## Extracellular vesicles released from chronic lymphocytic leukemia cells exhibit a disease relevant mRNA signature and transfer mRNA to bystander cells

B-cell chronic lymphocytic leukemia (CLL) is characterized by the clonal accumulation of mature apoptosis-resistant tumor cells in lymphoid tissues and the peripheral blood. Recently genetic and epigenetic aberrations associated with the pathogenesis, the progression, and the response to therapy were identified and have shed light on the genetic basis of this disease.[1] Cellular networks affected in CLL include Notch signaling, inflammatory pathways, B-cell receptor signaling, RNA processing, chromatin modification, and DNA damage response genes.[2] Of note, genetic aberrations do not act solely in a cell-autonomous manner as CLL cells do not proliferate or even survive when isolated from their environment. The bidirectional cross-talk between CLL and non-malignant bystander cells is mediated through a complex network of soluble factors, chemokine receptors, receptors of the tumor necrosis factor (TNF)-family, soluble factors and antigens that trigger B-cell receptor and toll-like receptor (TLR) signaling, constituting central pathogenic

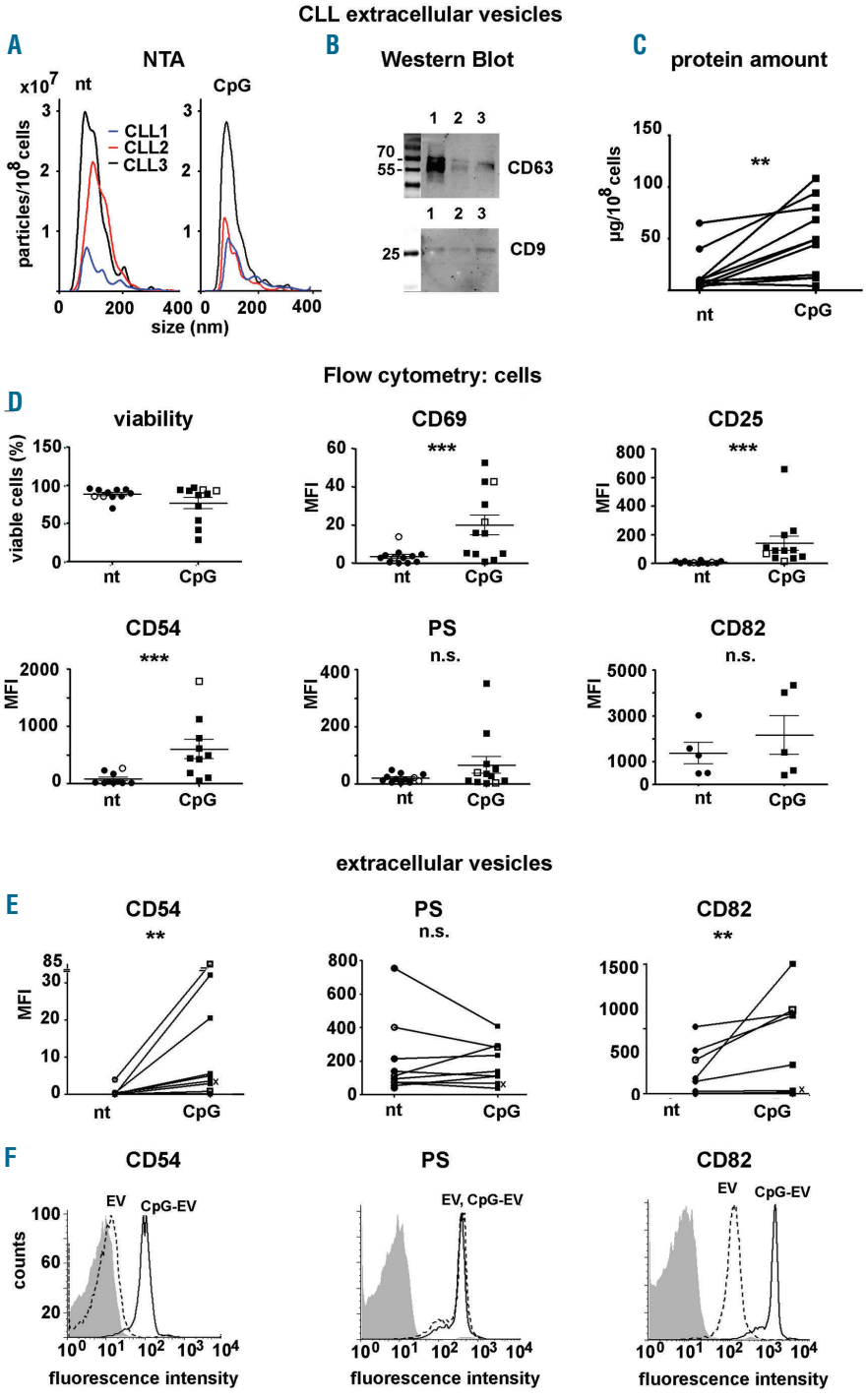


**Figure 1. Characterization of extracellular vesicles.** (A) Size distribution of CLL-derived vesicles (untreated, left panel, or treated with a TLR9 agonist (CpG, right panel) analyzed by NanoSight Technology (NS300, Malvern). Mean size diameter of vesicles from untreated/treated samples was 106/124 nm for CLL1, 115/99 nm for CLL2 and 97/98 nm for CLL3. All samples were similarly diluted and measured with a sample flow rate of 10 (n=6). (B) Western blot analysis of the exosome-enriched proteins CD63 and CD9 (both antibodies from BioLegend). EV released by $5x10^9$ CLL cells within 38-45 h were resuspended in 40 µL non-reducing lysis buffer; sodium