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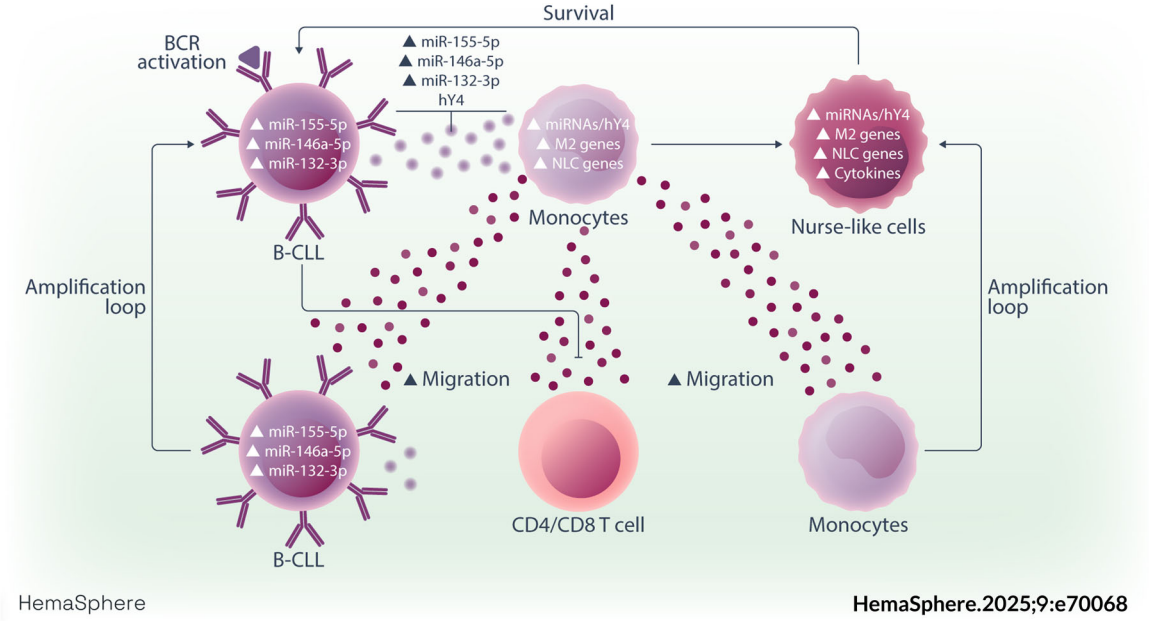
Graphical Abstract



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Extracellular vesicles from chronic lymphocytic leukemia cells promote leukemia aggressiveness by inducing the differentiation of monocytes into nurse-like cells via an RNA-dependent mechanism

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Abstract

Chronic lymphocytic leukemia (CLL) cells receive several stimuli from surrounding cells, such as B-cell receptor (BCR) stimulation, and can manipulate their microenvironment via extracellular vesicle (EV) release. Here, we investigated the small RNA content (microRNA and YRNA) of CLL-EVs from leukemic cells cultured with/without BCR stimulation. We highlight an increase of miR-155-5p, miR-146a-5p, and miR-132-3p in EVs and in cells after BCR stimulation ($p < 0.05$, $n = 25$). CLL-EVs were preferentially internalized by monocytes ($p = 0.0019$, $n = 6$) and able to deliver microRNAs and the hY4 RNA. Furthermore, BCR CLL-EV induced modifications in monocytes (shape change, microRNA and gene expression, secretome) suggesting nurse-like cell (NLC) differentiation, the tumor-associated macrophages of CLL. Functionally, monocytes treated with BCR CLL-EVs protect CLL cells from spontaneous apoptosis by pro-survival cytokine production and induce their migration as well as the migration of other immune cells. We finally reported by transfection experiments that hY4 is able to induce the expression of CCL24, a key gene in M2 macrophage differentiation. In conclusion, we showed that BCR stimulation modifies the small RNA content of CLL-EVs and that the addition of leukemic EVs to monocytes leads to monocyte differentiation into NLCs establishing a protective microenvironment that supports leukemic cell survival.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature $CD5^+CD23^+CD19^+$ B lymphocytes in the blood, bone marrow, and lymph nodes.[1] When removed from the body, CLL cells undergo rapid apoptosis, suggesting a crucial interaction between B cells and their supportive microenvironment.[2] Several reports have highlighted the bidirectional interaction between leukemic cells and bystander cells: CLL cells are indeed supported by stimuli received by the microenvironment, such as CD40,[3] Toll-like receptor (TLR),[4] or B-cell receptor (BCR)[5] stimulation. In addition, leukemic cells can influence and modify bystander cells to establish an environment that promotes their own survival.[6,7] Of these, monocyte-derived nurse-like cells (NLCs) are important players in CLL cell survival, chemoresistance, proliferation, and homing.[6,8] NLCs can be described as the tumor-associated macrophages (TAMs) of CLL and are close to M2 macrophages.[9] First described by Burger et al.,[6] these cells produce several soluble factors, including BAFF, APRIL,[10] and CXCL12.[6,11]

CLL cells cross-talk with their microenvironment via direct contact, the release of soluble factors, and the production of extracellular vesicles (EVs).[12] EVs are vesicles ranging from 50 nm to up to 1 µm in size.[13,14] Small vesicles (sEVs—from 50 to 100 nm), also sometimes called exosomes, are released from the endosomal compartment, while medium to large vesicles (m/lEVs—from 100 nm to 1 µm) are released directly from the cell membrane by budding.[15] EVs can contain different biological materials, such as surface markers, intracellular proteins, mitochondria, DNA, and RNA.[7,16–18] Encapsulated material or material present at the EV

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surface can travel and be delivered to the recipient cells, triggering several processes.[12]

High-throughput next-generation sequencing analyses have highlighted the accumulation of small noncoding RNAs (ribosomal RNA, transfer RNA, microRNA, and Y RNA) in exosomes.[17,19,20] In this report, we will focus on microRNAs (or miR) and Y RNAs. MicroRNAs play a role in several biological processes, including CLL pathophysiology.[21,22] Y RNAs are well-conserved noncoding RNAs 83 to 112 nucleotides in length transcribed by RNA polymerase III.[23,24] Four different Y RNAs, named hY1, hY3, hY4, and hY5, exist in humans.[25] These RNAs are involved in DNA replication and RNA quality control.[26,27]

More specifically, in the context of CLL microenvironment cross-talk, Paggetti et al. showed that leukemic EVs can induce the differentiation of mesenchymal stromal cells (MSCs) into cancer-associated fibroblasts (CAFs).[7] Yang et al. confirmed these results and demonstrated that miR-146a was responsible for the differentiation of MSCs into CAFs.[28] In 2017, Crompot et al. showed that EVs isolated from bone marrow MSCs can rescue CLL cells from spontaneous and drug-induced apoptosis, increase their migration capabilities, and change their gene expression profile.[29] In the same year, Haderk et al. reported that hY4 is enriched in CLL-derived exosomes and can be transferred to monocytes and activate TLR7 signaling.[17] Yeh et al. showed that BCR stimulation of leukemic cells can increase miR-150 and miR-155 in CLL exosomes,[30] showing for the first time that EV cargo can be modified following a stimulus. BCR triggering can occur in the microenvironment[31] and contribute to CLL cell survival, proliferation, adhesion, migration, and drug resistance.[32]

Here, we report a comprehensive analysis of primary CLL cell-derived EVs by establishing a small RNA content after BCR stimulation and investigating their potential role in the cross-talk between leukemic cells and monocytes.

METHODS

Patients, sample collection and preparation, and RNA extraction

This study was approved by the local ethics committee and was based on peripheral blood mononuclear cell (PBMC) samples collected at diagnosis after patients provided written informed consent. The cell purification and RNA extraction methods as well as the patient characteristics used for EV production, microRNA sequencing, and quantification in CLL serum are provided in Supporting Information S1: Data Text and Tables S1–S3, respectively.

Extracellular vesicle isolation and characterization

To isolate CLL-EVs, 100 million fresh purified B-CLLs were cultured in AIM V™ Medium (Thermo Fisher Scientific) for 24 h in a humidified atmosphere at 37°C and 5% CO_2. This medium does not contain any serum to prevent any serum EV contamination. Details about EV characterization, BCR stimulation, quantification and RNA extraction, RNA sequencing, and real-time validation are provided in Supporting Information S1: Data Text.

Analysis of monocytes: Morphology, gene expression comparison, secretome, migration, phagocytosis, CLL cell protection, cell transfection, and EV electroporation

Monocytes from healthy donors were cultured at a concentration of 1 million/mL with 10,000 control (unstimulated) or BCR CLL-EVs/cells. Cell morphology was analyzed, and total RNA was extracted after 5 days. The gene expression profiles of untreated cells and cells treated with BCR EVs were analyzed by RNA sequencing as described above. The EV gene signature was then compared to the NLC and M2 macrophage signatures based on available public data. Additional details about the monocyte culture, EV internalization, EV uptake mechanism, morphology analysis, signature generation, and bioinformatic analysis are provided in Supporting Information S1: Data Text. Details about secretome analysis, apoptosis, viability, migration, phagocytosis assay, cell transfection, and EV electroporation are provided in Supporting Information S1: Data Text.

Survival and statistical analysis

The Mann–Whitney test (for unpaired data) or the Wilcoxon matched pair test (for paired data) was used to analyze the statistical significance of the differences in the experimental results between the two groups. For small sample size experiments (e.g., RNAseq), a parametric test was used but was thereafter confirmed with a greater number of samples using a different technique. Survival analysis details are provided in Supporting Information S1: Data Text.

