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Dynamics of Microvesicle Generation in B Cell Chronic Lymphocytic Leukemia: Implication in Disease Progression

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Abstract

Previously, we reported that B-cell chronic lymphocytic leukemia (CLL) patients contained elevated levels of microvesicles (MVs). However, given the quiescent nature of CLL B-cells and the relative indolence of the disease, the dynamics of MV generation and their unique phenotypes are not clearly defined. In this study, we find that CLL B-cells generate MVs spontaneously and can be further induced by B-cell receptor-ligation. Most interestingly, CLL B-cells predominantly generate $CD52^+$ MVs, but not $CD19^+$ MVs *in vitro*, suggesting preferential usage of CD52 into leukemic-MVs and that the CLL plasma MV phenotypes corroborate well with the *in vitro* findings. Importantly, we detected increased accumulation of $CD52^+$ MVs in previously untreated CLL patients with progressive disease. Finally, sequential studies on MVs in pre- and post-therapy CLL patients demonstrate that while the plasma $CD52^+$ MV levels drop significantly after therapy in most and remain at low levels in some patients, a trend of increased accumulation of $CD52^+$ MVs was detected in majority of post-therapy CLL patients (25 of 33). In total this study emphasizes that dynamic accumulation of $CD52^+$ MVs in plasma can be used to study CLL progression and may be a useful biomarker for patients as they progress and require therapy.

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Authorship

Contribution: J.B. and M.N. performed experiments, analyzed data and created figures; G.M., G.P.M. and S.S. performed experiments, and created figures; M.G. performed experiments; SKV performed statistical analysis of the data; T.D.S. provided clinical samples with relevant information; N.E.K. provided clinical samples with relevant information, analyzed data and edited the manuscript; A.K.G. conceived and supervised the project, designed the research, analyzed data and wrote the manuscript; and all authors read and approved the final manuscript.

Disclosure of Conflicts of Interest

Authors declare no potential conflict of interest.

Keywords

Microvesicles; CLL; CD52; BCR; Biomarker

Introduction

Cancer cells emit a heterogeneous mixture of vesicular, organelle-like structures termed “microvesicles” (MVs) into their surroundings(1). Vesicle shedding is preceded by the budding of small cytoplasmic protrusions, which then detach by fission of their stalk. Some release of shedding vesicles takes place from resting cells however, the rate of the process increases dramatically upon stimulation. MVs are generated via diverse biological mechanisms including those featured by activated pathways involved in oncogenic transformation, microenvironmental stimulation, cellular activation, stress or even death(1). Different biological circumstances under which formation of MVs has been observed reflect the diversity of their biogenesis. Thus, cellular activation, transformation, stress or programmed cell death are associated with a varying MV production output and the nature of the vesicular structures(2). In addition, depending on the cell type, membrane MVs may be rich in cellular lineage markers(1).

While it is important to recognize the potential heterogeneity of MV populations, the release of MVs into blood and/or urine calls attention to their promise as circulating-biomarkers in the surveillance of disease progression and/or relapse. We identified increased levels of MVs consisting predominantly of platelet/megakaryocyte derived MVs(3) in early stage CLL (Rai 0–1) and observed a phenotypic shift of predominantly platelet/megakaryocyte derived MVs towards more leukemic B-cell derived MVs in the more advanced Rai stages(3). In the same study we documented that CLL MVs are able to integrate and activate the CLL bone marrow stromal cells with a dramatically increased level of vascular endothelial growth factor (VEGF), an important survival factor for CLL B-cells(3–5). At present, we remain focused in defining the mechanism and dynamics of MV generation and how they may relate to disease progression in CLL.

