Sphingosine kinase 1 participates in the activation, proliferation and survival of chronic lymphocytic leukemia cells

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doi:10.3324/haematol.2017.167353

SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Reagents, antibodies and cell line.

RPMI 1640, FCS, penicillin, and streptomycin were purchased from GIBCO. The Ficoll-Hypaque Plus used for cell separation was purchased from Amersham. MACS B cell isolation kit II was obtained from Miltenyi Biotec. BSA used for Ab staining buffer was obtained from Weiner Laboratorios (Santa Fé, Argentina). The TRIzol reagent and the SYBR Green PCR Master Mix used for RNA extraction or qRT-PCR, respectively, were obtained from Invitrogen. The MMLV RT used for cDNA synthesis was obtained from Promega. 3% SDS lysis buffer was purchased from Boehringer Mannheim (Mannheim, Germany). BCA protein assay was obtained from Pierce (Rockford, IL). PVDF membranes were purchased from Sigma-Aldrich (Dallas, TX) and the enhanced chemiluminescence (ECL) kit used for visualized protein bands was purchased from Amersham. SKI-II and DMSO were purchased from Sigma-Aldrich (Dallas, TX). The FIX & PERM Kit for BLC-2 intracellular staining was purchased from Caltag Laboratories (Burlingame, CA). S1P conjugated with human serum albumin (HSA) was purchased from Avanti Polar Lipids (Alabaster, AL). HSA was purchased from Universidad Nacional de Córdoba (Córdoba, Argentina) and the fatty acid–free BSA (BSA-FAF) was purchased from Sigma-Aldrich (St. Louis, MO). Ibrutinib was obtained from MedKoo Biosciences Inc (Chapel Hill, NC). Annexin-V-FITC was obtained from ImmunoTools (Friesoythe, Germany) and Propidium Iodide was purchased from Sigma-Aldrich (St. Louis, MO).

FITC- and PE-conjugated mAbs specific for anti-CD69 (clone FN50) or anti–CD38 (clone HB7), as well as control Abs with irrelevant specificities (isotype control), were purchased from BD Biosciences, Pharmingen (San Jose, CA). PC5-CD19 (PN IM2643) was purchased from Beckman coulter. PE-CD49d (clone 9F10) was obtained from BioLegend (San Diego, CA). Anti-BCL-2 for intracellular staining; anti-BCL-2 for western blot, HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-mouse IgG-FITC and anti-human-IgM Abs were obtained from Jackson ImmunoResearch (West Grove, PA). Anti-b-actin mAb and anti-SK1 Ab were purchased from Abgent. CD40L and CpG ODN were obtained from R&D Systems and IDT, respectively.

HS5 cell line was kindly provided by Dr Beverly Torok-Storb (Fred Hutchinson Cancer Research Center, Seattle, WA)

CLL patients and age-matched healthy donors.

This study included thirty five unrelated CLL patients and ten age-matched healthy donors. Peripheral blood samples were collected from CLL patients and healthy donors and bone marrow samples were collected from three CLL patients. The main clinical and biological characteristics of the patients enrolled in our study and the experiments performed with each sample were summarized in Table S1. All samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires, Argentina. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of analysis, all patients were free from clinically relevant infectious complications and either had received no treatment or had not received treatment for ≥3 months before the investigation began.

Cell separation procedures

Mononuclear cells were isolated from fresh blood (PBMC) or bone marrow samples by density centrifugation over a Ficoll-Hypaque Plus gradient, washed twice with saline solution, and suspended in complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin). Cells were used immediately or were cryopreserved in FBS 10% DMSO for further experiments.

B cells ($CD19^+$) were purified from PBMC by negative selection using the MACS B cell isolation kit II. The purity of B cells was checked using a FACS flow cytometer (BD Immunocytometry Systems) and anti-CD19-PC5 mAb and was always >98%.

In order to obtain *in vivo* subpopulations of leukemic cells, PBMCs from three CLL patients (Patient N° 1, 2 and 14 of Supplementary Table S1) were incubated with mAbs anti-IgG, anti-IgM, anti-CD5, and anti-CD19 for 20 minutes, washed twice, and sorted by FACS, as previously described[1]. Cells were maintained ice-cold throughout the isolation procedure. In the case of bone marrow samples, bone marrow mononuclear cells from three CLL patients (Patient N° 3, 13 and 28 of Supplementary Table S1) were incubated with mAbs

anti–CD19-PC5 and anti–CD38-PE for 20 minutes, washed twice, and sorted by FACS, as previously described[2]. Cells were maintained ice-cold throughout the isolation procedure.

