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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ABSTRACT

Interactions between chronic lymphocytic leukemia (CLL) B cells and the bone marrow (BM) microenvironment play a major function in the physiopathology of CLL. Extracellular vesicles (EVs), which are composed of exosomes and microparticles, play an important role in cell communication. However, little is known about their role in CLL / microenvironment interactions. In the present study, EVs purified by ultracentrifugation from BM mesenchymal stromal cell (BM-MSC) cultures were added to CLL B cells. After their integration into CLL B cells, we observed a decrease of leukemic cell spontaneous apoptosis and an increase in their chemoresistance to several drugs, including fludarabine, ibrutinib, idelalisib and venetoclax after 24 hours. Spontaneous (P=0.0078) and stromal cell-derived factor 1α-induced migration capacities of CLL B cells were also enhanced (P=0.0020). A microarray study highlighted 805 differentially expressed genes between leukemic cells cultured with or without EVs. Of these, genes involved in the B-cell receptor pathway such as CCL3/4, EGR1/2/3, and MYC were increased. Interestingly, this signature presents important overlaps with other microenvironment stimuli such as B-cell receptor stimulation, CLL/nurse-like cells co-culture or those provided by a lymph node microenvironment. Finally, we showed that EVs from MSCs of leukemic patients also rescue leukemic cells from spontaneous or drug-induced apoptosis. However, they induce a higher migration and also a stronger gene modification compared to EVs of healthy MSCs. In conclusion, we show that EVs play a crucial role in CLL B cells/BM microenvironment communication.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in the Western world and is characterized by the progressive accumulation of monoclonal, mature CD5$^+$/CD19$^+$/CD23$^+$ B cells in the blood, bone marrow, lymph nodes and spleen. The interactions between malignant B cells and the microenvironment (ME) play a major role in the physiopathology of CLL.[1] These interactions drive CLL B cells into the ME, where they receive advantageous signals from different populations of accessory cells mediated by diverse receptors such as CD40, Toll-like receptors (TLR), C-X-C chemokine receptor type 4 (CXCR4) and the B-cell receptor (BCR). CLL B cells present a defect of apoptosis *in vivo*; however, they rap-

idly undergo spontaneous apoptosis when they are cultured *in vitro*, suggesting the importance of the ME in their survival. Interactions with the ME, such as bone marrow mesenchymal stromal cells (BM-MSCs) or nurse-like cells (NLC), protect CLL B cells from spontaneous and drug-induced apoptosis.[2] However, the complex cellular and molecular mechanisms underlying this protection are not fully understood.

Previously, extracellular vesicles (EVs) were described as cellular debris, but today, EVs are known to play an important role in cell communication. EVs are plasma membrane released from the endosomal compartment (exosomes) or derived directly from the membrane (microparticles) of various cell types. Microparticles (MPs) are submicron vesicles from 100 nm to 1 μm, whereas exosomes have a smaller size from 30 nm to 100 nm.[3] To avoid confusion, "EVs" will be used to describe vesicles including exosomes and MPs.[4] These vesicles have an impact on several physio-pathological processes including immune responses, tissue regeneration, and blood coagulation. They contain selective patterns of microRNAs, proteins or DNA, which may be transferred into the target cells (reviewed by Abels and Breakefield[5]).

Here, we investigated the role of EVs derived from BM-MSCs in different biological processes critical for CLL cell survival. In addition, we broadened our understanding of the EV effect by studying the gene expression profile induced by EVs in leukemic cells.

Methods

Biological samples and cell culture

This study was approved by the Jules Bordet Institute ethics committee (Belgium). Peripheral blood mononuclear cells (PBMC) from CLL samples were obtained from patients after written informed consent. Additional details about sample isolation, CLL cell culture, patients' characteristics, BM-MSC isolation and characterization are available in the *Online Supplementary Table S1 and Online Supplementary Figure S1.*

Extracellular vesicle isolation and characterization

To obtain EVs, BM-MSCs were cultured with serum deprivation for 24 hours (h) to avoid contaminations by Fetal Bovine Serum (FBS)-derived vesicles. We also compared these conditions with exosome-depleted FBS and we did not observe any difference; serum deprivation over 24 h did not induce senescence and morphology modifications of BM-MSCs. We used 500 mL of BM-MSC supernatant to produce the EV pellets. Cell-free supernatants were obtained by 2 successive centrifugations at 300xg for 10 min-