RESULTS

CLL-EV characterization and small RNA sequencing

We first characterized CLL-EVs by transmission electron microscopy, which revealed typical features of EVs, such as a bilipidic layer and a cup-like shape (Figure 1A). The size and concentration were analyzed using nanotrack technologies: EV samples had an average size of 152 ± 4 nm (n = 25). A representative sample size distribution is shown in Figure 1B. EVs were also characterized by direct flow cytometry based on the protocol of Crompot et al.[33]: We observed clear expression of CD63 and CD9, two classical exosomal markers of EVs, but low expression of CD81 (Figure 1C). In addition, leukemic EVs were positive for leukemic cells (CD5), B cells (CD19, CD20), or hematopoietic markers (CD45) but negative for platelet (CD41), monocyte (CD14), T-cell (CD3), or NK cell (CD56) markers. In addition, as recommended by MISEV2024 guidelines, we confirmed the absence of cytochrome C, a protein that should be absent in EVs, and the presence of HSP70, a cytosolic protein, in EVs while both proteins were present in cells (Figure 1D). As previously reported,[17] EV RNA cargo is generally enriched in small RNAs, which was confirmed in our study by a representative Agilent profile of RNA size in EVs with a mode of approximately 25 nucleotides (Figure 1E). Therefore, to further characterize leukemic EVs, we performed small RNA sequencing of CLL-EV RNA (n = 4). The 20 most abundant RNAs expressed in the fraction of reads are provided in Figure 1F. The highest number of reads (37.7% ± 7.6%) were assigned to microRNAs, followed by ribosomal RNA (rRNA—19.6% ± 6.4%), Y RNA (18.9% ± 3.1%), PIWI-interacting RNA (piRNA—9.4% ± 2.7%), small nuclear RNA (snRNA—7.8% ± 1.7%), transfer RNA (tRNA—5.1% ± 1.3%), and small nucleolar RNA (snoRNA—1.5% ± 0.4%) (Figure 1G). When focusing only on microRNAs, the let-7 family was highly present, as were the other microRNAs previously described in CLL physiopathology (miR-150, miR-155) (Figure 1H). hY4 RNA was the most abundant Y RNA (63.6% ± 3.5%) in EVs, followed by hY5 (17.9% ± 3.8%), hY1 (16.8% ± 1.2%), and hY3 RNA (1.8% ± 0.5%) (Figure 1I).

BCR stimulation modulated EV microRNA cargo

BCR stimulation is an important stimulus received by CLL cells that induces cell survival and plays a crucial role in CLL clone selection.

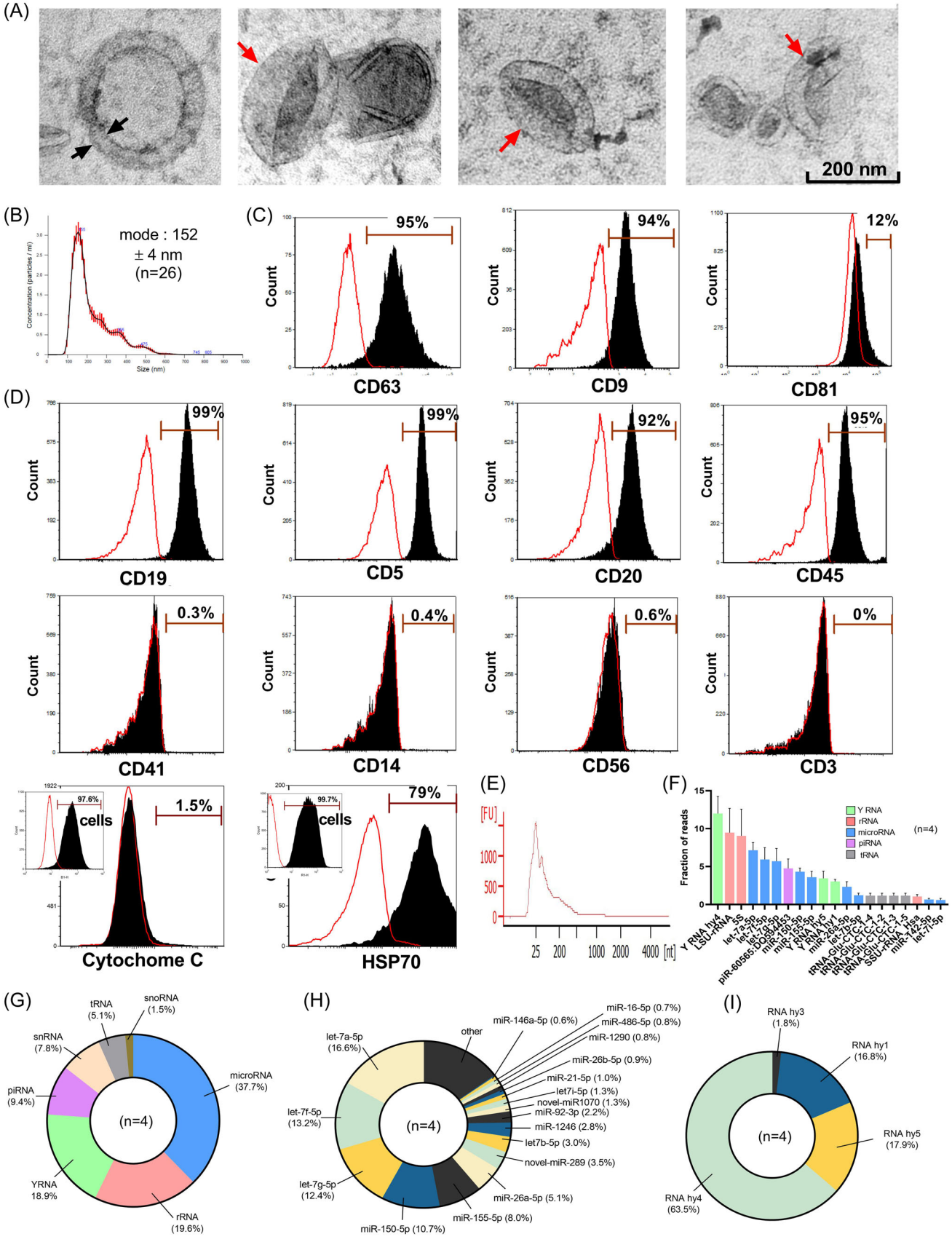

FIGURE 1 (See caption on next page).

FIGURE 1 **Characterization of CLL-EVs**. EVs produced by purified CLL cells in culture were characterized by different techniques. **(A)** Transmission electronic microscopy (TEM). EVs exhibit a classical spherical or cup-like shape with a lipidic bilayer. **(B)** Nanotrack analysis. The average mode of all analyzed samples was 152 ± 4 nm ($n = 26$). **(C, D)** Direct flow cytometry. EVs expressed CD63 and CD9 (EV markers). CD81 was slightly expressed. EVs were also positive for markers found on leukemic cells (CD19, CD5, CD20, and CD45) but negative for other markers (CD41, CD14, CD56, and CD3). As recommended by MISEV2024 guidelines, we also tested a protein that should be absent in EVs (cytochrome *C*) and a cytoplasmic protein (HSP70). Both proteins were present in the cells as shown in the respective upper left small panels **(E)** Agilent profile. A representative Agilent profile of EV RNA content shows that RNA species are mostly small RNAs (25 nucleotides). **(F)** Abundance in the fraction of reads of the 20 most abundant RNAs revealed by small RNA sequencing ($n = 4$). **(G)** Distribution of the proportion of reads in terms of RNA species. **(H)** Distribution of the proportion of reads in terms of microRNAs. **(I)** Distribution of the proportion of reads in terms of Y RNAs.

Therefore, CLL-EVs were produced in vitro from purified primary leukemic cells with or without BCR stimulation to highlight potential changes in the small RNA cargo of EVs. As shown in Figure 2A, BCR stimulation increased the production of EVs by leukemic cells ($n = 26$, $p = 0.0005$) but did not change their size ($n = 25$, $p = 0.1877$) (Figure 2B). MicroRNA expression comparison revealed that 31 microRNAs were differentially expressed between unstimulated and BCR-stimulated conditions ($n = 4$, $p < 0.05$) (Figure 2C). As shown in Figure 2D, we confirmed the higher abundances of miR-146a ($p < 0.0001$), miR-132 ($p = 0.0003$), and miR-155 ($p < 0.0001$) in a greater number of samples ($n = 25$). Interestingly, these three microRNAs which have a higher level in EVs after BCR stimulation also have a higher level in leukemic cells (Figure 2E). The choice of these three specific microRNAs was oriented by their high level in EVs and/or because these microRNAs have already been described in the CLL field.[28,34–37] Additional details about other microRNA analyses are provided in Supporting Information S1: Data Text and Supporting Information S1: Figure S1. We also quantified the level of hY4 within CLL-EVs and CLL cells but no significant difference was observed after BCR stimulation (Figure 2C,D). Interestingly, miR-155 in EVs is deregulated in CLL and is associated with a poor prognosis. Additional details can be found in the Supporting Information S1: Data Text and Supporting Information S1: Figure S2.

