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RESEARCH ARTICLE

Normal B cells express ZAP70 in chronic lymphocytic leukemia: A link between autoimmunity and lymphoproliferation?

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Abstract

ZAP70 has a prognostic value in chronic lymphocytic leukemia (CLL), through altered B-cell receptor signaling, which is important in CLL pathogenesis. A good correlation between ZAP70 expression in CLL cells and the occurrence of autoimmune phenomena has been reported. Yet, the great majority of CLL-associated autoimmune cytopenia is due to polyclonal immunoglobulin (Ig) G synthesized by nonmalignant B cells, and this phenomenon is poorly understood. Here, we show, using flow cytometry, that a substantial percentage of CD5⁻ nonmalignant B cells from CLL patients expresses ZAP70 compared with CD5⁻ B cells from healthy subjects. This ZAP70 expression in normal B cells from CLL patients was also evidenced by the detection of ZAP70 mRNA at single-cell level with polyclonal Ig heavy- and light-chain gene

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transcripts. ZAP70⁺ normal B cells belong to various B-cell subsets and their presence in the naïve B-cell subset suggests that ZAP70 expression may occur during early B-cell development in CLL patients and potentially before malignant transformation. The presence of ZAP70⁺ normal B cells is associated with autoimmune cytopenia in CLL patients in our cohort of patients, and recombinant antibodies produced from these ZAP70⁺ nonmalignant B cells were frequently autoreactive including anti-platelet reactivity. These results provide a better understanding of the implication of ZAP70 in CLL leukemogenesis and the mechanisms of autoimmune complications of CLL.

1 | INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a malignancy characterized by clonal accumulation of mature-appearing CD5⁺ B cells. The normal cell of origin from which CLL is derived has not yet been clearly identified.¹ Garcia-Munoz et al.^{2,3} proposed the *Burnet's "forbidden clone" theory*,⁴ implying that CLL cells originate from self-reactive B cells in which physiological immunologic tolerance mechanisms⁵ have induced additional genetic damage and may have immortalized and transformed them into leukemia cells.

Zeta-chain-associated protein of 70 kDa (ZAP70), a protein tyrosine kinase required for TCR signaling, is abnormally expressed in some CLL B cells and is a surrogate prognostic marker discriminating between mutated and unmutated immunoglobulin heavy variable (IGHV) gene CLL subsets.⁶⁻⁸ ZAP70⁺ CLL cells have survival advantages, possibly through ZAP70-related altered BCR signaling after BCR ligation,⁹⁻¹¹ which is known to play a role in CLL pathogenesis.¹²⁻¹⁷

Furthermore, CLL is associated with the development of autoimmune disorders, especially autoimmune cytopenia (AIC), such as autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia (ITP),¹⁸⁻²¹ that can occur at any time during CLL evolution, even in "preleukemic" monoclonal B-cell lymphocytosis.^{22,23} ZAP70 expression by CLL cells is the most discriminative parameter between CLL patients with or without AIC.²⁴ However, the mechanisms that lead to autoimmunity in CLL remain unknown as the great majority of autoantibodies in CLL-associated AIC are polyclonal IgG synthesized by nonmalignant B cells.²⁵

The consequence of abnormal ZAP70 expression in B cells on BCR signaling was recently highlighted.²⁶ By competing with the structurally similar spleen tyrosine kinase (SYK), ZAP70 diverts SYK from NFAT-dependent energy to PI3K-dependent survival signaling. It results in an escape of autoreactive or premalignant B-cell clones from negative selection with increased autoantibody production against multiple self-antigens and accelerated development of CLL B cells *in vivo*.²⁶

We hypothesized that ZAP70 expression may not be restricted to malignant B cells because of neoplastic transformation but may rather be an earlier phenomenon, which could predispose to malignancy and autoimmunity in CLL. The aim of our study was to assess ZAP70

expression in normal (nonmalignant) CD5⁻ B cells in CLL patients and their role in CLL-related AIC.

2 | METHODS

2.1 | Patients

Peripheral blood mononuclear cells (PBMC) from 63 CLL patients (preferentially with AIC) and from healthy subjects (healthy volunteers or buffy coats from blood donors) were isolated by Ficoll-Hypaque gradient and cryopreserved before analysis. Written consent was obtained from all patients, in accordance with Declaration of Helsinki and approved by the local ethics committee. CLL patients were not recently treated (<6 months), nor had other autoimmune disease or autoantibody besides AIC. Autoantibodies without associated cytopenia were not considered as AIC and not included in our cohort.

