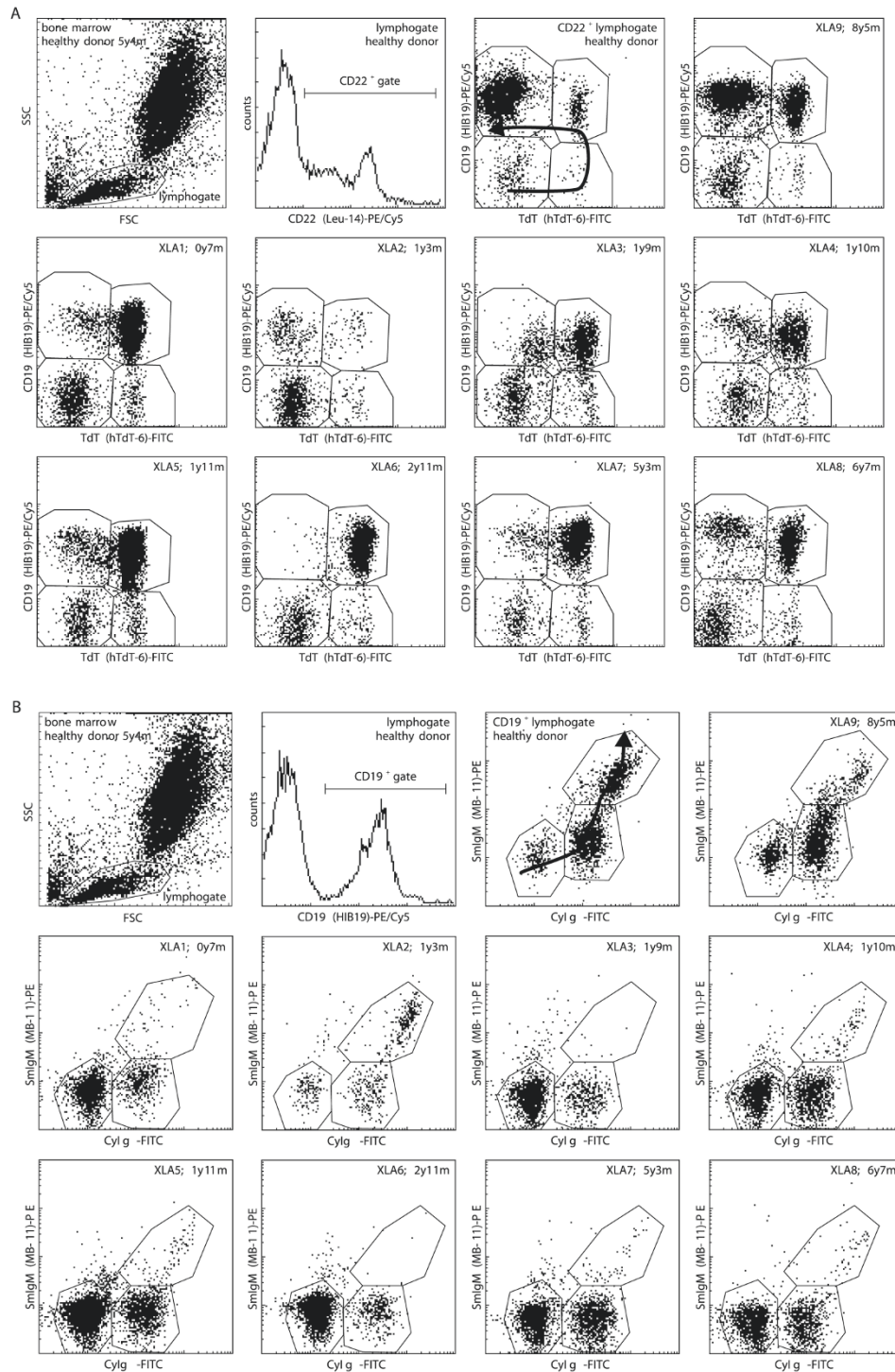


Figure 2. Flow cytometric analysis of BM samples from a healthy child and nine XLA patients, showing the normal composition of the precursor B-cell compartment and the differentiation arrests in XLA patients. The composition of the precursor B-cell compartment in the healthy child was representative of that in all other healthy children ($n = 19$). The composition of the precursor B-cell compartment was analyzed within a lymphogate and a CD22⁺ (A) or CD19⁺ histogram gate (B and C). The order of the B-cell differentiation stages in the healthy child is indicated with arrows. In general, XLA patients lacked or showed a severe reduction in the more mature B-cell differentiation stages with a relative increase in the immature B-cell differentiation stages.

the splice site sequences could be evaluated as well. All sequencing was performed as described before (32) and run on an ABI Prism 377 fluorescent sequencer (Applied Biosystems).