Total RNA Preparation, cDNA Synthesis and qRT-PCR

Total RNA was extracted from 2 x 10^6 purified B cell from PBMC using TRIzol reagent, and cDNA was generated by reverse transcription with MMLV RT according to the manufacturer´s instructions.

qRT-PCR was performed using SYBR Green PCR Master Mix in 20 µl reactions. Primers were designed using Primer3 software and purchased from Ruralex-Fagos: GAPDH Fw 5´GAGTCAACGGATTTGGTCGT 3´, GAPDH Rv 5´-TTGATTTTGGAGGGATCTCG-3´, SK1 Fw 5´ ACTGGGAGGAAGCTGTGAAGATGC 3´, SK1 Rv 5´ CAGGCCACAGACAGGAAGGAGAAA 3´, SK2 Fw 5´ GAGACCGCCATCCAGAAGC 3´, SK2 Rv 5´ CCGGCGGCACAGCAATAG 3´, S1PL Fw 5´ GAACCAGTTGCAGTTCCCACCCA 3´and S1PL Rv 5´ ACAGTTGTCTGGGCCATGCCATAGA 3´ and were used at a concentration of 250 nM. Reactions were carried out in a RotorGene (Quiagen). The cycling program used was 50 ˚C for 2 minutes, 95˚C for 1 minute, followed by 40 cycles of 95˚C for 15 seconds, 60˚C for 60 seconds. Data were analyzed using GAPDH as a reference gene.

Analysis of *IGHV* (immunoglobulin heavy chain variable region) mutational status

The *IGHV* gene sequences were determined as previously described[3]. Briefly, amplification of *IGHV* regions by PCR was performed on cDNA using VH framework region 1 consensus family specific primers (VH1-VH6) and JH primers. When amplifications of these primers were unsuccessful, an alternative set of primers that anneal to sequences in the leader region (LH1-LH6) and one antisense Cµ-primer were used. Thermal cycling conditions were 3 min at 93ºC, followed by 33 cycles at 94ºC for 30 s, 62ºC for 30 s, 72ºC for 30s, elongation at 72ºC for 7 min, and a final step at 4ºC for 10 min. PCR products were purified in 2% agarose gels, sequenced bi-directionally and analyzed on an automated DNA sequence analyzer (377 ABI Prism, PE Applied Biosystems, Foster City, CA). Sequence data were analyzed using IgBLAST (http://www.ncbi.nlm.nih.gov/igblast) and the ImMunoGeneTics database (IMGT; http://imgt.cines.fr). *IGHV* sequences with less than 98% homology with respect to the germline counterpart were considered as mutated while those with homology of 98% or higher were classified as unmutated.

Analysis of CD38 and CD49d expression by flow cytometry

CD38 and CD49d expression on CLL cells was evaluated by flow cytometry using a FACScan flow cytometer (BD Immunocytometry Systems). To this end, PBMCs were stained with anti-CD38 PE mAb, anti-CD49d PE mAb or PE-conjugated isotype control Abs and PC5-conjugated mAbs specific for CD19. Patients with ≥ 7 % of CLL cells expressing CD38 were considered $CD38^{+}$ [4]. On the other hand patients with ≥ 30 % of CLL cells expressing CD49d were considered $CD49d^{+}$ [5].

SK1 determination by western blot

Cell were harvested after different treatments and washed twice with cold PBS. Whole-cell lysates were prepared using a 3% SDS lysis buffer. Lysates were boiled for 5 minutes and protein was quantified using the BCA protein assay. Then, 25 μg of protein were separated on a standard 12% SDS–PAGE and transferred to PVDF membranes. Membranes were then blotted with antibodies against SK1 followed by HRP-conjugated anti-rabbit IgG. Specific bands were developed by enhanced chemiluminiscence (ECL). The same membrane was blotted with mAb anti-b-actin followed by HRP-conjugated anti-mouse IgG to compare the total amount of protein in each sample. Densitometric measurements of SK1 specific bands were normalized to β-actin by using ImageJ (NIH).

In vitro CLL cell cultures

CLL cell activation by anti-IgM, CD40L, CpG and HS5 cell line.

