SUPPLEMENTARY APPENDIX

Extracellular vesicles of bone marrow stromal cells rescue chronic lymphocytic leukemia B cells from apoptosis, enhance their migration and induce gene expression modifications

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ONLINE SUPPLEMENTARY APPENDIX

Online Supplementary Appendix Text

Biological samples and cell culture

All patients had the typical $CD19^+/CD5^+/CD23^+$ phenotype. PBMCs were isolated by Ficoll-Hypaque gradient centrifugation and B-cells were purified by negative selection, using a CLL B-cell isolation kit (Macs Miltenyi Biotec, Leiden, The Netherlands). The CLL B-cells were cultured at a density of 4×10^6 cells/500 µl in RPMI 1640 (Lonza, Verviers, Belgium) with 10% fetal bovine serum filtered through a 0.2-µm filter. These samples were cultured for 24 h before functional studies and RNA extraction. Online Supplementary Table S1 summarizes the patient characteristics. BM-MSCs were harvested from the sternum or iliac crest of healthy volunteers or CLL patients after written informed consent, isolated and cultured as previously described [1]. Additional details about MSC characterization are provided in Supplemental Information Figure S1.

Extracellular vesicle characterization

BM-MSC EVs were characterized by a latex bead technique; the EV pellet was incubated with 10µl of fluorochrome-conjugated monoclonal antibody for 15 minutes in the dark. Samples were washed with PBS and centrifuged at 150 000 × g for 1 hour at 4°C. Labeled EVs were resuspended in 1 ml of PBS and 1 µl of beads (aldehyde sulfate latex beads 4 µm-Invitrogen) was added. Samples were incubated overnight at 4°C with gentle agitation, followed by a wash step with centrifugation at 300 × g for 10 minutes. Beads were also incubated with the antibody washed at 150 000 × g (without sample) as the negative control. EVs labeled with a negative marker (a marker that is not present on the cells from which the EVs were derived; for example, CD45 for BM-MSC EVs) were used as a second negative control. The coated beads were resuspended in PBS before analysis with a MACSQuant Analyzer. Electron microscopy was used to study the morphology of the EVs. The samples were submitted for TEM (transmission electron microscopy). A total of 40 µl of vesicle suspension was placed on a carbon-coated EM grid, and 0.4 µl of 25% glutaraldehyde was added. Vesicles were then allowed to settle onto the grid overnight at 4°C. Grids were then blotted on filter paper and stained for 30 seconds with 2% uranyl acetate. After further blotting and drying, samples were directly observed on a Tecnai 10 TEM (FEI). Images were captured with a Veleta camera and processed with iTEM and Adobe Photoshop software.

Monitoring of BM-MSC EV uptake by CLL B-cells

EVs from BM-MSCs were labeled with PKH67, a green fluorescent dye that labels lipid membranes (Sigma Aldrich, Missouri, USA). EVs were incubated in 2 µl of PKH67 (2 µM) for 5 minutes, followed by incubation in 1% bovine serum albumin (BSA) at room temperature to stop the reaction and washed with PBS at 150 000 × g to remove excess dye. For the negative control, PBS was incubated with PKH67 and treated with the same procedure. A MACSQuant Analyzer (Miltenyi Biotec) was used to monitor the uptake of EVs by CLL B-target cells. CLL B-cells were cultured with BM-MSC EVs previously labeled with the fluorescent dye PKH67 for 10 minutes, 1 h, 3 h or 24 h. Internalization of EVs was detected within 10 minutes by flow cytometry, and an increased internalization of PKH67 positive EVs was detected with time, as shown with the Mean Fluorescence Intensity (MFI) in the Figure 1. To exclude false positive results due to dye contamination, we added PKH67-labeled PBS to samples. No "dye contamination fluorescence" was detected on samples, confirming that the washing steps in our protocol were efficient. We used flow cytometry to evaluate antigen expression in CLL B-cells after EV integration, and we observed the increased expression of CD63 (microvesicle marker), supporting the antigen transfer theory (data not shown).

Migration assay

Briefly, 10^6 CLL B-cells were resuspended in 200 µl of RPMI medium (without serum) and plated in the upper chamber of a 6.6-mm diameter Transwell culture insert in bare polycarbonate with a 5-µm pore size (Corning Incorporated, NY, USA). The lower chamber of each well contained 500 µl of RPMI medium with or without SDF-1α (stromal cell-derived factor 1α, a CXC chemokine, highly produced by BM-MSCs, that induces CLL cell migration) (100 ng/mL) (R&D Systems, Minneapolis, MN, USA). To evaluate the effect of EVs on cell migration, CLL B-cells were pre-incubated with 2 µg of BM-MSC EVs or with RPMI alone as a negative control for 4 h. This time of pre-incubation (4h) was chosen to allow the complete integration of EVs in CLL B-cells and the initiation of physiologic effect. After 4 hours of culture, cells were recovered from the lower chamber and concentrated by centrifugation. The absolute number of cells was then determined as follows: 100 µl of the cell suspension was counted with a MACSQuant Analyzer (Miltenyi Biotec) using the absolute volumetric cell counting function. The migration index was calculated as the number of cells transmigrating in the presence of SDF-1α divided by the number of transmigrating cells in the absence of SDF-1α and EVs. CLL B-cells were also pre-incubated during 1h with the CXCR4 antagonist AMD3100 (5µg/ml) and pertussis toxin (200ng/ml), a G-protein inhibitor, in order to evaluate action mechanism of EVs. The migration ratio represents the

number of migrating cells in each condition divided by the spontaneous migrating cells (in absence of EVs).

Measure of genes involved in apoptosis

Myeloid cell leukemia 1 (Mcl-1) intracellular expression was determined by flow cytometry using primary unconjugated antibody with a secondary antibody coupled to FITC (Immunoglobulin swine $F(ab')_2$ / DAKO, Denmark) and cell lymphoma extra-large (BCL-XL), X-linked inhibitor apoptosis protein (XIAP), B-cell CLL/lymphoma 2 (BCL2) and BCL2-associated X protein (BAX) expression were investigated by qPCR (quantitative real-time PCR) with specific primers.

Drugs for chemosensitivity assay

Flavopiridol (Sigma-Aldrich, Bornem, Belgium) and fludarabine (FLUDARA; Schering AG, Berlin, Germany) were dissolved to make stock solutions in water at 1 µM and 100 µM, respectively. Bortezomib (VELCADE) (Millennium Pharmaceuticals, Cambridge, MA, USA) was dissolved in dimethyl sulfoxide at a concentration of 1 mM. Cladribine (2CdA) (Sigma-Aldrich) and methylprednisolone (Sigma-Aldrich) were dissolved in absolute ethanol. Ibrutinib, idelalisib (CAL-101) and venetoclax (ABT-199) (Selleckchem.com, Munich, Germany) were diluted in water at a concentration of 1mM for ibrutinib and idelalisib and 1µM for venetoclax. Stock solutions in DMSO were stored at –20°C. CLL B-cells were pre-incubated with 2 µg of BM-MSC EVs or with RPMI as a negative control for 4 h and placed into 24-well plates with drugs. Apoptosis was then evaluated after 24 h by Annexin-V/7AAD labeling as described in the main manuscript. The results were normalized to the negative control (cells without drug and EVs).