Isolation of PCR products from agarose gels. RT-PCR products from XLA patient 9 were separated in a 1% agarose gel stained with ethidium bromide. Under UV illumination,

two bands of different sizes were cut out of the gel and frozen for 15 min at -20°C . After thawing, the PCR products were isolated from the gel fragments using ultrafree-mc Millipore columns (Millipore Corporation, Bedford, MA, U.S.A.) according to the manufacturer's instructions and used directly for fluorescent sequencing.

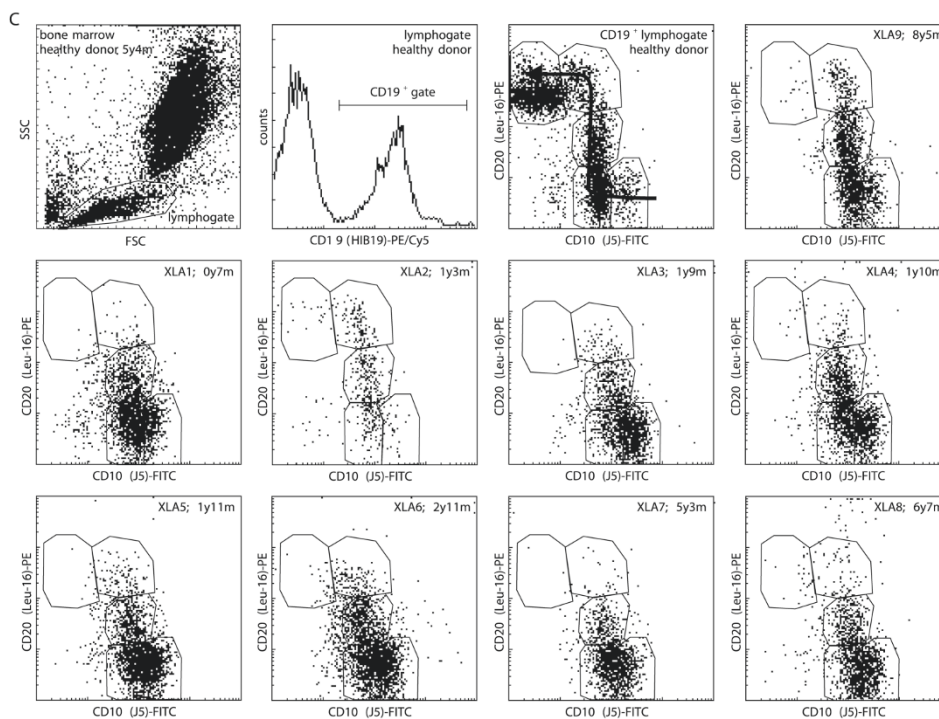


Figure 2. C.

RESULTS

Composition of the BM lymphogate. To characterize B-cell development in young children, BM samples from 19 healthy children and nine XLA patients were analyzed by flow cytometry, using 14 triple labelings (Table 1). The obtained percentages of CD22⁺ B cells in the BM lymphogate are shown in Table 2. This table illustrates the variation in composition of the lymphogate. This variation is caused by contamination of the lymphogate with T lymphocytes, NK cells, myeloid precursors, and normoblasts (Table 2). Therefore, reliable comparison of B-cell subpopulations in BM samples from healthy children and XLA patients requires analysis within a B-cell gate.

The use of CD22 as a pan-B-cell marker has been described previously (33, 34). To confirm the reliability of this marker we performed quadruple labelings using a mixture of PerCP-Cy5.5-labeled MAbs (CD3, CD33, and CD16) together with CD22. Analyses of BM samples from two healthy children showed that CD22 is rarely expressed on CD3⁺ T cells, CD33⁺ myeloid cells, or CD16⁺ NK cells (Fig. 1), thereby excluding the possibility that the CD22⁺ gate would include substantial numbers of non-B lineage cells. Furthermore, labelings containing both CD22 and CD79a showed a small CD22⁺/CyCD79a⁻ pro-B-cell population (data not shown). Therefore, we choose CD22 as a pan-B-cell marker instead of CyCD79a.

