Bruton's Tyrosine Kinase Is Essential for Human B Cell Tolerance

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Abstract

Most polyreactive and antinuclear antibodies are removed from the human antibody repertoire during B cell development. To elucidate how B cell receptor (BCR) signaling may regulate human B cell tolerance, we tested the specificity of recombinant antibodies from single peripheral B cells isolated from patients suffering from X-linked agammaglobulinemia (XLA). These patients carry mutations in the Bruton's tyrosine kinase (*BTK*) gene that encode an essential BCR signaling component. We find that in the absence of Btk, peripheral B cells show a distinct antibody repertoire consistent with extensive secondary V(D)J recombination. Nevertheless, XLA B cells are enriched in autoreactive clones. Our results demonstrate that Btk is essential in regulating thresholds for human B cell tolerance.

Key words: XLA • Bruton's tyrosine kinase • B lymphocytes • tolerance • autoantibody

Introduction

In humans, Ig gene recombination produces large numbers of self-reactive antibodies or B cell receptors (BCRs; reference 1). Most of the polyreactive antibodies and antinuclear antibodies (ANAs) are removed from the repertoire during B cell development, thereby ensuring self-tolerance (1). In mice, three mechanisms account for the silencing of newly arising autoreactive B cells: deletion, anergy, and receptor editing (2–5). These tolerance mechanisms are triggered and regulated by antigen binding to self-reactive BCRs. For example, transgenic and knockout mouse experiments have shown that BCR signaling thresholds are essential in B cell tolerance (6). Changes in tuning functions of the BCR coreceptors such as CD19 or CD22 and PD-1 and their associated signaling molecules Lyn and SHIP all impact on the tolerance response (7–10). Much less is known about the mechanisms that regulate B cell tolerance in humans.

Defects in BCR signaling have been reported in B cells from immunodeficient patients with common variable immunodeficiency who frequently develop autoimmunity, suggesting that BCR signaling may play an important role in counterselecting self-reactive B cells (11). To investigate the role of BCR signaling in the regulation of autoreactive B cells in humans, we analyzed tolerance in B cells from X-linked agammaglobulinemia (XLA) patients. XLA or Bruton's disease is characterized by a severe decrease of peripheral B cells and serum Ig (12, 13). Genetic studies in humans led to the identification of the defective gene, named Bruton's tyrosine kinase (*BTK*) gene, which encodes a cytoplasmic tyrosine kinase that plays an essential role in mediating BCR signaling (14, 15). Although B cell differentiation is severely affected at the pro- to pre-B transition, a few B cells develop and migrate to the periphery where they fail to accumulate (12, 16, 17). XLA has a more severe phenotype in humans than murine Xid, which results from loss of function of the murine *btk* gene (18). In $btk^{-/-}$ mice, peripheral B cells are observed but responses to T-independent antigen (type II) are impaired, whereas responses to T-dependent antigens remain normal (19). Therefore, mouse models for Btk deficiency cannot be extrapolated to humans. We report that in humans, central B cell tolerance checkpoints are abrogated in the absence of Btk.

The online version of this article contains supplemental material.

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Abbreviations used in this paper: ANA, antinuclear antibody; BCR, B cell receptor; Btk, Bruton's tyrosine kinase; RF, reading frame; XLA, X-linked agammaglobulinemia.

Materials and Methods

Patients. Patients 1 and 2 are adult first cousins who suffer from a mutation in exon 11 of the *BTK* gene consisting of an 18-bp insertion (XLA-Ins) resulting from the duplication of neighboring DNA. Patients 3 and 4 are adolescent and preadolescent brothers who show an adenosine to cytidine substitution in codon 117 that results in a proline instead of the wild-type threonine in the pleckstrin homology domain of Btk (XLA-T117P). All patients showed similarly decreased levels of peripheral B cells that were mostly $CD19^+$ $CD10^+$ IgM^+ $CD27^-$ new emigrant B cells. Control healthy donors were a 23-yr-old female (JH) and a 31-yr-old male (PE). All samples were collected after signed informed consent in accordance with IRB-reviewed protocols.