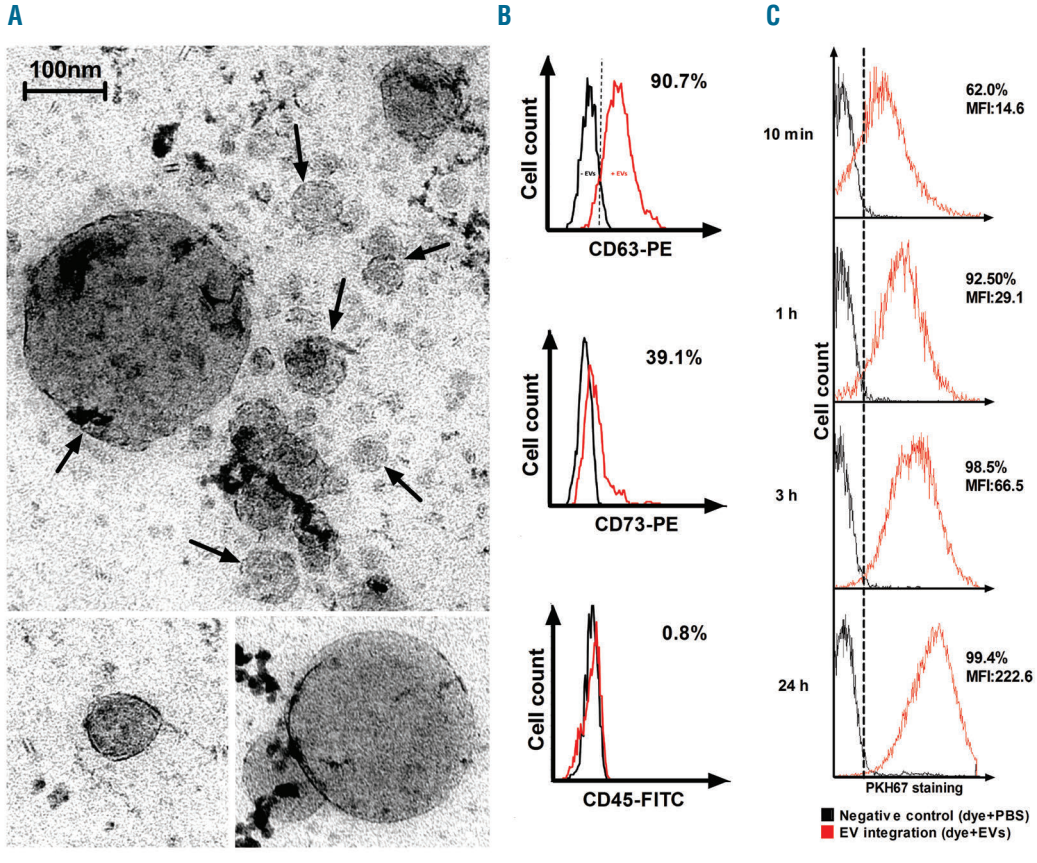

Figure 1. Characterization of bone marrow (BM) extracellular vesicles (EVs) and uptake by chronic lymphocytic leukemia (CLL) B cells. Bone marrow mesenchymal stromal cells (BM-MSCs) were characterized by electron microscopy (A) and flow cytometry (B) using the latex bead technique. EVs showed a classical spherical appearance by transmission electron microscopy (TEM). EVs expressed CD63 (EV marker) and CD73 (MSC marker) and were negative for CD45 (hematopoietic marker). (C) CLL B cells were incubated with BM-MSC EVs, previously labeled with PKH67, for 10 minutes (min), 1 hour (h), 3 h and 24 h. Flow cytometry showed a rapid increase of the mean of fluorescence intensity (MFI), depending on the incubation time. The uptake of BM-MSC EVs was a fast process; after 10 min, more than 60% of CLL B cells had integrated fluorescent vesicles. PBS: phosphate-buffered saline.

utes. The supernatants of BM-MSC cultures were then concentrated with a 3K Macrosep advance centrifugal device (Pall Life Science, New York, USA). The supernatants were then subjected to 150,000xg centrifugation for 1 h at 4°C (Ultracentrifuge MX 120+, Swinging Bucket rotor S50-ST, k-factor 77, Thermo Scientific, Waltham, MA, USA). EV pellets were washed using 0.2-μm-filtered phosphate buffered saline (PBS) by centrifugation for 1 h at 150,000xg. The pellets were finally reconstituted in 100 μL of PBS and stored at -80°C until use. The amount and size of BM-MSC EVs were determined using Nanosight technology (Nanosight Ltd., Minton Park, UK) and the concentration was determined using the Nanodrop (Thermo Scientific, Nanodrop 2000c) and BCA kit assay (Thermo Scientific). Details of EV characterization[6] and monitoring of BM-MSC EV uptake by CLL B cells are available in the *Online Supplementary Appendix*.

Apoptosis, cell viability, migration and chemosensitivity assay

Chronic lymphocytic leukemia B cells were cultured with or without 2 μg of EVs in 24-well plates (4x10^6 cells/well) in 10% FBS RPMI-1640. Apoptosis was determined using annexin V/7-Aminoactinomycin D (7AAD) staining and viability by 3,3′-dihexyloxacarbocyanine iodide (DiOC6)/propidium iodide (PI) staining as previously described.[7] CLL B cells cultured without EVs

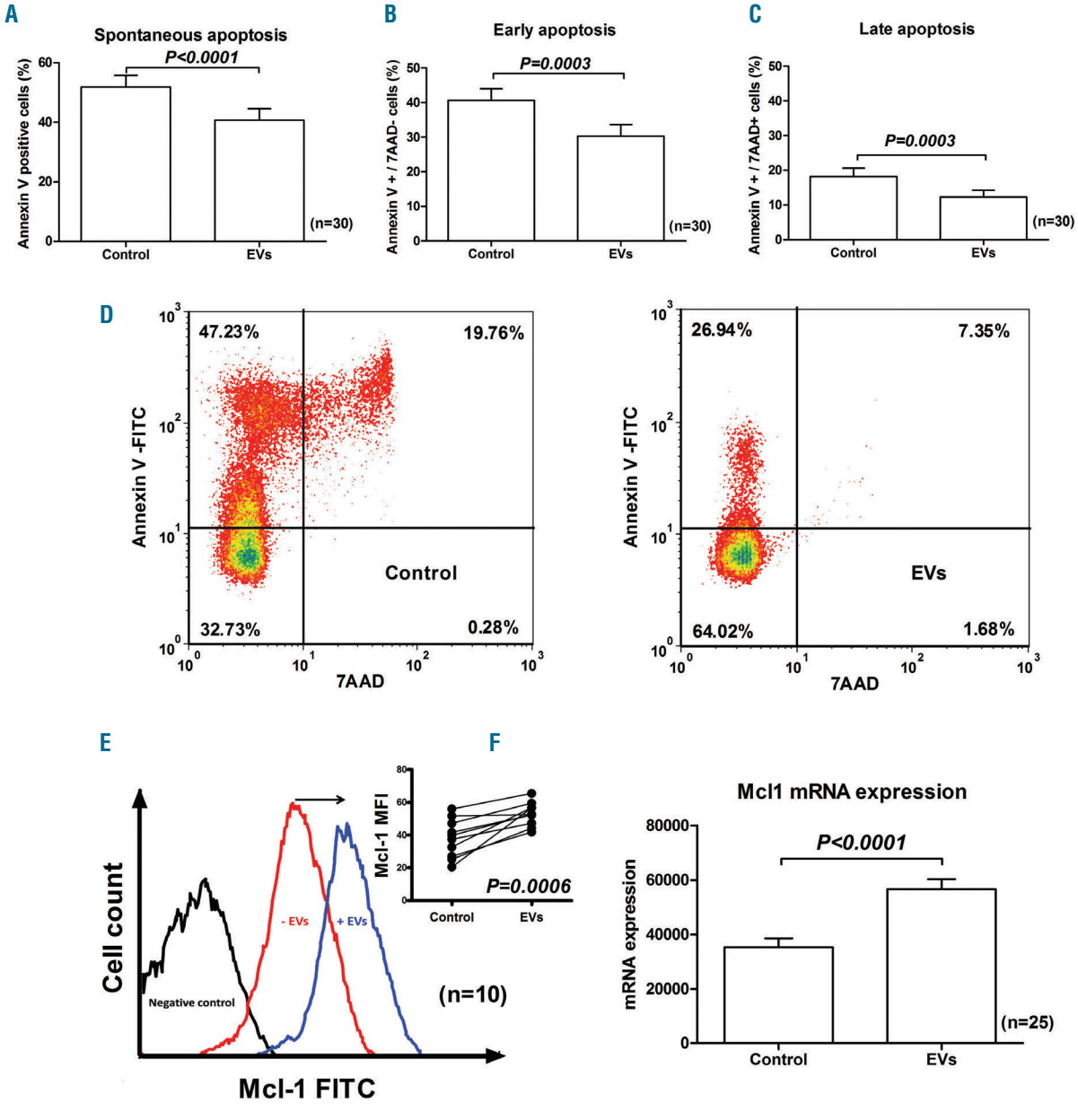

Figure 2. Bone marrow (BM) extracellular vesicles (EVs) protect chronic lymphocytic leukemia (CLL) B cells from spontaneous apoptosis. CLL B cells were incubated with bone marrow mesenchymal stromal cell (BM-MSC) EVs, and after 24 hours (h), cells were stained with annexin V-FITC/7AAD. We observed a decrease in spontaneous apoptosis (A) with a significant effect on early (B) and late (C) apoptosis. (D) A representative flow cytometry analysis of CLL B cells cultured with EVs for 24 h. (E) Expression of the anti-apoptotic protein Mcl-1 (myeloid cell leukemia 1). Red line: CLL B cells without EVs; blue line: CLL B cells cultured with EVs. The expression of secondary antibody was used as a negative control (n=10, *P*=0.0006). Results were also confirmed by qPCR (n=25, *P*=0.0001) (F).

were used as a negative control. The migration assay was performed as previously described.[7] Additional details can be found in the *Online Supplementary Appendix*. Cells were incubated with bortezomib, cladribine, fludarabine, flavopiridol or methylprednisolone at the half maximal (50%) inhibitory concentration (IC50) as previously established.[7,8] Ibrutinib, idelalisib and venetoclax were also used with specific concentration, as described in several studies.[9-11]

Additional details are provided in the *Online Supplementary Appendix* and *Online Supplementary Table S2*.