Gene expression profile and bioinformatic analysis

Gene expression profiles comparing CLL cells cultured with or without 5 µg of EVs were performed as previously described [2]. Total RNA was extracted from CLL B-cells cultured with or without 5 µg of EVs in a single step with TriPure Isolation Reagent (Roche Applied Science). Microarray analysis was performed using 120 ng of RNA with the Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, High Wycombe, UK). Amplification, hybridization, and scanning were performed according to standard Affymetrix protocols as described previously [2]. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE82051 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE82051). A comparative and explorative gene

expression profile was determined for 6 samples. CLL B-cells were obtained from 3 different patients and were incubated with or without EVs for 24 h. We identified significant differences between sample groups using the BRB Array Tools (Biometric Research Branch, National Cancer Institute, Bethesda, MA, USA). Only probe sets defined as present by the Affymetrix algorithm in at least 80% (5/6) of the cases were considered for further analysis. We performed two-sample paired t tests with a random variance model on the two groups, with or without EVs, for each gene. The false discovery rate (FDR) correction was applied but not used because of the low number of paired samples (n=3). However, genes of interest were further validated on a larger number of patient samples by real-time PCR. Using BRB gene set expression comparison tools, overrepresentation of gene ontology (GO) categories were investigated by the least squares (LS) permutation test. The ''SuperExactTest'' was applied to assess the statistical significance of intersections between different gene signatures. This test provides a p value characterizing the statistical difference between the expected and the obtained overlaps as described by Wang and colleagues [3]. To calculate this parameter, we used 12500 genes as total number of genes (obtained by removing the probesets considered as "absent" by the detection call algorithm in 80% of samples and by reducing this number to only one probeset per gene (the most expressed)).

Flow cytometry analysis

All harvested cells were analyzed on a MACSQuant Analyzer flow cytometer (Miltenyi Biotec). To establish the phenotype of CLL B-cells and BM-MSCs, the cells were washed with PBS, resuspended in 100 µl and labeled with specific fluorochrome-labeled monoclonal antibodies for 15 minutes in the dark. All antibodies are listed in the Online Supplementary Table S9. Cells were washed with PBS, fixed with a 4 % formaldehyde solution and a minimum of 20 000 cells were acquired. Collected data from all experiments were analyzed using FCS 3.0 (De Novo analysis software, Los Angeles, CA, USA). BM-MSCs were characterized by the positive expression of CD105, CD73, CD90, CD146 and CD166 and were negative for CD45 as presented in the Online Supplementary Figure S1. We compared the expression of these markers with the BM-MSCs obtained from healthy and CLL patients as presented in the Online Supplementary Table S10. CLL B-cells were characterized by the typical CD5+, CD19+ and CD23+ phenotype.

Validation by Real-time PCR analysis (qPCR)

We used 12.5 ng of cDNA produced by standard reverse transcription in a qPCR reaction with power SYBR ® Green PCR Master Mix (Life Technologies, Merelbeke, Belgium) and 0.32 µM gene-specific

forward and reverse primers (Life Technologies, Merelbeke, Belgium). Primer sequences are listed in the Online Supplementary Table S11. Standard real-time PCR was performed on an ABI VIAA7 (Life Technologies). To ensure that all expression levels were comparable, the data were normalized to the cyclophilin A gene (PPIA) as an endogenous control and calibrated by subtracting 10 (chosen arbitrarily) from the ΔCt value. The comparative ΔΔCt method was then applied for data analysis, and fold changes were subsequently calculated (fold change = $2^{-\Delta\Delta Ct}$) as described by Van Damme et al [4].

EV effect on CLL B-cell BCR activation

To evaluate if EVs can induce a BCR activation, Raji cell line or CLL cells were incubated with 2µg of EVs and BCR signaling was evaluated by examining intracellular calcium flux, ERK and AKT phosphorylation. Stimulation with a goat F(ab′)2 anti-human IgM (SouthernBiotech - final concentration 10 µg/ml) was used as positive control. Briefly, B cells at 1×10^6/mL were incubated in Hanks Balanced Salt Solution (HBSS) containing 5mM Fluo-8AM (Molecular Probes) and 0.02% (vol/vol) Pluronic F-127 (Sigma) for 45 minutes at 37°C. The mean of fluorescence intensity (MFI) was first measured by flow cytometry, normalized by the MFI of untreated cells and plotted every 10 seconds. Samples were subsequently treated with 1 µM of ionomycin (Sigma), which elicited robust calcium fluxes in all samples. The phosphorylation of ERK and AKT was evaluated by flow cytometry. After EV addition (or IgM stimulation as positive control), Raji cell line or CLL cells were fixed and permeabilized using the Cell Signaling Buffer Set A (Miltenyi Biotec). Cells were then stained with anti-ERK 1/2 pT202, pY204 PE (Miltenyi Biotec) and anti-AKT pS543 PE antibodies (Miltenyi Biotec) during 30 min. Flow cytometry (FC) data were acquired with a MacsQuant analyzer (MACS Miltenyi Biotec) and the analysis was performed with FCS 4 Express software (DeNovo Software). Data were presented as the mean fluorescence intensity ratio of IgM stimulation/unstimulated cells and EV treatment/untreated cells.

MicroRNA quantification

microRNA expression was measured using the TaqMan microRNA quantitative PCR (Life Technologies, Merelbeke, Belgium). Briefly, 10 ng of total RNA were reverse-transcribed using the microRNA reverse transcription kit (Life Technologies) and a specific reverse transcription stem-loop primer, according to the manufacturer's protocol. All reactions were run in duplicate. We measured expression of the endogenous control, RNU48 (Life Technologies), under the same conditions for all our samples. The expression of each microRNA relative to RNU48 was determined using the ΔΔcycle

threshold (Ct) method. MicroRNA levels are expressed in fold change of the target miR expression and calibrated by subtracting 10 (arbitrarily chosen) from the ΔCt. The fold changes were subsequently calculated (fold change = 2 exp (-ΔΔCt)).

Online Supplementary Appendix Tables

list	Sex	Age	Stade	IgVH	ZAP	LPL	CD38
P1	F	59	A	M	NEG	NEG	NEG
P2	F	75	A	M	NEG	NEG	NEG
P3	F	58	A	ND	NEG	NEG	NEG
P4	F	54	A	ND	POS	POS	POS
P5	F	53	A	M	NEG	NEG	NEG
P6	F	62	A	NM	POS	POS	POS
P7	F	61	A	M	NEG	NEG	POS
P8	F	60	A	NM	POS	POS	ND
P9	F	70	A	M	NEG	NEG	NEG
P10	F	59	A	ND	NEG	NEG	NEG
P11	F	63	A	M	NEG	NEG	NEG
P12	F	62	A	NM	POS	POS	POS
P13	F	58	A	M	POS	POS	NEG
P14	F	43	A	M	POS	POS	NEG
P15	F	67	A	ND	NEG	POS	POS
P16	F	56	A	NM	POS	POS	NEG
P17	M	75	A	ND	POS	POS	POS
P18	M	62	A	ND	ND	ND	NEG
P19	M	62	A	M	NEG	NEG	POS
P20	M	60	A	M	NEG	POS	NEG
P21	M	78	A	oligoclonal	POS	POS	POS
P22	M	51	A	M	NEG	POS	NEG
P23	M	66	A	M	NEG	NEG	NEG
P24	M	69	A	M	NEG	NEG	NEG
P25	M	68	A	oligoclonal	NEG	NEG	POS
P26	M	53	A	ND	NEG	POS	NEG
P27	M	42	A	NM	POS	POS	NEG
P28	M	57	A	NM	POS	POS	NEG
P29	M	59	A	M	NEG	NEG	NEG
P30	M	52	A	M	NEG	POS	NEG
P31	M	50	A	NM	ND	ND	POS
P32	F	51	B	ND	POS	POS	POS
P33	M	58	B	NM	POS	POS	NEG
P34	M	51	B	M	NEG	POS	POS
P35	M	54	B	NM	POS	POS	POS
P36	M	49	B	NM	POS	NEG	POS
P37	M	65	B	NM	POS	POS	POS
P38	M	62	B	ND	NEG	NEG	NEG
P39	M	65	C	NM	POS	POS	NEG
P40	M	68	C	oligoclonal	NEG	POS	ND

Table S1: Patient characteristics. The forty patients used in the study were diagnosed with CLL. Sex, age, stage, IgVH (Immunoglobulin variable heavy chain region) – ZAP70 (zeta-associated protein 70) – LPL (lipoprotein lipase) and CD38 (CD38 molecule) status (M=mutated/NM=unmutated/ND=not determined). These prognostic factors were assessed as previously described [5].