CLL-EVs are preferentially taken up by monocytes and able to deliver miR-155, miR-132, and miR-146a

Based on previous observations[17] and our findings, we postulated that the increased packaging of some specific microRNAs after BCR stimulation in CLL-EVs might have some biological and functional effects on recipient cells. To test this hypothesis, we first sought to identify the cell types in which these EV cargos are delivered. To monitor which cell type preferentially uptakes CLL-EVs, 10 million PKH67-labeled CLL cells producing green fluorescent EVs were co-cultured with 2 million CLL PBMCs separated by a filter allowing EV exchange (Figure 3A—additional details about the method can be found in Supporting Information S1: Data Text). Although they represented only 3.2% ± 1.1% ($n = 6$—Figure 3A), monocytes ($CD14^+$) rapidly integrated with CLL-EVs compared to other cell types, as shown by the increase in PKH67+ cells (Figure 3B). This preferential uptake was even more obvious when analyzing the increase in the mean intensity ratio (MFIR) (Figure 3C). EV uptake analysis pathways suggest that EV uptake is an energy-dependent process that requires a functioning cytoskeleton and clathrin-mediated endocytosis (Figure 3D and Supporting Information S1: Data Text). A representative histogram of the uptake-gated $CD14^+$ cells is shown in Figure 3E.

To investigate possible cargo transfer, we cultured purified monocytes from healthy donors without and with EVs produced under unstimulated (control CLL-EVs) and BCR-stimulated conditions (BCR CLL-EVs), and the levels of miR-155, miR-146, and miR-132 (all increased in CLL-EVs after BCR stimulation) were assessed in monocytes by qPCR after 24 h (Figure 3F). Incubation with control or BCR CLL-EVs significantly increased the levels of miR-155 (+2.2- and +7.2-fold, respectively), miR-146a (+5.0- and +9.2-fold, respectively), and miR-132 (+2.9- and +5.8-fold, respectively) ($n = 7$, $p = 0.0156$). We also observed a greater increase or a trend toward a greater increase when comparing monocytes treated with control or BCR CLL-EVs: miR-155 ($p = 0.0156$), miR-146a ($p = 0.2288$), and miR-132 ($p = 0.0608$), confirming that BCR indeed changed the EV microRNA content. In addition, no significant difference in terms of pri-microRNA expressions was observed between the different conditions, demonstrating that the increase in microRNA levels was not due to de novo transcription (Figure 3G).

CLL-EVs are able to deliver hY4 to monocytes

As hY4 RNA was also highly present in CLL-EVs, as demonstrated by others and confirmed by our sequencing (Figure 1I), we evaluated the impact of BCR stimulation on hY4 RNA levels. We observed an increase of hY4 in monocytes of healthy donors 24 h and 5 days after CLL-EV treatment. After 12 days, this increase was statistically higher in monocytes treated with BCR CLL-EVs compared to control CLL-EVs (Figure 3H).

CLL-EV modifications induced in monocytes isolated from healthy donors suggest NLC differentiation: Morphology and gene expression

After only one addition of 10,000 EVs per cell, we observed a morphological change in monocyte shape after 5 days (Figure 4A): monocytes took a more fibroblastic shape that was even more obvious on monocytes treated with BCR CLL-EVs, as confirmed by image quantification (Figure 4B).

To establish potential gene expression changes, monocytes from three different healthy donors who were not treated with BCR CLL-EVs or treated with BCR CLL-EVs were subjected to RNA sequencing. We found 914 genes that were differentially expressed ($p < 0.05$, $n = 3$ pairs) (a list of genes is available in Table S10). Interestingly, unsupervised clustering clearly separated the samples based on EV treatment (Figure 4C). When we performed a gene set enrichment analysis using the gene set published by Gavish et al.[38] corresponding to the hallmarks of transcriptional intratumor heterogeneity, the gene set with the highest significance ($q = 0.002$) and the highest normalized enrichment score (NES = 1.88) was the "metaprogramme macrophages" based on the glycolysis gene set, suggesting that this set of genes is associated with "tumoral" macrophages (Figure 4D).

The top 15 most highly expressed and deregulated genes in monocytes after treatment with BCR CLL-EVs are provided in Figure 4E. Interestingly, several upregulated genes were associated with macrophages or M2 macrophages, and the most downregulated gene was associated with M1 macrophages. The upregulation of three genes (CCL24, M-CSF/CSF1, and LILRB1) in M2 macrophages

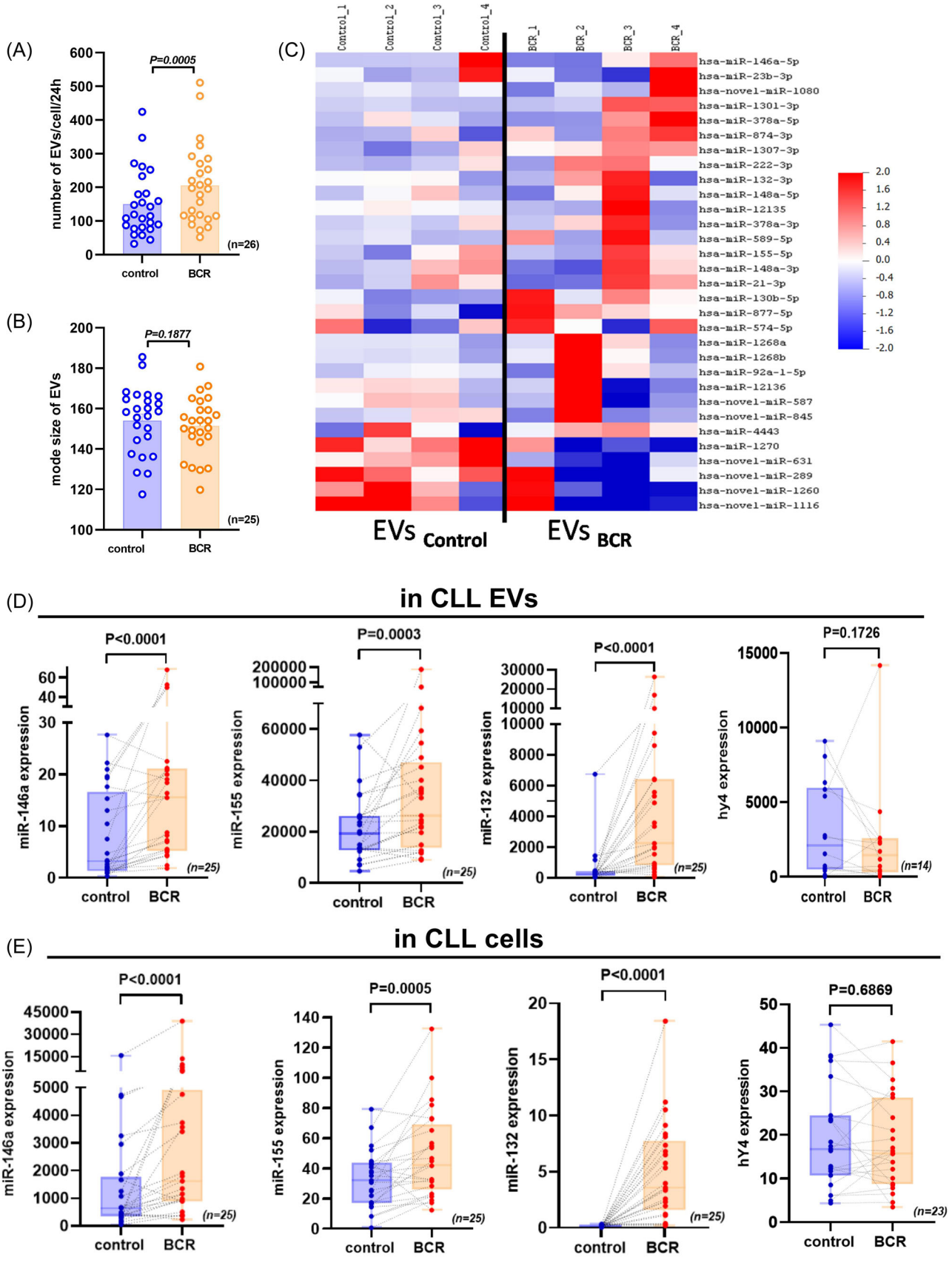

FIGURE 2 (See caption on next page).

FIGURE 2 **BCR stimulation modifies the microRNA content of CLL-EVs**. Distribution of CLL-EV production **(A)** and CLL-EV size **(B)** between the control and BCR stimulation conditions by nanotrack analysis. BCR stimulation does not influence EV size but significantly increases CLL-EV production. **(C)** Heatmap of the microRNA profiles of the EV cargo under control and BCR stimulation conditions determined by small RNA sequencing. A total of 31 microRNAs were differentially expressed between the two conditions ($p < 0.05$, $n = 4$). **(D)** We selected three differentially expressed microRNAs and confirmed their expression by real-time PCR in a cohort of 25–26 EV samples. The expression was normalized with a pool of four microRNAs as endogenous controls. Since the hY4 level was highly present in EVs, it was also measured in 14 EV samples. **(E)** The higher level of these three microRNAs and hY4 was also measured and confirmed in CLL cell samples by real-time PCR ($p < 0.05$, $n = 25$). hY4 expression was normalized to the average of RNU44 and RNU48 expression.

was confirmed by real-time PCR. Interestingly, a greater increase was observed in monocytes treated with BCR CLL-EV for CCL24 ($p = 0.0078$) and for M-CSF/CSF1 ($p = 0.0234$), suggesting that BCR CLL-EVs have a different impact than EVs produced under control conditions (Figure 4F). To investigate this hypothesis more deeply, we compared the EV signature with NLC and M2 signatures. These results are provided in Figure 4G,H, Supporting Information S1: Data Text and Tables S11–S13.