2.2 | Flow cytometry analysis

Viability staining was performed in all cells with Fixable Viability Dye eFluor 780 (eBioscience). Surface staining was performed with a mixture of anti-CD19-PE-Cy7, CD3-APC, λ-FITC, IgM-FITC, CD70-FITC, CD86-PerCPCy5.5, CD27-PerCPCy5.5, CD38-PerCPCy5.5, CD21-APC, CD43-APC (BD Pharmingen), CD5-ECD (Beckman Coulter), *kappa*-biotin (eBioscience), IgD-biotin (eBioscience) monoclonal antibodies. A streptavidin-APC was used as the secondary antibody. Intracellular ZAP70 staining was performed with anti-ZAP70-PE antibody (1E7.2) (BD Bioscience) after previous incubation with 2 mL of Lysing solution (BD Bioscience) for fixation and permeabilization. CLL samples were compared to an isotype control-PE antibody (mouse IgG1-PE, BD Bioscience). Cells were analyzed using Gallios flow cytometer (Beckman Coulter), and data were analyzed using Kaluza flow analysis software 1.2 (Beckman Coulter). Doublets and dead cells were excluded. CLL samples with less than 60 CD5⁻ B cells, and/or in which low CD5 expression did not permit a clear distinction between CD5⁻ B cells and CD5⁺ CLL cells were excluded. CLL samples were considered ZAP70 positive if ≥20% of CLL cells expresses ZAP70 compared with an isotype control.^{8,27} Results were expressed as percentage of positive cells^{7,28} and as mean fluorescence intensity (MFI) values.²⁹

2.3 | Single-cell reverse-transcription PCR (sc-rtPCR)

Sc-rtPCR was used to reverse-transcribe and amplify at the same time mRNA for rearranged Ig heavy and light chains and ZAP70 mRNA from single cells. Single switched (CD5-IgD-) B cells were isolated by Fluorescence-Activated Cell Sorting (FACS) on an Aria II sorter in a 96-well plate after surface staining with monoclonal antibodies, as described in flow cytometry method. Sorted single cells on dry well plates were sealed with aluminum foil and immediately placed on dry ice before storage at -80°C . cDNA was synthesized directly from total single-cell mRNA, without previous RNA extraction step. The reaction was carried out in a total volume of 20 μL per well in the original 96-well sorting plate. A mix of 140 ng of random hexamer primer (pd(N) 6) (Takara) and 1 μL of 10 μM ZAP70 gene-specific reverse primer, 0.5 μL of 100 mM DTT (Invitrogen, SuperScript III kit), 0.7 μL of 10% NP40 Surfact-Amps (Thermo Scientific), and 16 U of RNase out (Invitrogen) and H₂O was first added to each well, vortexed and then placed at 65°C for 1 minute, then on ice. A second mix was then added containing 5X first-strand buffer (Invitrogen, SuperScript III kit), 1 μL of 10 mM dNTP mix (Clontech), 1 μL of 100 mM DTT (Invitrogen, SuperScript III kit), 20 U of RNase out (Invitrogen), H₂O and 160 U of SuperScript III RT (Invitrogen). Reverse transcription was performed at 42°C for 5 min, 25°C for 10 min, 50°C for 55 min and 94°C for 5 min.

The gene-specific primers for ZAP70 mRNA isoform 1 (NM_001079.3 NCBI ref.) were designed in our lab with Vector NTI software (Life Technologies) and were highly specific (see Supplementary Table 1 in Supplementary data for sequences used). The primers used for *IgH*, *Ig λ* , and *Igk* amplification were the same as those published by Tiller et al.³⁰

IgH, *Ig λ* , *Igk*, and ZAP70 gene transcripts were amplified independently by nested PCR starting from 4 μL of cDNA for *Ig* or from 6 μL of cDNA for ZAP70, as template. All PCR1 reactions were performed in a total volume of 42 μL per well containing 1 μL of each primer (at 10 μM), 1 μL of 10 mM dNTP mix (Clontech), 2.2 U of HotStarTaq (Qiagen) with 10 \times PCR Buffer (HotStarTaq kit, Qiagen). All PCR2 reactions were performed with 4 μL for *Ig*, respectively, and 6 μL for ZAP70 of PCR1 product. PCR1 and PCR2 reactions were performed for *Ig* and ZAP70 in the same time and conditions: after an initial time at 94°C for 5 min, 50 cycles at 94°C for 30 s, 57°C (PCR1) or 59°C (PCR2) for 30 s, 72°C for 55 s (PCR1), or 72°C for 45 s (PCR2). The reaction was finished after a final elongation time at 72°C for 7 min, and then, the PCR products were placed on ice. The presence of amplified gene transcripts was then verified in agarose gel electrophoresis.