Drugs	Concentrations
Bortezomib	5 nM
Cladribine	500 nM
Flavopiridol	10 nM
Fludarabine	3 µM
Methylprednisolone	10 µM
Ibrutinib	10 µM
Idelalisib	10µM
Venetoclax	10nM

Table S2: Concentrations of drugs tested in the chemosensitivity assay. We used specific concentrations based on the half maximal (50%) inhibitory concentrations (IC50) as previously established by Stamatopoulos and colleagues [2]

	GO category	GO ontology	GO term	Number of genes	LS permutation p-value
1	GO:0007267	BP	cell-cell signaling	24	0.00658
2	GO:0003779	MF	actin binding	18	0.0107
3	GO:0007268	BP	synaptic transmission	16	0.01076
4	GO:0015629	CC	actin cytoskeleton	15	0.0125
5	GO:0045944	BP	positive regulation of transcription from RNA polymerase II promoter	25	0.01881
6	GO:0001228	MF	RNA polymerase II transcription regulatory region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription	5	0.01962
7	GO:0008289	MF	lipid binding	13	0.02454
8	GO:0045893	BP	positive regulation of transcription, DNA-templated	31	0.03033
9	GO:1902680	BP	positive regulation of RNA biosynthetic process	31	0.03033
10	GO:0000982	MF	RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity	6	0.03106
11	GO:0051254	BP	positive regulation of RNA metabolic process	32	0.03146
12	GO:0050804	BP	regulation of synaptic transmission	6	0.03507
13	GO:0008092	MF	cytoskeletal protein binding	31	0.03701
14	GO:1901652	BP	response to peptide	17	0.04614
15	GO:0034728	BP	nucleosome organization	6	0.04804
16	GO:0071824	BP	protein-DNA complex subunit organization	6	0.04804
17	GO:0045935	BP	positive regulation of nucleobase-containing compound metabolic process	36	0.04956
18	GO:0051173	BP	positive regulation of nitrogen compound metabolic process	36	0.04956
19	GO:0002790	BP	peptide secretion	5	0.0497
20	GO:0015833	BP	peptide transport	5	0.0497
21	GO:0005102	MF	receptor binding	29	0.05084

Table S3: Gene set enrichment (GO categories) using BRB array tools. Using BRB array tools, we performed gene set enrichment analysis investigating gene GO categories. The upregulated genes in CLL B-cells after EV treatment were highly represented in the categories of cell-cell signaling (GO:0007267), actin cytoskeleton organization (GO:0015629, GO:0007010, GO:0030036), receptor binding (GO:0005102) and positive regulation transcription (GO:0001228).

ProbeSet	Symbol	Parametric p-value	FDR Crompot	Geometric mean Crompot	Parametric p-value Burger	FDR Burger	Geometric mean Burger	Parametric p-value Guarini	FDR Guarini	Geometric mean Guarini
244480_at	TCF4	0.0016352	0.93	-2.38	0.0020926	0.03	-1.85	0.0231109	0.0752	-1.18
1558877_at		0.0034705	0.93	-2.13	0.0005616	0.02	-1.69	0.0004932	0.0129	-1.33
230364_at	CHPT1	0.0015667	0.93	-2.13	0.0031984	0.04	-1.89	0.0035465	0.0268	-1.27
209967_s_at	CREM	0.0007252	0.93	-2.13	0.0025909	0.03	-4.00	0.0023428	0.0223	1.26
236260_at		0.0066831	0.93	-2.08	0.0021637	0.03	-2.17	0.0022321	0.0218	-1.25
238983_at	NSUN7	0.0020277	0.93	-2.08	0.0036369	0.04	-1.72	0.0087642	0.0416	-1.25
231472_at	FBXO15	0.0014771	0.93	-2.08	0.0004168	0.01	-2.63	0.0461802	0.118	-1.22
231166_at	GPR155	0.0077568	0.93	-2.04	0.0009683	0.02	-2.27	0.0005218	0.0129	-1.39
243469_at	ELF2 E74	0.0044298	0.93	-2.00	0.0001376	0.01	-1.92	0.0006382	0.0144	-1.27
241861_at	SYCP3	0.0074234	0.93	-1.89	0.0244041	0.11	-1.45	0.0000919	0.011	-1.61
210377_at	ACSM3	0.0065966	0.93	-1.89	0.0002657	0.01	-2.38	0.0024557	0.0225	-1.37
244860_at	SIK3	0.0315227	0.93	-1.89	0.0401969	0.14	-1.35	0.0031007	0.0248	-1.18
243915_at		0.0012035	0.93	-1.85	0.0290873	0.12	-1.43	0.0031086	0.0248	1.33
222378_at	LOC378805	0.014539	0.93	-1.72	0.0050321	0.05	-1.92	0.0139713	0.0544	-1.22
233483_at	TBC1D27	0.0151178	0.93	-1.72	0.0335387	0.13	-1.33	0.006453	0.0347	-1.20
212094_at	PEG10	0.0164605	0.93	-1.69	0.0000114	0.01	-1.69	0.0038612	0.0275	-1.45
237187_at	HRK	0.0149487	0.93	-1.67	0.0010440	0.02	-2.04	0.0016088	0.0196	-1.32
236621_at	RPS27	0.0336616	0.93	-1.67	0.0376326	0.14	-1.33	0.0041761	0.0281	-1.18
210785_s_at	THEMIS2	0.0037767	0.93	-1.67	0.0000632	0.01	-2.50	0.0410023	0.109	-1.16
235765_at	TLE4	0.0320591	0.93	-1.64	0.0027831	0.04	-1.28	0.0148042	0.0567	-1.20
237741_at	SLC25A36	0.0128476	0.93	-1.61	0.0153590	0.09	-1.43	0.0107355	0.0463	-1.18
210069_at		0.0286858	0.93	-1.61	0.0112093	0.07	-1.27	0.0156074	0.0581	-1.14
233614_at		0.0320958	0.93	-1.59	0.0071181	0.06	-1.64	0.0048012	0.0296	-1.25
226164_x_at	RIMKLB	0.0065561	0.93	-1.59	0.0203715	0.10	-1.45	0.0125172	0.0512	-1.18
240174_at		0.0491966	0.93	-1.56	0.0209487	0.10	-1.30	0.00096	0.0169	-1.28
208078_s_at	SIK1	0.013732	0.93	-1.56	0.0029716	0.04	-2.17	0.0462271	0.118	-1.15
204194_at	BACH1	0.0171662	0.93	-1.52	0.0012402	0.02	-2.08	0.0024636	0.0225	-1.18
1557242_at		0.0204808	0.93	-1.49	0.0087357	0.06	1.23	0.011452	0.0483	-1.20