Purified CLL cells (2 x 10^6 cells/ml) were cultured in 24 well plate in complete medium alone (control) or in the presence of immobilized anti-IgM Abs (30 μg/ml) and CD40L (500 ng/ml), CpG ODN (500 nM) or HS5 as a feeder layer. After 24 hours of culture, CLL cells were collected, and activation was confirmed by flow cytometry by evaluating the surface expression of the activation marker CD69 using mAbs anti-CD69-FITC or the corresponding isotype control and anti-CD19-PC5. *SK1*, *SK2* or *S1PL* mRNA on control and activated purified CLL cells was assessed by qRT-PCR, as described above.

To assess the effect of the Ibrutinib in CLL cell activation and *SK1* mRNA expression, 2 x 10^6 CLL cells were pre-treated for 30 minutes in complete medium in the presence of 0.5 μM of Ibrutinib or the vehicle of the drug, DMSO (Control), and then cultured for 24 hours with anti-IgM and CD40L as described above. The expression of CD69 and SK1 mRNA was evaluated as previously described.

To assess the effect of the non-apoptotic doses of SKI-II in B cell activation, 1 x 10^6 CLL cells were cultured for 30 minutes in complete medium in the presence of SKI-II 15 μM or the vehicle of the drug, DMSO (Control), and then cultured for 24 hours with anti-IgM and CD40L as described above. The activation was confirmed by flow cytometry by evaluating the surface expression of the activation marker CD69 as previously described.

CLL cells proliferation assay.

To evaluate the effect of non-apoptotic doses of SKI-II in $CD19^+$ B cell proliferation, the CFSE dilution assay was used [6, 7]. To this aim, PBMC were labelled with CFSE 5 μM for 20 minutes at 37 °C and then washed three times with RPMI 1640 5 %. Afterward, cells were pre-treated with SKI-II 15 μM or the vehicle of the drug, DMSO (Control) and then transfer to a 48 well culture plate with or without anti-IgM and CD40L. After 5 days of culture, cells were collected, washed and stained with anti-CD19-PC5. CFSE dilution was measured on $CD19^+$ B cell population by flow cytometry. Viable cells were gated according to FSC and SSC parameters criteria. The number of cells that had proliferated was determined by gating on the $CD19^+$ $CFSE^{low}$ subset.

To evaluate the effect of the addition of S1P in the proliferation assay with SKI-II, PBMC were labeled with CFSE 5 μM for 20 minutes at 37 °C and then washed three times with serum-free RPMI 1640 with 1% BSA-FAF. Afterward, cells were pre-treated with SKI-II 15 μM or the vehicle of the drug, DMSO (Control) and then transfer to a 48 well culture plate with or without anti-IgM and CD40L in the presence of S1P-HSA 0.5 μM or HSA 0.25 μM (Control). After 5 days of culture, cells were collected, washed and stained with anti-CD19-PC5. CFSE dilution was measured on $CD19^+$ B cell population by flow cytometry as described above.

Evaluation of CLL cell death induced by SKI-II, fludarabine, bendamustine and ibrutinib.

To evaluate the effect of SKI-II on cell death, 1 x 10^6 CLL cells were cultured in complete medium in 96 well plates with different doses of SKI-II (5 μM, 15 μM , 30 μM or 50 μM) or vehicle alone (DMSO). The viability of CLL cells was daily evaluated by flow cytometric alterations of light-scattering properties (FSC-H)[8] or flow cytometry analysis of Annexin V-PI staining.

To evaluate the effect of the exogenous addition of S1P in the cell death induced by SKI-II, 1 x 10^6 CLL cells were cultured in serum-free RPMI 1640 with 1% BSA-FAF in 96 well plates with 50 μM of SKI-II or vehicle alone (DMSO) in the presence of S1P-HSA 0.5 μM or HSA 0.25 μM (control). The viability of CLL cells was daily evaluated as described above.

To evaluate if non-apoptotic doses of SKI-II may increase the susceptibility of CLL cells to die by the effect of other drugs, 1 x 10^6 cells from CLL patients were cultured in complete medium in 96 well plates with DMSO (Vehicle) or SKI-II (15 μM) in combination with Bendamustine (1μg/ml), Ibrutinib (0.5 μM) or Fludarabine (0.1 μg/ml). The survival of the cultures was daily evaluated as mentioned above.

