Membrane microdomain sphingolipids are required for anti-CD20-induced death of chronic lymphocytic leukemia B cells

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Online Supplementary Design and Methods

Reagents

RPMI-1640 medium, rhodamine (rho) 123, rifampicin, fluorescein isothiocyanate (FITC)-conjugated or biotinylated cholera toxin B were purchased from Sigma, and AB-human serum from Invitrogen. The unconjugated type II anti-CD20 monoclonal antibody B1 was generously donated by Dr Mark S Cragg (Southampton, UK), and the type I monoclonal antibody rituximab provided by Roche. The FITC-conjugated B1, phycoerythrin (PE)-conjugated anti-CD19, PE-conjugated anti-CD20, phycocyanin (PCy)5-conjugated anti-CD5 and PCy7-conjugated anti-CD19 monoclonal antibodies all came from Beckman-Coulter, along with their isotype controls. The mouse anti-P-gp monoclonal antibody was purchased from Millipore, the mouse anti-Csk binding protein (Cbp) monoclonal antibody from Interchim, and the rabbit antilysenin polyclonal antibody from PeptaNova. Rhodamine red X (RRX)-conjugated streptavidin, FITC-conjugated RRX-conjugated donkey anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, RRX-conjugated goat anti-mouse IgG, and FITC-conjugated goat anti-mouse IgM polyclonal antibody were from Jackson ImmunoResearch, and the horseradish peroxidase-labeled goat-anti-mouse IgG polyclonal antibody from Vector Laboratories.

Cell preparation

Blood samples were collected before any treatment. Mononuclear cells were separated by density-gradient centrifugation on Ficoll-Hypaque, and B lymphocytes isolated based on incubation with unconjugated anti-CD3, anti-CD4, anti-CD8 and anti-CD56 monclonal antibodies, followed by a negative selection using immunomagnetic BioMag goat anti-mouse IgG antibody-coated beads (Stem Cell Technologies).

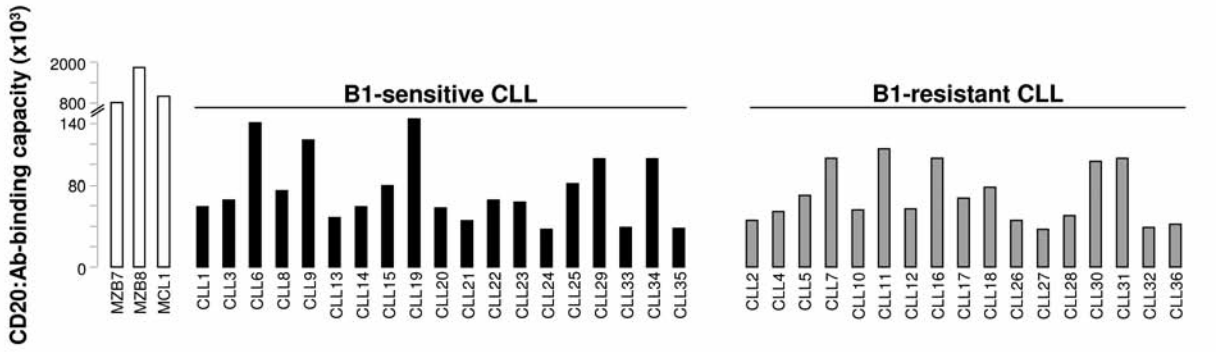
Western blotting

Proteins were extracted from 2×10^7 cells by a 30-min incubation at 4°C in a cell lysis buffer (Cell Signaling Technology), and the debris cleared by 10,000 rpm centrifugation for 10 min at 4°C. Thirty micrograms of samples were run in 7-13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE). Proteins from the PAGE were electroblotted onto polyvinylidene fluoride membranes (Amersham), and unoccupied sites saturated with 5% non-fat milk in 10mM Tris medium containing 0.1% Tween-20 for 1 h at room temperature. The membranes were then washed three times with phosphate-buffered saline, and incubated with biotin-cholera toxin B for 1 h. After another three washes with Tris, they were developed with 1:4,000 horse-radish peroxidase-labeled streptavidin, and intensities of the bands evaluated with an enhanced chemiluminescence (ECL) system kit (GE Healthcare).