235694_at	TCFL5	0.0149852	0.93	-1.45	0.0245322	0.11	-1.75	0.0002045	0.0123	-1.41
212960_at	TBC1D9	0.0242395	0.93	-1.45	0.0433257	0.15	-1.20	0.0004966	0.0129	-1.33
231911_at	ERMN	0.0418543	0.93	-1.45	0.0017055	0.03	-2.04	0.0013616	0.0183	-1.30
207237_at	KCNA3	0.0232545	0.93	-1.45	0.0273898	0.12	-1.27	0.0057739	0.0324	-1.28
201369_s_at	ZFP36L2	0.0200635	0.93	-1.45	0.0120842	0.08	-1.64	0.0085132	0.041	-1.22
212451_at	SECISBP2L	0.0223021	0.93	-1.43	0.0018268	0.03	-1.54	0.0037975	0.0275	-1.20
215102_at	DPY19L1P1	0.0395632	0.93	-1.41	0.0133389	0.08	-1.32	0.0078945	0.0393	-1.23
235459_at	RNF41	0.0489184	0.93	-1.39	0.0040892	0.04	-1.61	0.0073743	0.0375	-1.23
224707_at	CYSTM1	0.0299733	0.93	-1.37	0.0023188	0.03	-2.04	0.0222075	0.0733	1.19
226858_at	CSNK1E	0.0444659	0.93	-1.35	0.0050642	0.05	-1.67	0.0180606	0.0638	-1.20
206759_at	FCER2	0.0449327	0.93	1.34	0.0152915	0.09	2.17	0.0488493	0.122	1.22
213831_at		0.0489728	0.93	1.37	0.0426300	0.15	1.69	0.0464339	0.118	1.30
206206_at	CD180	0.0269908	0.93	1.43	0.0000097	0.01	2.91	0.0172653	0.0618	1.30
225540_at	MAP2	0.02693	0.93	1.43	0.0034505	0.04	1.90	0.0204279	0.0692	1.33
205291_at	IL2RB	0.0272784	0.93	1.43	0.0031467	0.04	1.63	0.0013416	0.0183	1.34
204674_at	LRMP	0.0355773	0.93	1.43	0.0001626	0.01	2.18	0.002204	0.0217	1.49
221581_s_at	LAT2	0.0396273	0.93	1.44	0.0165749	0.09	1.57	0.0003449	0.0123	1.43
1563674_at	FCRL2	0.023706	0.93	1.47	0.0206558	0.10	1.97	0.0130302	0.0522	-1.14
205659_at	HDAC9	0.0246864	0.93	1.48	0.0004732	0.02	2.16	0.0275198	0.0833	1.22
239975_at	HLA-DPB2	0.0384864	0.93	1.51	0.0012243	0.02	3.18	0.0071912	0.0371	1.33
204135_at	FILIP1L	0.0403201	0.93	1.54	0.0004489	0.01	2.77	0.0132365	0.0527	1.29
244413_at	CLECL1	0.015429	0.93	1.57	0.0000235	0.01	2.45	0.0404825	0.109	1.18
226147_s_at	PIGR	0.0353852	0.93	1.57	0.0001939	0.01	3.25	0.0050604	0.0302	1.56
204032_at	BCAR3	0.0451759	0.93	1.58	0.0000281	0.01	2.61	0.0003419	0.0123	1.99
226517_at	BCAT1	0.0251515	0.93	1.71	0.0419284	0.15	1.60	0.0014015	0.0184	2.20
201577_at	NME1	0.0456957	0.93	1.73	0.0239437	0.11	1.86	0.0008131	0.0158	1.95
203471_s_at	PLEK	0.0296043	0.93	1.79	0.0034663	0.04	2.92	0.0095134	0.0437	1.42
201694_s_at	EGR1	0.0049216	0.93	1.83	0.0000579	0.01	2.39	0.0002389	0.0123	2.49
203504_s_at	ABCA1	0.0101293	0.93	1.84	0.0303216	0.12	1.81	0.0352116	0.0985	-1.16

237753_at	IL21R	0.0295439	0.93	1.84	0.0361478	0.14	1.67	0.0015811	0.0196	1.79
223751_x_at	TLR10	0.0142748	0.93	1.88	0.0004388	0.01	2.76	0.0060941	0.0334	1.46
200953_s_at	CCND2	0.0135025	0.93	1.94	0.0104396	0.07	2.21	0.0010871	0.0173	1.64
236099_at		0.0059956	0.93	1.96	0.0000409	0.01	3.83	0.0027729	0.024	2.30
202391_at	BASP1	0.0128482	0.93	2.00	0.0017401	0.03	1.72	0.0003798	0.0124	1.61
212295_s_at	SLC7A1	0.0153344	0.93	2.03	0.0032142	0.04	1.60	0.0007901	0.0157	1.77
200629_at	WARS	0.0113106	0.93	2.24	0.0037782	0.04	2.41	0.0006497	0.0144	2.46
205249_at	EGR2	0.0165075	0.93	2.25	0.0000728	0.01	4.35	0.0001687	0.0123	3.60
201761_at	MTHFD2	0.0110963	0.93	2.65	0.0099564	0.07	1.81	0.0002699	0.0123	2.40
205114_s_at	CCL3	0.0207883	0.93	3.75	0.0095773	0.07	6.11	0.0000441	0.011	7.91
206115_at	EGR3	0.01632	0.93	3.77	0.0000780	0.01	7.80	0.0008653	0.0164	3.63
204103_at	CCL4	0.0116537	0.93	4.62	0.0036825	0.04	8.74	0.0000673	0.011	7.79

Table S4: List of 69 genes common in CLL B-cells activated by 3 different microenvironmental stimuli. We compared our microarray results with 2 previously published studies on CLL B-cells activated by NLC culture [6] or BCR stimulation using anti-IgM [7]. We obtained many similarities between the differentially expressed genes, and 69 genes were common among all three studies. (FDR= False discovery rate)

	Crompot	Burger	Guarini
Crompot et al. 2016		9%	11%
Burger et al. 2009	28%		27%
Guarini et al. 2008	22%	17%	

Table S5: Percentage similarity between signatures of CLL B-cells activated by 3 different microenvironmental stimuli. We used public microarray signatures from 2 different studies on CLL B-cells activated by NLC culture or by BCR stimulation [6;7]. Guarini shared 11 and 27% of the common differentially expressed genes with microarray signatures from Crompot and Burger, respectively, whereas Burger shared 9 and 17 % of the common differentially expressed genes with signatures from Crompot and Guarini, respectively. In total, 28% and 22 % of the differentially expressed genes in CLL B-cells pre-treated with EVs were in common with signatures obtained by Burger and Guarini, respectively.