Interestingly, when comparing the expression of three selected microRNAs increased in EVs after BCR stimulation, we also observed an increase of these microRNAs when monocytes were differentiated in NLCs (Supporting Information S1: Figure S3). In addition, some genes described as targets of the transferred microRNAs (CRISPLD2, KLF4, and S1PR3 are targets of miR-150, miR-146a, and miR-146a/miR-155, respectively) decreased while others, specific to NLC (CCL24, CSF1, MMP9, and SPP1), are increased. Taken together, these data suggest that monocytes treated with BCR CLL-EVs are close to NLCs, which are likely M2-like macrophages, according to the gene expression profile. Using different algorithms for the prediction of microRNA targets (miRDB, miRTar, TargetScan), we highlighted potential targets of miR-155, miR-146a, and miR-132 and made the intersection of these potential targets with the gene downregulated in monocytes after EV treatment (Figure 5A). As shown in this gene–microRNA interconnection diagram, some genes are targeted by multiple microRNAs. We thereafter confirmed the downregulation of some of these targets by real-time PCR ($n = 10$, $p = 0.0020$) demonstrating as proof of concepts that transferred microRNAs could have an impact on monocyte gene expression profile (Figure 5B).

BCR CLL-EVs modify the monocyte secretome toward the NLC secretome

The differentiation of healthy donors' monocytes into NLCs is accompanied by drastic changes in their secretome. However, since NLCs exist only by definition in coculture with leukemic cells, different cytokines can be secreted by both cell types, making it difficult to investigate the NLC secretome itself. In an attempt to reveal the specific NLC secretome, we investigated the mRNA levels of several cytokines between monocytes and NLCs using available public data. We selected significantly upregulated (FDR < 0.05) mRNAs of key cytokines for NLC differentiation (M-CSF, GM-CSF, CCL2) or CLL cell survival (SDF1α, IL6, IL10, IFNγ), as presented in Figure 6A. We subsequently measured the levels of these cytokines in the supernatants of monocytes and monocytes treated with control CLL-EVs or BCR CLL-EVs after 5 days using a bead-based immunoassay (Luminex). As shown in Figure 6B, the levels of all these cytokines were significantly greater in the supernatants of monocytes treated with BCR CLL-EVs than in those of untreated monocytes. However, while control EVs induced a significant increase in IL6 (12.2×), IL10 (2.8×), IL1Ra (4.9×), CCL3 (8.0×), and TNFβ (2.2×) compared to those in untreated monocytes, this effect was not observed for the majority of cytokines, suggesting that without BCR stimulation, EVs are less able to induce these modifications. This was confirmed when comparing cytokine levels between the control CLL-EV and BCR CLL-EV treatment groups: a significant increase was observed for almost all cytokines (except CCL2 and SDF1α), with increases ranging from 2.3× (IFNγ) to 23.7× (IL6) ($p < 0.05$). These results are in line with the transformation of the monocyte secretome to the NLC secretome, especially after BCR CLL-EVs. In addition to this NLC signature, we also evaluated other cytokines previously described as prosurvival factors in CLL. As shown in Figure 6C, all these cytokines were highly secreted by monocytes upon CLL-EV treatment, with a trend toward increased secretion (IL-2, IL-8) or a significant increase (IL-4, IFNα2, IL-9) with BCR CLL-EVs compared to that with control CLL-EVs.

CLL-EV-transformed healthy donors' monocytes protect CLL cells from apoptosis and increase the migration of immune cells

To investigate whether CLL-EVs have any functional impact, monocytes were treated (or not) with control CLL-EVs or BCR CLL-EVs. After 5 days, the medium was replaced with fresh medium, and PBMCs from CLL patients were then cocultured with these untreated or control-EV or BCR-EV-treated monocytes. After 24 h, apoptosis and viability were analyzed. As shown in Figure 7A, the apoptosis of leukemic cells was significantly reduced (decrease of 60%, $p = 0.0313$) when monocytes were treated with BCR EVs, while no significant reduction was observed with control CLL-EVs. These results were confirmed by viability analysis. A representative flow cytometry biparametric dot plot is provided in Figure 7A. Migration in response to conditioned medium and phagocytosis capabilities were also investigated and discussed in Supporting Information S1: Data Text and Figure 7B,C.

RNA contained within CLL-derived EVs is essential for the induction of monocyte differentiation into NLCs: The importance of hY4 RNA

To determine whether the different effects of CLL-EVs on healthy donors' monocytes are due to the presence of RNA, CLL-EVs were treated with RNAse according to the protocol of Otsuru et al. (details about the method can be found in Supporting Information S1: Data Text). Therefore, the expression of mRNAs and the levels of secreted cytokines upregulated in M2 macrophages or during NLC differentiation were measured by RNA-seq after monocytes were treated with CLL-EVs. CCL24, CSF1, and LILRB1 expression increases induced by BCR-derived CLL-EVs were decreased or completely abolished when EVs were treated with RNase (Figure 8A). These results were also confirmed for secreted cytokines such as IL6, CCL4, and IL10 (Figure 8B).

Since miR-155, miR-146a, and miR-136 are more abundant in EVs after BCR activation and because miR-150 is one of the most abundant microRNA EVs, we transfected specific microRNA mimic or microRNA inhibitors in untreated or BCR CLL-EV treated monocytes, respectively. While several putative targets of these microRNA are

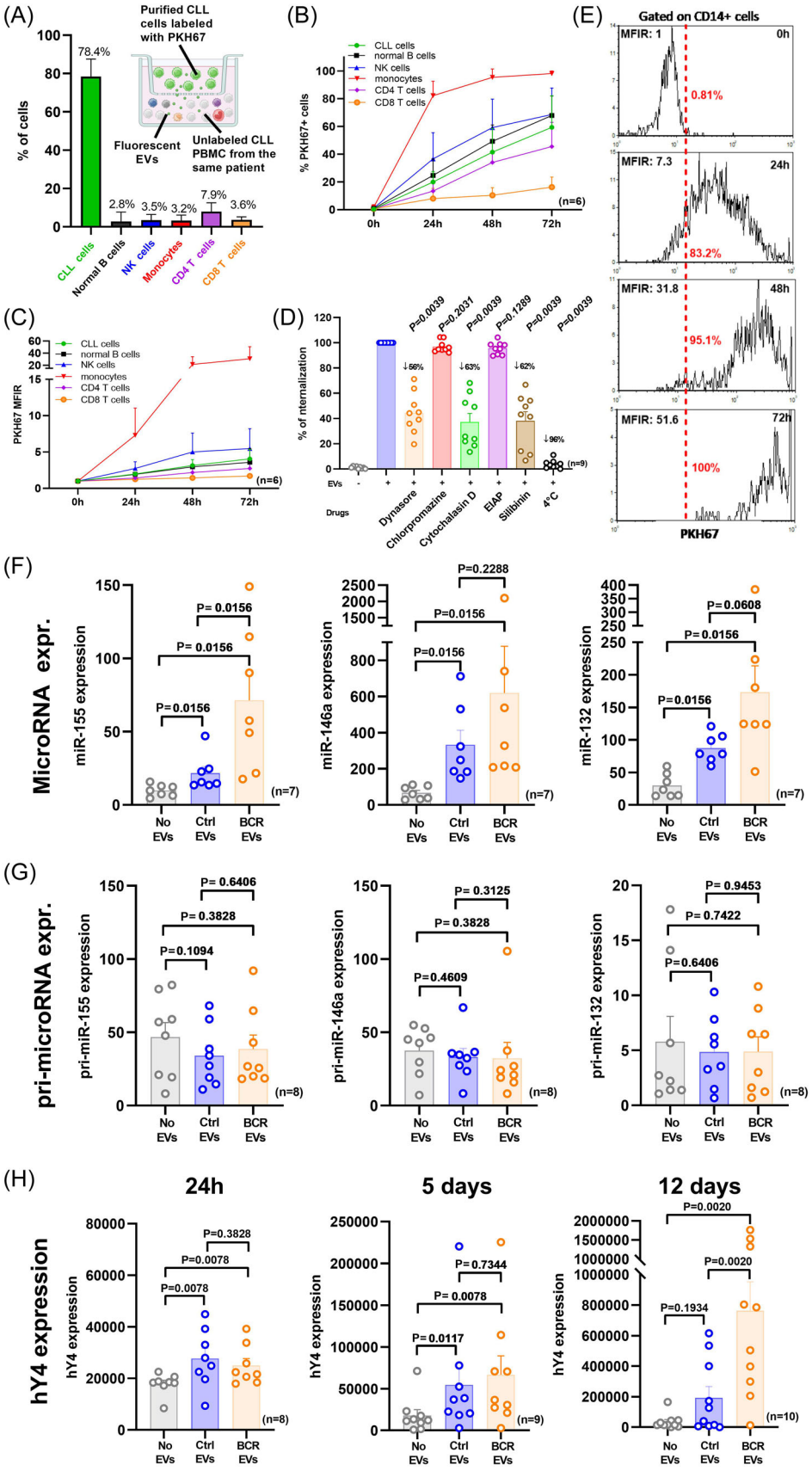

FIGURE 3 (See caption on next page).