Single Cell Sorting. Peripheral B cells were purified from the blood of XLA patients and from the blood of two nonrelated healthy donors by negative selection using the RosetteSep procedure (StemCell Technologies, Inc.). Enriched B cells were stained with FITC anti–human CD27, PE anti–human CD10, anti–human IgM-biotin, and allophycocyanin anti-CD19 (BD Biosciences and Becton Dickinson). Biotinylated antibodies were revealed using Streptavidin-Red613 (GIBCO BRL). Single $CD19^+$ $CD10^+$ IgM^+ $CD27^-$ new emigrant B cells from XLA patients and control donors were sorted on a FACSVantage (Becton Dickinson) into 96-well PCR plates containing 4 μl of lysis solution (0.5× PBS containing 10 mM DTT, 8 U RNAsin [Promega], and 0.4 U 5′-3′ RNase Inhibitor [Eppendorf]) and immediately frozen on dry ice. All samples were stored at −70°C.

cDNA, RT-PCR, Antibody Production, and Purification. RNA from single cells was reverse transcribed in the original 96-well plate in 12.5 μl of reactions containing 100 U Superscript II RT (GIBCO BRL) for 45 min at 37°C. RT-PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as described previously (1). Ig sequences were analyzed by Ig BLAST comparison with GenBank.

ELISAs and Immunofluorescence Assays. Antibody concentration, reactivity against specific antigens, and indirect immunofluorescence were as described previously (1). High (polyreactive ED38) and weak (mGO186) ANA-reactive and -nonreactive mGO53 and iGO13 were used as positive and negative controls in self-reactivity and polyreactivity ELISAs (1, 20).

Statistical Analysis. Two-tailed p-values were calculated by the Fisher Exact Test. Bonferroni corrections were used when analyzing unpredictable D gene usage frequency by multiplying the p-value by the number of parameters. Although some data from individual patients could reach statistical significance, the frequency of self-reactive clones was calculated after pooling data from patients carrying the same *BTK* gene mutation. Because of the very large number of Ig gene segments, antibody repertoire analyses were performed after pooling Ig sequences from all XLA patients.

Online Supplemental Material. Antibody characteristics from control new emigrant, XLA-Ins, and XLA-T117P B cells are presented in Tables S1–S3. Fig. S1 shows antibody features that differ between nonreactive and polyreactive antibodies from XLA B cells. Tables S1–S3 and Fig. S1 are available at http://www.jem.org/cgi/content/full/jem.20040920/DC1.

Results

Peripheral B Cell Selection in XLA Patients. *BTK* gene encodes a cytoplasmic tyrosine kinase that plays an essential role in BCR signaling in humans (14, 15, 21, 22). XLA patients are characterized by a severe decrease in peripheral B cells and serum Ig levels (12, 13). In line with this observation, we found that most peripheral blood B cells from XLA patients were $CD19^+$ $CD10^+$ IgM^+ $CD27^-$ new emigrant B cells that fail to develop into $CD19^+$ $CD10^-$ IgM^+ $CD27^-$ mature naive B cells (Fig. 1; reference 23).

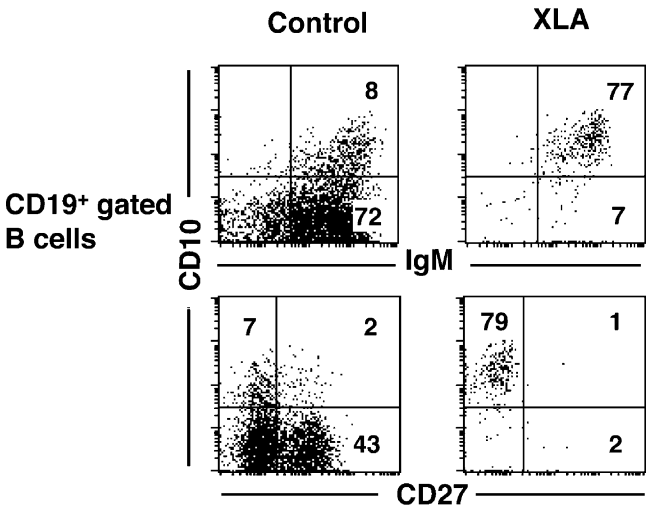

Figure 1. Peripheral XLA B cell are new emigrant B cells. Dot plots show CD10, IgM (top), CD10, and CD27 (bottom) expression on gated $CD19^+$ B cells in a control (left) and XLA patient (right). Most peripheral blood B cells from XLA patients were $CD19^+$ $CD10^+$ IgM^+ $CD27^-$ new emigrant B cells that fail to develop into $CD19^+$ $CD10^-$ IgM^+ $CD27^-$ mature naive B cells.