ProbeSet	Parametric p-value	FDR	GM	Symbol	Parametric p-value Burger	FDR Burger	GM Burger	Parametric p-value Guarini	FDR Guarini	GM Guarini	Parametric p-value Herishanu BM	FDR Herishanu BM	GM Herishanu BM	Parametric p-value Herishanu LN	FDR Herishanu LN	GM Herishanu LN
1558882_at	0.0081721	0.93	1.87	HTATSF1P2												
1552742_at	0.0019666	0.93	1.87	KCNH8												
226560_at	0.0353017	0.93	1.87	SGPP2				0.0053063	0.0308	1.39						
223751_x_at	0.0142748	0.93	1.88	TLR10	0.0004388	0.01	2.76	0.0060941	0.0334	1.46				0.0000001	4.76E-07	1.63
204440_at	0.0198748	0.93	1.92	CD83				0.0008872	0.0165	2.03	0.0003388	0.000504	1.47	0.0000001	0.0000001	2.89
200953_s_at	0.0135025	0.93	1.94	CCND2	0.0104396	0.07	2.21	0.0010871	0.0173	1.64	0.0000103	0.0000358	1.4	0.0000001	0.0000001	2.33
202391_at	0.0128482	0.93	2	BASP1	0.0017401	0.03	1.72	0.0003798	0.0124	1.61				0.0000001	0.0000001	1.48
212295_s_at	0.0153344	0.93	2.03	SLC7A1	0.0032142	0.04	1.60	0.0007901	0.0157	1.77						
241278_at	0.0011709	0.93	2.05	FCRL3	0.0014897	0.03	1.96									
202431_s_at	0.0073695	0.93	2.12	MYC				0.0024242	0.0225	2.05				0.0000059	0.0000131	2.21
206641_at	0.0081022	0.93	2.16	TNFRSF17	0.0000138	0.01	7.63							0.0004591	0.000564	1.41
224405_at	0.0069175	0.93	2.19	FCRL5	0.0008401	0.02	3.30									
200629_at	0.0113106	0.93	2.24	WARS	0.0037782	0.04	2.41	0.0006497	0.0144	2.46						
205249_at	0.0165075	0.93	2.25	EGR2	0.0000728	0.01	4.35	0.0001687	0.0123	3.60	0.0000051	0.0000234	2.42	0.0000001	4.76E-07	3.69
201669_s_at	0.0009462	0.93	2.33	MARCKS				0.0056968	0.0322	1.50						
201761_at	0.0110963	0.93	2.65	MTHFD2	0.0099564	0.07	1.81	0.0002699	0.0123	2.40				0.0000001	0.0000001	2.25
235122_at	0.0105866	0.93	3.58	HIVEP3	0.0046659	0.04	2.18									
205114_s_at	0.0207883	0.93	3.75	CCL3	0.0095773	0.07	6.11	0.0000441	0.011	7.91	0.0051931	0.0425	1.61	0.0000001	0.0000001	8.33
206115_at	0.01632	0.93	3.77	EGR3	0.000078	0.01	7.80	0.0008653	0.0164	3.63	0.0000003	0.00000433	2.65	0.0000014	0.00000421	2.9
204103_at	0.0116537	0.93	4.62	CCL4	0.0036825	0.04	8.74	0.0000673	0.011	7.79	0.0000399	0.0000979	1.95	0.0000001	0.0000001	6.16

Table S6: Comparison of microarray profiles between CLL B-cells activated by EVs from BM-MSC, by NLC culture, and CLL B-cells from lymph nodes (LN) and bone marrow (BM) —the 20 most increased genes. We listed the 20 most differentially expressed genes in our microarray signatures compared with signature of CLL B-cells activated by NLC culture and with signature of CLL B-cells coming from LN and BM. We observed multiple common genes; between 5 and 50% of genes were similar.

ProbeSet	Parametric p-value	FDR	GM	Symbol	Parametric p-value Burger	FDR Burger	GM Burger	Parametric p-value Guarini	FDR Guarini	GM Guarini	Parametric p-value Herishanu BM	FDR Herishanu BM	GM Herishanu BM	Parametric p-value Herishanu LN	FDR Herishanu LN	GM Herishanu LN
203474_at	0.000852	0.93	-3.03	IQGAP2												
214481_at	0.000282	0.93	-2.70	HIST1H2AM										0.0028611	0.00312	1.37
230505_at	0.000822	0.93	-2.63	LOC145474	0.000035	0.01	-2.22									
208547_at	0.000245	0.93	-2.63	HIST1H2BB	0.036171	0.14	-1.47									
210387_at	0.000521	0.93	-2.56	NCALD				0.0215648	0.0716	-1.19						
226430_at	0.000930	0.93	-2.56	RELL1												
214472_at	0.004179	0.93	-2.50	HIST1H3D	0.001371	0.03	-1.37							0.0000001	0.00453	1.57
214469_at	0.000281	0.93	-2.44	HIST1H2AE										0.0000297	0.0000502	1.58
244480_at	0.001635	0.93	-2.38	TCF4	0.002093	0.03	-1.85	0.0231109	0.0752	-1.18						
215779_s_at	0.001441	0.93	-2.38	HIST1H2BG										0.000001	0.000254	1.92
225767_at	0.000847	0.93	-2.33	LOC283501	0.000682	0.02	-5.00							0.0000001	0.0178	1.49
236561_at	0.007771	0.93	-2.33	TGFBR1	0.003767	0.04	-1.61									
202254_at	0.001188	0.93	-2.27	SIPA1L1	0.001121	0.02	-1.89							0.0000003	0.0000012	-1.67
208575_at	0.000520	0.93	-2.27	HIST1H3A												
1556055_at	0.006130	0.93	-2.22	LOC283501												
215967_s_at	0.001343	0.93	-2.22	LY9	0.013431	0.08	-2.08									
235683_at	0.001753	0.93	-2.22	SESN3				0.015922	0.0589	-1.20						
1561469_at	0.003764	0.93	-2.17	OC101928865	0.021923	0.10	-1.22				0.000002	0.0000122	1.45			
1556750_at	0.004939	0.93	-2.17	LOC153577												
209967_s_at	0.000725	0.93	-2.13	CREM	0.002591	0.03	-4.00	0.0023428	0.0223	1.26						

Table S7: Comparison of microarray profiles between CLL B-cells activated by EVs from BM-MSC, by NLC culture, and CLL B-cells from lymph nodes (LN) and bone marrow (BM) —the 20 most decreased genes. We listed the 20 most differentially expressed decreased genes in our microarray signatures compared with signature of CLL B-cells activated by NLC culture and with signature of CLL B-cells coming from LN and BM. We observed multiple common genes; between 30 and 85% of genes were similar.