B-cell subsets in bone marrow from healthy donors. The markers CD22 and CD19 were used to identify the total B-cell population in BM, because these two markers are regarded as pan-B-cell markers, which are expressed by virtually all precursor B cells. In each triple labeling, the expression pattern of the two other MAbs was analyzed within the CD22 or CD19

gate, resulting in normal differentiation pathways (Fig. 2). The apparent heterogeneity of the CD22⁺ gate in Figure 2A was caused by different expression levels (CD22⁺ and CD22^{bright}) on precursor B-cells, which was also clear from Figure 1.

Virtually all events within the B-cell gates were grouped using different regions, bringing the total percentage of identified B-cell subpopulations in each gate to approximately 100%. For example, the CyIgμ-SmIgM-CD19 labeling was analyzed within the CD19 gate (Fig. 2B). Three subpopulations could be distinguished (from immature to mature): CyIgμ⁻/SmIgM⁻, CyIgμ⁺/SmIgM⁻, and CyIgμ⁺/SmIgM⁺. An identical approach was followed for other markers within the CD22 or CD19 gates of all 14 triple labelings, resulting in the differentiation scheme in Table 3.

Several human B-cell differentiation schemes with five to six precursor B-cell subsets have been published (18–22). Pro-B, pre-B, immature B, and mature B cells were designated according to Ghia *et al.* (21). The CD19⁻ fraction was termed pro-B cells, and was further subdivided on the basis of TdT expression. Pre-B-I cells were defined as CD19⁺, CD10⁺, TdT⁺, CD34⁺, and CyIgμ⁻ and were further subdivided on the basis of the level of CD10 expression. Pre-B-II cells were CD19⁺, CD10⁺, TdT⁻, CD34⁻, and CyIgμ⁺, and were further subdivided on the basis of the presence or absence of CD20 expression. Immature B cells were CD19⁺, CD10⁺, SmIgM⁺, and SmIgD⁻, whereas mature B cells were CD19⁺, CD10⁻, SmIgM⁺, and SmIgD⁺.

All percentages of precursor-B cell subpopulations in healthy children within a CD22⁺ or CD19⁺ gate are listed in the lower part of Table 3. It has been published that the relative composition of the precursor B-cell compartment is age related with several differences between children and adults (22). We

Table 3. B-cell differentiation stages in BM from healthy children (<16 y)

Immunologic markers		1	2	3	4	5	6	7	8
		Pro-B	Pre-B-I	Pre-B-II	Immature B	Mature B			
CD19		–	–	+	+	+	+		
CD22		+	+	+	+	+	+	+	++
CD34		+	+	+	+	–	–	–	–
TdT		–	+	+	+	–	–	–	–
CD10		–	–	++	+	+	+	+	–
CD20		–	–	–	–	–	+	++	++
CD38		NA	NA	++	++	++	++	++	+
CyIgM		–	–	–	–	+	+	+	+
SmIgM		–	–	–	–	–	–	+	+
SmIgD		–	–	–	–	–	–	–	+
Mean % within CD22 gate (SD)	<5 y (n = 6)	6.0 (1.7)	1.2 (1.4)	7.6 (4.0)		83.8 (7.0)			
	5–10 y (n = 7)	8.2 (4.8)	1.0 (0.6)	9.1 (1.5)		79.7 (6.2)			
	10–16 y (n = 6)	6.8 (2.2)	0.8 (0.3)	8.3 (3.1)		82.6 (4.6)			
Mean % within CD19 gate (SD)	<5 y (n = 6)	–		3.7 (2.4)	3.2 (2.3)	23.2 (16.3)	22.4 (5.0)	19.6 (8.0)	25.6 (14.0)
	5–10 y (n = 7)	–		4.6 (1.4)	4.9 (2.1)	34.8 (12.7)	18.2 (4.7)	15.2 (3.2)	24.4 (11.5)
	10–16 y (n = 6)	–		5.7 (2.3)	4.9 (3.1)	25.8 (8.0)	20.4 (6.7)	18.1 (3.8)	28.8 (13.4)

NA = not analyzed. Because CD38 was not used in combination with CD22 (Table 1), no precise information about CD38 expression on pro-B cells was available. However, analysis of CD34 and CD38 expression in the lymphogate showed that >99% of CD34⁺ cells were also CD38⁺. Hao *et al.* (53) showed that the CD38[–] population comprised 3.53% ± 0.68% of the CD34⁺ cells.