B-cell receptor (BCR) signaling plays a critical role in the behavior of CLL B-cells. CLL B-cells can be subdivided into two major subsets based on the level of hypermutation for the tumor immunoglobulin variable heavy chain (IGVH) genes(6). Currently it is believed that CLL B-cells with unmutated IGVH (UM-CLL) status derive from naïve $CD5^{+}CD27^{-}$ B-cells, while CLL B-cells with mutated IGVH genes (M-CLL) status may derive from post germinal center $CD5^{+}CD27^{+}$ cells(7, 8). Importantly, these subsets have distinct clinical behavior where UM-CLL has a more aggressive clinical course. Antigen signaling is thought to be ongoing in both subsets and, rather than the presence or absence of signaling, it is the balance between distinct types of antigen responses in these two subsets that appears to determine clinical behavior(9). While, anergy, a state of cellular lethargy that is induced following antigen engagement in the absence of T cell help(10), is observed in all CLL B-cells, it is particularly prominent in M-CLL(9). By contrast, antigen signaling leading to proliferation and survival appears more evident in UM-CLL. A recent report demonstrated that antigen-engagement induced production of increased levels of exosomes from CLL B-

cells(11), however no report is available on MV generation under similar *in vitro* experimental conditions. Given the critical role of MVs in modulating CLL tumor microenvironment(3), we sought to determine the dynamics of MV generation by the leukemic B-cells, and their relevance to the clinical course of CLL.

Our study herein identifies a unique leukemic derived MV marker, CD52, via both *in vitro* and *in vivo studies*. Results from this study also suggest that leukemic B-cell derived MV plasma levels in CLL are biomarker candidates for changes in the clinical status in CLL. Specifically for the latter we found that $CD52^+$ MV plasma levels are associated with disease progression/time to therapy and rise in certain patients following therapy.

Materials and Methods

Reagents

All the chromogen-conjugated antibodies used in flow cytometry and BD Trucount beads were purchased from BD Biosciences. Fluorescein isothiocyanate (FITC)-conjugated annexin V was purchased from Life Technologies. Goat $F(ab')_2$ anti-human IgM was purchased from SouthernBiotech. Ibrutinib (PCI-32765) was purchased from Selleck Chemical(12).

Collection of plasma and primary cells

All CLL patients provided written informed consent according to the Declaration of Helsinki to the Mayo Clinic Institutional Review Board and the University of Oklahoma Institutional Review Board, which approved these studies. Primary CLL B-cells or normal B-cells were purified from blood samples of previously untreated CLL patients or normal, healthy individuals using the RosetteSep B-cell enrichment kit (StemCell Technologies)(12, 13) as needed. Cells were cultured in serum-free AIM-V medium (GIBCO). Of note, we did not supplement fetal bovine serum (FBS) to CLL B-cell cultures as prior study found that FBS induces spontaneous apoptosis in CLL B-cells(14); instead, we used serum-free AIM-V basal media that contain human serum albumin to support primary CLL B-cell growth. Therefore, for comparison and as controls, we cultured purified B-cells isolated from healthy, normal individuals in serum-free AIM-V media, instead of RPMI+10% FBS(15). Plasma samples were collected from CLL patients or age-matched normal, healthy individuals as described earlier(3). Plasma samples were also collected from CLL patients (n=33) who entered onto a clinical trial PCR-B (pentostatin, cyclophosphamide and rituximab [PCR] with bevacizumab [anti-VEGF antibody])(16) conducted at Mayo Clinic at baseline (before therapy) and then sequentially (after therapy) every 6-months until the end of the mandated observation period. In addition, we also collected plasma from treatment naïve CLL patients (n=9) at the time of diagnosis and a second sample before therapy. Finally, CLL plasma was obtained from a limited number of CLL patients (n=5) undergoing ibrutinib-therapy at baseline (before initiation of therapy) and after 3 months of therapy. Freshly collected plasmas were made “platelet-free” as described earlier(3) and kept frozen in aliquots in −80°C until use.

In vitro BCR stimulation

Purified CLL B-cells (5.0×10^6/mL) from treatment naïve CLL patients with mutated (n=15) or unmutated (n=15) IGVH status were stimulated *in vitro* with 10μg/mL goat anti-human IgM for 72 hours or left untreated(17). Purified normal B-cells from healthy individuals (n=5) were also stimulated for comparison. “Used” culture media (serum-free AIM-V) were collected by centrifugation in order to make them cellular debris free and stored in aliquots in −80°C until use.