ProbeSet	Symbol	Parametric p-value	FDR Crompot	GM Crompot	Parametric p-value	FDR Herishanu	GM Herishanu	Parametric p-value	FDR Herishanu	GM Herishanu
222139_at	ERV3-2	0.0120762	0.93	-1.96	0.0000071	0.00	-1.54	0.0003011	0.000458	-1.33
242163_at	THRAP3	0.0127298	0.93	-1.92	0.0000469	0.00	1.54	0.0016418	0.00196	1.34
243996_at	#N/A	0.0420794	0.93	-1.75	0.0000026	0.02	-1.41	0.000131	0.00428	-1.49
222378_at	LOC378805	0.014539	0.93	-1.72	0.0000001	0.00	2.81	0.0039845	0.0358	1.35
223044_at	SLC40A1	0.0113652	0.93	-1.72	0.0000004	0.00	2.26	0.0000002	0.0000034	2.38
238987_at	B4GALT1	0.01053	0.93	-1.64	0.0007103	0.00	1.60	0.0009489	0.0012	1.34
208078_s_at	SIK1	0.013732	0.93	-1.56	0.0000012	0.00	2.16	0.033681	0.137	1.36
202499_s_at	SLC2A3	0.0106537	0.93	-1.52	0.0000001	0.00	2.89	0.000301	0.000458	1.51
222557_at	STMN3	0.015219	0.93	-1.52	0.0000001	0.00	-2.04	0.0000065	0.0000278	-1.37
218113_at	TMEM2	0.0148242	0.93	-1.52	0.0000001	0.00	1.74	0.0000107	0.0000366	1.34
231085_s_at	PCBD1	0.0392184	0.93	-1.49	0.0002861	0.00	-1.82	0.0001789	0.00514	-1.41
235286_at	CKLF	0.0298872	0.93	-1.49	0.0000003	0.00	2.20	0.0000157	0.00126	1.39
242325_at	YWHAH	0.0269495	0.93	-1.49	0.0009843	0.00	-1.64	0.0000001	0.00000238	-1.85
208891_at	DUSP6	0.0118315	0.93	-1.47	0.0000003	0.00	3.07	0.000002	0.0000122	2.58
208553_at	HIST1H1E	0.0171241	0.93	-1.47	0.0125070	0.01	1.51	0.0000019	0.0000121	-1.52
242918_at	NASP	0.0362146	0.93	-1.47	0.0000014	0.00	2.10	0.0001015	0.000194	1.38
222088_s_at	SLC2A14	0.0182502	0.93	-1.47	0.0000038	0.00	2.27	0.000476	0.00923	1.39
237318_at	SLC43A3	0.0444614	0.93	-1.45	0.0001234	0.00	-1.82	0.0000002	0.000114	-1.59
241996_at	RUFY2	0.0388297	0.93	-1.45	0.0000373	0.00	-1.47	0.0002698	0.000418	-1.32
1560926_at	PPP4R2	0.0370256	0.93	-1.43	0.0000003	0.00	1.47	0.0000682	0.00289	1.49
239328_at	#N/A	0.046883	0.93	-1.35	0.0030601	0.00	-2.33	0.0000013	0.000329	-1.56
1559044_at	EXOSC1	0.0495316	0.93	-1.35	0.0003943	0.00	1.39	0.0002241	0.000365	1.31
226397_s_at	#N/A	0.046678	0.93	1.51	0.0000001	0.00	2.28	0.0000001	0.0000001	1.79
204135_at	FILIP1L	0.0403201	0.93	1.54	0.0000026	0.00	1.74	0.0001045	0.000199	1.56
204032_at	BCAR3	0.0451759	0.93	1.58	0.0000001	0.00	2.25	0.0000836	0.000172	1.35
237753_at	IL21R	0.0295439	0.93	1.84	0.0000001	0.00	1.72	0.0000002	0.0000034	1.39
1557049_at	BTBD19	0.049729	0.93	1.85	0.0000001	0.00	2.78	0.0007296	0.000949	1.48
204440_at	CD83	0.0198748	0.93	1.92	0.0000001	0.00	2.89	0.0003388	0.000504	1.47
200953_s_at	CCND2	0.0135025	0.93	1.94	0.0000001	0.00	2.33	0.0000103	0.0000358	1.40
236099_at	#N/A	0.0059956	0.93	1.96	0.0000001	0.00	1.35	0.0003059	0.00717	1.31
205249_at	EGR2	0.0165075	0.93	2.25	0.0000001	0.00	3.69	0.0000051	0.0000234	2.42
205114_s_at	CCL3	0.0207883	0.93	3.75	0.0000001	0.00	8.33	0.0051931	0.0425	1.61
206115_at	EGR3	0.01632	0.93	3.77	0.0000014	0.00	2.90	0.0000003	0.00000433	2.65
204103_at	CCL4	0.0116537	0.93	4.62	0.0000001	0.00	6.16	0.0000399	0.0000979	1.95

Table S8: List of 39 genes common in CLL B-cells treated with BM-MSC EVs and from lymph nodes (LN) and bone marrow (BM). We compared our microarray results with previously published study on CLL B-cells coming from bone marrow and lymph nodes [8]. We obtained many similarities between the differentially expressed genes, and 39 genes were common among all three signatures. (FDR= False discovery rate)

Name	Fluorochrome	Provider
CD19	PE	Miltenyi Biotec
CD5	FITC	Miltenyi Biotec
CD23	FITC	Beckman Coulter
CD20	PE	Miltenyi Biotec
CD105	FITC	Ancell Corporation
CD166	PE	BD Biosciences Pharmingen
CD184	PE	BD Biosciences Pharmingen
Annexin-V	FITC	BD Biosciences Pharmingen
7AAD		BD Biosciences Pharmingen
CD146	PC5	Beckman Coulter
CD73	PE	Miltenyi Biotec
CD90	PE	Miltenyi Biotec
CD45	PE-cy7	BD Biosciences Pharmingen
Mcl-1	Uncoupled	Santa Cruz Biotechnology
Immunoglobulin swine anti-rabbit F(ab')2	FITC	Dako A/S
CD63	PE	Miltenyi Biotec
CD69	PE	Miltenyi Biotec

Table S9: Antibodies used for flow cytometry analyses. Primary and secondary antibodies used for flow cytometry and PCR manipulations.

Type	CD90	CD146	CD166	CD73	CD105	CD45
Healthy MSC (%)	99.0	98.0	96.0	96.0	96.3	0.9
SEM (n=5)	0.3	1.0	1.5	1.5	1.4	0.1
CLL MSC (%)	96.3	96.0	97.7	96.7	95.9	0.8
SD (n=3)	0.3	2.1	0.3	1.5	1.10	0.2

Table S10: Expression of classical markers of MSC obtained from healthy and CLL patients. We determined the expression of CD90/CD146/CD166/CD73/CD105/CD45 on BM-MSCs from healthy and CLL patients (presented by percentage of positive cells with the standard error of the mean (SEM) calculated on 5 and 3 samples for healthy and CLL BM-MSCs, respectively. We did not observe any difference about the expression of these markers and any CD19 positive cells have been detected confirming the purity of the culture.

Name	Forward	Reverse
PPI	GCTCGTGCCGTTTTGCA	GCAAACAGCTCAAAGGAGACG
FCRL5	CCCAGCGCAGTGAGACAGT	GCAAAAGGGCCACTTCTGTTC
CD69	TGGTGATGAAGACCACATTCA	AGAACAGCTCTTTGCATCCG
CCL4	CGTGTATGACCTGGAACTGAACTG	TCCCTGAAGACTTCCTGTCTCTGA
HRK	CAGGCGGAACTTGTAGGA	CTCTGGAAACGGGAACC
PDE8A	AGGCGCCCATCACCAA	TGTCACAGGCATGGGACTACTT
LY9	TTCAGCTGGTGCATTTGGAA	TCAGCTTGGCTGGAACAGAA
CCR7	CAGATGCAATGACTCAGGAC	CTGTTTCCCAGTGTTGTCTG
HDAC9	CTCATGCCGAGCAAATGGTT	GACTTAGCAGGTTCATGGAATCTTC
MCL1	GAGACCTTACGACGGGTT	TTTGATGTCCAGTTTCCG
LPL	CCGCCGACCAAAGAAGAGAT	TTCCTGTTACCGTCCAGCCAT
MYC	CTTCTCTCCGTCCTCGGATTCT	GAAGGTGATCCAGACTCTGACCTT
BCL2	GGCTGGGATGCCTTTGTG	GCCAGGAGAAATCAAACAGAGG
XIAP	AGTGGTAGTCCTGTTTCAGCATCA	CCGCACGGTATCTCCTTCA
BAX	CCTGTGCACCAAGGTGCCGGAACT	CCACCCTGGTCTTGGATCCAGCCC
BCLXL	GGGGTAAACTGGGGTCGCATT	ACCTGCGGTTGAAGCGTTC

Table S11: Used primer sequences in the qPCR study. Forward and reverse primer sequences of genes studied in CLL B-cells by qPCR.