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.[12] Additional details and bioinformatics analysis can be found in the *Online Supplementary Appendix*.

EVs effect on CLL B-cell BCR activation

Measurement of intracellular calcium flux, mitogen-activated protein kinase 1 (ERK) and v-akt murine thymoma viral oncogene homolog 1 (AKT) phosphorylation were investigated by flow cytometry and are detailed in the *Online Supplementary Appendix*.

Statistical analyses

The Wilcoxon matched pairs test was used to analyze the statistical significance of experimental results. $P<0.05$ was considered statistically significant. Data were analyzed and graphics were constructed using GraphPad Prism v.5.0 (GraphPad Software, San Diego, CA, USA).

Results

Characterization of BM-MSC EVs and uptake by CLL B cells

We first verified the presence of BM-MSC EVs by characterization using transmission electron microscopy, and they presented classical aspects (Figure 1A). EVs were also characterized by latex bead flow cytometry technique, as previously described,[6] and we observed weak expression of CD73 (marker commonly found on MSCs) and high expression of CD63, a classical marker of EVs.[13] CD45 was used as a negative control (Figure 1B). EV uptake by CLL cells was verified and monitored by PKH67 fluorescent labeling (Figure 1C and the *Online Supplementary Appendix*).

BM-MSC EVs protect CLL B cells from spontaneous apoptosis

The addition of EVs resulted in a decrease of spontaneous apoptosis after 24 h of culture ($P<0.0001$) (Figure 2A). The integration of EVs also induced a decrease of both early and late apoptosis (Figure 2B and C), and this protection against apoptosis was dose-dependent (*Online Supplementary Figure S2*). Figure 2D shows a representative flow cytometry analysis of the EV effect on CLL B cells after 24 h. We observed a significant decrease of annexin V positive cells. We also studied the expression of Mcl-1, an anti-apoptotic protein (see the *Online Supplementary Appendix* for details). The addition of EVs increased Mcl-1 expression as measured by flow cytometry (n=10, $P=0.0006$) (Figure 2E) and qPCR (n=25, $P<0.0001$) (Figure 2F). We also observed an increase in the expression of B-cell lymphoma extra-large (BCLXL) (n=15, $P=0.0067$) and X-linked inhibitor apoptosis protein (XIAP) (n=10, $P=0.0020$), two other anti-apoptotic proteins. However, no difference was observed for B-cell CLL/lymphoma 2 (BCL2) and BCL2-associated X protein (BAX) expression (*Online Supplementary Figure S3*). Finally, viability was studied by DiOC6-PI labeling; EV addition significantly increased CLL cell viability (n=15, $P<0.0001$/n=10, $P<0.01$) (*Online Supplementary Figure S4*). These different observations demonstrated the capacity of BM-MSC EVs to protect CLL B cells from spontaneous apoptosis, thereby increasing their viability.

BM-MSC EVs increase CLL B-cell migration capacity

When unstimulated, CLL B cells were plated in the upper chamber of a Transwell, low spontaneous migration capacity was observed. The pre-incubation of CLL cells with EVs for 4 h significantly increased the level of migrating cells ($P=0.0078$) (Figure 3A). When CLL B cells were pre-incubated with BM-MSC EVs for 4 h, a significant increase of migration was observed in the presence of the SDF1α (n=10, $P=0.0020$) (Figure 3B). These results demonstrate that EVs from BM-MSCs can enhance the spontaneous and SDF-1-induced migration of CLL B cells. Interestingly, we observed no effect of CXCR4 receptor antagonist AMD3100 ($P=0.9143$) and the addition of pertussis toxin ($P=0.0634$) did not significantly decrease the number of migrating cells (n=25) (Figure 3C).

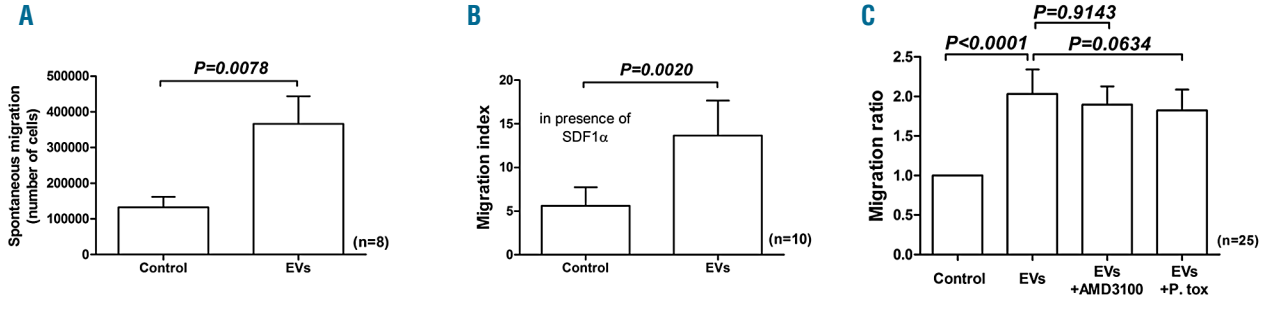

Figure 3. Bone marrow (BM) extracellular vesicles (EVs) increase chronic lymphocytic leukemia (CLL) B-cell migration capacity. CLL B cells were incubated with bone marrow mesenchymal stromal cell (BM-MSC) EVs in a Transwell assay. EV addition increased the spontaneous migration (A). The migration index was also calculated as the number of cells transmigrating in the presence of SDF-1α divided by the number of cells transmigrating in the absence of SDF1α. A significant increase in migration was observed in the presence of the chemoattractant SDF-1α (B) (n=10, $P=0.0020$). (C) Migration of CLL B cells in the presence of AMD3100 and pertussis toxin (*P. tox*). We did not observe a decrease in the migrating cells in the presence of AMD3100 or pertussis toxin (*P. tox*).