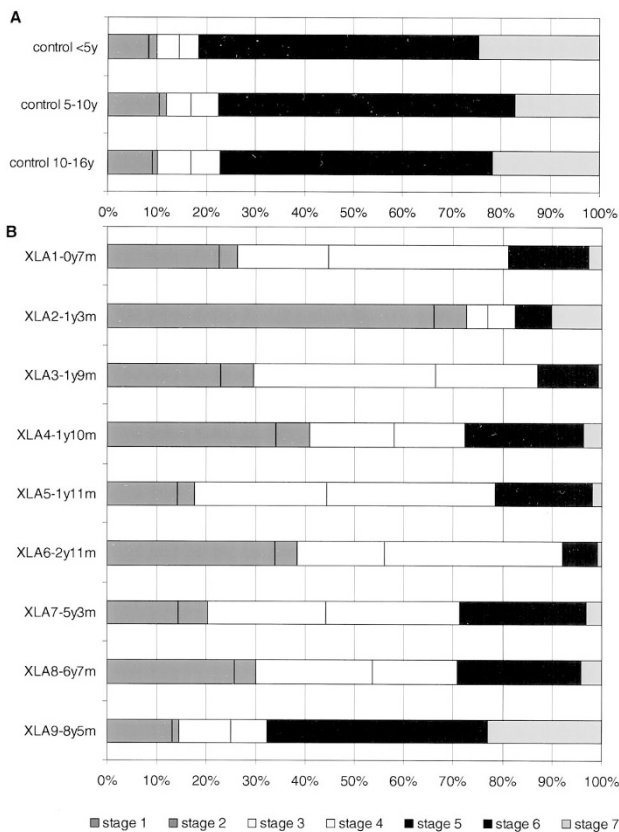


Figure 3. Composition of the precursor B-cell compartment in healthy children (A) compared with XLA patients (B), corrected for the composition of the lymphogate. The precursor B-cell compartment (Table 2) was set to 100%. To this aim, CD10[–]/SmIgM⁺/SmIgD⁺ mature B cells were excluded (see text for details). Seven of nine XLA patients showed a similar differentiation arrest with approximately 80% of the precursor B cells in the pro-B and pre-B-I stages, thereby revealing an arrest at the transition from CyIgμ[–] to CyIgμ⁺ pre-B cells, with a variable degree of leakiness. XLA patients 2 and 9 showed different compositions of their precursor B-cell compartments.

subdivided the healthy children into three age groups to check for differences among children of different age groups. Our results showed only minor nonsignificant differences in the composition of the precursor B-cell compartment in BM from children of different age groups.

Definition of CD22⁺ and CD19⁺ B-cell compartments. We identified the entire B-cell compartment as being CD22⁺. In healthy children younger than 5 y of age, between 5 and 10 y of age, and between 10 and 16 y of age, the CD19⁺ fraction within this CD22⁺ B-cell compartment was very large (labelings 1 and 8), *i.e.* 91.4%, 88.7%, and 90.9%, respectively, implying that the CD19[–]/CD22⁺ pro-B-cell population was small in all three age groups (Table 3). Consequently, the percentages of the different subpopulations within the CD19 gate could be recalculated into percentages of the CD22⁺ population, by multiplying with 0.914, 0.887, and 0.909, respectively. The same approach was followed for XLA patients.

Correction of the B-cell compartment in BM for blood contamination. The mature B-cell population (stage 8) varied between healthy children mainly because of blood contamination with CD10[–]/SmIgM⁺/SmIgD⁺ B-lymphocytes: 1–30% within the lymphogate (Table 2) and 8–42% within the CD19⁺ gate. This variable degree of blood contamination was also clear from the comparable variation of CD3⁺ T lymphocytes within the lymphogate, ranging from 9 to 35% (Table 2). To correct for this variance in our comparative studies between precursor B cells in normal BM and in BM from XLA patients, we excluded the mature CD10[–]/SmIgM⁺/SmIgD⁺ B-cell population from our calculations (Table 2). Consequently, the percentages of stages 1–7 were recalculated to 100% (Fig. 3). From this recalculation, the percentages of precursor B cells in Table 2 were set to 100%.