2.4 | Sequence analysis, cloning, and monoclonal antibodies production

PCR products were purified with Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced with the respective forward and reverse primers used in PCR2, by GATC Biotech services. Before analysis, each forward and respective reverse sequence was aligned in

Vector NTI software. ZAP70 sequence was analyzed by NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm human ZAP70 isoform 1 (NM_001079.3). Each Ig gene, was analyzed by IgBLAST comparison with IMGT Ig gene database (<http://www.ncbi.nlm.nih.gov/igblast/>) and by IMGT/V-QUEST (<http://www.imgt.org/IMGTvquest/vquest?livret=0&Option=humanlg>). The strategies to clone and express human recombinant monoclonal antibodies (rAb) were the same as those used by Tiller et al.³⁰

2.5 | Enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assays

Binding to nuclear and cytoplasmic antigens (HEp-2 cell line extract) was assessed using ELISA.³¹ For indirect immunofluorescence assays, HEp-2 cell coated slides (Bion Enterprises, LTD) were incubated in a moist chamber at room temperature with purified rAb at 50–100 mg/mL and detected with FITC-conjugated goat anti-human IgG.³² Poly-reactivity was assessed by ELISA against single-stranded and double-stranded DNA (ssDNA and dsDNA), insulin, and lipopolysaccharide (LPS). Antibodies that bind to all three antigens are considered poly-reactive.³² ED38 antibody was used as a positive control.³¹ The cutoff used to define the positivity of OD405 was twice the average OD405 ELISA value of all tested clones (approximately 0.5).

2.6 | Indirect anti-globulin (IAT) and monoclonal antibody immobilization of platelet antigens (MAIPA) test

rAb from ZAP70⁺CLL associating AIC were tested for reactivity against red blood cells by IAT (DC-ScreeningII, Bio-Rad, ref:004836) and against platelets by MAIPA (Complete MAIPA Kit, apDia, ref:900006).

3 | RESULTS

3.1 | Nonmalignant CD5- B cells from CLL patients express ZAP70

Gating strategy and CLL patients' characteristics are provided, respectively, in Supplementary Figure 1 and Supplementary Table 2 in Supplementary data. Among the 63 CLLs, 75% were ZAP70+. A higher percentage of CD5- B cells from CLL patients expressed ZAP70 compared to those from healthy subjects ($n = 36$) ($p < .0001$, Figure 1A,B). Accordingly, ZAP70 MFI in CD5- B cells from CLL patients was higher (1.72 vs. 1.03, $p < .0001$, Figure 1C). We observed a positive correlation between the level of ZAP70 expression in CD5- B cells and CLL B cells from the same patient, both in terms of percentage of ZAP70+ cells and of ZAP70 MFI ($p < .0001$ for both) (Figure 1D,E). Hence, ZAP70 expression is not restricted to malignant CLL B cells, as it is also detected in CD5- B cells from CLL patients.