BM-MSC EVs increase chemoresistance of CLL B cells

We determined whether EVs are implicated in the drug resistance of CLL B cells. Eight different drugs were tested (Figure 4). The cytotoxicity of these drugs was studied after 24 h of incubation on CLL B cells cultured alone (as a negative control) or pre-treated with EVs for 4 h. CLL B cells presented significant resistance to these 8 drugs in the presence of BM-MSC EVs; they significantly decreased the number of apoptotic CLL B cells after 24 h (Figure 4). Interestingly, for cladribine and bortezomib, EVs completely suppressed drug-induced apoptosis.

Impact of BM-MSC EVs on CLL B-cell gene expression profile

The transcriptomic profiles of CLL B cells from 3 different patients were determined to obtain a global view of gene expression differences in leukemic cells cultured with or without BM-MSC EVs. A *P* value less than 0.05 allowed us to identify 805 genes differentially expressed between leukemic cells cultured with or without EVs. Among them, 152 (19%) were up-regulated and 653 (81%) were down-regulated in CLL B cells cultured with BM-MSC EVs (Figure 5A). Gene set enrichment analysis investigating gene GO categories demonstrated that up-regulated 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 of transcription (GO:0001228); all significant categories with the LS-permutation *P*-value selection are listed in the *Online Supplementary Appendix Table S3*. To validate the robustness of our microarray analysis, a panel of 7 genes implicated in CLL physiopathology was selected for qPCR validation on an extended cohort (n=18) (Figure 5B). All tested genes were confirmed as differentially expressed in CLL B cells after BM-MSC EV addition, highlighting the robustness of our microarray data.

Comparison of microarray signatures with other studies

We compared our microarray results with 2 previously published studies of CLL B cells co-cultured with NLC culture[14] or stimulated by anti-IgM stimulation.[15] The intersection of differentially expressed genes after these different ME stimulations showed important overlaps. In total, 177 (22% of our study) and 226 (28%) genes were shared between our study and that of Burger *et al.* and Guarini *et*

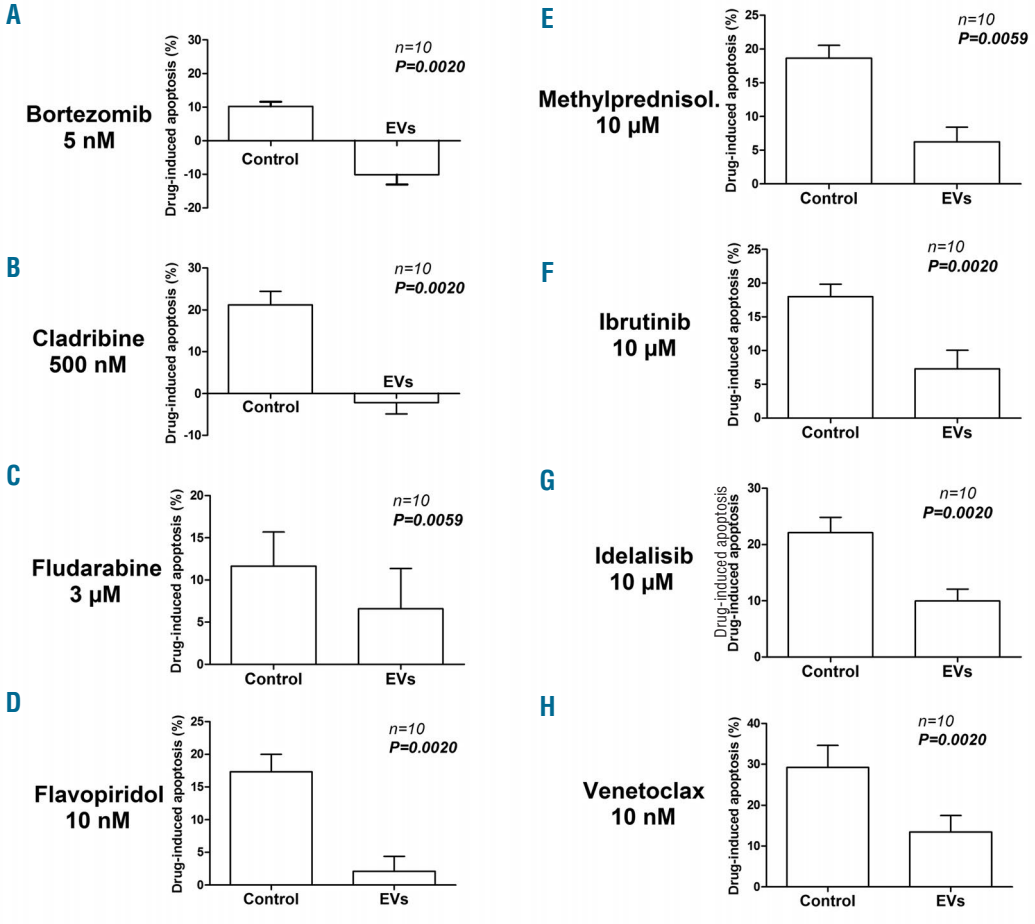

Figure 4. Bone marrow (BM) extracellular vesicles (EVs) increase chemoresistance of chronic lymphocytic leukemia (CLL) B cells. CLL B cells were incubated with bone marrow mesenchymal stromal cell (BM-MSC) EVs for 4 hours (h) and then treated with drugs for 24 h: bortezomib (A), cladribine (B), fludarabine (C), flavopiridol (D), methylprednisolone (E), ibrutinib (F), idelalisib (G) and venetoclax (H). Cells were then stained with annexin V-FITC/7AAD. The addition of BM-MSC EVs in CLL B-cell culture could protect them from drug-induced apoptosis; we observed a decrease in annexin-positive cells in the presence of each of the 8 drugs. The results were normalized by the subtraction of spontaneous apoptosis (cells without drug and EVs).

al., respectively. A total of 69 genes (8.5%) were common in the 3 studies. Using a "super exact test",[16] we obtained a *P* value of $7.04x10^{-65}$ indicating that the 69 genes in common between the 3 studies were not due to hazard (Figure 5C). Among them, CCL4/3, early growth response (EGR) family, TLR10, IL21R, and HDAC9 were all up-regulated after the different ME stimuli. The complete list of common genes is provided in *Online Supplementary Table S4*. The percentages of similarities between studies are shown in *Online Supplementary Table S5*, and summarized in Figure 5C. We observed 20% and 50% of common genes in the 20 most decreased genes in CLL B cells after BCR stimulation and NLC co-culture, respectively. A total of 70% of genes were common within the 20 most increased genes from both signatures (*Online Supplementary Tables S6* and *S7*). We conclude that EVs alone can induce a significant part of the gene expression modifications induced by NLC culture or BCR stimulation. We finally compared our microarray results with the study of Herishanu *et al.*[17] who analyzed the differential gene expression of CLL cells obtained from different compartments: peripheral blood (PB), bone marrow (BM) and lymph node (LN). We observed significant overlap between an EV treatment and a stimulation provided by an LN microenvironment (Figure 5D). In addition, 34 genes were in common between our study and CLL cells receiving stimuli from BM and LN ($P=8.89x10^{-15}$) (*Online Supplementary Tables S6-S8*). Of these, we again noticed genes linked to BCR pathway. Interestingly, EV gene signature shared 3 times more genes with LN than BM signature.