Online Supplementary Appendix Figures

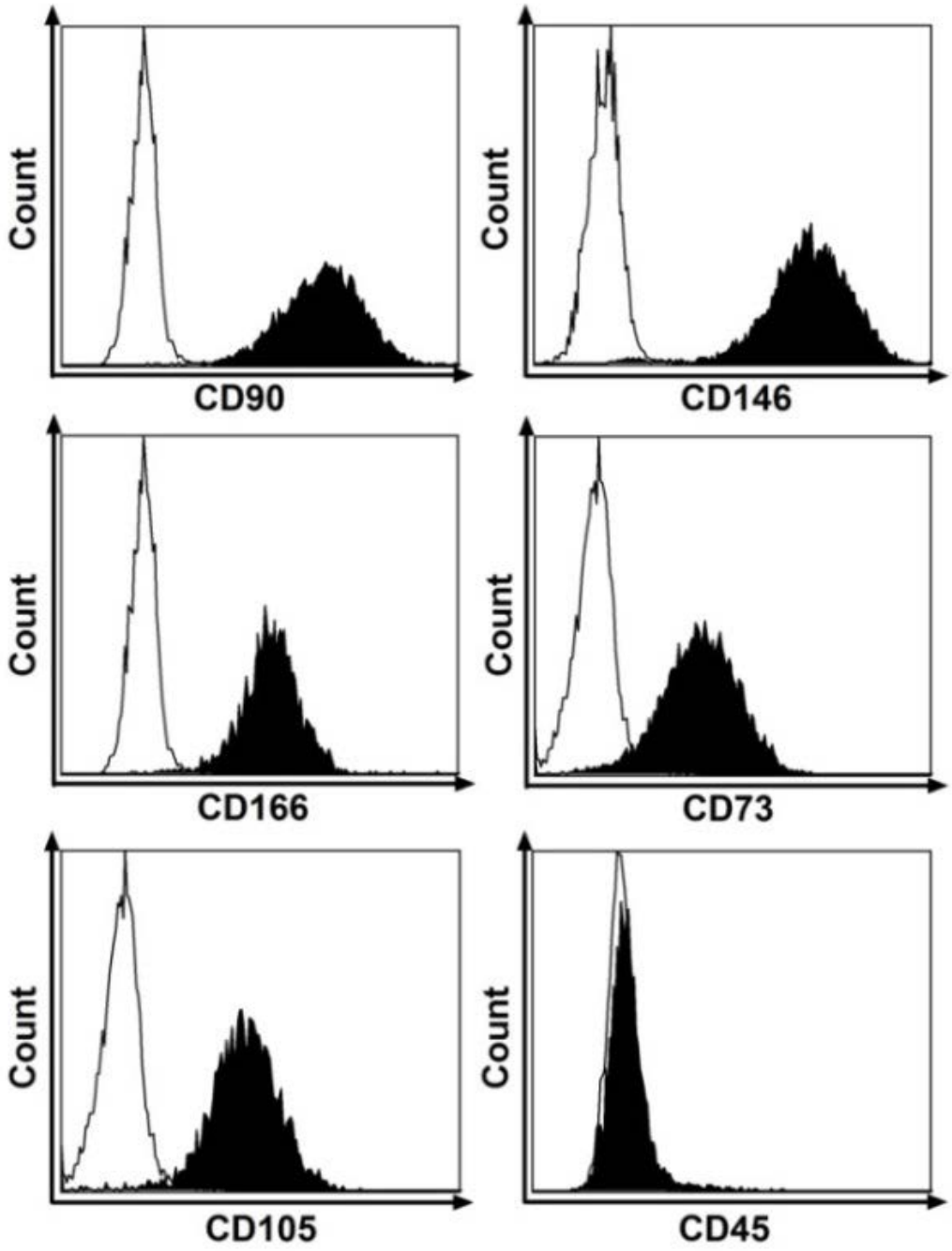

Figure S1: Characterization of BM-MSCs. BM-MSCs were characterized by flow cytometry, they expressed classical markers of MSCs (CD105, 73, 146, 90 and 166) and were negative for CD45. The fibroblast-like morphology was confirmed by optical microscopy.

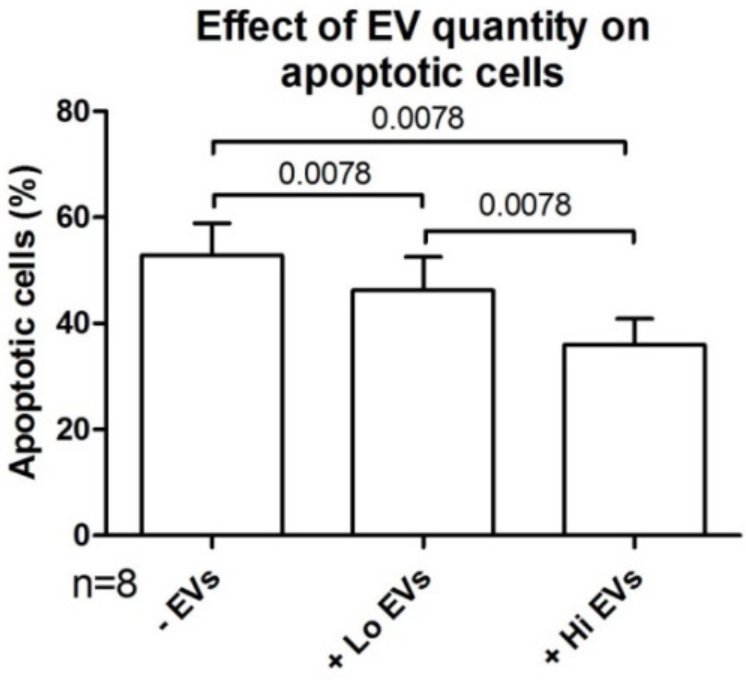

Figure S2: EV quantity effect on CLL B-cell apoptosis. Apoptosis assay was performed on CLL B-cells that received two different quantities of EVs or nothing as a negative control. Low (Lo) EVs means between 0.25 and 1µg while High (Hi) EVs means more than 1µg for 4 million of cells. We observed a significant impact of higher quantity of EVs on CLL B cell apoptosis.