Isolation of MVs

MVs were isolated from the plasma samples or used culture media by differential centrifugation as described(3). Purified MVs resuspended in PBS and stored in −80°C for subsequent analysis.

Determination of MV levels and phenotypes

Levels and phenotypes of MVs were determined by flow cytometry (BD Canto-I) after staining the MVs with annexin V-FITC(3) and chromogen-conjugated antibodies to various relevant cell surface marker proteins of CLL B-cells and platelet/megakaryocyte. These included antibodies to CD5, CD9, CD19, CD20, CD23, CD37, CD41a, CD52, CD61, PD1, PDL1 and PDL2. BD Trucount beads were used to measure absolute MV levels according to the manufacturer’s instructions.

Determination of CD19 and CD52 expression levels on normal B- and CLL B-cells by flow cytometry

Expression levels of CD19 and CD52 on CLL B-cells and normal B-cells were determined by flow cytometric analysis after staining the cells with chromogen-conjugated isotype control antibodies or antibody to CD19 or CD52. Mean fluorescent intensity (MFI) of the stained cells was used to represent expression levels of each membrane receptor after normalizing with the isotype controls.

Transmission electron microscopy

Purified CLL B-cells (5.0×10^6/mL) were cultured in AIM-V medium, harvested after 48 hours and placed in fixative solution containing 4% formaldehyde and 1% glutaraldehyde in a phosphate buffer, pH 7.3. The fixed sample was manually cut into small pieces and then subjected to a series of chemical solutions that preserved ultrastructure, added stain, removed water and infiltrated with resin. The chemical procedure was as follows: the sample was rinsed with phosphate buffer, followed by treatment with Osmium tetroxide, rinsed with water and then treatment with Uranyl acetate followed by a water rinse. Sample was then treated with the Ethanol series (60–100%; desiccant), 100% Acetone (desiccant) and finally, added Epoxy resin (Spurr)/acetone mix, then pure resin. The sample was then placed into small plastic “BEEM” capsules filled with resin and kept in a 60°C oven to polymerize overnight. The sample was sectioned and subjected to transmission electron microscopy (TEM).

Purified MVs were stained with Uranyl acetate for TEM. A 10μl MV preparation was allowed to settle on glow discharged copper grids for 3 min. The grids were washed and 10μl of Uranyl acetate, pH 4.4 was added to the grids for 30 seconds. The grids were washed, air-dried and observed under a Hitachi H7600 Transmission Electron Microscope equipped with a 2K × 2K AMT digital camera.

Statistical analysis

To compare the MV generation by CLL B-cells between stimulated and unstimulated pathways the Wilcoxon matched-pairs signed-rank test was used. Wilcoxon matched-pairs signed-rank tests were also used to compare levels of CD19 versus CD52 expression on the cell surface. To compare MV generation between the CLL cohorts (i.e. mutated vs unmutated IGVH; normal vs CLL; high vs low Rai-risk; high vs low-risk FISH) the Mann-Whitney test was performed. An alpha of 0.05 was used. Graphs were produced using the PRIZM software. Statistical tests were performed using SAS version 9.4 software (SAS Institute Inc., 2012).

