Calreticulin as a novel B-cell receptor antigen in chronic lymphocytic leukemia

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Supplemental Materials and Methods

Patients samples and reagents

Peripheral blood (PB) samples were obtained from previously untreated patients fulfilling diagnostic and immunophenotypic criteria for CLL at the Leukemia Department at MD Anderson Cancer Center after informed consent and Institutional Review Board approval (Supplemental Table 1). Sera from patients with active systemic lupus erythematosus (SLE) were obtained from patients fulfilling diagnostic criteria for SLE at the Ospedale Vita-Salute San Raffaele (Milano, Italy) after informed consent and approval by the Institutional Ethical Committee. CLL- PBMCs were isolated by density gradient centrifugation over Ficoll-Plaque (GE Healthcare), and cultured at 1.5 x 10^7cells/mL in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS; Gibco), and penicillin-streptomycin-L-Glutamine solution (Corning Inc.) (complete RPMI).

Immunofluorescence and confocal microscopy

CLL-PBMCs were cultured at 1.5 x 10^7cells/ mL in complete RPMI for 14 days onto 8-well microscope-compatible chamber slides (ibidi). Media was then removed, and NLC outgrowth (1) confirmed by phase contrast microscopy. Cells were then washed twice in PBS, then fixed with 4% paraformaldehyde (PAF) for 10 minutes at room temperature. PBS washes were repeated prior to permeabilization with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. PBS washes were again repeated, then cells were blocked for one hour in blocking solution (3% BSA + 1% horse serum) at room temperature. Rabbit monoclonal anti-Calreticulin primary antibody (Cell Signaling Technology, 1:400) and mouse monoclonal anti-CD68 (Abcam, 1:200) were added and incubated overnight at 4ºC. Cells were then washed twice with PBS and secondary anti-rabbit AlexaFluor488 and anti-mouse AlexaFluor594 were added at 1:1000 concentration, and incubated

for 1 hour at room temperature. Cells were again washed, then stained with DAPI (1:10.000) for 3 minutes at room temperature, prior to addition of 300μL mounting media (Dako) to the chamber, and imaged through an Olympus FV-1000 laser confocal microscope (60X objective). For surface immunofluorescence staining, anti-Calreticulin antibody (1:400) and mouse monoclonal anti-IgM (Abcam, 1:200) were added prior to fixation and permeabilization of the CLL-NLC preparations.

Immunohistochemistry

For double immunostain, section of a lymph node effaced by CLL underwent two-step procedure. During the first step, anti-CD68 (clone KP1, Leica Biosystem, dilution 1:400) mouse monoclonal IgG1 followed antigen retrieval with Tris-EDTA at pH9 and was revealed with DAB. The second step utilized anti-Calreticulin rabbit monoclonal IgG at 1:200 dilution following antigen retrieval for 5 minutes of microwave treatment with citrate buffer at pH6 and the reaction was revealed with FastRed.

Western Blot and flow cytometry

Immunoblot (2) and flow cytometry (3) were performed as previously described. After 14 days of CLL-NLC co-culture, CLL cells were washed off of the NLC layer by repeated washes with complete RPMI medium. To detach NLC, 5mM EDTA in PBS was added for 2 minutes, prior to scraping. Membrane and cytosolic fractions were isolated with the MEM-PER extraction kit from Thermo Fisher Scientific, according to the manufacturer's instructions. Anti-Calreticulin, anti-HSP90 and anti-GAPDH primary antibodies, all of them used at a 1:1000 concentration, were purchased from Cell Signaling Technology. For flow cytometry, cells were incubated for 20 minutes with PE-conjugated anti-Calreticulin (1:100) from Enzo Life Sciences at 4°C, or isotype

control. Samples were acquired on a FACSCalibur (Beckton Dickinson) and data analyzed using FlowJo Software version 9.6.4. (TreeStar).