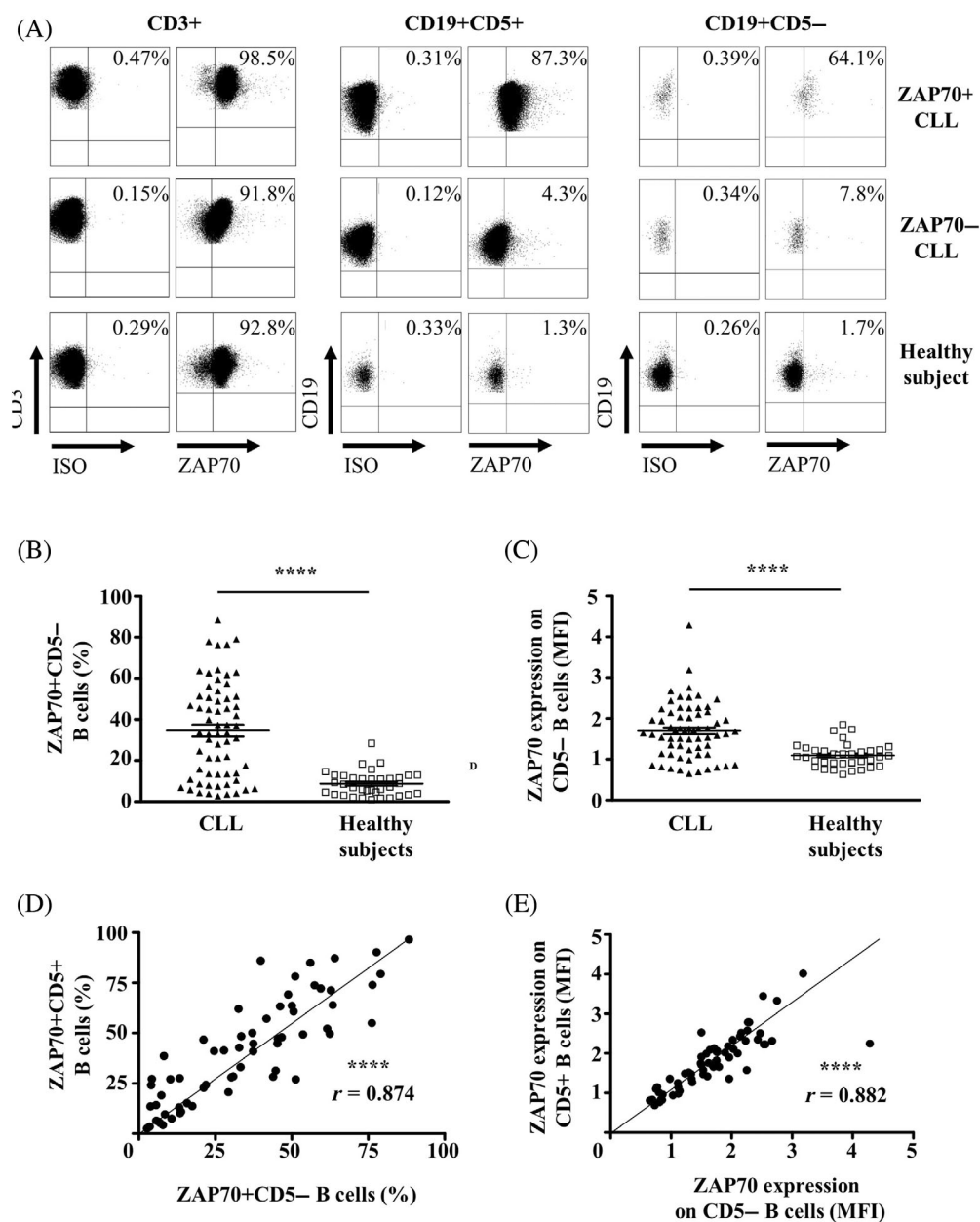


FIGURE 1 Normal B cells from CLL patients frequently express ZAP70. (A) Representative FACS plots of ZAP70 expression compared with isotype control (ISO) on ZAP70+ CLL case (upper), ZAP70- CLL case (middle), and healthy subject (bottom). On the left panels, we showed positive control expression on CD3+ cells, in the middle ZAP70 expression on CD5+ B cells, and on the right ZAP70 expression on normal B cells (CD5-). (B, C) Comparison of percentage of ZAP70+ cells among CD5- B cells (B) and ZAP70 MFI in CD5- B cells (C) from CLL patients ($n = 63$) and healthy subjects ($n = 36$) (Mann-Whitney test). (D, E) Correlations between the percentage of ZAP70+ cells among CD5+ and CD5- B cells in CLL patients (D) ($n = 63$) and ZAP70 expression (MFI) and ZAP70 expression (MFI) in the same cells (E) (Spearman test); **** $p < .0001$; MFI, mean fluorescence intensity.

3.2 | ZAP70 + CD5- B cells are distinct from CLL clones, polyclonal, and do not belong to a specific B-cell subset

To determine that ZAP70 expression in CD5- B cells did not represent contamination by a contingent of malignant cells that would have lost CD5 expression, we assessed CD19 expression levels and Ig kappa and Ig lambda light chain expression. CD19 expression in ZAP70 + CD5- B cells (MFI = 16.2) was similar to that in normal B cells from healthy subjects (MFI = 17.4, $p = .6$) and higher than that in CLL B cells (MFI = 9.75, $p < .001$), low levels of CD19 being a characteristic of CLL cells (Figure 2A).³³ ZAP70 + CD5- B cells were polyclonal as they expressed normal Ig κ/λ light chain ratios (1.19 ± 0.44) (see Supplementary Figure 2A in Supplementary data), whereas

ZAP70 + CD5+ CLL B cells expressed either Ig κ or Ig λ (Figure 2B). Moreover, the level of expression of surface Ig light chains in ZAP70 + CD5- B cells was also similar to that of B cells in healthy controls and higher than that in CLL B cells in which BCR expression is low (Figure 2B). Finally, we documented clear ZAP70 expression in CD5- B cells not expressing the same light chain as the clonal population (see Supplementary Figure 2B in Supplementary data). ZAP70 + CD5- and ZAP70-CD5- B cells were equally distributed in the different B-cell subsets (Table 1). In addition, ZAP70 + CD5- B cells were not enriched in autoimmune-prone CD21low B cells, neither in B1-like cells (CD27 + CD43 + CD70-).³⁴ Hence, we conclude that nonmalignant ZAP70 + CD5- B cells from CLL patients are distinct from CLL clones, polyclonal, and not part of a particular B-cell subset.