Results

CLL B-cells generate MVs *in vitro*

Only a small fraction (varying from 0.1% to greater than 1.0% of the entire clone) of CLL B-cells proliferates *in vivo* each day while the remainder of CLL B cells remain largely quiescent(18). However, CLL is not a static disease rather, it is now known to be a dynamic process composed of leukemic cells that proliferate and undergo apoptosis(18). Cells when activated or tumor cells which are highly proliferative and active shed MVs(19). Thus, here we explored whether the nonproliferative leukemic B-cells from CLL patients (n=30) shed MVs spontaneously and/or upon *in vitro* BCR stimulation. We detected highly variable levels of spontaneous MV production by the leukemic B-cells after a 72-hour culture (Fig. 1A). However, *in vitro* stimulation of the BCR signaling pathway significantly increased MV production over spontaneous levels from the leukemic B-cells (p=0.0005) obtained from majority of the CLL patients (24 of 30; Fig. 1A). Of note, an *in vitro* time-dependent activation of the BCR signaling in leukemic B-cells detected increased accumulation of MVs after 72 hours when compared to 24- or 48-hour BCR stimulation (Supplementary Fig. 1). Based on published studies, survival(20, 21), rather than apoptosis induction(22), seems to be the more common response of CLL B-cells to anti-IgM stimulation (23). Further analysis demonstrated that while both the M-IGVH and UM-IGVH CLL clones produced increased levels of MVs in response to BCR-ligation compared to unstimulated control cells (M-IGVH, p=0.1688, Fig. 1B; UM-IGVH, p=0.0002, Fig. 1C), the majority of UM-IGVH clones had a trend to be more sensitive to BCR-simulation than M-IGVH clones. Thus, the median difference of MV levels between stimulated and unstimulated UM-IGVH clones was 14474 vs 2761 although the difference did not reach statistical significance (p=0.1688 by Wilcoxon two-sample test).

CLL B-cells generate predominantly $CD52^+$ MVs *in vitro*

MVs generated from the purified CLL B-cells *in vitro* with or without BCR-stimulation described above were phenotyped using antibodies to various cell surface markers and

analyzed on BD Canto-I flow cytometer using standard counting beads. We found that CLL B-cells spontaneously shed primarily $CD52^+$ MVs ($p<0.0001$), but not the B-cell lineage specific marker $CD19^+$ MVs (Fig. 1D). However, we also found that the levels of $CD52^+$ MVs were increased ($p=0.0002$) but not the $CD19^+$ MV levels ($p=0.7254$) upon BCR-stimulation of CLL B-cells (Fig. 1D). Of note, we did not find any significant levels of MVs carrying CD5, CD20, CD23 or CD37(24) markers, nor did we detect MVs simultaneously expressing both CD19 and CD52 proteins (data not shown). Interestingly, we found that normal B-cells also preferentially shed $CD52^+$ MVs (Fig. 1E) under similar *in vitro* experimental conditions.

Although we found that BCR-ligation augments *in vitro* MV production, inhibition of the tonic BCR signal in CLL B-cells by ibrutinib(12) did not have any impact on spontaneous production of MVs by the leukemic B-cells *in vitro* (Fig. 1F); suggesting that other yet to be defined intrinsic mechanisms in the leukemic B-cells are involved in regulating spontaneous MV production. Given the *in vitro* findings, we then examined the status of plasma $CD52^+$ MV levels in CLL patients before and after ibrutinib-therapy. To begin to do this, we collected plasma MVs from CLL patients (n=5) before and 3 months after ibrutinib-therapy and determined $CD52^+$ MV levels. We found that 4 of 5 CLL patients exhibited reduced levels of plasma $CD52^+$ MVs after 3 months of therapy as compared to the pre-therapy CLL plasma samples (Fig. 1G). It is possible that the reduction of $CD52^+$ MV levels after 3 months of ibrutinib-treatment resulted from reduced tumor burden in the CLL patients. To support this possibility we found that the Pearson correlation measurement between the changes in $CD52^+$ MV levels and absolute lymphocyte counts (ALC) was 0.98 (95% Confidence Interval 0.76–1.00) suggesting a positive association between the two parameters in this small cohort.

To further validate that CLL B-cells are able to shed MVs spontaneously, we performed transmission electron microscopy (TEM) of purified CLL B-cells with UM-IGVH cultured *in vitro* for 48 hours without exogenous stimulation. Observation of the cells under EM suggests that indeed CLL B-cells shed MVs (Fig. 2, A,B,C). In addition it would appear based on size determination that both exosomes and MVs are being produced by CLL B-cells. Similarly, we were also able to detect MVs purified from “used” media (72 hours) of *in vitro* CLL B-cell culture (Fig. 2D) as well as CLL plasma (Fig. 2E) obtained from the same CLL patient as heterogeneous populations under electron microscope as reported earlier(3).