To examine the self-reactivity of the antibodies developing in the absence of Btk, we cloned antibodies from isolated $CD19^+$ $CD10^+$ IgM^+ $CD27^-$ new emigrant B cells from four XLA patients and two healthy donors (refer to Materials and Methods). Repertoire analysis revealed that two specific V_H genes, V_H1-3 and the self-reactive V_H4-34 used in all cold agglutinin antibodies (24, 25), were increased in Btk-deficient B cells and encoded up to 25% (21 out of 91) of all antibodies in XLA patients (Fig. 2, A and B). In contrast, V_H1-3 and V_H4-34 genes represented only 7.3% (8 out of 109) of the antibodies from control new emigrant B cells ($P = 0.002$; Tables S1–S3, available at http://www.jem.org/cgi/content/full/jem.20040920/DC1). In addition, the antibody repertoire of XLA B cells was devoid of V_H4-59, a gene commonly used in B cells from healthy donors ($P = 0.008$; Fig. 2 B; references 1 and 26). Human antibodies show preferential use of certain D segments that are mostly used in the hydrophilic reading frame (RF; reference 27). We found that D6 gene usage was increased from 15.8% in control new emigrant to 35% in XLA B cells ($P = 0.028$ after Bonferroni correction; Fig. 2 C). When combined, D2, D5, and D6 family members in hydrophobic RFs were increased from 35.5% in control new emigrant to 58.3% in XLA B cells ($P = 0.038$), whereas those that used the hydrophilic RFs decreased from 57.8% in control to 31.25% in XLA B cells ($P = 0.013$; Fig. 2 D). We conclude that XLA B cells are selected to express a unique antibody repertoire using distinct

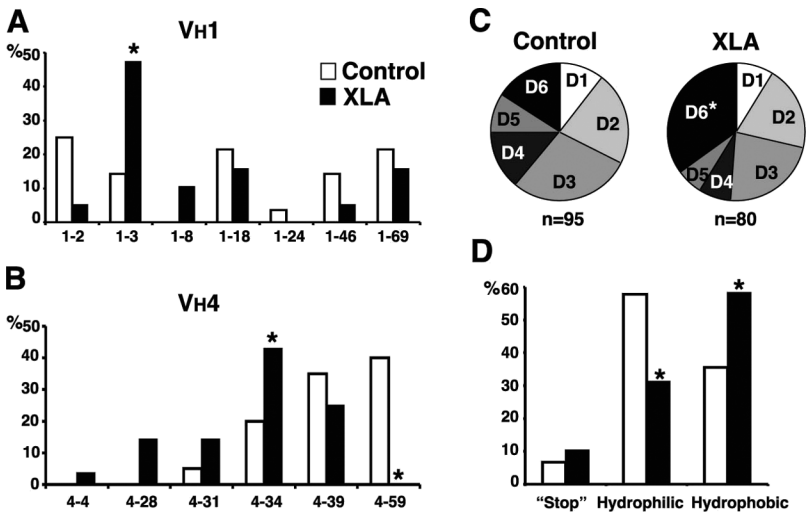

Figure 2. XLA B cells are a highly selected B cell population with a unique V_H and D gene usage. Proportions of V_H1 (A), V_H4 (B), D family (C) gene, and RF (D) usage in control new emigrant (open bars) and XLA B cells (closed bars). Control new emigrant B cell sequences included sequences shown in Table S1 and as reported previously (reference 1). D RF usage for combined D2, D5, and D6 family gene segments represented in panel D was assigned according to Corbett et al. (reference 27). *, statistically significant difference. P-values for differences between fractions are stated in the text and were calculated by the Fisher Exact Test.

V_H and D genes that favor hydrophobic RFs that are normally counterselected in B cells from healthy donors.

Increased Secondary V(D)J Recombination in XLA B Cells. In addition to their unique heavy chain repertoire, XLA B cells also display remarkable light chains. We found that Igκ genes from XLA patients had decreased Jκ1 usage (11.5% in XLA vs. 28.5% in control new emigrant B cells; P = 0.038) and increased Jκ5 usage (28% in XLA vs. 8.5% in control new emigrant B cells; P = 0.0086), suggesting extensive secondary V(D)J recombination on the Igκ locus (Fig. 3 A). In contrast, control new emigrant B cells preferentially use Jκ1 and Jκ2 segments (n = 70 from four healthy donors; Fig. 3 A and Table S1). XLA B cells also showed a Vκ gene usage biased to upstream Vκs consistent with secondary recombination events (P = 0.0045; Fig. 3 B). An apparent exception to this rule was the increased Vκ4-1 usage, the most downstream of all Vκs (16.25% in XLA patient vs. 1.5% in control new emigrant B cells; P = 0.0047; Fig. 3 B; references 20 and 28). However, Vκ4-1 rearrangements may correspond to secondary recombination events because of their inverse orientation on the Igκ locus (29). IgL gene rearrangements follow an ordered process where Igκ genes rearrange before Igλ genes (30). In addition to the apparent increase in Igκ gene secondary re-