Patient characteristics and mutations in the BTK gene. The nine boys were clinically diagnosed as XLA on the basis

Table 4. Characteristics of XLA patients

Patient	Age at diagnosis*	Serum Ig levels at diagnosis†			Age at BM puncture	Genomic mutation‡		Predicted effect at protein level	
		IgM (g/L)	IgG (g/L)	IgA (g/L)		Exon	Nucleotide	Amino acid	Affected domain
XLA1	0 y 7 mo	0.11	0.3	0.01	0 y 7 mo	17	C53T	R562W	Kinase
XLA2	1 y 2 mo	0.26	0.27	0.17	1 y 3 mo	7	26delG	L198stop	TH
XLA3	1 y 9 mo	<0.1	0.36	<0.1	1 y 9 mo	2	142delTC	Y40stop	PH
XLA4	0 y 7 mo	0.13	0.41	0.04	1 y 10 mo	15	G78A	M509I	Kinase
XLA5	1 y 10 mo	0.13	0.01	0	1 y 11 mo	15	C106A	Y485stop	Kinase
XLA6	2 y 11 mo	<0.05	<0.1	<0.06	2 y 11 mo	3	75insA	E84stop	PH
XLA7	1 y 8 mo	NA	<0.3	NA	5 y 3 mo	14	C98A	Y425stop	Kinase
XLA8	6 y 7 mo	<0.1	3.0	<0.05	6 y 7 mo	18	T10C	M587T	Kinase
XLA9§	8 y 4 mo	0.05	4.7	0.4	8 y 5 mo	3	G99A	L119stop	PH

* XLA diagnosis was based on strongly reduced or absent numbers of B lymphocytes in the PB and hypogammaglobulinemia.

† Lower level normal serum Ig values according to Cejka *et al.* (35): 5–12 mo: IgM 0.47, IgG 4.34, IgA 0.15; 1–2 y: IgM 0.39, IgG 3.69, IgA 0.15; 2–3 y: IgM 0.53, IgG 5.10, IgA 0.25, 6–9 y: IgM 0.54, IgG 6.83, IgA 0.32.

‡ The beginning of each exon is numbered as 1.

§ Mutation in donor splice site of exon 3 with mutant and wt *BTK* mRNA detectable.

NA = not analyzed.

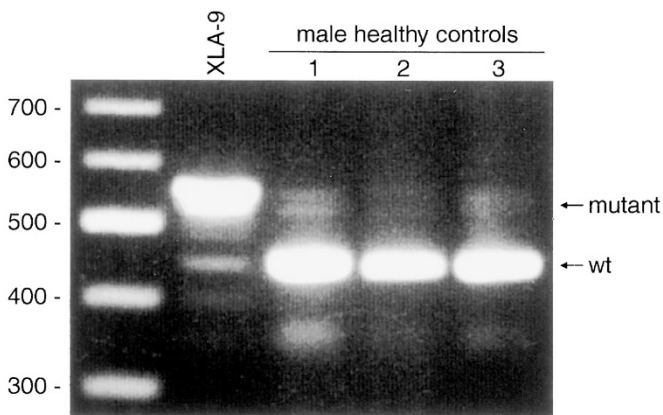


Figure 4. RT-PCR analysis of *BTK* mRNA from PB granulocytes of XLA patient 9 and three healthy male control children. XLA patient 9 predominantly expressed the mutated form (*upper band*) with 106 nucleotides inserted from intron 3. Small amounts of wt *BTK* mRNA (*lower band*) could be detected.

of hypo- or agammaglobulinemia (35) and strongly reduced or undetectable numbers of mature B lymphocytes in the PB. Analysis of PB mature B lymphocytes for SmIgM/SmIgD expression showed that XLA patients 1, 4, 5, and 7 had very low percentages (<0.1% of lymphocytes) of SmIgM⁺⁺/SmIgD⁺ B lymphocytes. In XLA patients 3 and 6, no SmIg⁺ B lymphocytes could be detected. XLA patient 2 had 0.6% SmIgM^{+/++}/SmIgD⁺ B lymphocytes at the age of 6 y 5 mo, whereas XLA patient 8 had 0.3% SmIgM⁺⁺/SmIgD⁺ B lymphocytes at the age of 6 y 7 mo. XLA patient 9 was diagnosed at 8 y of age and had 1.0% of SmIgM^{+/++}/SmIgD⁺ B lymphocytes in the PB and almost normal levels of IgG and IgA, but decreased levels of IgM (Table 4).

Mutation analysis of the *BTK* gene was performed at the DNA level. We found five point mutations, one splice site mutation, two small deletions, and one insertion. Five mutations (XLA patients 1, 4, 5, 6, and 7) had already been observed in other patients (5, 36–41), whereas the other four mutations (XLA patients 2, 3, 8, and 9) were previously unknown. The entire *BTK* gene of each patient was sequenced to exclude additional mutations. The predicted effects of these

mutations on the protein level are summarized in Table 4. Except for XLA patient 9, all *BTK* mutations would lead to absence of functional BTK proteins, either because of aa substitutions in the BTK kinase domain or because of premature stop codons.