CLL B-cells and normal B-cells express higher levels of CD52 than CD19

Given that we had found CLL B cells shed primarily $CD52^+$ MVs but not $CD19^+$ MVs, we then wished to determine the comparative expression levels of CD19 and CD52 on normal and leukemic B-cells. For this, both cell types were stained with an antibody to CD19 or CD52 and analyzed by flow cytometry. Our findings suggest that both normal B- and CLL B-cells express significantly higher levels of CD52 than CD19 ($p<0.0001$) (Fig. 2F). Interestingly, the mean expression level of CD52 on normal B-cells was ~3-fold higher than that on the leukemic B-cells ($p<0.0001$). Given this latter data we hypothesized that lower

expression levels of CD52 on CLL B-cells could be a result of increased shedding of $CD52^+$ MVs by the leukemic cells.

Shedding of $CD52^+$ MVs results in reduction of CD52 expression levels on CLL B-cells

To address the above hypothesis, we next investigated whether shedding of $CD52^+$ MVs *in vitro* either spontaneously or upon BCR stimulation reduced expression levels of CD52 on CLL B-cells. For this, expression levels of CD52 and CD19 on freshly isolated, purified CLL B-cells were determined by flow cytometric analyses immediately before culture (time 0) or after 72 hours of *in vitro* culture with or without BCR stimulation. Interestingly, we found that while there was no detectable alteration of the CD19 membrane expression levels on CLL B-cells, significant reduction of CD52 levels on CLL B-cells was discernible after 72 hours of *in vitro* culture with ($p<0.0001$) or without ($p=0.006$) BCR stimulation (Fig. 2G). This finding is consistent with the possibility that shedding of $CD52^+$ MVs is, at least in part, responsible for the lower levels of CD52 on CLL B-cells. To this end we examined if there is an inverse association between shedding of $CD52^+$ MVs by CLL B-cells and their cell surface CD52 expression levels. For this, purified CLL B-cells from previously untreated CLL patients (n=11) were stimulated by BCR-ligation or left unstimulated *in vitro* for 72 hours. Levels of $CD52^+$ MVs in the used culture media and CD52 expression levels on CLL B-cells with or without BCR-stimulation were then determined. Consistent with our observation above (Fig. 2G) we found a decrease of CD52 membrane levels on CLL B-cells following BCR-stimulation when compared to the unstimulated control cells (Fig. 2H). The median decrease of membrane CD52 expression assessed by change in CD52 MFI on CLL B-cells after BCR-stimulation was −75 (MFI ranged from −498.2 to −2). In contrast, increased shedding of $CD52^+$ MVs, albeit at variable levels (median increase was 7291 with a range from 282 to 103,768), by the leukemic B-cells was noted after *in vitro* BCR engagement. While the difference was variable and not normally distributed, a Spearman correlation analysis between these differences found a Spearman rho of −0.60 (a p-value of 0.0510) indicating an inverse relationship between the CD52 expression on CLL B-cell surface and the $CD52^+$ MV shedding by CLL B-cells.

Shedding of $CD52^+$ MVs is a property of human B-lymphocytes

Having detected predominantly $CD52^+$ MVs in the culture media of purified CLL B-cells or normal B-cells, we investigated whether CLL plasma or normal plasma from healthy individuals had primarily $CD52^+$ MVs versus $CD19^+$ MVs. To address this, MVs were isolated from CLL (n=33) or normal (n=10) plasma and phenotyped. Indeed we detected significantly higher levels ($p<0.0001$) of $CD52^+$ MVs as compared to $CD19^+$ MVs in CLL circulation (Fig. 3A). MVs isolated from plasma of normal, healthy individuals also showed similar patterns of MV phenotypes ($CD19^+$ vs. $CD52^+$ MV levels had a median difference of 15896; $p=0.002$) albeit at significantly lower levels than those in CLL plasma. When we compared differences between $CD52^+$ and $CD19^+$ MV levels for normal and CLL plasma samples we found a p-value of 0.0002 comparing the median values in both; normal plasma=15896, CLL plasma=115624 (Fig. 3A). Thus, the phenotype pattern of plasma MVs in CLL patients is similar to the phenotypes of *in vitro* generated MVs from leukemic B-cells.