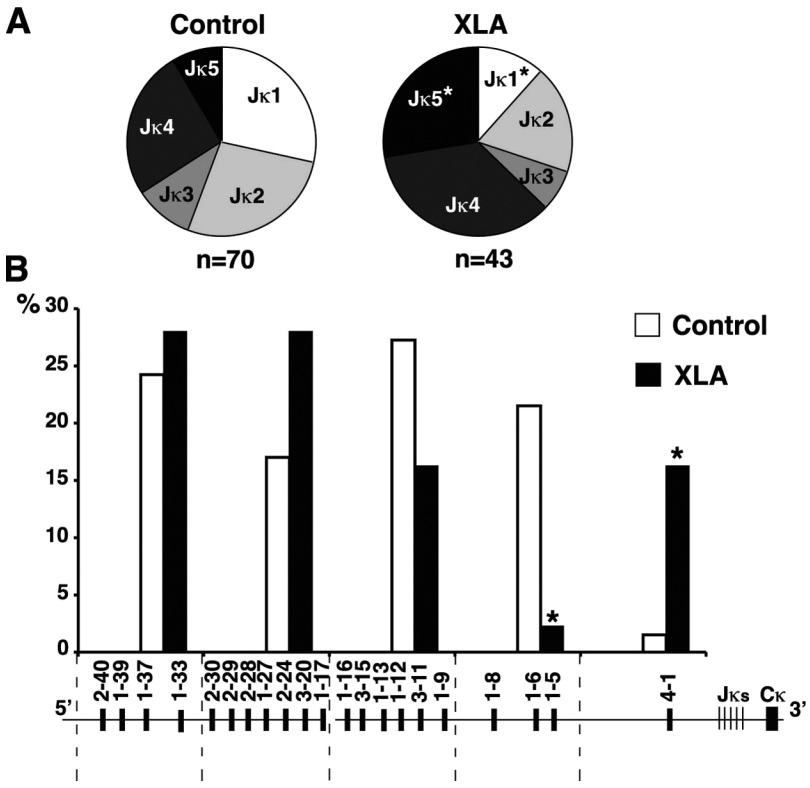

Figure 3. Extensive Igκ secondary recombination in XLA B cells. Jκ (A) and Vκ (B) usage in control new emigrant and XLA B cells. Control new emigrant B cell sequences included sequences shown in Table S1 and as reported previously (reference 1). Pie charts show proportion of different Jκ genes. The number of sequences analyzed in each fraction is indicated below the pie charts. The proximal Vκ locus (B) involved in 95% of Vκ–Jκ rearrangements is shown clustered into groups of V genes. The percent of each Vκ group is indicated on the y axis. *, statistically significant difference. P-values for differences between fractions are stated in the text and were calculated by the Fisher Exact Test.