Expression of recombinant CLL monoclonal antibodies and ELISA assays

Soluble CLL monoclonal antibodies (mAbs) were prepared as recombinant human IgG1 paired with kappa or lambda light chain depending on the isotype expressed by the leukemic clone, using standard methodology (4). In brief, heavy and light chain variable regions were amplified by PCR using gene-specific primers and cloned into a human IgG1, IgK, or IgL expression vector containing the human Igγ1, Igκ or Igλ constant regions and a murine Ig gene signal peptide sequence (GenBank accession no. DQ407610). Recombinant mAbs were expressed in human embryonic kidney (HEK) 293 cells by co-transfection with immunoglobulin (IG) heavy chain and corresponding IG light chain encoding plasmids using Jet Pei reagent (Polyplus transfections). Twelve hours after transfection, cell culture medium was replaced with serum-free DMEM supplemented with 1% Nutridoma SP (Roche Diagnostics). Supernatants were collected after 7 days of culture and cell debris was removed by centrifugation at 800 x g for 10 minutes. Recombinant mAbs were purified using Protein G beads (GE Healthcare), eluted in 0.1M glycine (pH 3.0) and neutralized with 1M Tris-HCl (pH 8.0). Their integrity and purity was confirmed by SDS-PAGE analysis and Coomassie blue staining and their concentration were determined by quantitative ELISA (Human IgG ELISA Quantitation Kits, Bethyl laboratories Inc.). Reactivity against Calreticulin was assessed by ELISA. Briefly, 96-well plates (Corning) were coated with 100µl of 5 µg/ml of purified Calreticulin (Abcam) overnight at 4°C, and then blocked with 150 µl 3% BSA in PBS for 3 hours at room temperature. CLL mAbs were used as primary antibodies at a concentration of 20 µg/mL and incubated overnight at 4°C. In parallel, sera from systemic lupus

erythematosus (SLE) patients were used as positive control in a 1:100 dilution. Next, 100µl of horseradish peroxidase (HRP) conjugated goat anti-human IgG antibody (Bethyl laboratories) at 1:200.000 dilution was added for 1 hour at room temperature and assays were developed using 50µl of TMB Peroxidase Substrate (Bethyl laboratories). The reaction was stopped after 15 minutes by adding 50µl 0,18 M H_2SO_4 (Bethyl laboratories) and the absorbance was measured at 450 nm. All samples were tested in triplicates. The analysis was performed on a BioTeK ELx800 ELISA reader (BioTek), and data analyzed by GraphPad Prism 5 software. Anti-Calreticulin binding of CLL mAbs was plotted as the signal-to-background ratio. CLL mAbs with a ratio >2.5 were considered as Calreticulin binders. The titration of anti-Calreticulin reactivity of the positive mAbs was performed by ELISA as described above, and CLL mAbs were used at various concentrations (2,5- 80 µg/ml).

Epitope mapping

To reconstruct epitopes of the target molecule a library of peptides was synthesized. An amino functionalized polypropylene support was obtained by grafting with a proprietary hydrophilic polymer formulation, followed by reaction with t-butyloxycarbonyl-hexamethylenediamine (BocHMDA) using dicyclohexylcarbodiimide (DCC) with Nhydroxybenzotriazole (HOBt) and subsequent cleavage of the Boc-groups using trifluoroacetic acid (TFA). Standard Fmoc-peptide synthesis was used to synthesize peptides on the amino-functionalized solid support by custom modified JANUS liquid handling stations (Perkin Elmer). Synthesis of structural mimics was done using Pepscan's proprietary Chemically Linked Peptides on Scaffolds (CLIPS) technology. CLIPS technology allows to structure peptides into single loops, double loops, triple loops, sheet-like folds, helix-like folds and combinations thereof. CLIPS templates are coupled to cysteine residues.

The side-chains of multiple cysteines in the peptides are coupled to one or two CLIPS templates. For example, a 0.5 mM solution of the P2 CLIPS (2,6-bis(bromomethyl)pyridine) is dissolved in ammonium bicarbonate (20 mM, pH 7.8)/acetonitrile (1:3(v/v)). This solution is added onto the peptide arrays. The CLIPS template will bind to side-chains of two cysteines as present in the solid-phase bound peptides of the peptide-arrays (455 wells plate with 3 µl wells). The peptide arrays are gently shaken in the solution for 30 to 60 minutes while completely covered in solution. Finally, the peptide arrays are washed extensively with excess of H_2O and sonicated in disrupt-buffer containing 1% SDS/0.1 %beta-mercaptoethanol in PBS (pH 7.2) at 70°C for 30 minutes, followed by sonication in H_2O for another 45 minutes. The T3 CLIPS carrying peptides were made in a similar way but now with three cysteines. The binding of antibody to each of the synthesized peptides was tested in a PEPSCAN-based ELISA. The peptide arrays were incubated with primary antibody solution (overnight at 4°C). After washing, the peptide arrays were incubated with a 1:1000 dilution of an appropriate antibody peroxidase conjugate (SBA) for one hour at 25°C. After washing, the peroxidase substrate 2,2' azino-di-3-ethylbenzthiazoline sulfonate (ABTS) and 20 µl/ml of 3% H_2O_2 were added. After one hour, color development was measured and quantified with a charge coupled device (CCD) - camera and an image processing system. Values obtained from the CCD camera range from 0 to 3000 mAU, similar to a standard 96-well plate ELISA reader. The results were quantified and stored into the Peplab database.