Next, we explored whether the levels of $CD52^+$ MVs in CLL plasma were associated with known prognostic CLL risk factors. To that end, further analysis of the CLL plasma MV phenotypes (as shown in Fig. 3A) detected no significant difference in the levels of $CD19^+$ (p=0.4824) or $CD52^+$ (p=0.6525) MVs between the CLL patients with M-IGVH vs. UM-IGVH status (Fig. 3B). While the levels of $CD52^+$ MVs in CLL circulation did not show any positive association to high Rai-risk (Rai-III/IV) vs. low Rai-risk (Rai-0/I) CLL patients (Fig. 3C; median 145511 vs 138317, p=0.9661), we did detect a trend for $CD52^+$ MV levels to increase in CLL patients with high-risk FISH (17p-/11q-) when compared to CLL cohort with low-risk FISH (13q-/Tri12 or no genetic abnormalities) (Fig. 3D; median 166186 vs 94402, p=0.1635).

Accumulation of $CD52^+$ MVs may predict CLL progression or time of therapy

We next attempted to determine whether leukemic B-cell derived MV levels in CLL patients were associated with disease progression and time to therapy. Initially, we isolated MVs from the plasma of a small CLL patient cohort (n=9) at or near diagnosis and at time points close (0.4–1.8 months) to initiation of therapy (n=6) or ~2–8 months before start of therapy (n=3). We found that $CD52^+$ MV levels in CLL plasma increased substantially a few weeks before clinical detection of disease progression (Fig. 4). In most cases, the progression event was accompanied by an increase in Rai-stage, ALC values and/or a decrease in platelet counts (Fig. 4; Supplementary Table 1) however, all patients were subsequently treated. It was also found that increase of plasma $CD52^+$ MV levels was associated with increases of ALC levels (7 of 9 patients) and/or progression to higher Rai-stages (6 of 9 patients). Interestingly, platelet-derived ($CD61^+$) MV levels were also increased despite moderate to severe thrombocytopenia (Fig. 4). Of note, MV parameters in plasma from two CLL patients with stable disease did not show a significant increase of leukemic B-cell derived MVs in their circulation over a period of 9–12 months (Supplementary Fig. 2). Together these observations suggest that accumulation of $CD52^+$ MVs in CLL may be associated with disease progression in previously untreated patients.

Dynamics of leukemic B-cell derived MVs in pre- and post-therapy CLL patients

To further define the dynamics of MV levels in CLL we assessed sequentially the plasma levels of MV generation in CLL patients who were treated. Here MVs were isolated from plasma of CLL patients (n=33) who were treated with 6 cycles of a PCR-B therapeutic regimen previously reported(16) at baseline (just before therapy) and then every 6-months for a maximum of 4 years after therapy. Leukemic B-cell derived MV ($CD52^+$) levels were plotted against the blood ALC values and shown graphically in Fig. 5 and in Supplementary Figs. 3A,B,C. $CD52^+$ MV levels dropped after therapy within 6–12 months in most (27 of 33) of the CLL patients, while in 6 of 33 (18%) CLL patients (P16, P17, P19, P20, P22, P25) we observed an initial rise of $CD52^+$ MV levels after therapy which was then followed by a decrease in MV levels (Fig. 5, Supplementary Fig. 3 A&B). In addition, we noted rising levels of plasma $CD52^+$ MVs in post-therapy CLL plasma after an initial drop with no obvious disease progression (16 of 33) (Fig. 5, Supplementary Figs. 3A,B,C). Of importance, 7 of these 16 patients progressed at later time points with a median relapse time of 45 months from the time of therapy.