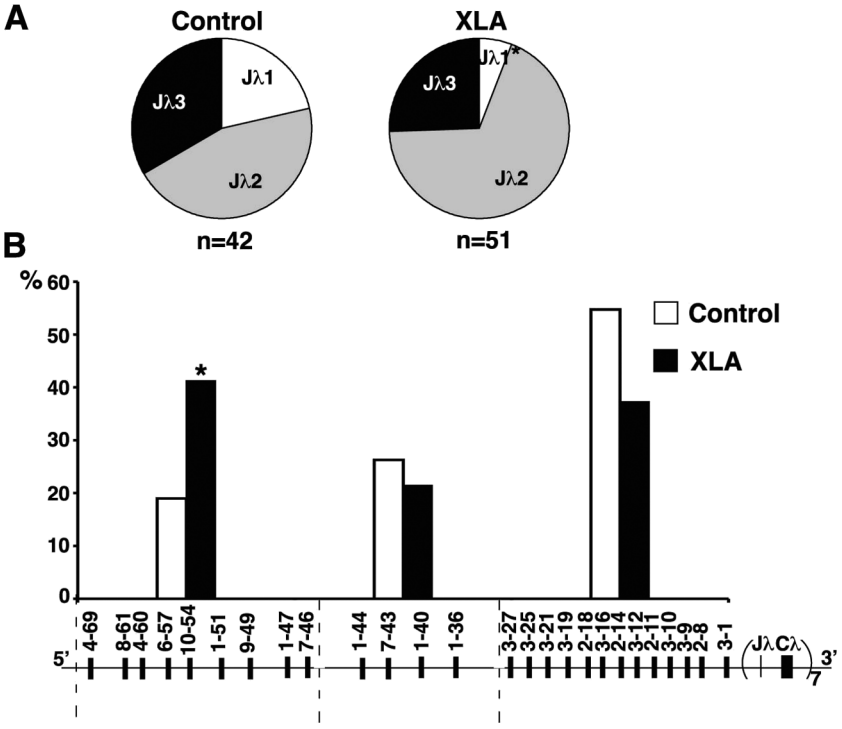

Figure 4. Extensive Igλ secondary recombination in XLA B cells. Jλ (A) and Vλ (B) usage in control new emigrant and XLA B cells. Control new emigrant B cell sequences included sequences shown in Table S1 and as reported previously (reference 1). Pie charts show proportion of different Jλ genes with the number of sequences analyzed indicated below. The Vλ locus (B) is shown clustered into groups of V genes. *, statistically significant difference. P-values for differences between fractions are stated in the text and were calculated by the Fisher Exact Test.

combination in XLA B cells, we also found an increase in Igλ-expressing B cells (43 Igκ/51 Igλ in XLA vs. 70 Igκ/42 Igλ in control new emigrant B cells; P = 0.017). Further, Igλ genes from XLA patients showed evidence of increased secondary V(D)J recombination in that there was a significant decrease of upstream Jλ1 usage in XLA B cells (5.9% in XLA vs. 21.4% in control new emigrant B cells; P = 0.032) and a proportional increase in more downstream Jλ usage (Fig. 4 A). When compared with control new emigrant B cells, XLA B cells showed a significant decrease in downstreamVλ3 family gene usage (P = 0.005) and an increase in upstream Vλ gene usage including unusual Vλ4, Vλ7, Vλ8, Vλ9, and Vλ10 genes (P = 0.026; Fig. 4 B). Our results are consistent with the possibility that XLA B cells suffered extensive secondary recombination on both Igκ and Igλ loci.

XLA B Cells Express Self-reactive Antibodies. To determine whether antibodies expressed by XLA B cells were self-reactive, we initially tested 65 antibodies cloned from single XLA new emigrant B cells and 29 antibodies from control new emigrant B cells for binding to nuclear and cytoplasmic antigens (HEp-2 cell line extract), using a standard clinical ELISA for ANAs (1). Additional control antibodies included a strong (ED38) and a weak (mGO186) positive control as well as nonself-reactive mGO53 and iGO13 antibodies (1, 20). In agreement with our previous report, we found that the proportion of HEp-2 lysate-reactive antibodies expressed by new emigrant B cells (31%) was similar to that of previous controls (40.7%; P = 0.48; Fig. 5 A; reference 1). In contrast, we found an increase in the frequency of B cells that expressed HEp-2 lysate-reactive antibodies in both XLA-Ins and XLA-T117P patients (62.1 and 55.6%, respectively; P = 0.025; Fig. 5 A). When control antibodies were pooled with the previously reported control antibodies isolated from new emigrant B cells (1), the proportion of HEp-2 lysate-reactive clones in this compartment reached 37.3% (31 out of 81) and the p-value for the difference of HEp-2 ELISA-reactive antibodies between control and XLA B cells slightly increased up to 0.013.

To determine whether XLA self-reactive antibodies were true ANAs or anti-cytosolic antibodies, we performed indirect immunofluorescence assays on fixed HEp-2 cells (Fig. 5 B). Autoreactive antibodies expressed by XLA B cells showed either nuclear (X2-20) or nuclear and cytoplasmic (X1-30, X2-53, X3-40, X3-69, and X-4-13) staining patterns (Fig. 5 B). Our observations suggest that peripheral XLA B cells contained a slightly increased proportion of lymphocytes expressing ANAs.