Analysis of BCL-2 protein expression by flow cytometry and western blot

For intracellular detection of BCL-2 protein expression, 5 x 10^5 CLL cells were collected after 48 hours of culture with DMSO (Vehicle), SKI-II 15 μM or 50 μM and were fixed and permeabilized by using FIX & PERM Kit, according to the manufacturer's instructions, before the addition of anti-BCL-2 (4 μg/ml) or isotype-matched mAb (4 μg/ml). Then, the BCL-2 expression in leukemic cells was revealed with anti-mouse IgG-FITC in the $CD19^+$ population.

For western blot detection of BCL-2 protein, cell were harvested after different treatments and washed twice with cold PBS. Whole-cell lysates were prepared using a 3% SDS lysis buffer. Lysates were boiled for 5 minutes and protein was quantified using the BCA protein assay. Then, 25 μg of protein were separated on a standard 12% SDS–PAGE and transferred to PVDF membranes. Membranes were then blotted with antibodies against BCL-2 followed by HRP-conjugated anti-rabbit IgG. Specific bands were developed by enhanced chemiluminiscence (ECL). The same membrane was blotted with mAb anti-b-actin followed by HRP-conjugated anti-mouse IgG to compare the total amount of protein in each sample. Densitometric measurements of BCL-2 specific bands were normalized to β-actin by using ImageJ (NIH).

Statistical analysis

Statistical significance was determined using the nonparametric tests: Mann–Whitney test, Column Statistics followed by Wilcoxon's signed rank test, Kruskal-Wallis test, one way ANOVA followed by Dunn's multiple comparison test, Spearman correlation test. The analysis of the contingency tables for the

association between SK1/S1PL ratio and different clinical and biological features of CLL patients was made using Fisher's exact test. In all cases, $p<0.05$ was considered statistically significant.