dodecyl sulfate (SDS) gel electrophoresis was performed using 10 µL EV/lane in 12.5% SDS gels and subsequently proteins were transferred to a nitrocellulose membrane. (C) The amount of purified EV protein released by $10^8$ cells was measured with Nanodrop1000 (Thermo Scientific) (n=11). (D) Flow cytometric analysis of CLL cells after 38-45 h culture in Panserin medium with or without 200 nM CpG. Most patients had IgHV mutations (see black circles or squares). Samples without mutations are represented by non-filled circles or non-filled squares. Cell viability was assessed with an apoptosis assay, staining with annexin V-PE and 7-AAD (BioLegend) (PS = phosphatidylserine). Cells were stained with CD69-FITC (BD Biosciences), CD25-FITC (BD Biosciences), purified CD54 and CD82 (both BioLegend), which were detected with goat-anti-mouse-PE (BioLegend). The analysis was performed with a FACS Calibur (BD Biosciences) or FACS Gallios (BeckmanCoulter). Dead cells were excluded by gating for 7AAD (BD Biosciences) negative cells (n=5-14). (E) Flow cytometric analysis of EV: EV were bound to polystyrene beads (Polysciences) and subsequently stained and analyzed as described in (D). Only EV derived from cell samples with a viability of more than 90% were used for further analysis (n=5-9). The IgHV status of one patient analyzed for CD54 expression (left panel) is unknown (labeled with an x). (F) One representative histogram for FACS data depicted in (E).

events in CLL.[3] Recently the relevance of extracellular vesicles (EV) to the intercellular cross-talk and the establishment of a tumor-promoting CLL microenvironment was investigated in several studies.[4-7] EV are either shed from the cell surface or released as exosomes via exocytosis upon generation in multivesicular bodies. Tumor cell-derived EV enable the transfer of cancer-associated proteins and RNA which are able to alter the phenotype