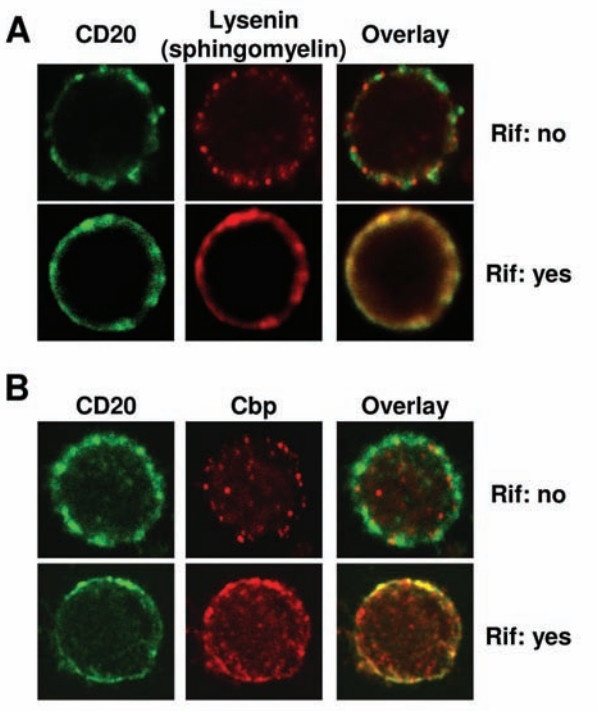
Online Supplementary Table S1. Clinical features and immunological characteristics of the 36 chronic lymphocytic leukemia patients included in this study.

Patient number	Gender	Age (years)	Lymphocytes ($x10^9$/L)	Binet stage	% of CD38-positive cells	Follow-up (years)
B1-sensitive CLL						
1	M	77	79	A	1	7
3	M	76	24	C	2	6
6	M	68	50	A	ND	7
8	M	72	41	C	4	6
9	F	50	24	A	1	1
13	F	79	124	C	0	15
14	F	77	93	A	1	5
15	F	68	101	B	8	1
19	F	81	85	A	ND	7
20	F	68	133	A	1	2
21	M	91	70	A	66	1
22	M	80	43	A	0	4
23	M	87	186	A	1	2
24	M	81	151	C	3	6
25	M	75	42	A	7	6
29	F	60	29	C	5	11
33	F	83	14	A	1	9
34	M	71	30	A	2	6
35	F	59	45	A	49	6
B1-resistant CLL						
2	M	52	45	A	0	7
4	M	60	66	B	31	5
5	F	70	142	A	2	3
7	M	77	20	A	1	4
10	F	77	172	B	65	1
11	F	59	36	A	2	4
12	M	71	53	C	10	5
16	M	50	48	A	<1	3
17	M	35	55	B	3	2
18	F	71	97	A	62	5
26	F	86	54	A	1	1
27	M	65	55	A	ND	6
28	F	72	74	A	ND	7
30	M	61	71	A	0	5
31	F	83	78	C	65	5
32	M	63	61	A	44	3
36	F	73	33	A	3	3

ND: not determined



Online Supplementary Figure S1. Quantification of CD20 expression on B lymphocytes from NHL, B1-sensitive and B1-resistant CLL patients. The numbers of CD20 molecules per cell were indirectly quantified by determining the amount of monoclonal antibody binding to the cells at saturating concentrations, using the Quantum kit (Flow Cytometry Standards Corp).



Online Supplementary Figure S2. Rifampicin (rif) permits sphingomyelin (SM) and Csk-binding protein (Cbp) association with CD20. (A) CD20 stained with FITC-conjugated anti-B1 monoclonal antibody, and SM stained with rhodamine-conjugated anti-lysenin monoclonal antibody were analyzed by confocal microscopy in B cells from B1-resistant CLL patients. At the start, green CD20 and red SM did not co-localize, whereas they did so after treatment with rif, as shown by their yellow overlay. (B) Co-localization of B1 and Cbp was also restored after stimulation with rif in B1-resistant B cells. Surface expression of SM was increased in B1-resistant B cells. These were normalized by rif (magnification x100).