References

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Supplemental Table 1

Clinical and biological features of the analyzed CLL patient samples.

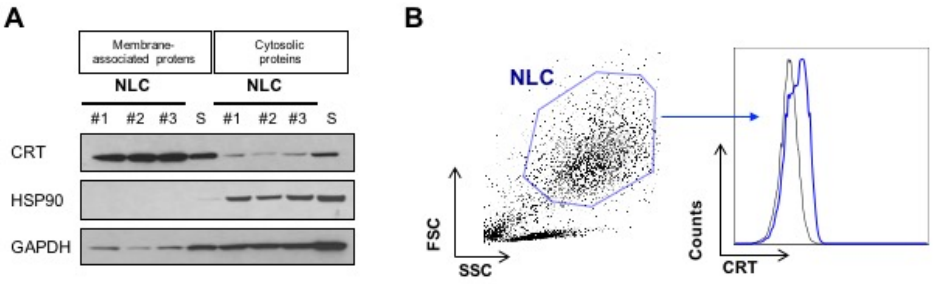
Rai stage	Age	Sex	IGHV status	CD38	ZAP70	Cytogenetics	CRT expression analysis
I	67	M	M	Negative	Negative	del (13q)	sIF, FC,WB
0	55	M	M	Negative	Negative	Negative	IF, sIF, FC
0	72	M	nd	Positive	Positive	tri12	IF,WB
0	42	M	U	Negative	nd	del (13q)	IF, WB
IV	73	M	U	Negative	nd	Negative	IF, sIF, FC
II	62	M	U	Positive	Negative	del (13q)	sIF, FC

Rai stage of disease, age at diagnosis, sex, IGHV gene mutational status (M, M-CLL; U, U-CLL), CD38 expression (pos, positive; neg, negative), ZAP70 expression (pos, positive; neg, negative), cytogenetic abnormalities (del, deletion; tri, trisomy), and type of analysis conducted to assess CRT expression. IGHV gene mutational analysis was performed by PCR, followed by direct sequencing, and 98% cutoff was used for mutational status assessment. CD38 expression was determined by flow cytometry, and 30% positivity was used as cutoff. ZAP70 expression was evaluated by immunohistochemical staining of bone marrow biopsies. Cytogenetic abnormalities were determined by fluorescence in situ hybridization analysis. IF: immunofluorescence; sIF: surface immunofluorescence; FC: flow cytometry; WB: Western Blot.

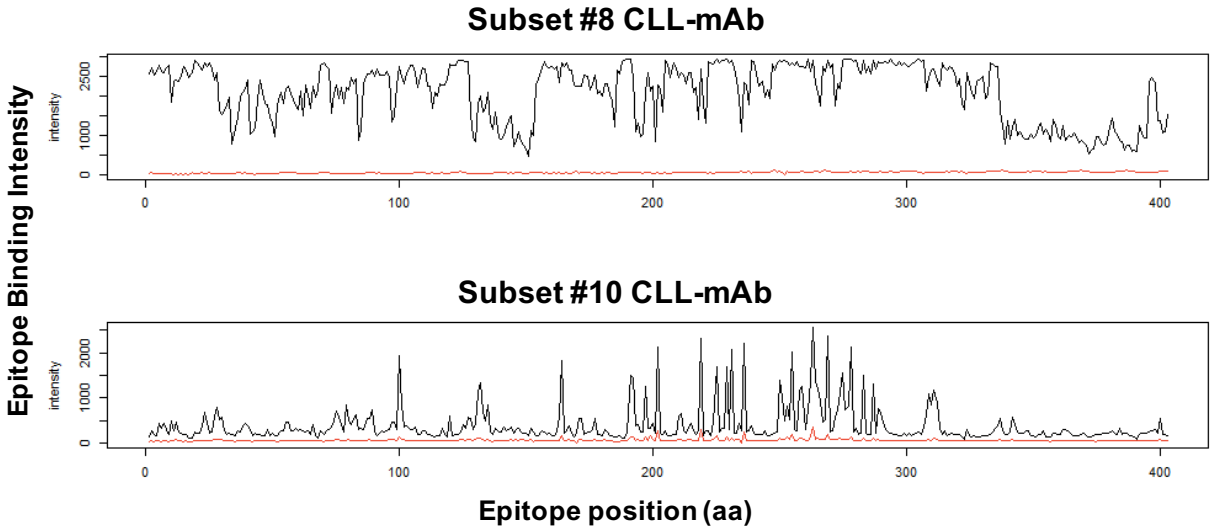
Supplemental Table 2. Molecular characteristics and stereotyped subset assignment of recombinant CLL monoclonal antibodies tested for anti-Calreticulin reactivity