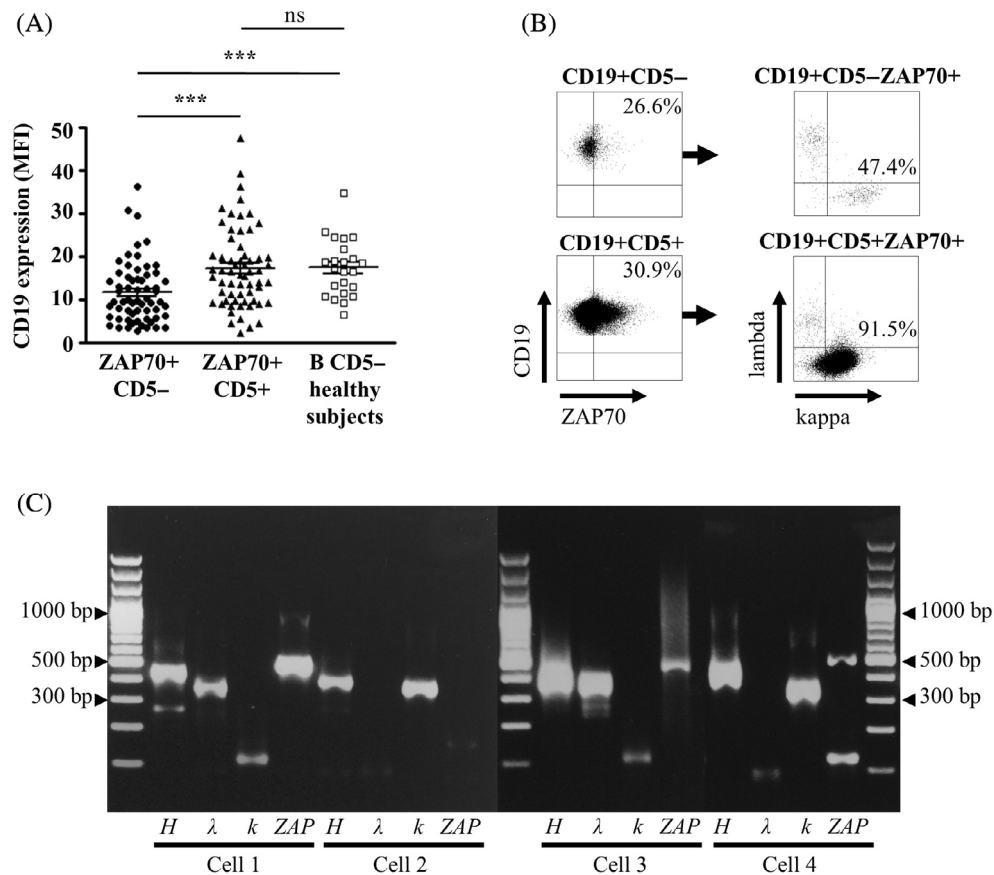


FIGURE 2 ZAP70+ normal B cells do not represent contamination of normal B cells with CLL. (A) CD19 MFI in ZAP70 + CD5-, ZAP70 + CD5+ B cells, and B cells from controls. (B) Representative FACS plot showing that ZAP70 + CD5- B cells express high level and polyclonal surface kappa or lambda light chains contrary to CLL cells (CD5+), which express weak and monoclonal kappa light chains. (C) Representative sc-rtPCR for *IgH*, (≈ 400 bp), *Igλ* (≈ 380 bp), *Igκ* (≈ 390 bp), and *ZAP70* (487 bp) in 4 normal B cells from two ZAP70 + CLL patients. Left panel: cells n°1 (ZAP70+) and n°2 (ZAP70-, negative control for ZAP70 mRNA) are normal B cells from patient A (with monoclonal kappa CLL). Right panel: cells n°3 and n°4 are both ZAP70+ normal B cells with different light chains Ig from patient B (with monoclonal kappa CLL). Cell n°4 is different from the CLL clone, as they express different IgH and Vk rearrangements (IgH 3-30, Vk 1-33 for CLL clone and IgH 3-23, Vk 3-20 for cell n°4). *** $p < .001$; ns, nonsignificant.

TABLE 1 ZAP70 + CD5- B cells do not belong to a specific B-cell subset and do not express the activation marker CD86.

Characteristics	ZAP70 + CD5- B cells	ZAP70-CD5- B cells	<i>p</i>
CD86 MFI (mean \pm SEM)	0.52 \pm 0.02	0.46 \pm 0.01	0.0646
B-cell subsets (mean \pm SEM), %			
Naive (IgM + IgD + CD27-)	24.43 \pm 3.03	26.26 \pm 3.36	0.6880
Switched memory (IgM-IgD-CD27+)	28.84 \pm 2.56	31.68 \pm 2.57	0.4373
Nonswitched memory (IgM + IgD-CD27+)	3.93 \pm 0.72	3.89 \pm 0.79	0.9647
Marginal zone (IgM + IgD + CD27+)	12.89 \pm 2.23	11.00 \pm 1.97	0.5287
Memory CD27- (IgM-IgD-CD27-)	20.21 \pm 2.18	19.82 \pm 2.36	0.9030
Anergic (IgM-IgD + CD27-)	3.92 \pm 0.87	3.51 \pm 0.75	0.7266
CD27+	52.67 \pm 4.40	54.29 \pm 4.86	0.8066
CD21low	16.70 \pm 2.26	11.72 \pm 2.06	0.1140
B1-like (CD27 + CD43 + CD70-)	9.19 \pm 2.62	7.10 \pm 1.45	0.4917

Abbreviation: SEM, standard error of the mean.