SUPPLEMENTARY TABLES

Supplementary Table S1

Table S1. Clinical and biological features of CLL patients enrolled in our study

CLL Patient #	Age (years)	Gender	Binet	Leucocytes, x 10^3/µl	Lymphocytes, x 10^3/µl	CD19$^+$, % *	CD38$^+$, % †	CD49d$^+$, % ‡	β2micro, µg/ml	LDH, U/l	HGB, g/dl	Platelets, x 10^3/µl	Treatment	Progression	IGVH mutational status ¥	SK1/S1PL Ratio §	SK1/S1PL High/Low £	Other experiments perfomed
1	51	M	C	65.20	40.00	79	7.5	ND	ND	ND	12.1	225	FC	Yes	U	ND	ND	QF/PF sorter experiment
2	55	M	C	220.00	120.00	82	29	ND	ND	ND	ND	ND	FCR	Yes	U	ND	ND	QF/PF sorter experiment
3	57	M	B	61.79	56.10	96	82	25	ND	320	11.7	111	FCR	Yes	U	6,30	High	CD38 sorter experiment. SKI-II experiments
4	59	M	A	170.00	158.00	98	43	ND	2.4	540	13.9	210	B	Yes	ND	ND	ND	*In vitro* CLL activation
5	59	M	C	77.60	69.20	95	1	3	2.1	454	14.5	179	FCR	Yes	U	3,10	Low	*In vitro* CLL activation. Proliferation assay. SKI-II experiments
6	60	M	A	24.50	19.60	96	2	2	1.7	320	15.6	101	FR	No	M	3,76	Low	-
7	60	F	A	11.80	8.00	78	98	98	2.4	177	12.6	125	-	No	U	40,79	High	*In vitro* CLL activation. Proliferation assay
8	61	M	C	44.00	38.70	86	12	99	ND	740	8.3	90	BR	Yes	U	30,27	High	*In vitro* CLL activation. SKI-II experiments
9	61	M	C	416.40	345.40	97	97	99	3.5	580	2.8	262	FCR	Yes	U	8,31	High	SKI-II experiments
10	62	F	A	53.00	46.00	93	0.5	22	244	1.6	14.3	126	-	No	M	1,16	Low	SKI-II experiments
11	65	M	B	76.70	67.50	96	37	4	3.6	290	15.4	178	BR	Yes	U	11,59	High	SKI-II experiments
12	65	F	C	270.00	243.00	97	86	56	3.8	654	8.8	90	BR	Yes	M	2,06	Low	*In vitro* CLL activation. Proliferation assay. SKI-II experiments
13	65	M	B	41.20	37.10	61	17	ND	3.6	380	12.3	210	-	No	M	37,20	High	CD38 sorter experiment
14	66	F	B	150.00	80.00	75	45	ND	ND	ND	14.8	206	FC	No	U	ND	ND	QF/PF sorter experiment
15	68	M	B	70.90	62.40	94	16	0.6	3.0	181	14.2	163	-	No	U	1,50	Low	-
16	70	F	A	96.80	91.00	97	0.5	2	2.7	161	11.3	130	-	Yes	M	2,08	Low	*In vitro* CLL activation. Proliferation assay. SKI-II experiments
17	70	M	B	34.50	31.20	95	6	1	3.2	390	6.8	145	BR	No	M	4,48	Low	SKI-II experiments
18	70	M	A	10.30	9.80	90	99	0.5	6.4	332	14.0	69,4	-	No	ND	3,31	Low	SKI-II experiments
19	70	M	C	96.00	86.40	92	50	ND	4.3	670	6.7	73	FCR	Yes	U	41,93	High	-
20	71	M	B	558.00	535.70	98	15	95	4.4	305	12.0	126	I	Yes	U	13,09	High	*In vitro* CLL activation. SKI-II experiments
21	72	M	B	59.60	40.60	82	19	4	2.2	392	13.4	199	-	No	ND	9,38	High	*In vitro* CLL activation. Proliferation assay.
22	72	M	A	49.90	42.90	91	0.5	1	2.7	339	13.6	216	-	No	M	2,41	Low	*In vitro* CLL activation. Proliferation assay. SKI-II experiments
23	72	F	A	25.30	4.00	85	3	9	ND	ND	13.5	234	-	No	M	1,00	Low	SKI-II experiments
24	73	M	B	98.70	80.90	99	0.6	1.8	3.5	380	11.5	165	-	No	ND	0,54	Low	*In vitro* CLL activation. Proliferation assay. SKI-II experiments
25	73	M	B	114.90	110.00	85	44	6	1.9	385	14.0	282	-	No	M	59,71	High	SKI-II experiments
26	75	F	C	187.00	179.50	97	0.2	99	4.4	163	9.6	113	-	Yes	M	60,55	High	Proliferation assay. SKI-II experiments
27	75	M	A	36.40	28.40	81	16	8	2.3	356	13.6	199	-	No	M	4,20	Low	*In vitro* CLL activation. Proliferation assay
28	77	F	A	20.50	10.50	84	3	93	2.3	300	14.3	397	-	No	U	ND	ND	CD38 sorter experiment
29	80	M	A	36.00	31.10	90	5	ND	2.7	373	13.7	176	-	No	M	4,81	Low	*In vitro* CLL activation
30	80	M	C	14.40	10.40	60	99	16	4.2	467	8.6	94	BR, I	Yes	M	115,36	High	Proliferation assay.
31	82	M	A	27.00	25.60	95	0.6	2	3.1	340	11.5	116	ChP	No	M	0,50	Low	*In vitro* CLL activation. Proliferation assay. SKI-II experiments
32	82	M	A	27.95	5.90	85	2	3	2.0	391	14.3	192	-	No	U	0,95	Low	*In vitro* CLL activation
33	84	M	A	27.50	21.40	84	1.3	32	1.7	268	13.7	151	-	No	M	17,51	High	*In vitro* CLL activation. SKI-II experiments
34	84	M	B	70.38	59.80	93	73	ND	2.1	519	11.9	134	Ch	No	M	4,98	Low	*In vitro* CLL activation
35	87	F	C	40.10	26.90	88	3	99	ND	550	7.7	109	-	No	U	8,00	High	SKI-II experiments

M indicates male; F, female; ND, not determined; β2micro, beta-2 microglobulin; LDH, lactate dehydrogenase; HGB, hemoglobin; F, fludarabine; C, cyclophosphamide; R, rituximab; I, Ibrutinib; Ch, chlorambucil; P, prednisone; QF, quiescent fraction; PF, proliferative fraction; and BM,

* Percentage of CD19$^+$ cells (B cells, more than 99% CLL cells) in peripheral blood lymphocytes