XLA B Cell Antibodies Are Polyreactive. Because the HEp-2 ELISA is a crude assay for self-reactivity, we tested the reactivity of 67 antibodies isolated from XLA B cells and 31 antibodies from control new emigrant B cells to single-stranded DNA, double-stranded DNA, insulin, and LPS (1, 20). In agreement with our previous reports, we found that only a small minority (9.4%) of antibodies cloned from control new emigrant B cells was polyreactive (Fig. 6; P = 0.7 when our 31 clones are compared with previously characterized new emigrant antibodies). When combined with previously characterized new emigrant antibodies, the proportion of polyreactive clones in the new emigrant B cell compartment of healthy donors averaged 8.2% (Fig. 6; reference 1). In contrast, 50 and 37.8% of antibodies expressed by XLA-ins and XLA-T117P B cells

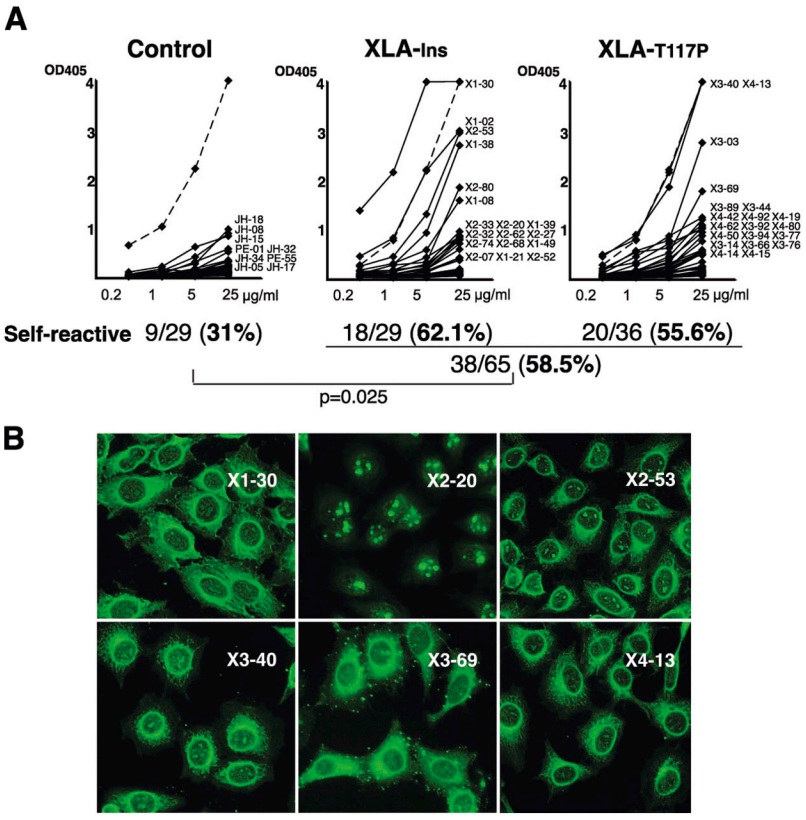

Figure 5. A majority of XLA B cells express self-reactive antibodies. (A) Data shown are from ELISAs for anti–HEp-2 cell reactivity using recombinant antibodies from 29 control new emigrant (left), 29 XLA-Ins (middle), and 36 XLA-T117P B cells (right). Reactive antibodies are indicated by clone number. Dotted lines show ED38 positive control (reference 20). The percentage of autoreactive clones for each fraction is indicated. P-values for differences between fractions are shown and were calculated by the Fisher Exact Test. The p-value increases up to 0.013 when control new emigrant antibodies reported previously were included (reference 1). (B) XLA B cells express ANAs. Antibodies from XLA B cells show various patterns of ANA including nucleolar (X2-20) and nuclear and cytoplasmic patterns (X1-30, X2-53, X3-40, X3-69, and X-4-13).

were polyreactive ($P \leq 0.0001$; Fig. 6). In addition, polyreactive antibodies from XLA B cells showed higher levels of self-reactivity than polyreactive antibodies from control new emigrant B cells (Fig. 6).

XLA polyreactive antibodies were enriched in long IgH CDR3s, positively charged amino acids, aromatic residues such as tyrosines encoded by the J_H6 gene segment, and hydrophobic amino acids encoded by hydrophobic D2 and D6 RF. These features have been associated with polyreactivity (Fig. S1 and Tables S2 and S3, available at http://www.jem.org/cgi/content/full/jem.20040920/DC1; references 1, 20, and 31). XLA nonself-reactive antibodies significantly favored V_H3 and J_H4 gene usage and shorter IgH CDR3s with hydrophilic D RF (Fig. S1 and Tables S2 and S3). We conclude that almost half of the antibodies expressed by XLA B cells are highly polyreactive and that Btk is essential for the removal of autoreactive B cells in humans.