3.3 | Ig V(D)J gene sequence analysis by sc-rtPCR confirms the polyclonality of ZAP70 + CD5- B cells

V(D)J rearrangement amplification cannot be performed on single sorted ZAP70+ cells as its intracellular localization requires prior fixation and permeabilization procedures with paraformaldehyde, which intercalates within nucleic acids and inhibits subsequent PCR reactions. Pretreatment kits reverting formaldehyde modifications on RNA are designed for large quantities of nucleic acids but are not sensitive enough to be used on single cells. We then developed a sc-rtPCR method to amplify ZAP70 and V(D)J gene transcript on single cell, sorted as CD19 + CD5-IgD-, from 7 ZAP70+ CLL patients with AIC. Gating strategy for sorting is provided in Supplementary Figure 3 in Supplementary data.

We first confirmed that a proportion of CD5- B cells actively transcribed ZAP70 in each patient and that ZAP70 + CD5- B cells were polyclonal, since either *Igk* or *Igλ* rearranged V genes were amplified (Figure 2C). Sequencing confirmed the polyclonal nature of ZAP70 + CD5- B cells, which displayed V(D)J rearrangements that differed from those of malignant clones (Supplementary Table 3 in Supplementary data). Sequencing of the ZAP70 transcripts found 100% identical with the classical isoform 1 of ZAP70 described in T cells (NCBI Reference Sequence: NM_001079.4) and CLL.³⁵

We then compared *IgH/L* CDR3 sequences used by ZAP70 + CD5- and by CLL B cells. CDR3 from clonal CLL cells was longer than CDR3 from nonmalignant B cells in 6 out of 7 patients (Supplementary Table 3 in Supplementary data). Of note, few ZAP70 + CD5- B cells (e.g., GEN KA and HIT31) displayed the same *IgH V(D)J* rearrangement and CDR3 to that of their respective CLL B cells but had a different light chain (Supplementary Table 3 in Supplementary data). Overall, Ig repertoire analysis confirms the polyclonality of ZAP70 + CD5- B cells.

3.4 | ZAP70 + CD5- B cells may be involved in the development of autoimmunity in CLL patients

Only patients with ZAP70+ CLL developed AIC (autoimmune hemolytic anemia and immune thrombocytopenia) in our cohort ($n = 19/63$). Moreover, all these patients demonstrated a significant percentage of ZAP70 + CD5- B cells (see Supplementary Table 2 and Supplementary Figure 4A,B in Supplementary data).

To assess whether autoreactive antibodies could arise from ZAP70 + CD5- B cells, we cloned and expressed in vitro 18 monoclonal recombinant antibodies (rAb) from ZAP70 + CD5-IgD- cells from 7 patients with ZAP70 + CLL and AIC and tested their potential auto-reactivity. We selected only class-switched (i.e., IgD-) normal B cells because these cells are responsible for the great majority of autoimmune phenomena in CLL.²⁵ rAb heavy and light chain complete sequences are available at GenBank, accession numbers ON156401-ON156438 (submission ID 2558184). Nine (50%) rAb, from 5 of the 7 CLL patients, were autoreactive (2 with anti-nuclear pattern, 5 with cytoplasmic pattern, and 7 positive in ELISA) (Figure 3A,B;

Supplementary Table 4 in Supplementary data). Among the 18 rAb, 4 (22.2%), from 3 of the 7 CLL patients, were polyreactive (Figure 3C; Supplementary Table 4 in Supplementary data). One rAb was positive in MAIPA and none in IAT (Supplementary Table 4 in Supplementary data).

4 | DISCUSSION

In humans, ZAP70 is detected in early medullar stages of B-cell development, with unknown significance.^{26,36} Its expression in peripheral circulating B cells is null or very low and seems limited to some activated B-cell subsets from tonsils and spleen.³⁷⁻³⁹ Conversely, our study demonstrates for the first time that circulating nonclonal CD5- B cells from CLL patients express high levels of ZAP70 and correlated with that on CLL B cells.