EV effects on CLL B-cell BCR activation

Regarding the significant overlap between IgM stimulation and EV treatment, we investigated the potential activation of the BCR pathway by studying Ca^{2+} flux using FLUO8, the phosphorylation of ERK and AKT by flow

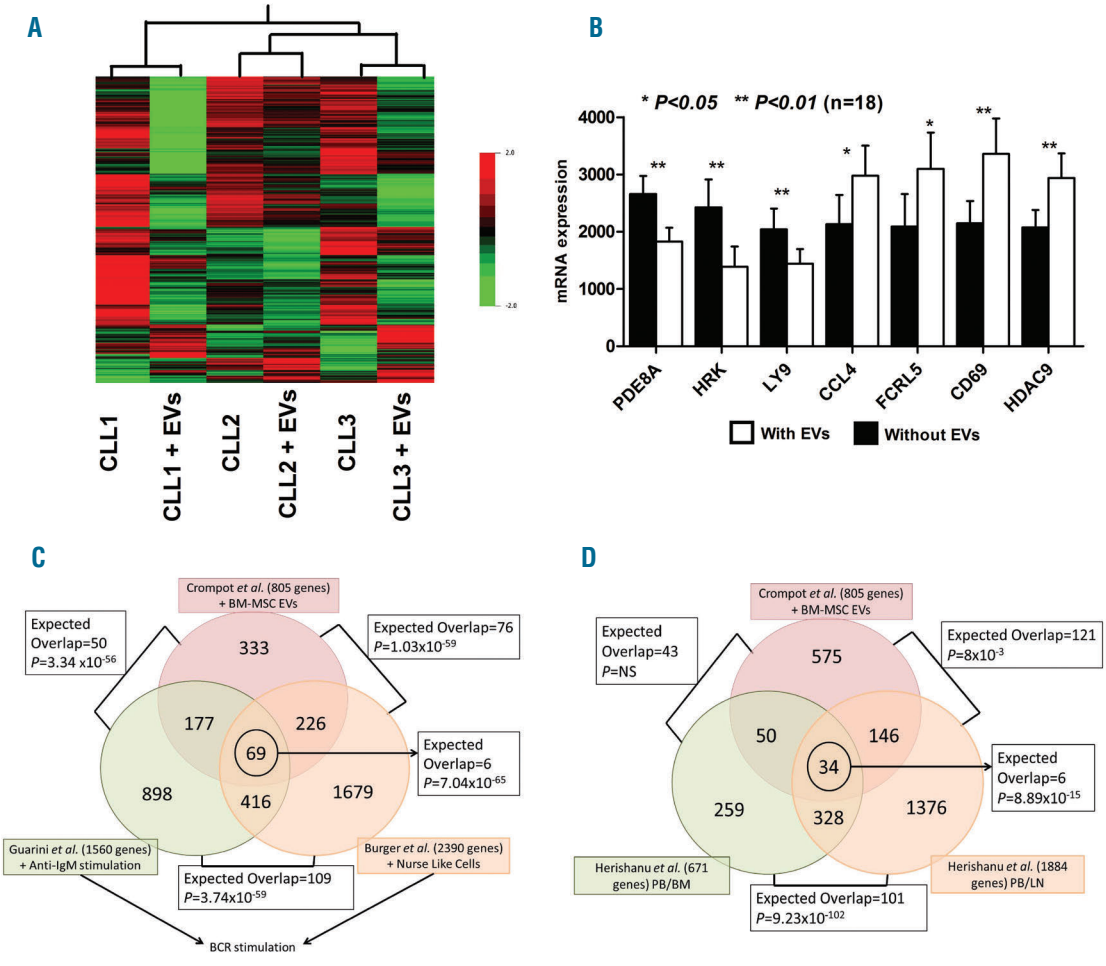

Figure 5. Impact of bone marrow (BM) extracellular vesicles (EVs) on chronic lymphocytic leukemia (CLL) B-cell gene expression profile and comparison with other microarray studies. The genetic profiles of CLL B cells from 3 different patients were determined to obtain a global view of gene expression differences in leukemic cells with or without bone marrow mesenchymal stromal cell (BM-MSC) EV treatment. (A) A total of 805 genes were differentially expressed between leukemic cells cultured with or without EVs ($P<0.05$). (B) We selected 7 differentially expressed genes in our microarray analysis and confirmed their expression by real-time PCR. All genes were validated, confirming our microarray results. (C) We compared our microarray results with 2 other published studies on CLL B cells activated by NLC culture[14] or IgM stimulation.[15] We obtained a substantial overlap between the differentially expressed genes; 177 and 226 genes were shared between our study and those of Burger *et al.*[14] and Guarini *et al.*,[15] respectively, and 69 genes were common among all 3 studies. (D) Our microarray results were also compared with the study of Herishanu *et al.*[17] who analyzed the differential gene expression of CLL cells obtained from different compartments: peripheral blood (PB), bone marrow (BM) and lymph node (LN). A significant overlap was observed between an EV treatment and a stimulation provided by an LN microenvironment. In addition, 34 genes were in common between our study and CLL cells receiving stimuli from BM and LN ($P=8.89x10^{-15}$). The "SuperExactTest'' was applied to evaluate the statistical value of the intersections between 3 studies as indicated by the expected overlap and the *P* values. BCR: B-cell receptor.

cytometry. In order to work with 'responding' cells, we performed these experiments with the Raji cell line known to be an excellent responder to IgM stimulation. Despite a BCR-like signature, we did not observe calcium flux in Raji cells (Figure 6A). While an IgM stimulation increases the ERK and AKT phosphorylation, EV treatment did not, neither in Raji cells (Figure 6B and C), nor in primary CLL cells (Figure 6D and E). We also studied other targets known to be regulated after BCR activation: CCL4, lipoprotein lipase (LPL) and MYC expression by qPCR and CXCR4 (CD184), and CD69 expression, by flow cytometry. After 24 h of an EV treatment, we observed in all cases a modulation of these targets in the same way as a BCR activation (increase in CD69, LPL, MYC, CCL4 and a decrease in CXCR4) (Figure 6F-H). Interestingly, while we did not observe a calcium flux and ERK/AKT phosphorylation, inhibition of BCR pathway using ibrutinib or idelalisib abolished EV-induced migration (Figure 6I). In addition, ibrutinib treatment also abolished gene modifications induced by EVs (Figure 6 J).