† Percentage of CD38$^+$ cells in CD19$^+$ lymphocytes

‡ Percentage of CD49d$^+$ cells in CD19$^+$ lymphocytes

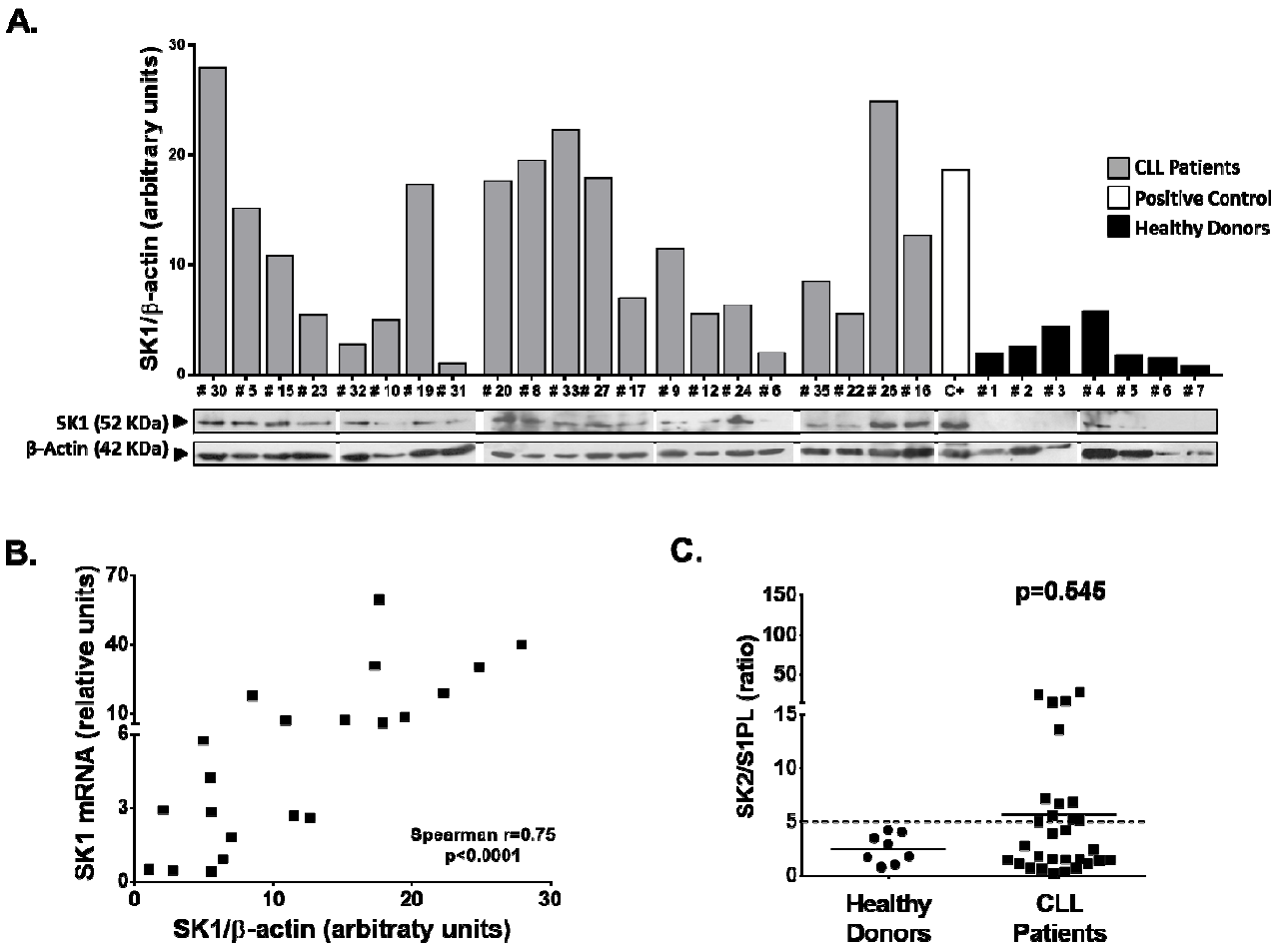
¥ U, unmutated; M, mutated.

§ Ratio between SK1 mRNA and S1PL mRNA levels evaluated by qRT-PCR in purified B cells from CLL patients. Results were normalized to GAPDH human gene and were represented as relative units ($2^{-\Delta Ct} \times 10^3$).

£ High indicates ratio > 5, Low indicates ratio ≤ 5. A cut/off was established between patients with less or more than -3 standard deviation (SD) respect to the healthy control group.