FIGURE 3 **CLL-EVs are preferentially internalized by monocytes and deliver their microRNA content**. **(A)** Proportion of the different cell types in the six analyzed CLL patients and schematic representation of the coculture experiment using a 0.4 μm filter. **(B)** Incorporation of PKH67-labeled EVs by different cell types in terms of PKH64-positive cells and the MFIR **(C)**. Monocytes preferentially internalized CLL-EVs. **(D)** EV uptake analysis pathways. Monocytes pretreated with dynasore hydrate (80 μM), cytochalasin D (20 μM), chlorpromazine (10 μM), 5-(N-ethyl-N-isopropyl)amiloride (EIPA) (5 μM), and silibinin (100 μM) or incubated at 4°C were treated with PHK67-labeled CLL-EVs. After 24 h, they were analyzed by flow cytometry, and the percentage of fluorescent cells was determined. Results were normalized to the condition "monocytes with EV." **(E)** Representative flow cytometry experiment showing the increase in monocyte fluorescence in this coculture system. **(F)** Quantification of miR-155, miR-146a, and miR-132 in untreated monocytes or monocytes treated with control CLL-EVs or BCR CLL-EVs after 24 h. The difference in microRNA expression increased with CLL-EV treatment, with higher expression of the BCR CLLEVs. MicroRNA expression in cells was normalized to the average expression of the endogenous controls RNU44 and RNU48. **(G)** Quantification of pri-microRNAs of these different microRNAs. No significant difference was observed between the different conditions, demonstrating that the increase in microRNA levels was not due to de novo transcription. **(H)** Quantification of hY4 in untreated monocytes or monocytes treated with control CLL-EVs or BCR CLL-EVs after 24 h, 5 days, and 12 days. hY4 expression was normalized to the average RNU44 and RNU48 expression.

downregulated in monocytes after BCR CLL-EV treatment, the transfection of microRNA inhibitors or miR mimic did not have any significant effect ($p > 0.05$) on CCL24 expression, the most expressed and upregulated gene highlighted by our RNAseq experiment after BCR CLL-EV treatment (Figure 8C) that we used to monitor NLC differentiation. Since hY4 RNA is highly abundant in EVs, transferred in monocytes, and since its expression after BCR CLL-EVs is higher compared to untreated cells after 5 days, we transfected a synthetic hY4 RNA directly in monocytes and observed already after 24 h, the upregulation of CCL24 mRNA, a key gene in M2 macrophage polarization (Figure 8D). Compared to lipofectamine alone, monocytes transfected with hY4 showed a 3.0-fold increase in CCL24 expression ($n = 6$, $p = 0.0313$). In order to verify the specificity of this increase, another Y RNA, the hY5, was also transfected and did not induce any modification of the CCL24 level ($n = 7$, $p = 0.1094$). Inhibition of hY4 was also investigated by transfection of a siRNA against hY4 in monocytes followed by a BCR CLL-EV treatment: 24 h after the transfection, a significant reduction of CCL24 mRNA was observed compared to cells transfected with a control siRNA ($n = 7$, $p = 0.0078$). In addition, when the siRNA against hY4 was directly electroporated into BCR CLL-EVs, similar results were observed ($n = 4$, $p = 0.0092$) (Figure 8D).

DISCUSSION

Several studies have shown that EV content can be influenced by different stimuli received by cells. Here, we report the first complete microRNA screening of CLL cells after BCR stimulation. In 2015, Yeh et al. showed that BCR stimulation can increase EV release by CLL cells and that these EVs displayed a distinct microRNA signature with an increase in miR-150 and miR-155.[30] Our results confirmed the increased production of EVs upon BCR stimulation. In addition, we showed here that several other microRNAs (mR-146a, miR-132, miR-16, miR-34a, miR-29c, and miR-223) are modified in CLL-EVs; in the majority of cases, the microRNA levels observed in EVs reflect their cellular level upon BCR stimulation. As previously reported[7,17] and shown in our study, EVs are enriched in small RNAs. We observed that 38% of the RNA EVs were microRNAs. This finding is consistent with the proportion of microRNAs found in other small RNA sequencing studies (33% in Paggetti et al.[7] and 17% in Haderk et al.[17]). In addition, under unstimulated conditions, high levels of miR-155 and miR-146a were already observed in CLL patient exosomes.[7] Interestingly, the miR-146a content in CLL-EVs is involved in the transition of mesenchymal stromal cells into cancer-associated fibroblasts,[28] indicating that microRNAs can have a functional impact when transferred to surrounding cells. The different Y RNA proportions we observed were also similar to those reported by Haderk, who reported a predominant amount of hY4, which was validated by a method other than our small RNA sequencing method.

In CLL patients' blood, the leukemic compartment is generally predominant compared to other cell types, especially in advanced stages. This is also true in the lymphoid tissue compartment, such as in the bone marrow or the lymph nodes where cells from the microenvironment are surrounded by a high proportion of leukemic cells. By coculturing CLL PBMCs with purified PKH67-labeled CLL cells and by respecting the in vivo cell proportion range, we showed that monocytes are the first to incorporate CLL-EVs, while the other cell types are also targeted by CLL-EVs but with slower kinetics and a smaller proportion. Interestingly, we observed an increase in miR-155, miR-146a, and miR-132 in monocytes treated with CLL-EVs, while their respective pri-miRs did not increase, indicating that this increase was due to transfer and not de novo transcription. The increase in microRNAs tended to always be greater with BCR CLL-EVs. These results are in line with previous studies demonstrating the transfer of EV cargo into monocytes. Claben et al. showed the intracellular location of CFSE-stained EVs as well as the transfer of miR-155.[39] Njock et al. observed the transfer of anti-inflammatory microRNAs to monocytes via endothelial cell EVs.[40] We also highlighted the increase of hY4 in monocytes treated with CLL-EVs. This increase was statistically higher after 12 days in BCR CLL-EV-treated monocytes compared to control CLL-EVs but was not observed in control EVs at this late time point. This could be explained by the differences in EV content after BCR stimulation. In addition, as shown in Figure 3C, EV internalization slows down after 48–72 h and higher expression of hY4 after 12 days could therefore potentially be explained by a de novo transcription.

To mimic the in vivo microenvironment in which monocytes are surrounded by a high proportion of leukemic cells, we treated monocytes with 10,000 EVs per cell. We previously observed that leukemic cells can produce an average of 150–200 EVs per cell in 24 h. Given that the number of leukemic cells is often greater (especially in the tissue where it can reach >90% of the cells) than the number of monocytes, we concluded that this amount is close to the physiological doses received in vivo. Under these conditions, we observed a morphological change in monocytes accompanied by a modification of their gene expression profile and secretome. In addition, gene set enrichment analysis associated these gene signatures with macrophages found in the tumor microenvironment according to the publication of Gavish et al.[38] Since important genes associated with the M2 macrophage signature were increased and validated by real-time PCR, we compared this EV signature to M2 macrophages and the NLC signature. A significant overlap was found between the EV/NLC, EV/M2, and NLC/M2 signatures, suggesting that NLCs are indeed M2-like macrophages and that EV-treated cells tend to differentiate monocytes into NLCs. When comparing the levels of several cytokines in EV-treated monocytes to those in untreated controls, we observed an increase, and in almost all the patients, there was a statistically greater increase when BCR CLL-EVs were used than when EVs were produced under unstimulated conditions.