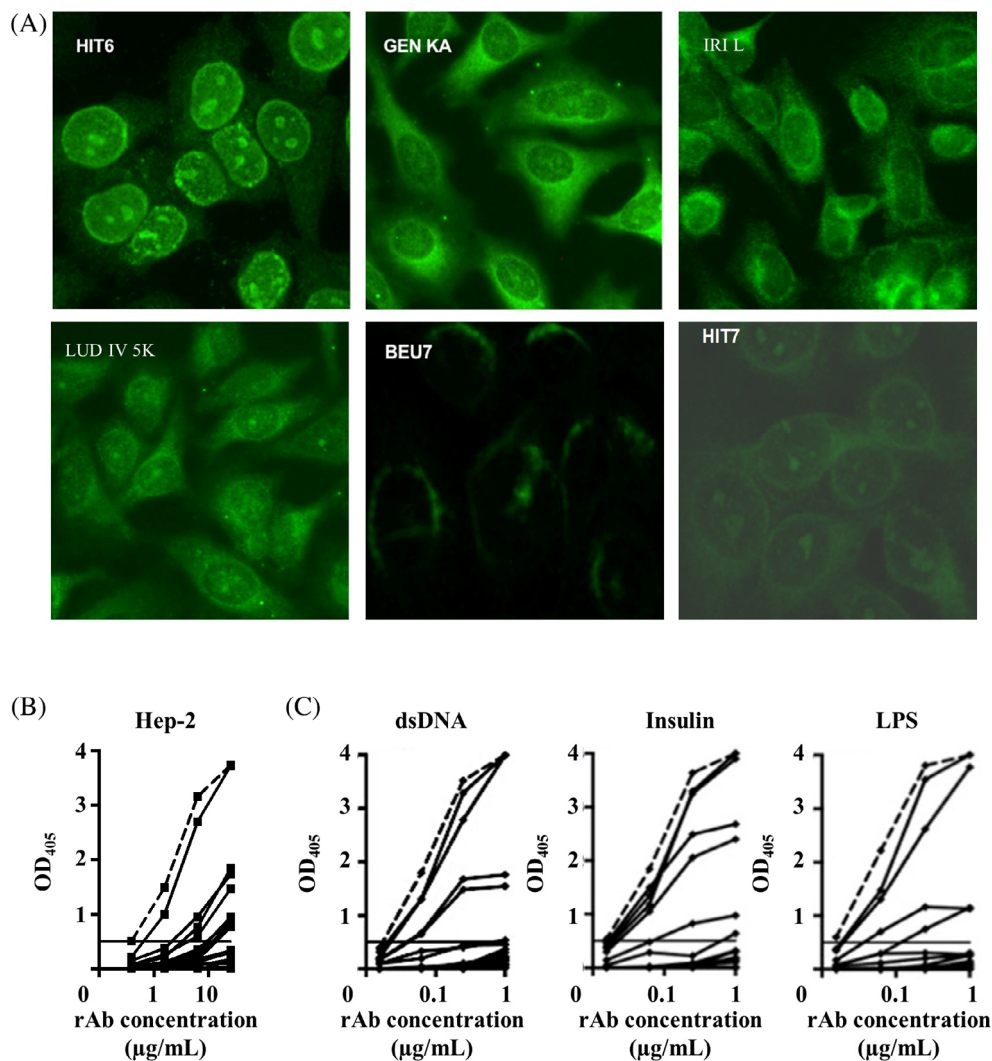
The presence of ZAP70 among various B-cell subsets including naive B cells suggests that its expression is an early process in B-cell development, which may occur before malignant transformation. The recently described behavior of CLL BCR as pre-BCR, which has antigen-independent tonic signaling,¹⁵ together with the fact that ZAP70 has been demonstrated to have a role in the pre-BCR signaling,⁴⁰ supports the role of ZAP70 in malignant B-cell ontogeny. This is in line with very recent data showing that ZAP70 is aberrantly co-expressed with SYK in a broad range of B-cell malignancies. ZAP70/SYK co-expression suppresses chronic autonomous BCR activity, allowing B-cell clones to evade NFAT-mediated induction of anergic state, and to retain responsiveness upon BCR engagement.²⁶ In addition, ZAP70/SYK co-expression is associated with significantly accelerated development of CLL B cells in vivo.²⁶ However, precise assessment of ZAP70 expression at earlier developmental stages in the bone marrow remains challenging as bone marrow examination is not part of the diagnostic procedure in CLL patients.

The mechanisms that contribute to the elevated expression of ZAP70 in nonmalignant B cells are unknown. By preliminary experiment of the culture of PBMC and serum from ZAP70+ CLL patients or healthy subjects, we did not observe an increased expression of ZAP70 in CD5- B cells (see Supplementary Figure 5 in Supplementary data). However, ZAP70 expression after direct in vitro stimulation or mediated by extrinsic cellular interaction (i.e., stromal cells) has not yet been studied.