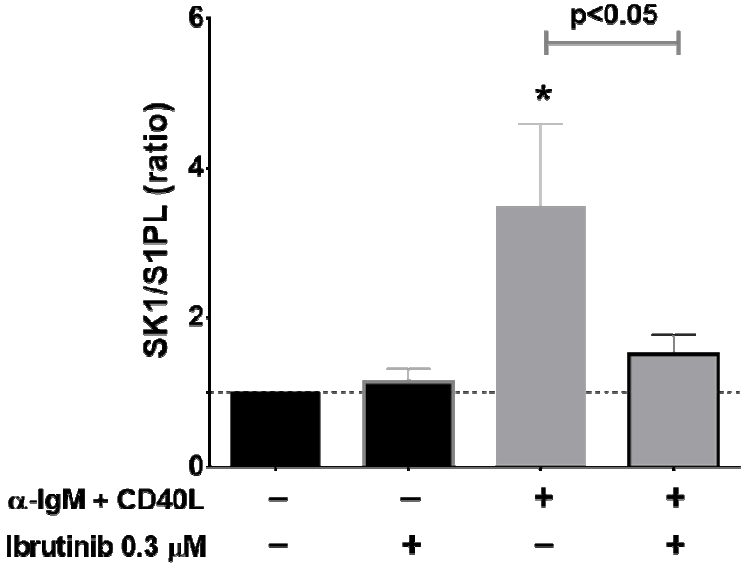
SUPPLEMENTARY FIGURES

Supplementary Figure S1



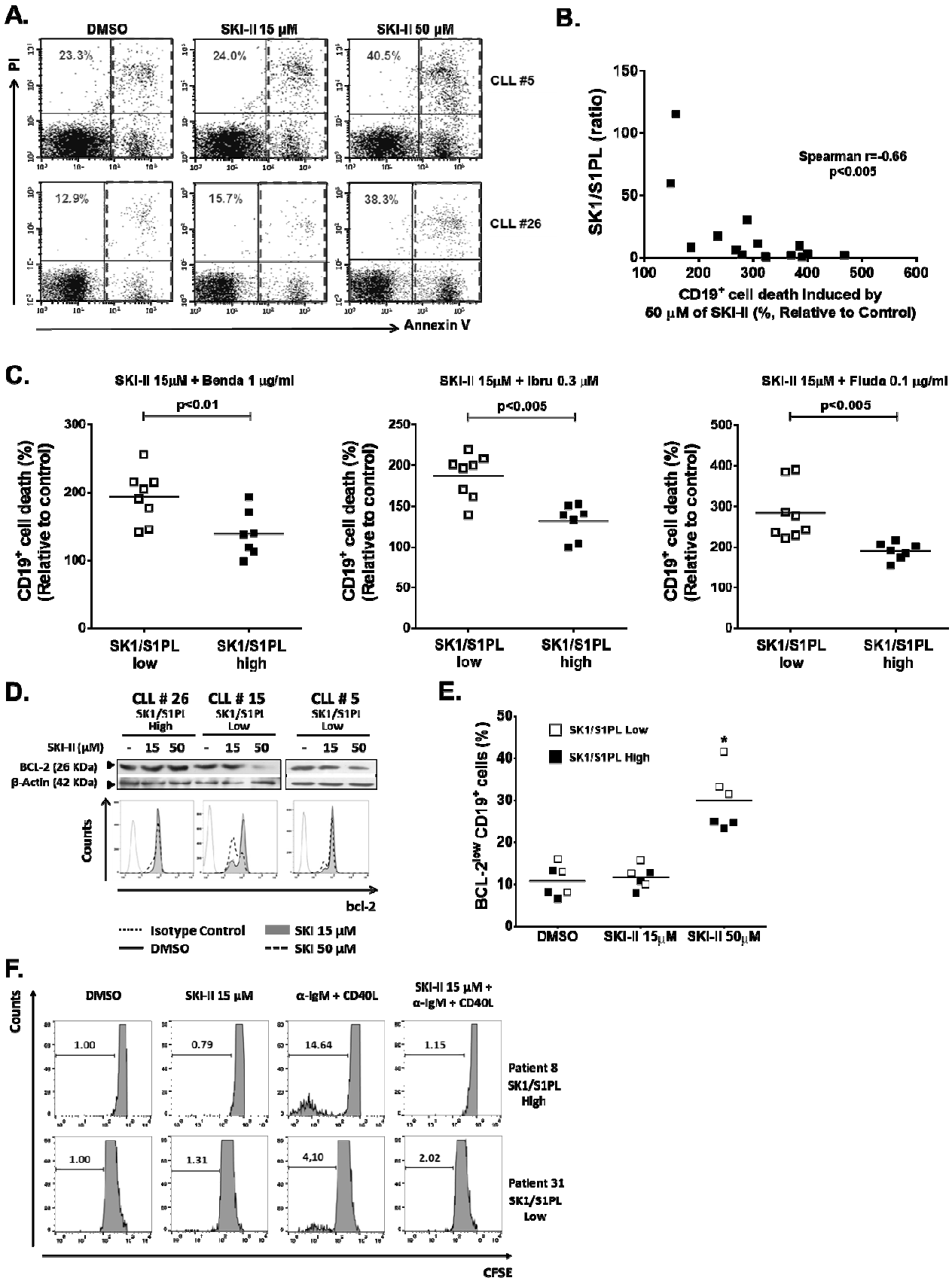
Supplementary Figure S1: **(A)** Western blot analysis (bottom) and quantitative densitometry protein expression (top) of *SK1* relative to β-actin in B cells from 21 CLL patients and 7 age-matched healthy donors. Protein extract from Jurkat cell line was used as a positive control. **(B)** The figure shows the correlation between the *SK1* mRNA levels measured by qRT-PCR as described in materials and methods and the quantitative densitometry protein expression of SK1 relative to β-actin in B-cells from CLL patients measured by western blot. **(C)** The figure shows the ratio of *SK2* mRNA and *S1PL* mRNA in B cells from CLL patients and age-matched healthy donors. Statistical analysis was performed using Mann–Whitney test.