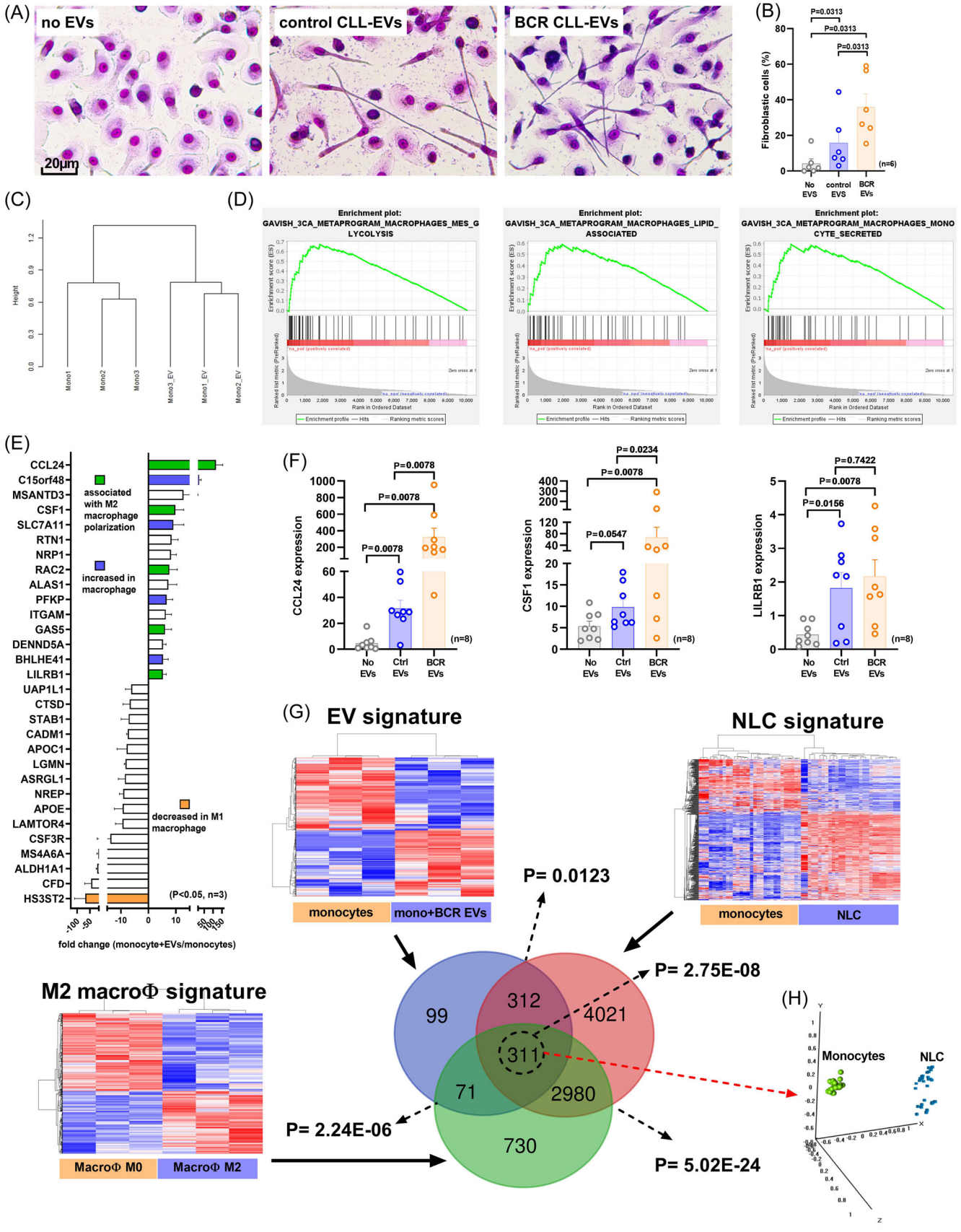

FIGURE 4 (See caption on next page).

FIGURE 4 **BCR CLL-EVs induce modifications in monocytes compatible with an NLC phenotype**. **(A)** Monocytes were treated without or with control CLL-EVs or BCR CLL-EVs, and images were acquired using a microscope. An increased number of fibroblast-shaped monocytes was observed under CLL-EV conditions. **(B)** Evaluation of the proportion of fibroblast-shaped monocytes compared to that of round monocytes. A significant increase in fibroblast-shaped monocytes was observed with BCR CLL-EVs. **(C)** Unsupervised clustering based on RNA-seq data of samples treated or not treated with BCR CLL-EV. **(D)** Gene set enrichment analysis using the gene set published by Gavish et al. corresponding to the hallmarks of transcriptional intratumor heterogeneity. This GSEA highlights three sets of genes related to tumor macrophages. **(E)** The 15 genes most highly expressed and deregulated in monocytes after treatment with BCR CLL-EVs. **(F)** Confirmation by real-time PCR of three different genes highlighted by RNA-seq ($n = 8$). **(G)** Venn diagram of the NLC, EV, and M2 macrophage signatures. "SuperExactTest" was applied to evaluate the statistical value of the intersections between three signatures, as indicated by the expected overlap and the p values. **(H)** The intersection of the three signatures (311 genes) was used to classify monocyte and NLC samples via 3D hierarchical clustering.

These increases are compatible with the RNA overexpression observed between monocytes (on Day 0) and the same monocytes differentiated in NLCs (after 14 days in culture with CLL cells). Interestingly, after the addition of BCR CLL-EVs, the levels of anti-inflammatory cytokines such as IL-1Ra (+14.9-fold compared to those in untreated controls), IL-6 (+289-fold), and IL-10 (15.4-fold) were clearly increased. These cytokines are also significantly increased compared to those in control EVs, underscoring the additional effect of BCR CLL-EVs. Other cytokines (previously described as prosurvival factors for CLL cells), such as IL4,[41] SDF1α,[6,8] IL2,[42] IL8,[43] IFNγ,[44], and IL9,[45] also display significantly increased secretion by monocytes after BCR CLL-EV treatment.

Previous studies have shown that EVs can induce differentiation into M2 macrophages. Njock showed that endothelial EVs promote tumor growth by tumor-associated macrophage reprogramming and microRNA-mediated targeting of PTEN.[46] Yao et al. reported that circRNAs transferred by EVs can induce macrophage M2 polarization in lung cancer.[47] Additionally, in hematological malignancies, leukemic

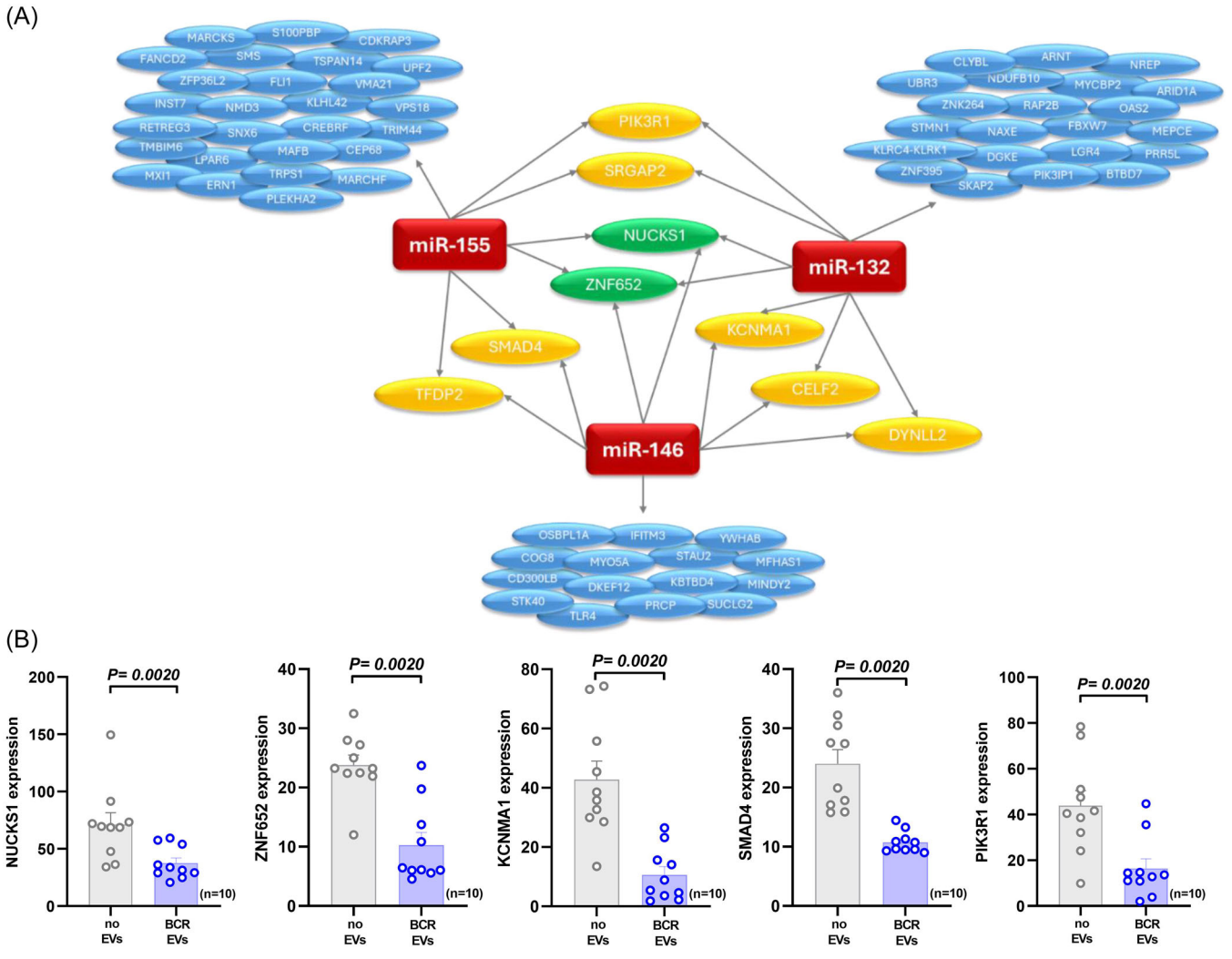

FIGURE 5 **Targets of EV-transferred microRNAs are downregulated in monocytes treated with CLL-EVs**. **(A)** Connection diagram between miR-155, miR-146a, and miR-132 and their potential targets predicted by MiRDB, miRTar, or TargetScan and downregulated in monocytes treated with BCR CLL-EVs. **(B)** Five selected targets were quantified by real-time PCR on monocytes and monocytes treated with BCR CLL-EVs. Genes were normalized with the mean of glyceraldehyde 3-phosphate dehydrogenase and beta-actin genes.