We describe herein a reverse-transcription scPCR method allowing successful detection of ZAP70 mRNA at single-cell level in CD5- B cells, together with Ig heavy and light chain gene transcripts. The presence of ZAP70 transcript was not a false positive product as the primers for ZAP70 mRNA were highly specific and most of the CD5- B cells were ZAP70-. Moreover, ZAP70 transcripts amplified from single cell were found 100% identical to the sequence previously described in T cells and in CLL cells.³⁵

CDR3 from CLL cells were longer than those from CD5- B cells, a feature often associated with self-reactivity.³¹ This is in line with the hypothesis that clonal CLL cells may originate from autoreactive B cells.⁵ Given that BCR V regions are involved in the transformation/

FIGURE 3 Reactivity for rAb cloned from ZAP70 + CD5-IgD-B cells from AIC-associated CLL. (A) Representative IFA on Hep-2 cell-coated slides for 6 autoreactive rAb with cytoplasmic reactivity: GEN KA, IRI L, LUD IV 5 K, BEU 7, with nuclear reactivity: HIT 6, or with both: HIT 7. (B) ELISA using Hep-2 cell lysates allowed the identification of 7 autoreactive rAb out of 18 rAb tested (see Supplementary Table 4 in Supplementary data for more details). (C) ELISA for polyreactivity towards DNA (ss and dsDNA), insulin, and LPS. Four out of 18 rAb were considered polyreactive (when they recognized all three antigens). Highly polyreactive ED38 antibody was used as a positive control. Fluorochromes: goat anti-human IgG-FITC; original magnification $\times 40$ for panel A.



proliferation of CLL B cells, and that ZAP70 diverts SYK from NFAT-dependent energy state to PI3K-dependent survival signaling and retains responsiveness upon BCR engagement, resulting in escape from negative selection,²⁶ ZAP70 expression may facilitate the transformation of CD5- B cells, and also through the protective role of microenvironment.⁴¹ However, SYK/ZAP70 co-expression levels and molecular interactions of ZAP70 in ZAP70 + CD5- B cells from CLL patients remain to be explored.

In our cohort, all CLL-AIC patients had a significant percentage of ZAP70 + CD5- B cells. In ZAP70+ CLL, the presence of AIC was not associated with a higher level of ZAP70, as compared with ZAP70+ CLL without AIC (see Supplementary Figure 4A,B). Thus, the co-expression of ZAP70 and SYK in CD5- B cells seems a necessary but not sufficient condition to AIC occurrence in CLL patients. The fact that only a certain level of ZAP70 protein is necessary to alter BCR signaling was previously demonstrated for CLL clones.¹⁰ Moreover, patients with an intermediate level of ZAP70 in CLL B cells have the same risk of aggressive disease as those with high levels.²⁸

We recently demonstrated, in a *Zap70*^{Mb1-Cre}-knockin mouse model, that co-expression of ZAP70 and SYK was associated with

reduced apoptosis rate of pre-B and immature B cells and increased autoantibody production against multiple self-antigens, supporting defective negative selection of autoreactive B cells.²⁶ Here, we found that 50% and 22.2% of rAb from ZAP70 + CD5- class-switched memory B cells were, respectively, autoreactive and polyreactive. These percentages are lower than CLL clone from UM-CLL cases,⁵ but higher than those of naive B cells^{31,42} and similar to those of class-switched memory B cells from normal subjects.^{32,42}

The limited number of tested rAb did not allow statistical analysis. However, given the low sensitivity of the MAIPA test (53%)⁴³ and the scarcity of circulating anti-platelet antibody-producing B cell (5.4% in primary ITP),⁴⁴ we assume that 1 positive rAb towards platelets of a total of 18 is a significant event. Thus, ZAP70 + CD5- B cells from CLL patients could be nonanergic autoreactive cells that may escape tolerance mechanisms, due to advantages conferred by early and abnormal ZAP70 expression altering BCR signaling. Interestingly, a role for ZAP70 expression in B cells in the occurrence of systemic autoimmune diseases was suggested by another group that demonstrated that B cells in synovial fluid obtained from rheumatoid arthritis patients could express ZAP70.^{45,46}

In conclusion, we show for the first time that nonmalignant B cells from CLL patients frequently express ZAP70, are distinct from CLL clone, distributed among various B-cell subsets, and frequently autoreactive and polyreactive. However, relationships between ZAP70 expression in B cells, BCR signaling, leukemogenesis, and tolerance breakdown remain to be further explored, as well as the molecular mechanisms that promote aberrant ZAP70 expression in CD5- B cells.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

For original data, please contact dana.ghergus@chu-lyon.fr. rAb heavy and light chain complete sequences are available at GenBank, accession numbers ON156401- ON156438 (submission ID 2558184).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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