In XLA patient 9, the last nucleotide of exon 3 was mutated. This mutation did not give rise to an aa substitution. However, RT-PCR analysis on mRNA from PB granulocytes generated two bands of different sizes, which were isolated and sequenced. We identified a dominant mutated form of *BTK* mRNA with 106 nucleotides inserted from intron 3, but also low levels of wt *BTK* mRNA (Fig. 4). Quantitative analysis by real-time quantitative PCR with TaqMan technology of wt *BTK* mRNA expression in PB granulocytes of this patient indicated that <5% of normal levels were present (data not shown).

Flow cytometric analysis of BM precursor B cells from XLA patients. Because of lack of BM cells, a few triple labelings could not be performed in some XLA patients. Particularly labeling 7 (SmIgD/SmIgM/CD19) could not be performed on BM samples from XLA patients 2, 3, 4, and 7. In these four XLA patients we used the CD10⁻/SmIgM⁺ population to identify mature B cells. In five XLA patients we used thawed BMBC because no fresh BM samples were available (see “Methods”).

In BM from healthy children, approximately 20% of the B-cell compartment consisted of CyIgμ⁻ precursor B cells, equally distributed over pro-B cells and pre-B-I cells (pro-B/pre-B-I ratio of 1.2 ± 0.8), whereas in BM from eight of nine XLA patients approximately 80% (71–92%) of the B-cell compartment was located in these stages (Fig. 3). Therefore, our results indicate that the differentiation arrest in the eight XLA patients resulted in a 4-fold relative accumulation of B-cell subpopulations located before the transition from CyIgμ⁻ to CyIgμ⁺ pre-B cells. The relative accumulation of CyIgμ⁻ precursor B cells was somewhat lower for pro-B cells (18–42%) compared with pre-B-I cells (34–63%; Fig. 3B), resulting in a mean pro-B/pre-B-I ratio of 0.6 ± 0.3 in seven of the eight XLA patients. Although this differentiation arrest was

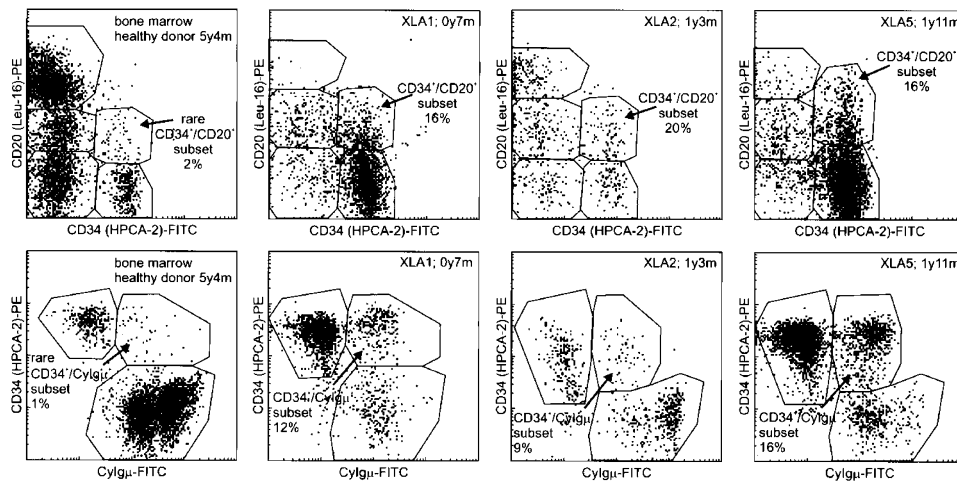


Figure 5. Flow cytometric analysis of CD34/CD20 coexpression within the CD19⁺ lymphogate of BM from a healthy child and three XLA patients. In all healthy children the markers CD34 and CD20 were virtually exclusive (CD34⁻/CD20⁻ population, stage 5). However, most XLA patients showed a dominant CD34⁺/CD20⁺ population (*upper panel*). This CD34⁺/CD20⁺ population originated from prolonged CD34 expression, inasmuch as in XLA patients a large proportion of CyIgμ⁺ pre-B-II cells was also CD34⁺ (*lower panel*). XLA patients 2 and 5 showed the smallest and largest CD34⁺/CyIgμ⁺ populations, respectively. XLA patient 1 was selected as a representative example of all other XLA patients (except for XLA patient 9).

virtually identical in seven of the eight XLA patients, the percentage of B-cell subpopulations located behind the arrest (CyIgμ⁺ pre-B-II cells and immature B cells) was variable, indicating leakiness (Fig. 3B).