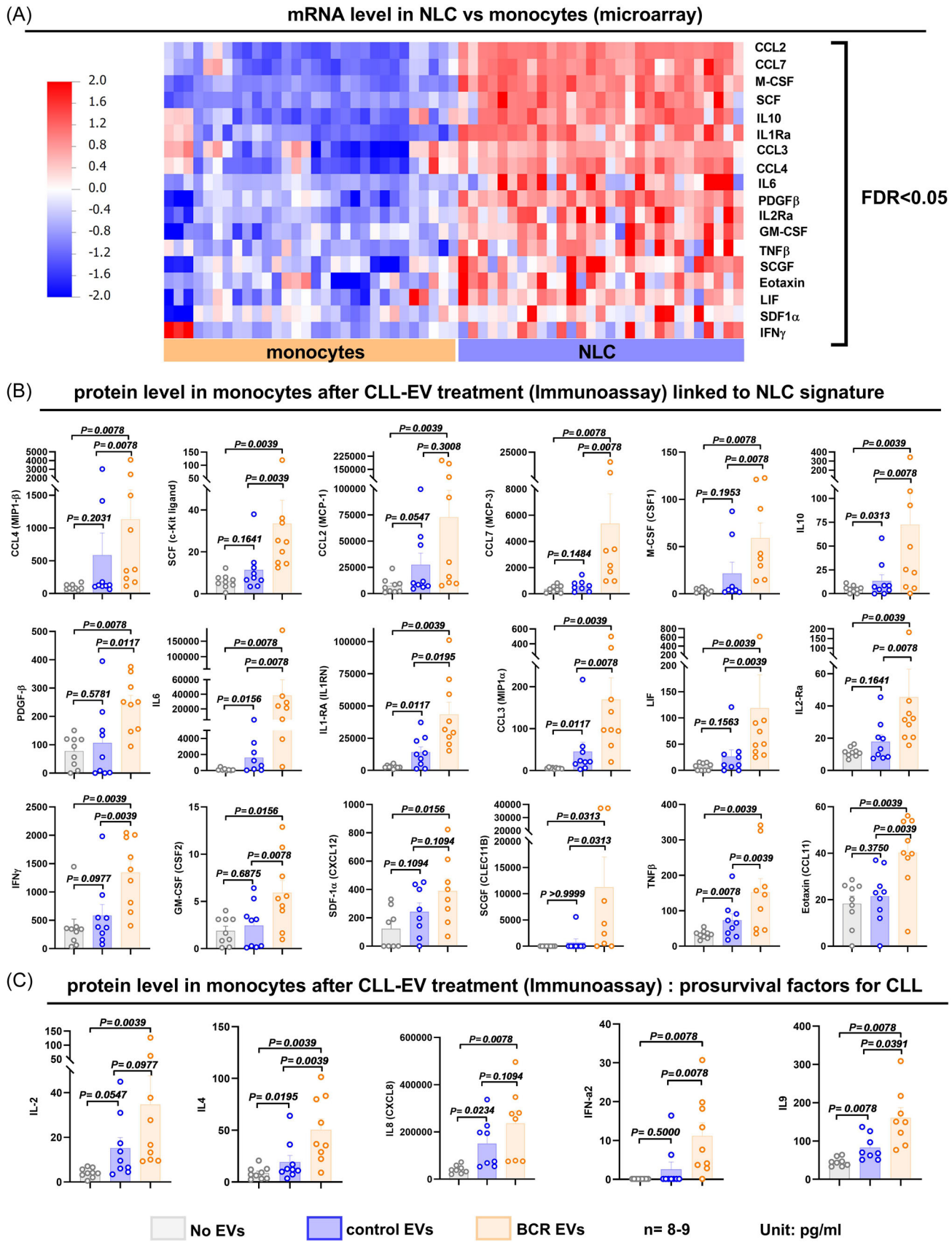

FIGURE 6 **CLL-EVs modify the monocyte secretome toward an NLC secretome.** **(A)** Heatmap of key cytokines based on public Affymetrix gene expression data for monocytes ($n = 30$) and NLCs ($n = 29$). **(B)** Protein levels of cytokines linked to the NLC signature. The supernatants of monocytes treated without or with control CLL-EVs or BCR CLL-EVs were analyzed by immunoassay. **(C)** Protein levels of cytokines already described as prosurvival factors for CLL cells. The supernatants of monocytes treated without or with control CLL-EVs or BCR CLL-EVs were analyzed by immunoassay.

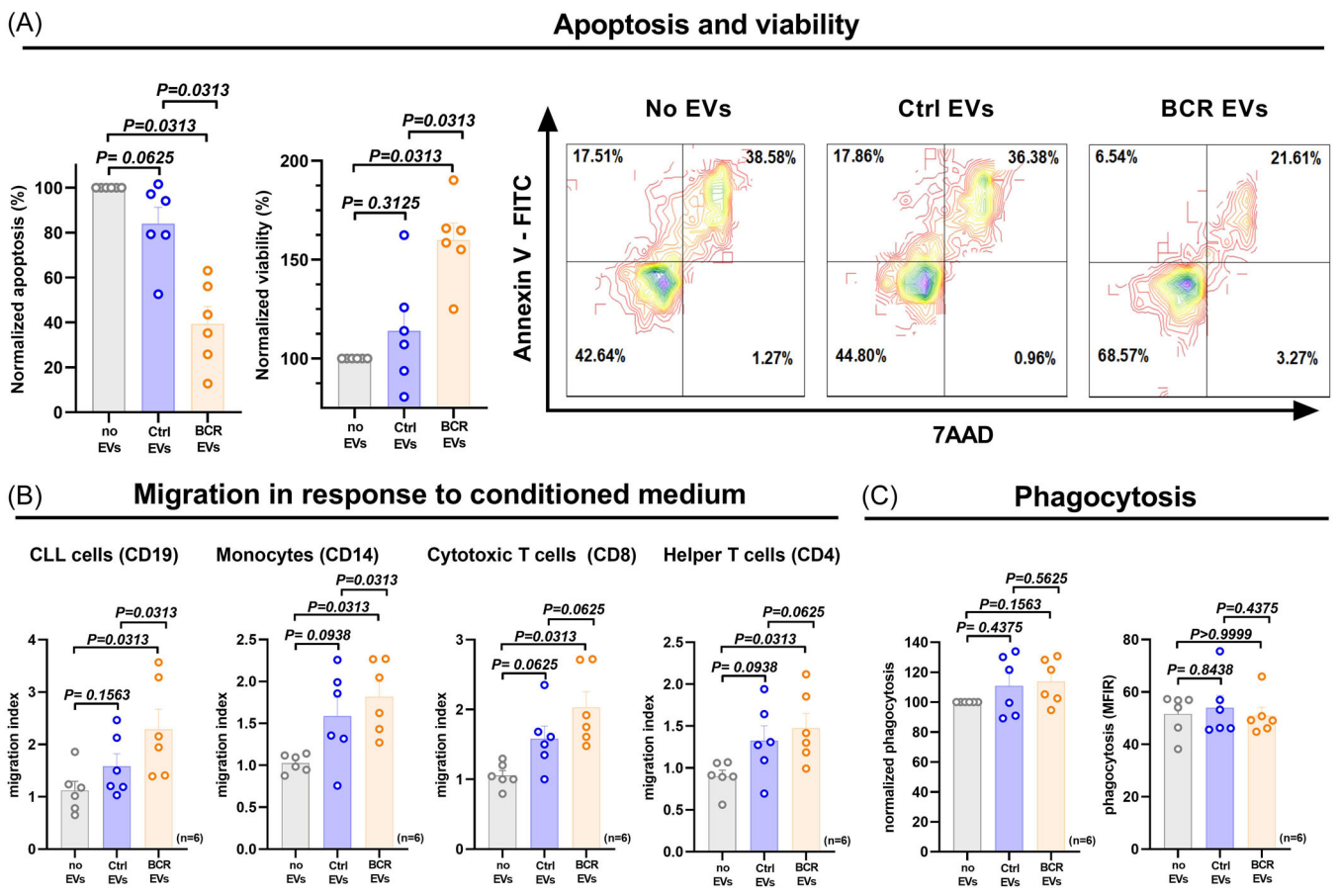

FIGURE 7 **Monocytes treated with BCR CLL-EVs protect CLL cells from apoptosis and induce the migration of immune cells but do not change their phagocytic ability. (A)** Apoptosis and viability of CLL cells cocultured with monocytes treated without or with control CLL-EVs or BCR CLL-EVs. CLL BCR-EVs significantly protect CLL cells from spontaneous apoptosis and increase their viability. Apoptosis and viability were measured by flow cytometry using Annexin V/CD19/7AAD staining. A representative sample presented in a biparametric dot plot under the three different conditions is provided. **(B)** Migration of different immune cells (CD19, B cells; CD14, monocytes; CD8, cytotoxic T cells; CD4, helper T cells) was measured using a Boyden chamber and in response to conditioned medium from monocytes treated without or with control CLL-EVs or BCR CLL-EVs. The migration index was calculated as the number of cells transmigrating in the presence of a conditioned medium divided by the number of transmigrating cells in the RPMI medium. **(C)** Phagocytosis of monocytes treated without or with control CLL-EVs or BCR CLL-EVs. No significant differences were detected between the different conditions in terms of the number of positively phagocytosing cells or in terms of the MFIR of the phagocytosed fluorescent bioparticles.