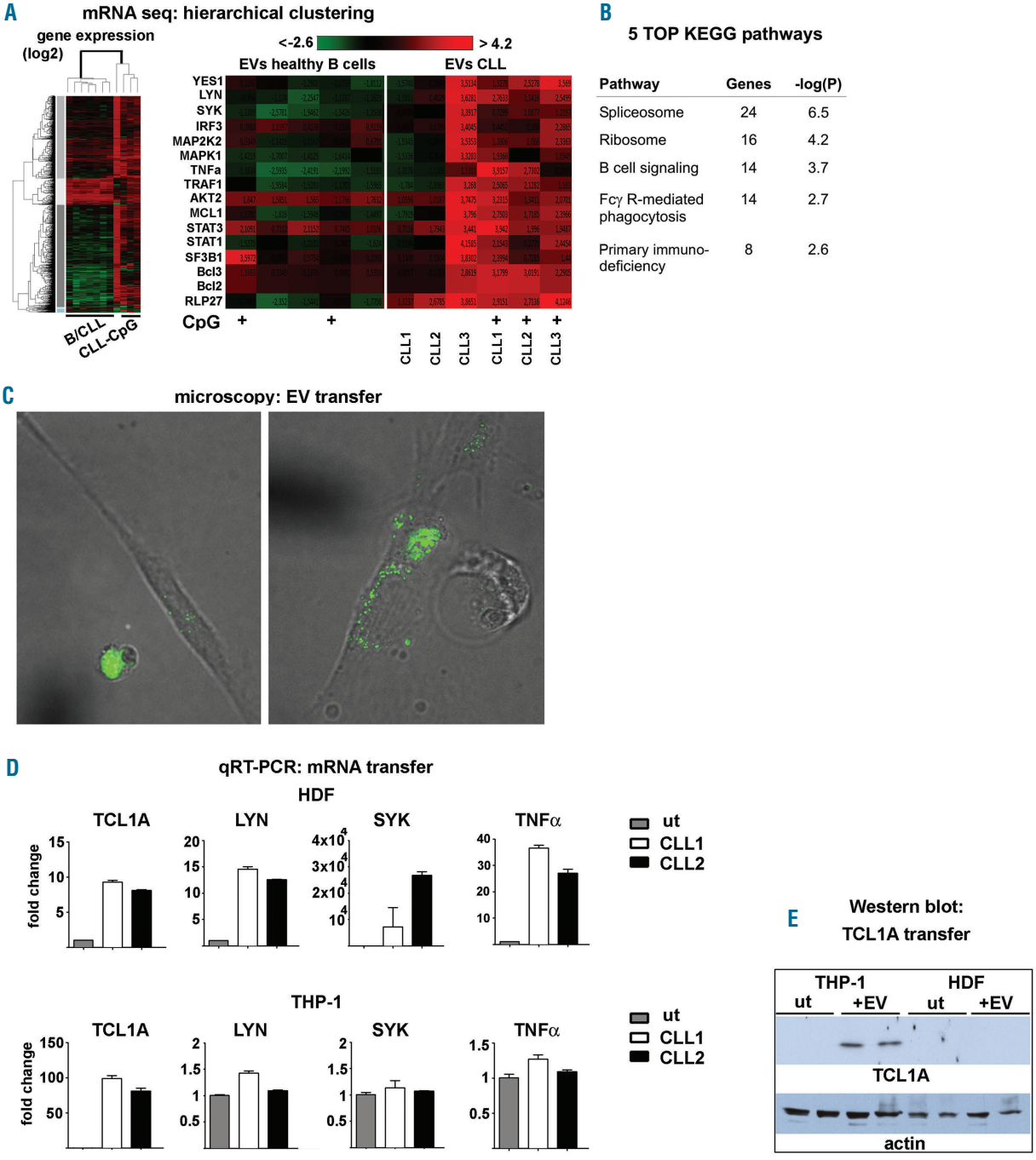


**Figure 2. mRNA signature of CLL-derived vesicles.** (A) Hierarchical clustering of mRNAseq data (left: all; right: selected for CLL-relevant genes) obtained using next-generation sequencing of RNA isolated from vesicles of normal B cells (obtained from 3 healthy donors) and CLL cells (obtained from 3 patients) cultured in Panserin medium with or without 200 nM CpG (maximal cell density of $2.5x10^7$ cells/mL). Vesicles were purified after 38-45 h from supernatant by serial ultracentrifugation to extract RNA using RNAeasy Mini Kit (Qiagen). B: B-cell derived vesicles, CLL: CLL-derived vesicles, CpG: stimulated with CpG. (B) KEGG pathway analysis of sequencing data to identify top canonical pathways was performed with DAVID 6.7. The functional annotation clustering was performed with the highest classification stringency. The KEGG-pathway function included in DAVID 6.7 was used to interpret the data in the context of biological processes, pathways and networks. Both up- and down-regulated identifiers were defined as value parameters for the analysis. (C-E) Uptake of CLL-vesicles results in TCL1A, LYN and SYK expression in fibroblasts and macrophages. (C) CLL cells were labeled with 5 μM DiO for 10 min and incubated together with HDFn fibroblasts for 16 h. Live cell imaging was performed using a Nikon spinning disc. (D-E) HDFn fibroblasts or THP-1 cells (monocytic cell line) were incubated with purified CLL-EV from two different patients (43 and 64) for 48 h to exclude any contribution or interference with CLL debris. (D) Expression of TCL1A, SYK and LYN in untreated HDFn fibroblasts and THP-1 and in cells treated with CLL-vesicles was measured using quantitative real-time polymerase chain reaction (see also *Online Supplementary Table S4*). (E) Western blot of cell lysates from THP-1 and HDFn cells untreated (ut) or cultured with CLL-EV (n=2, two independent patients) detecting TCL1A protein.

of recipient cells. The aim of this study was to characterize the mRNA cargo of CLL vesicles and to confirm its vesicle-associated transfer to non-malignant bystander cells.