Discussion

Human autoreactive B cells generated during early B cell differentiation fail to be removed when BCR signaling is altered in the absence of functional Btk. The majority of XLA B cells express autoreactive and polyreactive antibodies that display sequence features that favor DNA binding. These include long IgH CDR3s enriched in aromatic and positively charged amino acids (1, 20, 28, 31–33). In XLA patients, long IgH CDR3s and positively charged amino acids may result from increased nontemplate nucleotide addition at V-D and D-J junctions (34). XLA B cells resemble bone marrow early immature B cells from healthy donors in that both B cell populations express similar percentages of autoreactive and polyreactive antibodies (1). However, XLA B cells differ from early immature B cells by expressing high levels of surface IgM, whereas early immature B cells are surface IgM^- (Fig. 1 and reference 1). XLA B cells also display a unique antibody repertoire favoring specific V_Hs and Ds using hydrophobic RF, whereas early immature B cells from healthy donors do not, suggesting that Btk-deficient B cells might be selected to express such autoreactive antibodies (1, 26). This apparent positive selection for self-reactive BCRs in XLA B cells might be explained by the fact that B cells are unable to survive in the absence of BCR signaling (35, 36). In the absence of Btk, BCR signaling is severely impaired in humans and the few XLA B cells that migrate to the periphery may express autoreactive BCRs that are triggered by self-antigens and may generate the intracellular signals required for their survival. Similarly to Btk-deficient mice, XLA B cells that have undergone receptor editing may have received additional signals that rescue maturation defects, resulting from the absence of functional Btk (37). The recent development of antileukemic drugs inhibiting Btk signaling to promote the apoptosis of lymphoproliferative B cells would have to be studied carefully because these drugs will interfere with normal BCR signaling and affect B cell selection, and may allow the release of autoreactive B cells in treated patients (38).