EV effects are due to *de novo* cell regulation rather than RNA transfer

In order to investigate if the EV increases of gene expression are due to RNA transfer or *de novo* transcription, we treated CLL cells with actinomycin D (5 μg/mL) prior to EV incubation. After 4 h of EV integration, we did not observe any significant change in gene expression of some representative targets [CCL4 (Figure 6 H), FCRL5 and TLR10 (*data not shown*)] indicating that the increase of these genes was not due to an RNA transfer. After 24 h, we confirmed the increase in these genes but this increase was abolished by an actinomycin D treatment. In addi-

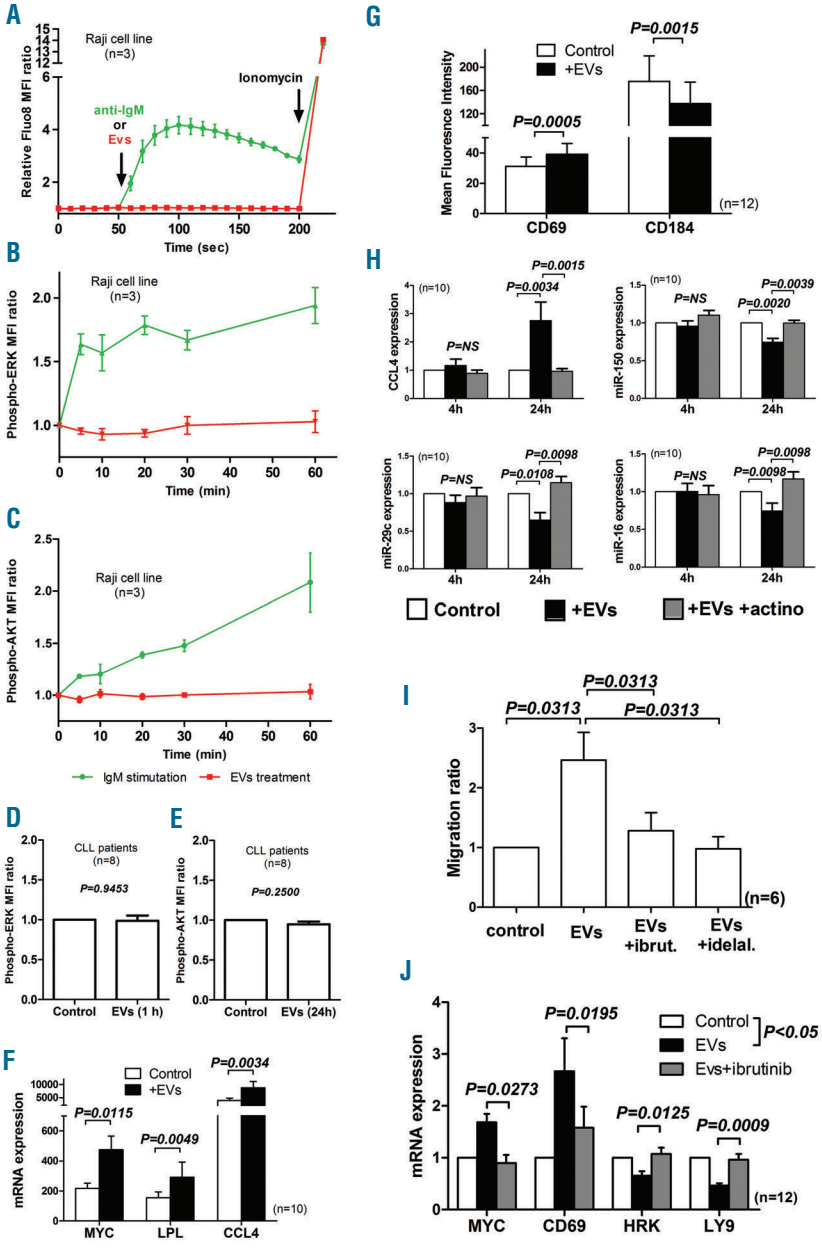

Figure 6. Extracellular vesicles (EV) effects on chronic lymphocytic leukemia (CLL) B-cell receptor (BCR) activation. (A) Calcium flux in Raji cell line (n=3) after anti-IgM or EV treatment measured using FLUO8 by flow cytometry. The mean of fluorescence intensity (MFI) was normalized by the MFI of untreated cells and plotted every ten seconds. The phosphorylation of ERK (B and D) and AKT (C and E) was evaluated by flow cytometry. Data were presented as the mean fluorescence intensity (MFI) ratio of IgM stimulation/unstimulated cells and EVs treatment/untreated cells. Downstream targets of BCR activation in CLL B cells after EV integration were analyzed by real-time PCR (MYC, LPL, CCL4) (F-H) and flow cytometry (CD69, CXCR4 or CD184) (G). (H) Four targets of BCR activation (CCL4, miR-229c, miR-150, miR21) after 4 hours (h) or 24 h of EV integration treated or not with actinomycin D (5 μg/mL). Genes were normalized using cyclophilin A (PPIA) as endogenous control while microRNAs were normalized using RNU48. (I) CLL B cells were incubated with BM-MSC EVs in a Transwell assay. Cells were treated with or without ibrutinib (ibrut.: 10 μM) or idelalisib (idelal.: 10 μM), with or without EVs. The migration ratio represents the number of migrating cells in each condition divided by the spontaneous migrating cells (in absence of EVs or drugs). (J) Cells were treated with or without ibrutinib (10 μM) and with or without EVs. Gene expression for a selection of genes was evaluated by real-time PCR. Data were normalized with the expression of untreated cells (without EVs and without ibrutinib).

tion, we tested, in the same conditions, 3 microRNAs previously investigated in CLL (miR-29c, miR-150, miR-16)[18-20] and known to be decreased after BCR stimulation.[21,22] Again, we did not observe expression modification at 4 h, but after 24 h, we observed that EVs are able to induce an miR-29c, miR-150 and miR-16 decrease, as is the case after BCR stimulation (Figure 6H). This decrease was abolished by actinomycin D, demonstrating that this decrease was due to *de novo* regulation.

Quantitative and qualitative comparison between healthy and CLL-derived EVs

In this study, we used EVs from MSC culture established from healthy donors. In order to complete this work, we performed the same experiments with EVs produced by BM-MSCs obtained from CLL patients. Nanoparticle tracking analysis (NTA) was used to evaluate size distribution and the concentration of EVs. Despite the difficulty to maintain them in 'long-term culture', CLL BM-MSCs seem to be a 'higher producer' of EVs. After concentration of the collected medium, we indeed obtained a mean of 955±172 EVs/MSC/day (n=17) while this number reached 1634±387/MSC/day for CLL MSC (n=5) (*P*=0.0417) without any significant change in their size (Figure 7A-D). Furthermore, CLL BM-MSC EVs induced similar effects on apoptosis (Figure 7E) and viability (Figure 7F) of CLL B cells. In addition, we also compared the protective effect of both EV types after ibrutinib,