For this purpose, vesicles were collected from the supernatants of primary CLL cells (see *Online Supplementary Table S1A* for the patients' characteristics). The average diameter of purified vesicles from non-treated cells (Figure 1A, left panel, nt) and from cells treated with CpG to induce TLR signaling (Figure 1A, right panel, CpG) was about 100 nm, which corresponds to the size of exosomes as measured by nanoparticle tracking analysis. Markers of exosomes, including CD63 and CD9, could be detected in western blots (Figure 1B). However, the purified fraction was collectively referred to as EV since it also included larger vesicles of up to 200 nm and exclusive exosome-specific markers are not yet available. Next, the impact of TLR signaling, a pathway with high relevance for CLL growth and progression, on the release of EV was analyzed.[8] To minimize non-CpG-related differences among samples we included only IgHV-mutated cases, which are known to be more indolent than the IgHV-unmutated type of CLL. The specific cargo of vesicles from different CLL subsets and the role of TLR signaling on these CLL subsets remains to be analyzed. Interestingly, treatment with the TLR9-agonist CpG-ODN2006[9] triggered significant release of EV (Figure 1C). Signaling of TLR9 converges, via MyD88, with the nuclear factor-kB pathway, also activated upon B-cell receptor or CD40/interleukin (IL)-4 engagement, which was used in other studies to promote EV release from CLL cells.[6,10] As expected, activation markers CD69, CD25, and CD54 (ICAM-1) were elevated on CLL cells upon CpG-ODN2006 treatment (Figure 1D). Cell surface CD54 corresponded to an increased CD54 expression on released CLL-CpG-EV, but expression of CD69 and CD25 was beneath the detection level as measured by flow cytometry of (bead-coupled)-vesicles (Figure 1E, mean fluorescence intensity and Figure 1F, one representative histogram). Tetraspanin CD82 was significantly induced on CLL-CpG-extracellular vesicles, but not at the cell surface, together indicating that the surface extracellular vesicle cargo does not simply mirror the expression level of membrane proteins. CD54 was identified on exosomes from mature dendritic cells as a critical molecule for the vesicle-mediated priming of naïve T cells[11] and the tetraspanin CD82 is known as an exosome marker also involved in sorting of exosomal cargo.[12] Changes in the expression of phosphatidylserine were not observed on either cells or vesicles. Based on these data we speculated that CLL-CpG-EV are functionally distinct compared to EV from unstimulated cells or from normal B cells.