Discussion

In this study we evaluated the *in vitro* dynamics of MV generation by CLL leukemic B-cells known to be largely quiescent(18). We found that while *in vitro* BCR-stimulation significantly increased MV production from CLL B-cells with UM-IGVH status, leukemic B-cells with M-IGVH status also responded well to BCR-ligation based on *in vitro* MV production. There was a subset of CLL clones with M-IGVH status where we found a decrease in the *in vitro* MV production upon BCR-stimulation. It is unclear why this occurred but may be a result of cellular anergy in CLL B-cells upon antigen engagement in the absence of T cell help that is more prominent in M-CLL(9). Of interest, further analysis suggests that the tonic BCR signal may not be the only factor influencing spontaneous shedding of MVs by the leukemic B-cells, as *in vitro* ibrutinib-treatment could not block basal level production of MVs from CLL B-cells while it is known that ibrutinib can block exosome production(11). Given this it is likely that other intrinsic and/or tumor microenvironmental factors yet to be elucidated are critically involved in regulating the spontaneous generation of MVs from CLL B-cells. However, the reduction of plasma $CD52^+$ MVs by ibrutinib reinforces the possibility that MV plasma levels can be biomarkers for CLL disease status.

This work also detected significantly higher levels of $CD52^+$ MVs compared to $CD19^+$ MVs in our *in vitro* CLL B-cell culture system. In many cases secreted MVs carry surface receptors characteristic of the originator cells(25). Of note, while we detected a lower level of *in vitro* generated MVs carrying CD19 antigen there were also remarkably low or undetectable levels of $CD5^+$, $CD23^+$ or $CD37^+$ MVs in the *in vitro* generated MV pool. We also found that CLL B-cells and normal B-cells both express significantly higher levels of CD52 than CD19 on cell surface however; normal B-cells express ~3-fold higher CD52 levels compared to that on leukemic B-cells. Given this, we hypothesized that increased shedding of $CD52^+$ MVs by the leukemic B-cells is a process that might result in the reduced expression of CD52 on the cell surface. Indeed, we detected significant reduction of CD52 levels on CLL B-cell surface after 72 hours of culture while no significant alteration of the CD19 levels under similar experimental conditions was observed. Thus, it appears that spontaneous or induced production of $CD52^+$ MVs can contribute to a reduction of CD52 expression levels on CLL B-cells. As further confirmation of the latter possibility we detected an inverse correlation between decrease of CD52 expression levels on CLL B-cells and increased shedding of $CD52^+$ MVs by the leukemic B-cells after BCR-stimulation. Of importance, *in vivo* findings on MV phenotypes isolated from CLL plasma also corroborate well with the *in vitro* observation that CLL B-cells primarily generate $CD52^+$ MVs. Further analysis suggests that normal B-cells also generate primarily $CD52^+$ MVs *in vitro* and *in vivo* albeit at much lower levels than the leukemic B-cells suggesting that the generation and shedding of $CD52^+$ MVs is a physiologic characteristic of human B-lymphocytes. To our knowledge this is the first report to demonstrate that CLL B-cells/normal B-cells tend to shed predominantly $CD52^+$ MVs, not $CD19^+$ MVs.

In explaining why CD52, but not CD19, is preferentially a component of leukemic-MVs it is important to take into context the known function of each. CD52 is a small, heavily glycosylated protein of 12 amino acids anchored to glycosylphosphatidylinositol (GPI) and

expressed at high levels (5×10^5 molecules per cell) on human lymphocytes, sperm cells and monocytes(26, 27). CD52 is also a very effective target for CD52 specific monoclonal antibodies (i.e. alemtuzumab) in the induction of complement mediated lysis, antibody-mediated cellular cytotoxicity(28), and results in very dramatic depletion of T- and B-cells *in vivo* (29). Thus, leukemic B-cells may escape complement mediated lysis and/or cytotoxic effects of the antibody-based therapeutic agent, alemtuzumab, by shedding increased levels of $CD52^+$ MVs in circulation. It is reported in earlier studies that CLL plasma contain increased levels of free circulating soluble CD52(30) however given our current work we believe CD52 is bound to plasma MVs(3). Since CD52 is highly negatively charged and present on sperm cells and lymphocytes, it has been conjectured that its function is related to anti-adhesion activity, allowing cells to freely circulate(31). Of note, CD52 binds the ITIM (Immunoreceptor tyrosine-based inhibitory motif)-bearing sialic acid-binding lectin SIGLEC10(32). In contrast CD19 is critically involved in establishing intrinsic B-cell signaling thresholds through modulating both BCR-dependent and independent signaling(33, 34). CD19 acts as a vital co-receptor for BCR signal transduction and, recruits and amplifies the activation of Src-family protein tyrosine kinases such as Lyn and Fyn(35–38). Upon BCR activation, CD19 also enhances BCR-induced signaling crucial for B-cell expansion, through recruitment and activation of PI3K and downstream Akt kinases(39). It also functions as the dominant signaling component of a multimolecular complex on the surface of mature B-cells, alongside complement receptor CD21 (CD2), and the tetraspanin membrane protein CD81 (TAPA-1), as well as CD225(35, 36, 40, 41). CD19 is critical for B-cell signaling and expansion while the role of CD52 in B-cell survival is not yet well defined but given the above we would propose that it is likely that CLL B-cells or normal B-cells need to preferentially retain CD19 as it is a key factor involved in B-cell survival signaling complex.