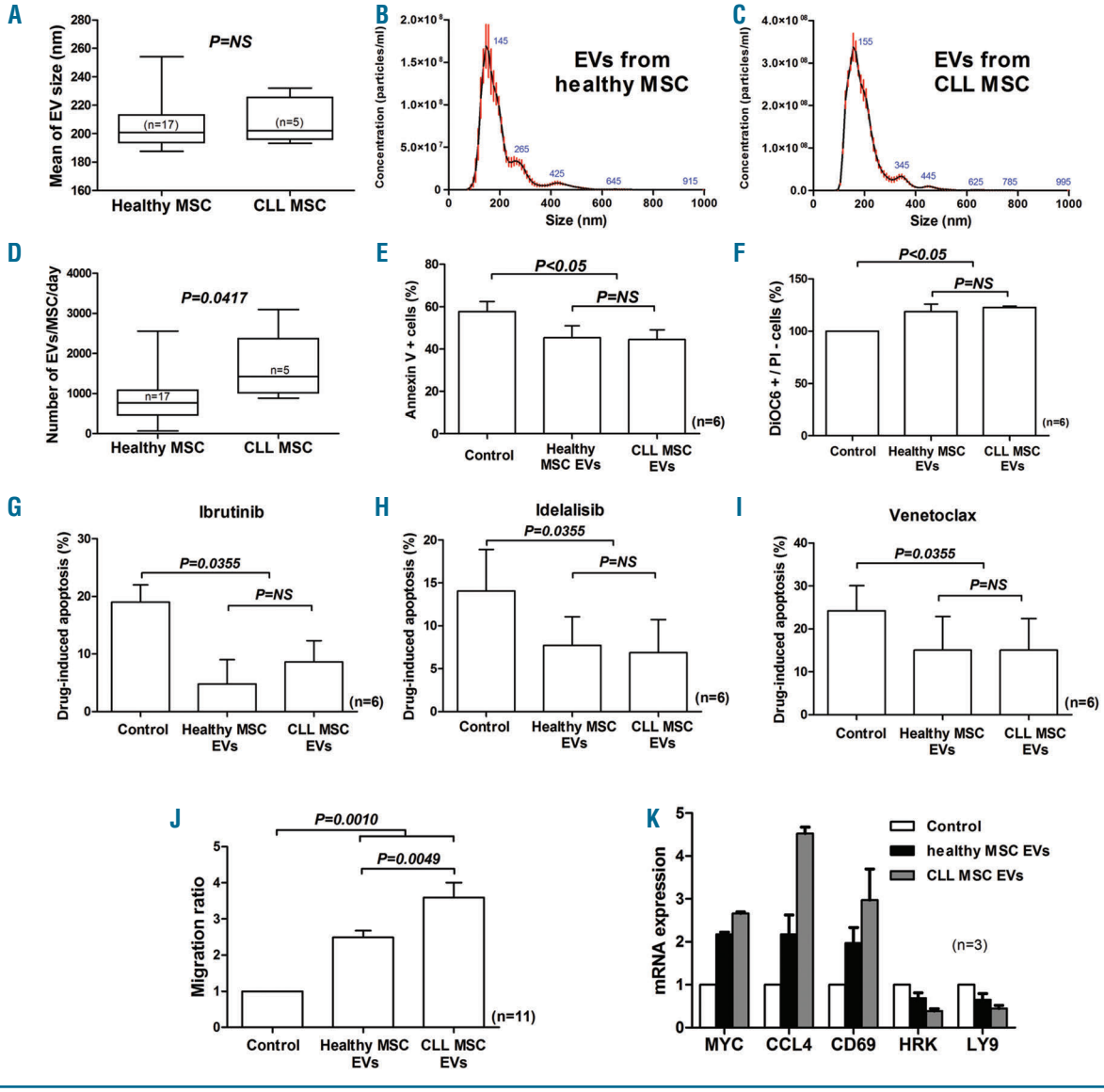

Figure 7. Quantitative and qualitative comparison between healthy and chronic lymphocytic leukemia (CLL)-derived extracellular vesicles (EVs). (A) Measure of mean size of EVs from healthy and CLL MSC. (B and C) A representative size distribution of healthy and CLL mesenchymal stromal cell (MSCs), respectively. CLL B cells were incubated with healthy or CLL-MSC EVs for 24 hours (h) and cells were stained with annexin V-FITC/7AAD to measure apoptosis (E) or DioC6/propidium iodide to measure cell viability (F). We observed a decrease of spontaneous apoptosis. CLL B cells were incubated with healthy or CLL-MSC EVs for 4 h and then treated for 24 h with ibrutinib (G), idelalisib (H) or venetoclax (I). Cells were then stained with annexin V-FITC/7AAD. The results were normalized by the subtraction of spontaneous apoptosis (cells without drug and EVs). (J) CLL B cells were incubated with healthy or CLL MSC EVs in a Transwell assay. The migration ratio represents the number of migrating cells in each condition divided by the spontaneous migrating cells (in absence of EVs). (K) Cells were incubated with healthy or CLL MSC EVs for 24 h. Gene expression for a selection of genes was evaluated by real-time PCR. Data were normalized with the expression of untreated cells (without EVs).

idelalisib and venetoclax treatment, and we did not observe any difference (Figure 7G-I). However, spontaneous migration of CLL B cells after addition of BM-MSC EVs from CLL patients is higher compared to healthy EVs (P=0.0049) (Figure 7J). Interestingly, when we investigated EV effect on gene expression, both EV types are able to induce gene modifications described above, but CLL MSC-derived EVs display a stronger action as is shown for MYC, CCL4, CD69 increase or HRK, LY9 decrease (Figure 7K).

Discussion

In hematologic malignancies, the microenvironment interacts with tumor cells principally through cell-cell contact and soluble factor production to support leukemic development.[23] Recently, EVs were proposed as a new mechanism of cross-talk and cellular communication.[24] PB, BM, and LN-derived B cells present different RNA signatures, confirming that the ME gives signals activating specific pathways.[17] The production of soluble factors by MSCs can attract CLL B cells in the ME and protect them from spontaneous apoptosis,[2] but it is now widely accepted that MSCs can also communicate by EVs.[25] MSCs show bidirectional cross-talk with normal B cells,[26] neoplastic B cells[27] or other malignant cells, such as multiple myeloma and chronic myelogenous leukemia cells.[28,29] Roccaro *et al*. demonstrated that BM-MSC EVs contribute to disease progression in multiple myeloma.[28]

Because the exact role of BM-MSC EVs remains unknown in CLL, we investigated modifications induced by EVs in CLL B cells using microarray analyses and determined their impact on CLL B-cell survival, migration and chemoresistance. We studied EVs (microparticles and exosomes together) because this is more similar to physiological conditions. To maintain EVs as close as possible to their native state, we did not use any activator to increase EV production, and serum deprivation was applied on BM-MSC cultures to avoid any fetal bovine serum vesicle contamination. Numerous authors used a concentration of EVs between 30 and 50 μg/mL.[29-31] In the present study, we used 10 times lower concentrations (between 2 and 5 μg/mL) to be closer to the physiological condition, and observed significant effects. Furthermore, the addition of conditioned medium *versus* EV-depleted conditioned medium from BM-MSC culture already induces a protective effect, illustrating the implication of EVs in cell functions in more physiological conditions (*Online Supplementary Figure S5*).