Supplementary Figure S2



Supplementary Figure S2: Purified CLL cells were cultured for 24 hours without (Control) or with anti-IgM plus CD40L in the presence or absence of Ibrutinib and the expression of *SK1* and *S1PL* mRNA were evaluated as described above. Bars represent the mean ± SEM of *SK1/S1PL* ratios relative to control cultures (n=10). Statistical analysis was performed using Wilcoxon's signed rank test (*p<0.01) and Friedman test followed by the Dunn post test.

Supplementary Figure S3



Supplementary Figure S3: **(A)** PBMCs from CLL patients (>85% leukemic B-cells) were cultured with DMSO (vehicle) or different doses of SKI-II. The figure shows representative dot plots of Annexin V-PI

staining at 24 hours. The percentages shown in each dot blot corresponds to the Annexin V positive cells. **(B)** The figure shows the correlation between the percentage of cell death induced by SKI-II 50 μM for 72 hours (relative to control cultures) and the basal *SK1/S1PL* ratio of each patient. **(C)** PBMCs from CLL patients (>85% leukemic B-cells) were cultured with DMSO (Vehicle) or SKI-II (15 μM) for 30 minutes and then non-apoptotic doses of bendamustine (Benda), ibrutinib (Ibru) or fludarabine (Fluda) were added to the cultures. The figure shows the percentage of cell death relative to control at 72 hours with the different drug combinations in CLL patients segregated by low or high *SK1/S1PL* ratio. Statistical analysis was performed using Mann–Whitney test. **(D)** PBMCs from CLL patients (>85% leukemic B-cells) were cultured with DMSO (vehicle) or different doses of SKI-II and after 24 hours of culture BCL-2 expression was evaluated by western blot (top) and flow cytometry (bottom) as detailed in materials and methods. The results of three representative patients are shown. **(E)** PBMCs from CLL patients (>85% leukemic B-cells) were cultured with DMSO (vehicle) or different doses of SKI-II and after 24 hours of culture BCL-2 expression was evaluated by flow cytometry as detailed in materials and methods. The figure shows the percentage of $CD19^+$ BCL-2^{low} cells from CLL patients with high or low *SK1/S1PL* ratios in each condition. Statistical analysis was performed using Friedman test test (*$p<0.05$). **(F)** CFSE-labeled PBMC from CLL patients were pre-treated with SKI-II (15 μM) or DMSO for 30 minutes and then cultured without (Control) or with α-IgM plus CD40L. After 5 days the proliferation of CLL cells was analyzed using the CFSE dilution assay. Figure shows representatives CFSE histograms showing the percentages of $CD19^+$ cells with low CFSE expression.

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