Of the eight XLA patients with absence of functional BTK proteins, only XLA patient 2 displayed an atypical differentiation pattern with 73% CD19⁻/CD22⁺ pro-B cells and 10% pre-B-I cells (pro-B/pre-B-I ratio of 7.4). Apparently, in this patient a major differentiation arrest was located at a very early stage, *i.e.* at the transition from pro-B cells to CyIgμ⁻ pre-B-I cells (Fig. 3B). However, the SmIgM⁺ B-cell population was extensive compared with other XLA patients, suggesting a second arrest at the transition from immature SmIgM⁺ to mature SmIgM⁺/SmIgD⁺ B cells.

XLA patient 9 showed an apparently normal composition of the precursor B-cell compartment. Nevertheless, this patient had diminished percentages of mature B cells in his BM (Table 2) and in his PB.

In general, seven of the eight XLA patients with absence of functional BTK proteins showed a similar differentiation arrest at the transition from CyIgμ⁻ to CyIgμ⁺ pre-B cells, with a variable degree of leakiness (Fig. 3B).

Atypical B-cell differentiation patterns in BM from XLA patients. In BM samples from healthy children, coexpression of the markers CD34 and CD20 was virtually absent (Table 3), as <4.4% CD34⁺/CD20⁺ cells were detectable (Fig. 5). However, in BM from most XLA patients a substantial CD34⁺/CD20⁺/CD19⁺ population could be identified. This atypical B-cell population varied among XLA patients from 11 to 20%, but was smaller (6%) in XLA patient 9. The atypical marker expression was predominantly caused by prolonged expression of CD34, because in BM samples from XLA patients 9–16% of CyIgμ⁺ pre-B-II cells was CD34⁺ (4% in XLA patient 9), compared with <2.4% in healthy children (Fig. 5).

DISCUSSION

We have analyzed BM samples from 19 healthy children by flow cytometry, using 14 triple labelings, revealing the normal composition of the precursor B-cell compartment (Fig. 2). This resulted in an accurate human B-cell differentiation scheme, which resembles the classification according to Ghia *et al.* (21). Our scheme, in which Ig expression patterns are dominant variables, consists of eight consecutive stages and is summarized in Table 3. Similar analysis of the composition of the precursor B-cell compartment in BM from nine XLA patients showed that approximately 80% (71–92%) of the B-cell compartment in eight of these patients consisted of CyIgμ⁻ precursor B cells (pro-B cells and pre-B-I cells), in contrast to approximately 20% in healthy children. This is in line with the results of Campana *et al.* (11), who described a maturation arrest at the transition between CyIgμ⁻ and CyIgμ⁺ pre-B cells in BM of XLA patients. The localization of this arrest at the transition between CyIgμ⁻ and CyIgμ⁺ pre-B cells in seven of the eight XLA patients with absence of functional BTK proteins suggests that BTK proteins are required for the expansion of the CyIgμ⁺ pre-B-II population by signaling via the pre-BCR, which is expressed on CyIgμ⁺ pre-B cells (21, 42). This is supported by the finding that BTK is constitutively phosphorylated in a pre-B-cell line, which implicates a functional role for BTK in pre-BCR signaling during B-cell development (43).

B-cell differentiation arrests have been analyzed in BM samples from patients with non-X-linked causes of agammaglobulinemia. These patients have mutations in genes that encode other components of the pre-BCR, such as the μ heavy chain or λ5/14.1 (44, 45), or in genes that encode proteins involved in signaling via the pre-BCR, such as CD79a or B-cell linker protein (BLNK) (46, 47). BLNK is an adapter protein, which plays a role in the same signal-transduction

pathway as BTK (48). The B-cell differentiation arrest in BM from these non-XLA patients seems to be comparable to the arrest in XLA patients and is also located at the transition from $\text{CyIg}\mu^-$ pre-B-I cells to $\text{CyIg}\mu^+$ pre-B-II cells (49). These results strongly support a role for BTK at the pre-BCR checkpoint in human B-cell development.

The flow cytometric data of the XLA patients clearly show a relative accumulation of $\text{CyIg}\mu^-$ precursor B cells, particularly of pre-B-I cells (Fig. 3B). This is in contrast with the studies by Campana *et al.* (11), who described normal frequencies of $\text{CyIg}\mu^-$ precursor B cells, and Pearl *et al.* (10), who described normal frequencies of $\text{CyIg}\mu^+$ precursor B cells in their XLA patients. These discrepancies are probably related to differences in techniques and in age of the studied patients. Our results are in line with the study by Nomura *et al.* (15), who described significantly increased percentages of precursor B cells located before the blockade.