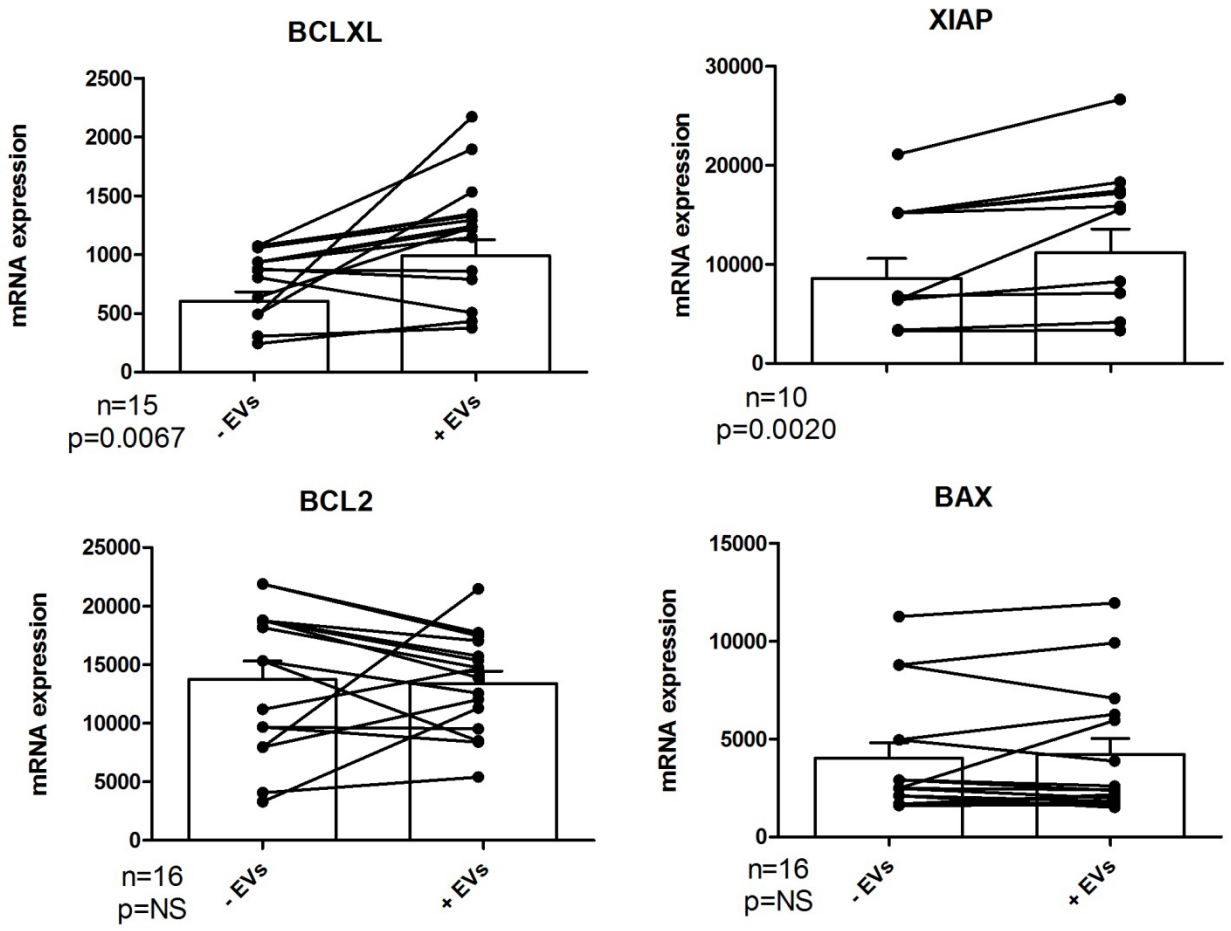

Figure S3: Expression of genes involved in apoptosis after EV integration in CLL B-cells. We studied the expression of BCLXL, XIAP, BCL2 and BAX in CLL B-cells after EV integration. We noted an increased expression of BCLXL and XIAP mRNA by qPCR but no difference was observed for BCL2 and BAX.

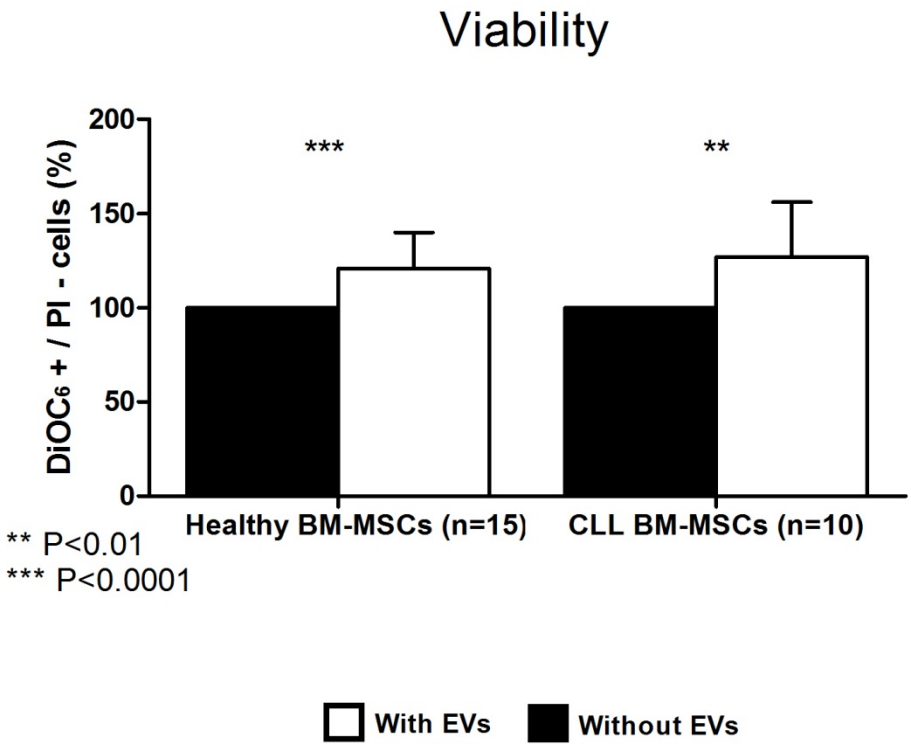

Figure S4: EV effect on CLL B-cell viability. Cell viability was analyzed by DiOC6/PI labeling assay on CLL samples treated by EVs derived from healthy (n=15) and CLL BM MSCs (n=10). Results were normalized using the viability of cell without EVs (100%). The addition of EVs from BM-MSC can significantly increase this parameter.

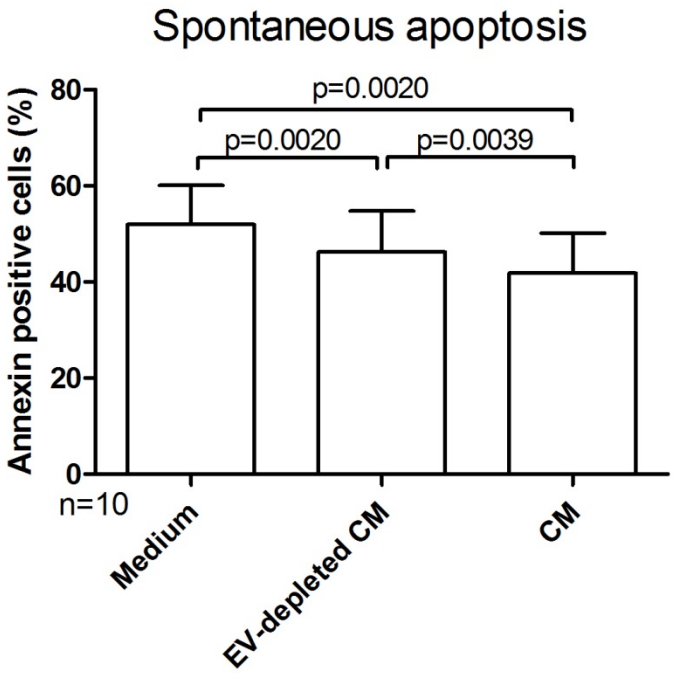

Figure S5: Effect of MSC conditioned medium and EV depleted conditioned medium on CLL B-cell apoptosis. The addition of conditioned medium versus EV depleted conditioned medium from BM-MSC culture exhibited a significant difference of annexin positive cell percentage, demonstrating that conditioned medium, without concentration of EVs already induce protective effect on CLL B-cells.

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