To analyze differences in the mRNA content of EV from B cells isolated from healthy donors and CLL B cells, which were either untreated or stimulated with CpG, next-generation sequencing was applied. This revealed a distinct cluster of CLL-CpG-EV (3 patients' samples), separated from unstimulated CLL-EV or B-cell-EV (irrespectively of whether the B cells were stimulated or not) (Figure 2A left panel and *Online Supplementary Table S2*). Thus normal B cells do not respond to the TRL9 agonist CpG-ODN2006 as CLL cells do. This was expected, since CLL cells, but not B cells selectively express high levels of TLR9 mRNA and proteins.[13]

Of note, one of the samples not treated with CpG, collected from a patient with a more advanced disease stage and thus undergoing therapy, clustered together with the CLL-CpG-EV (see *Online Supplementary Table S1B* for this patient's characteristics).

Disease-related mRNA were enriched in CLL-CpG-extracellular vesicles and among these were B-cell receptor kinases (LYN, SYK, MAPK1, MAPK2), apoptosis-related genes (BCL2, BCL3) and SF3B1, a splicing factor frequently mutated in CLL (Figure 2A, right panel). The top KEGG pathways identified in CLL-CpG-EV (see legend to Figure 2B for details) were related to spliceosome and ribosome as well as to B-cell receptor or FcγR signaling and to primary immunodeficiency. CLL-relevant molecules were covered by the top upstream regulators (TNFα, CD40, STAT3, IL1, IL6).

To test whether these disease-related mRNA are transferred to non-malignant cells in the tumor microenvironment, we co-incubated DiO-labeled (green) CLL patients' cells with primary fibroblasts ($HDF_n$) or a monocytic cell line (THP-1). The uptake of CLL-released (DiO-labeled) microvesicles (Figure 2C, HDFn) was easily detectable in the co-cultures. Moreover, mRNA of the CLL-associated kinase coactivator and oncogene TCL1A, normally not expressed in these cell types, was detectable at marked levels upon co-incubation with CLL-EV from two independent patients, arguing for a vesicle-dependent transfer of TCL1A-mRNA (Figure 2D, left panels; the primer sequences and quantitative reverse transcriptase polymerase chain reaction conditions are summarized in *Online Supplementary Table S3*). The TCL1A protein was detectable in CLL-EV-incubated THP-1 cells, indicating successful translation of transferred mRNA (Figure 2E). The fact that TCL1A protein was not detectable in fibroblasts correlated with a far less effective TCL1A mRNA uptake of these cells compared to monocytes. Recently a TCL1-transgenic mouse model was used to show that monocytes were indispensable for the development of CLL.[14] Uptake and expression of mRNA encoding the kinases LYN and SYK as well as TNFα was observed for fibroblasts, while in THP-1 the expression remained unchanged (Figure 2D and *Online Supplementary Table S4*). This might reflect cell-specificity of vesicle uptake or rather be attributed to the high endogenous expression of these genes in THP-1 cells, which may mask the transfer of EV-mRNA. Please note that untreated HDF cells have low, but detectable levels of TCL1 and LYN, while SYK and TNF are almost not expressed. Untreated THP-1 cells, on the other hand, do not express TCL1, while LYN, SYK and TNFα are detectable.

To summarize, we identified vesicle-associated disease-relevant mRNA, including splicing factors, the TCL1A oncogene, and tyrosine kinases, which were enriched in CLL-vesicles compared to vesicles from healthy B cells. This data set complements studies on proteins and microRNA transferred by CLL-vesicles[5-7] and might finally contribute to a detailed understanding of tumor-related aberrant gene expression in stromal cells, known to be crucial for CLL survival.[15] Interestingly, the disease-related mRNA signature was pronounced upon TLR engagement used as a milieu-mimic. Moreover, the transfer of enriched mRNA to monocytes and fibroblasts was demonstrated and the otherwise strictly lymphocyte-restricted protein expression of TCL1A was detectable in fibroblasts upon vesicle uptake. These data show that CLL-released vesicles transfer mRNA molecules to non-malignant bystander cells to spread tumor-milieu instructions within the CLL microenvironment.

Thus, TLR signaling may contribute to CLL progression via different mechanisms, including regulation of the mRNA expression profile in vesicles, making this pathway a promising target for novel therapeutic approaches.

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