We hypothesized that accurate characterization of the B-cell differentiation arrest in BM of XLA patients would help to explain the level of immunologic variation previously reported (10, 11). In seven of the eight XLA patients with absence of functional BTK proteins, this differentiation arrest appeared to be very similar, with a large $\text{CyIg}\mu^-$ precursor B-cell compartment and a more prominent accumulation of pre-B-I cells compared with pro-B-cells, resulting in a pro-B/pre-B-I ratio of 0.6 ± 0.3 , which is significantly lower (Mann-Whitney *U* test for unrelated groups with unequal SDs; $p < 0.03$) than in normal BM (1.2 ± 0.8). Although the arrest was similar, the degree of leakiness, indicated by the percentage of B-cell subpopulations located behind the arrest, varied among these seven XLA patients (Fig. 3B). The age of the XLA patients was comparable and the percentage of myeloid cells in the lymphogate did not differ significantly between patients, indicating that none of the patients suffered from infections at the time of BM donation. It seems therefore most likely that other genetic factors influence the degree of leakiness, as has also been suggested with respect to the heterogeneity of the clinical picture of XLA (7, 8).

In the eighth XLA patient (XLA-2), the $\text{CyIg}\mu^-$ precursor B-cell compartment mainly consisted of pro-B cells (pro-B/pre-B-I ratio of 7.4). This particularly concerned the most immature $\text{CD}22^+/\text{CD}19^-/\text{TdT}^-$ pro-B cells (stage 1), which represented 66% of the total precursor B-cell compartment in this patient (Figs. 2A and 3B). Curiously, the percentage of SmIgM^+ B cells was also increased in this patient compared with other XLA patients, indicating a second arrest at the transition from immature to mature B cells. We do not have an explanation for this unique composition of the precursor B-cell compartment.

It is interesting to note that the precursor B cells of XLA patient 9, which can generate low levels of wt *BTK* mRNA (Fig. 4), apparently can overcome the differentiation arrest at the pre-BCR level, as $\text{CyIg}\mu^+$ pre-B-II cells were present in near normal frequencies in the BM of this patient (Fig. 3B). However, the reduced numbers of mature B lymphocytes in the PB of this patient indicate that the differentiation arrest at the BCR level is more severe. This means that if BTK proteins are indeed involved in signaling via the pre-BCR, lower amounts

of BTK are needed for signal transduction from the pre-BCR compared with the BCR. The phenotype of this patient resembles that of X-linked immunodeficiency mice, which have a point mutation in the pleckstrin homology domain or a targeted deletion of *BTK*. These mice show normal precursor B-cell compartments in BM, but reduced numbers of mature B lymphocytes in PB, and predominantly lack IgM and IgG_3 in the serum (50–52).

The marker expression on precursor B cells in BM samples from the seven similar XLA patients seemed to be normal until the pre-B-II stage, in which $\text{CyIg}\mu$ is expressed, was reached. The markers CD34 and CD20 showed virtually no coexpression in healthy children, but were coexpressed in these XLA patients, resulting in a clearly detectable $\text{CD}34^+/\text{CD}20^+/\text{CD}19^+$ population (Fig. 5). The coexpression appeared to be caused by prolonged CD34 expression, as a large proportion of $\text{CyIg}\mu^+$ pre-B-II cells was $\text{CD}34^+$. This suggests that normally CD34 down-regulation is mediated via pre-BCR signaling.

In conclusion, the B-cell differentiation arrest in the majority of XLA patients appeared to be homogeneous, with approximately 80% of the precursor B-cell compartment being negative for $\text{CyIg}\mu$ expression. The size and nature of the residual more mature B-cell population (leakiness) varied among patients, independent of the type of *BTK* mutation. Furthermore, it appears that the composition of the precursor B-cell compartment in BM of some XLA patients can be influenced by low levels of wt *BTK* mRNA, as was particularly illustrated by XLA patient 9. It would be interesting to analyze the presence of wt *BTK* mRNA levels in other XLA patients with splice site mutations in the *BTK* gene. Because low levels of wt *BTK* mRNA appear to be sufficient for a seemingly normal composition of the precursor B-cell compartment, one might expect more mature B lymphocytes in the PB and higher serum Ig levels in these patients.

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