We showed that CLL B cells can rapidly integrate EVs from BM-MSCs. This active uptake of the BM-MSC EVs by target cells was shown in other studies, indicating the *in vivo* relevance of EV transfer.[32] BM-MSCs increase the migration capacity,[33] decrease apoptosis,[2] and increase chemoresistance[7] of CLL B cells after direct contact. Here, we report for the first time that EVs alone can induce similar effects as their cell counterparts in CLL. Indeed, EVs protect CLL cells from spontaneous apoptosis similar to a stromal layer.[2] In addition, it is now well known that CLL B cells can escape from chemotherapy by migrating into stromal niches.[34] Here, we observed that EVs increase the migratory capacity of CLL cells and, subsequently, could play a role in *in vivo* survival. Interestingly, the effect of EVs on CLL-cell migration is independent of CXCR4 receptor or other G protein-coupled receptors. Currently, CLL remains incurable with classical treatments, and relapse remains the major cause of death. In this study, we demonstrated that the addition of BM-MSC EVs to CLL B-cell cultures increases their resistance against 8 different drugs, including purine nucleoside analogs, corticosteroids, ibrutinib, idelalisib or venetoclax. A study showed the same effect against bortezomib in multiple myeloma cells after the integration of exosomes from BM-MSCs.[35] These important data highlight the crucial role of EVs in the context of chemoresistance.

Our microarray study revealed 805 genes differentially expressed between CLL B cells treated with or without EVs. Lymphocyte antigen 9 (LY9), which presents decreased expression (-2.2-fold) after EVs integration, is a tumor antigen targeted by T cells in CLL B cells.[36] Moreover, phosphodiesterase 8A (PDE8A), which presents decreased expression in CLL B cells treated with EVs, is a predictor of treatment-free survival (TFS) and overall survival (OS).[12] By contrast, some mRNAs were increased. We observed the doubled expression of CD83 (+1.9-fold), which is associated with a shorter TFS.[37] Furthermore, HDAC9, which was significantly increased in our microarray data (+1.5-fold), is correlated with ZAP70-positive CLL patients.[38] Based on these different observations, we suggest that EVs from the microenvironment can promote the development of a poor prognosis profile and influence the clinical outcome of CLL patients. Interestingly, we also observed that BM-MSC EVs increase the CD69 mRNA expression (+1.7-fold). This result was also confirmed by flow cytometry, as previously reported.[17] This antigen on activated B cells is well described in the CLL literature. In 2001, D'Arena *et al*. hypothesized that CD69 is a prognostic factor in CLL disease due to more genetic abnormalities in CD69 positive patients, and thereafter, Del Poeta showed its independent prognostic power.[39] Importantly, other authors had previously investigated the impact of other stimuli from the microenvironment on CLL B-cell gene expression. Burger *et al*. studied CLL B cells activated by NLC,[14] whereas Guarini and colleagues analyzed the effect of BCR stimulation using anti-IgM.[15] In total, 226 (28% of the genes of our study) and 177 (22%) differentially expressed genes in our study were described as differentially expressed in the studies by Burger *et al*. and Guarini *et al*., respectively. When we compared our microarray results with the gene modifications induced in CLL cells obtained from different compartments (BM and LN),[17] we observed that these different stimuli could induce similar gene expression modifications compared to EV treatment. These results indicate that a non-negligible part of the ME effects are also mediated through EVs. Among the common increased genes, we identified CCL4 (+4.6-fold), CCL3 (+3.8-fold), EGR family members (+1.8-fold/+3.8-fold), FCRL 5 (+2.2-fold), and MYC (+2.1-fold), which are genes that are increased after BCR activation.[40,41] We hypothesize that BM-MSC EVs induce BCR-like activation, leading to cell survival and drug resistance. Complementary to our study, other authors showed that BCR activation induces CLL EV overproduction and leads to a worse prognosis.[42] A possible activation loop mediated by EVs could potentially lead to BCR activation, survival factor production and association with a poor prognosis in CLL. Furthermore, Yeh *et al*. reported that the number of exosomes is not directly correlated with the

number of B cells, but with BCR activation, contrary to the findings of Ghosh *et al.*,[42,43] another finding that supports our hypothesis. To further investigate the hypothesis of BCR activation, we studied CCL4, CD69, CXCR4 (CD184), lipoprotein lipase (LPL) and MYC expression or protein level: EVs increase the expression of CCL4, CD69, LPL and MYC, and decrease the level of CXCR4 similar to BCR activation, as previously described.[14,22] Similarly, miR-29c, miR-150 or miR-16 are decreased after EV treatment as is also the case after a BCR activation.[21] To evaluate the potential BCR activation, we measured intracellular calcium flux, AKT and ERK phosphorylation by flow cytometry, but we did not observe phosphorylation of these targets or calcium flux with EV treatment after 24 h (nor in a shorter period of time). Moreover, several authors observed a BCR signature but none of them demonstrated a direct BCR activation using calcium flux or ERK phosphorylation.[14,17] Surprisingly, inhibition of BCR pathway is able to decrease/abolish the gene modifications induced by EVs as well as the EV-related migration, indicating that BCR pathway is linked to EVs. Based on these observations, we could hypothesize that EVs are not able to activate BCR as is classically shown in the literature (no calcium flux, no AKT or ERK phosphorylation). However, we observed a BCR-like gene signature, microRNA BCR-related modifications, inhibition of gene signature modifications, and migration by BCR pathway inhibitor suggesting that the BCR pathway could be activated by EVs in an intracellular manner.

Finally, we also observed that BM-MSCs of CLL patients produce a higher amount of EVs, suggesting that EVs could even be a more important actor in leukemic cells/microenvironment interaction in a pathological context. Similarly to BM-MSC EVs from healthy donors, we demonstrated that BM-MSC EVs of CLL patients also rescue CLL B cells from spontaneous apoptosis. However, CLL MSC-derived EVs induce a higher migration compared to healthy MSC-derived EVs, and also a stronger gene modification. All these data taken together indicated that EVs from CLL MSC could even play a more important role in a CLL *in vivo* context.

In conclusion, we report for the first time that EVs from ME can increase viability, rescue CLL B cells from spontaneous and drug-induced apoptosis, and enhance their migratory capacities in a contact-independent manner. In addition, EVs induced gene expression profile modifications of CLL cells that substantially overlap with transcriptomic signatures obtained after BCR stimulation, NLC co-culture or CLL in a lymph node ME. In this work, we focused on EVs from bone marrow ME, specifically from MSC, but vesicles from other cells (T cells, NK cells, NLC, etc.) may also interfere with the communication between CLL B cells and the ME. Collectively, we provide evidence that EVs alone can modulate several key cellular processes, suggesting that interference with EV production/internalization may be a new target for therapeutic strategies.

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