EVs were shown to induce macrophage polarization. Jafarzadeh et al. reported that chronic myeloid leukemia-derived EVs induce changes in the expression of genes associated with macrophage polarization in line with M2 tumor-associated macrophage characteristics.[48] Furthermore, multiple myeloma-derived EVs influence the fate of macrophages by transferring miRs able to drive macrophage M2 polarization through the transfer of overexpressed miR-let-7c.[49]

One important feature of NLCs is their ability to support CLL cell survival.[6,8] Here, we showed for the first time that monocytes treated with BCR CLL-EVs are able to decrease the spontaneous apoptosis of CLL cells and increase their viability. Interestingly, this effect was not observed with control EVs. This could be explained by differences in cytokine production and RNA content between these two types of EVs. This finding also suggested that when CLL cells receive stimuli from the microenvironment, the content of their EVs and their effect on surrounding cells are different. Binder et al. reported that the vimentin and calreticulin present in NLCs can, for example, trigger the BCR signaling of CLL cells.[31] In addition, gene expression profile analyses of CLL cells after CLL-NLC coculture showed that NLC activated the BCR signaling pathways in CLL cells.[11] CM from monocytes treated with BCR CLL-EVs can increase the migration of CLL cells but also the migration of other immune cells, such as monocytes and T cells. This phenomenon represents an amplification loop establishing a protective microenvironment: indeed, the chemoattraction of CLL cells toward transformed monocytes will increase CLL cell survival and BCR stimulation, leading to a greater release of BCR CLL-EVs. These EVs will in turn transform new monocytes that are also attracted to the tissue microenvironment, which will ultimately increase the number of NLCs. As T cells are also attracted to these sites, their antitumoral response is hampered, as described by Gargiulo et al.[50] and Böttcher et al.,[51] which promotes CLL progression.

To elucidate whether monocyte differentiation into NLCs is related to RNA transfer, we treated CLL-EVs with a high dose of RNAse according to a protocol previously described.[52] We observed that modifications induced by CLL-EVs, such as gene expression or cytokine production, were decreased/abolished by RNase A treatment, suggesting that the RNA content is responsible for these changes. Several authors have shown that microRNA transfer via EVs can induce an M2 macrophage phenotype. Saha et al. reported that EV

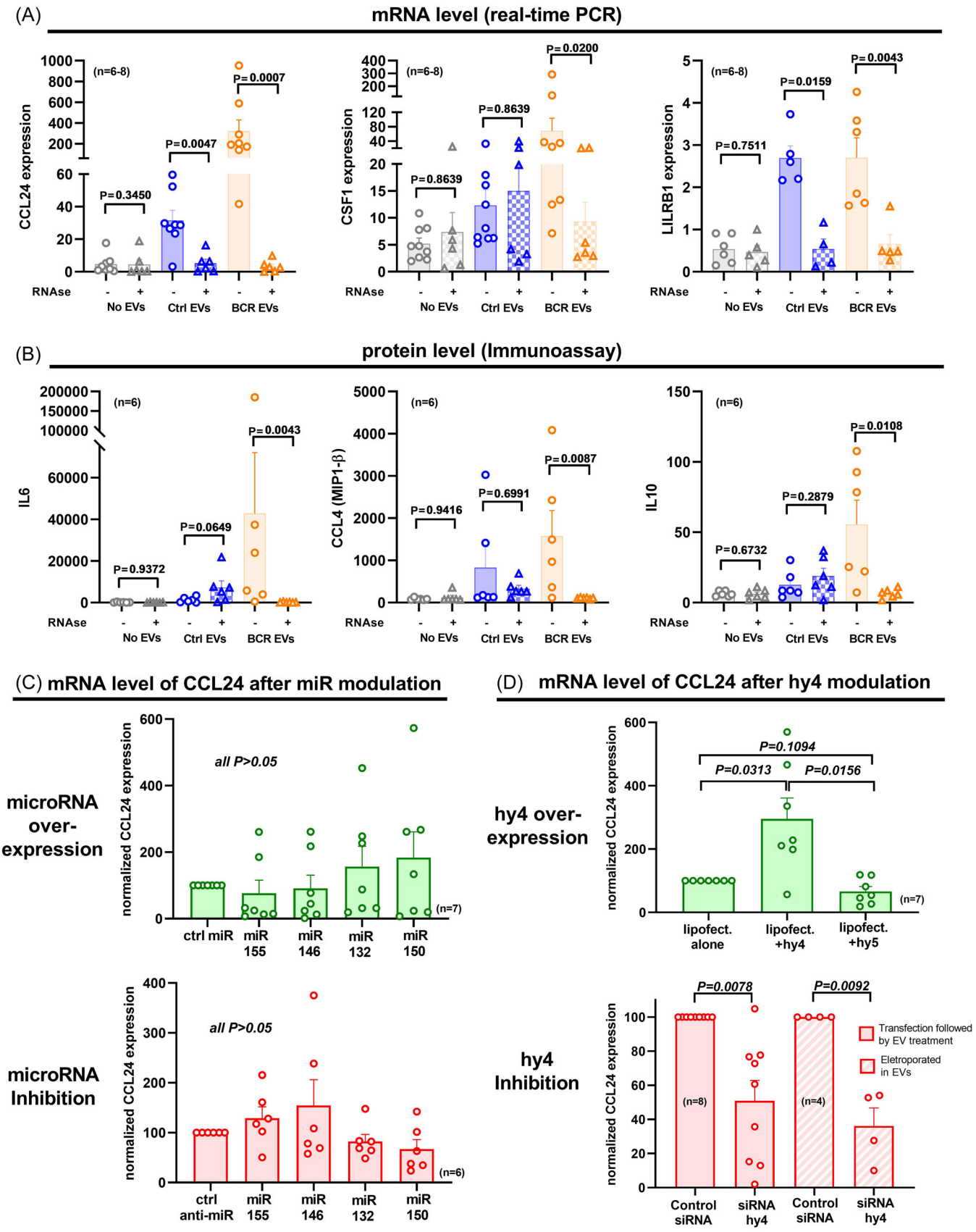

FIGURE 8 (See caption on next page).

FIGURE 8 **The RNA contained within CLL-EVs is essential for the induction of monocyte differentiation into NLCs**. Upregulated genes **(A)** highlighted by RNA-seq and confirmed by real-time PCR (CCL24, CSF1, and LILRB) as well as increased cytokine levels **(B)** (IL6, CCL4, and IL10) were detected in monocytes and in the conditioned medium of cells treated without or with control CLL-EVs or BCR CLL-EVs. For each condition, EVs were also treated with high-dose RNase A for 3 h at 37 °C. RNAse treatment decreased/abolished the effects of CLL-EVs on monocytes. **(C)** MicroRNA inhibitors and microRNA inhibitor negative control, microRNA mimics, and microRNA mimic negative control were transfected into monocytes and the expression of CCL24 was quantified as the reflection of NLC differentiation. No statistical difference was observed compared to control conditions. **(D)** Synthetic hY4 and hY5, siRNA against hY4, and siRNA negative control were transfected into monocytes. Overexpression of hY4 was associated with an upregulation of CCL24, while this increase was not observed with hY5 transfection. The inhibition of hY4 induced the decrease of CCL24 when a siRNA against hY4 was directly transfected into monocyte electroporated into BCR CLL-EVs used to thereafter treat monocytes.

microRNA cargo from alcohol-exposed monocytes signals naive monocytes to differentiate into M2 macrophages.[53] However, inhibition or overexpression of microRNA did not show in our hands the modulation of key genes in monocyte differentiation. However, the level of miR-155, -146a, -132, or -150 targets is significantly downregulated in monocytes treated with BCR CLL-EVs suggesting that microRNA could contribute to the gene expression modification but are not sufficient for monocyte differentiation into NLC. Other authors reported that long noncoding RNAs[54] or circular RNAs,[55] via exosome-mediated transfer, also promote M2 macrophage polarization. In the context of CLL, Haderk et al. reported that hY4 can be transferred via CLL exosomes and induce PD-L1 expression in monocytes as well as the release of several cytokines via TLR7 triggering.[17] These results are in line with our observations showing that inhibition or overexpression of hY4 in monocytes can, respectively, decrease or increase the level of CCL24 expression, a key gene in monocyte differentiation into NLC. Taken together, these data suggest that multiple RNA species (microRNA, long noncoding RNA, circular RNA) can cooperate to induce monocyte differentiation.[56]

In conclusion, our study revealed a sophisticated communication pathway within the CLL microenvironment: CLL cells activated by BCR stimulation exhibit increased EV release and modified RNA content in terms of microRNAs. These BCR CLL-EVs have the ability to induce an NLC phenotype in terms of gene and microRNA expression as well as cytokine release via an RNA-dependent mechanism. Compared with untreated monocytes, BCR CLL-EV-modified monocytes are able to support CLL cell survival and induce the migration of leukemic cells. Our results also highlight that the released cytokines also induce the migration of other immune cells, such as monocytes and T cells, reinforcing the establishment of a protective microenvironment for CLL cells. The depletion, reprogramming, and molecular targeting of TAMs are being evaluated as new therapeutic strategies for hematological malignancies[57] and solid cancers.[58] Our work provides evidence that EVs represent a non-negligible part of intercellular communication and that modulating the number and extent of CLL-EVs to inhibit the formation of NLCs could represent a potential new therapeutic approach.

AUTHOR CONTRIBUTIONS

Nathan Dubois, Laurence Lagneaux, and Basile Stamatopoulos conceived and designed the experiments. Nathan Dubois performed the experiments. Nathan Dubois, Laurence Lagneaux, and Basile Stamatopoulos analyzed the data. Dominique Bron contributed patient samples and collection. Filip Van Nieuwerburgh and Laurentijn Tilleman contributed to the RNA-seq and bioinformatic analyses. Nathan Dubois and Basile Stamatopoulos wrote the paper. All the authors reviewed the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data are available in Supporting Information Tables. All data eventually not included in the supplemental can be provided upon request to bstamato@ulb.ac.be.

ETHICS STATEMENT

This study was approved by the Jules Bordet Institute ethics committee (Belgium). This study was based on peripheral blood mononuclear cell (PBMC) samples collected at diagnosis after patients provided written informed consent.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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