CLL mAb	#	IGHV	IGHV %	IGHD	IGHJ	HCDR3 amino acid sequence	IGLV	IGLV %	IGLJ	LCDR3 amino acid sequence
1	#1	IGHV1-03*13	100,00	IGHD6-19*01	IGHJ4*02	CAREQWLVLNYFDYW	IGKV1-39*01	100,00	IGKJ2*01	CQQSYSTPPYTF
2	#1	IGHV1-2*02	100,00	IGHD6-19*01	IGHJ4*02	CARGQWLVQLNFDYW	IGKV1-39*01	100,00	IGKJ2*02	CQQSYSTPPYTF
3	#1	IGHV5-a*03	100,00	IGHD6-19*01	IGHJ4*02	CAREQWLVLEHFDYW	IGKV1-39*01	100,00	IGKJ3*01	CQQSYSTPRFTF
4	#2	IGHV3-21*01	97,22	IGHD1-1*01	IGHJ6*02	CASDKNGMDVW	IGLV3-21*01	99,57	IGLJ3*02	CQVWDSSSDHPWVF
5	#2	IGHV3-21*01	98,61	ND	IGHJ6*02	CARDQNAMDVW	IGLV3-21*01	97,85	IGLJ3*02	CQVWDSGSDHPWVF
6	#6	IGHV1-69*06	100,00	IGHD3-16*02	IGHJ3*02	CARGGNYDYIWGSYRSNDAFDIW	IGKV3-20*01	100,00	IGKJ4*01	CQQYGSSPPLTF
7	#6	IGHV1-69*06	100,00	IGHD3-16*02	IGHJ3*02	CARGGDYDYVWGSYRSNDAFDIW	IGKV3-20*01	100,00	IGKJ4*01	CQQYGSSPTF
8	#8	IGHV4-39*01	100,00	IGHD6-13*01	IGHJ5*02	CARHNLGYSSSWYSRNNWFDPW	IGKV1-39*01	100,00	IGKJ1*01	CQQSYSTPRTF
9	#8	IGHV4-39*07	100,00	IGHD6-13*01	IGHJ5*02	CARRF_GYSSSWYGLD_WFDPW	IGKV1-39*01	99,64	IGKJ1*01	CQQSYSTPRTF
10	#8	IGHV4-39*01	99,31	IGHD6-13*01	IGHJ5*02	CASKT_GYSSSWYGRD_WFDPW	IGKV1-39*01	99,64	IGKJ1*01	CQQSYSTPRTF
11	#9	IGHV3-21*01	100,00	IGHD3-3*01	IGHJ6*02	CARGVLNYDFWSVYYYYGMDVW	IGKV4-1*01	100,00	IGKJ4*01	CQQYYSTPLTF
12	#10	IGHV4-39*01	100,00	IGHD2-2*01	IGHJ6*02	CARHRLGYCSSTSCYYYYYGMDVW	IGLV1-40*01	98,96	IGLJ2*01	CQSYDSSLSVVF
13	#4	IGHV4-34*01	96,84	IGHD3-10*01	IGHJ6*04	CARGYADTPVFRRYYYYGMDVW	IGKV2-30*02	97,62	IGKJ2*01	CMQGTHWPPYTF
14	#4	IGHV4-34*01	96,84	IGHD5-18*01	IGHJ6*02	CARGYGDTPTIRRYYYYGMDVW	IGKV2-30*02	98,62	IGKJ2*01	CMQGTHWPPYTF

Stereotyped CLL-mAb heavy (IGHV, IGHD, IGHJ) and light chain (IGLV, IGLJ) characteristics, percentage (%) identity to germline IGHV and IGLV genes, and CDR3 aminoacidic sequences of heavy (HCDR3) and light (LCDR3) chains.



Supplemental Figure 1. Calreticulin is expressed on the surface of NLC. (A) Western blot analysis of Calreticulin (CRT) expression after membrane to cytosolic fractionation of 3 NLC preparations. hTERT stroma (S) is used as positive control. HSP90 expression is analyzed to exclude cytosolic contamination in membrane fractions. GAPDH is tested, as loading control. **(B)** Flow cytometry staining of Calreticulin expression on the surface of NLC, gated based on FSC-SSC parameters. Isotype histogram is shown, as control.



Supplemental Figure 2. Intensity profiles recorded for one subset #8 (CLL mAb #8 as in Supplemental Table 2) and one subset #10 (CLL mAb #12 as in Supplemental Table 2) antibody under high (red traces) and moderate stringency conditions (black traces) with linear 15-mer peptides.