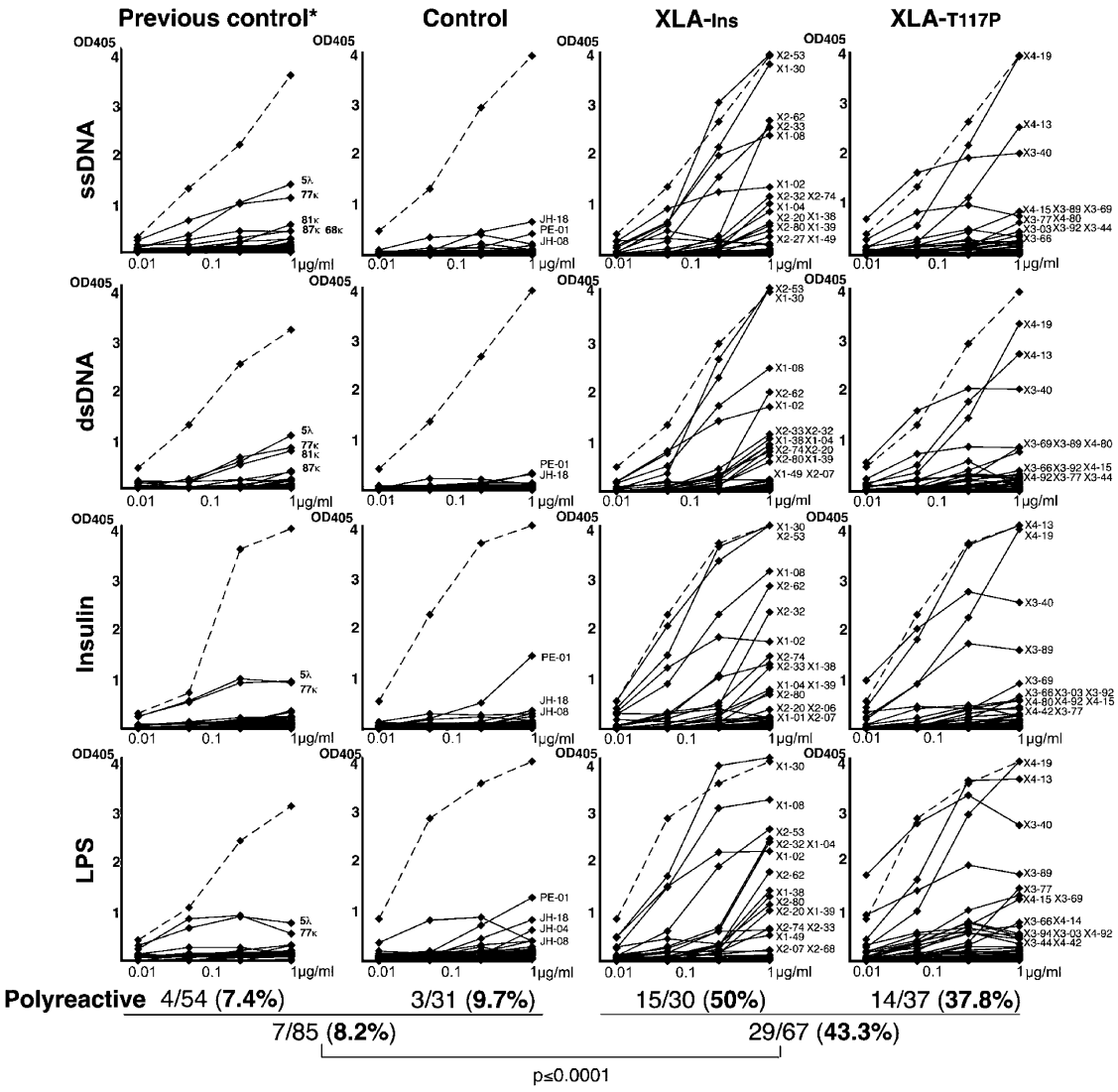

Figure 6. XLA B cells express polyreactive antibodies. Data shown are from ELISAs for reactivity with single-stranded DNA, double-stranded DNA, insulin, and LPS. Percentages represent frequency of polyreactive antibodies. Dotted lines show ED38 positive control (reference 20). Reactive antibodies are indicated by clone number. The frequency of polyreactive antibodies is significantly increased in XLA B cells ($P \leq 0.0001$). *, control new emigrant clones reported previously (reference 1).

XLA B cells show a distinct IgL repertoire characterized by increased upstream VL and downstream JL usage consistent with extensive IgL secondary recombination (39). A similar bias of the Igκ repertoire can be observed in antibody sequences obtained from EBV-transformed B cells from other XLA patients (40). As a consequence of extensive secondary recombination, we found an increased proportion of λ transcripts. However, XLA B cells have been reported to favor Igκ expression (12). This apparent discrepancy may result from different mutations of the *BTK* genes that would allow residual Btk signaling and the down-regulation of secondary recombination in some cases but not others. The bias in the IgL repertoire that we observed in XLA B cells may result from the negative selection of clones that expressed innocuous BCRs and did not undergo IgL secondary recombination, but failed to escape deletion in the absence of Btk. Alternatively, newly arising XLA B cells may not sense the expression of a BCR on their cell surface and may remain equivalent to pre–B cells in that they would keep recombining their IgL genes in the absence of Btk-dependent signals initiated by the BCR. In contrast, mouse Btk-deficient B cells do not show extensive IgL secondary recombination potentially because of Btk redundancy with another tyrosine kinase from the same family, Tec, in mouse but not in humans (41, 42). We previously reported that IgL secondary recombination was prominent in human Igμ–deficient pro–B cells (26). IgL gene secondary recombination appears to be a default mechanism in the absence of IgM signaling and this regulation suggests that receptor editing at the pre–B/immature B cell stage may require appropriate BCR signaling to be down-regulated (26). Developing B cells that fail to generate such a signal

may remain trapped in this editing compartment when insufficient BCR density is expressed on the cell surface or when expressing self-reactive BCRs (43–46).

The mechanisms that result in the development of autoimmunity in humans remain poorly understood. XLA as well as common variable immunodeficiency patients whose B cells show defects in BCR signaling may develop autoimmune diseases, suggesting that BCR signaling may play an important role in counterselecting self-reactive B cells (11, 47). In this report, we provide the first evidence that the alteration of BCR signaling threshold in XLA patients results in the release of self-reactive B cells in the periphery. Interestingly, the alteration of TCR signaling threshold in SKG ($ZAP70^{W163C}$) mice allows the release of self-reactive T cells in the periphery and the development of a rheumatoid arthritis–like disease (48). Because TCR and BCR signaling pathways share many molecules, it is likely that the alteration of the signaling ability of one or more of those molecules may result in the modification of TCR and BCR thresholds and in the release of both self-reactive T and B cells, thereby predisposing to autoimmunity.

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