Having established that CLL B-cells preferentially shed $CD52^+$ MVs both *in vitro* and *in vivo*, we interrogated whether increasing plasma accumulation of leukemic B-cell derived MVs would be predictive of disease progression. Indeed in CLL patients (n=9) with evidence for progression we detected an increase of $CD52^+$ MVs in circulation of 7 of 9 CLL patients as they progressed from a more indolent phase, while no significant alteration of the $CD52^+$ MV levels was found in 2 CLL patients with stable disease. These early findings, while seen in only a small cohort of CLL patients, do indicate that further validation in a much larger cohort will be of value in continuing to pursue the possibility that $CD52^+$ MVs can be a biomarker of disease status in CLL.

To further our knowledge of *in vivo* MV dynamics in CLL patients, we studied MV parameters in pre- and post-therapy CLL plasma in a cohort of 33 CLL patients treated with the PCR-B protocol. While plasma $CD52^+$ MV levels dropped in the majority of CLL patients 6–12 months after therapy, in ~48% cases, $CD52^+$ MV levels started to rise with time with no apparent sign of clinical progression. The observed rise of post-therapy plasma $CD52^+$ MV levels appears to be independent of the status of the clinical response of the patients to therapy as determined at 6 months of treatment, e.g., complete remission (CR), partial remission (PR) or nodular(n)PR (Fig. 5, Supplementary Fig. 3A,B,C). Two relevant observations in the PCR-B patient study were: (i) elevation of $CD52^+$ MV levels in post-therapy CLL plasma at early time points with clinical manifestation of the disease only

noted at a later time point (n=10) and subsequent treatment; and (ii) elevation of $CD52^+$ MV levels in post-therapy CLL plasma (n=16) where there were no signs of clinical progression over the time of observation (Fig. 5, Supplementary Fig. 3A,B,C). Although intermittent elevations of $CD52^+$ MV levels can be a result of leukemic B-cell activation in patients who have infections or inflammatory complications, a steady and consistent rise of $CD52^+$ MV levels in the latter scenario is most likely an early indication of leukemic growth and future relapse. We believe that collection of sequential blood samples for MV evaluation at shorter time intervals could be critical in assessing the value of $CD52^+$ MVs as biomarker for CLL disease. Since MVs are released from leukemic B-cells regardless of IGVH status and which tissue site they reside in and ultimately circulate into the plasma pool, it is feasible that serial, timely and accurate measurements of plasma MVs will be found to be extremely sensitive biomarker measures of tumor burden and/or progression. Further studies are needed to validate this possibility as it does hold the promise of predicting occurrence of relapse earlier than clinical manifestation of the disease.

In summary our studies have found that CLL B cells whether of mutated or unmutated IGVH status can produce and secrete $CD52^+$ MVs. The stimulation of MV generation by BCR-ligation can accentuate the spontaneous production of MVs from CLL B cells whether of mutated or unmutated status indicating that the microenvironment can modulate MV production in CLL. In addition the levels of MVs appear to be associated with disease progression for previously untreated CLL. Future studies are now focused on a more extensive analysis of plasma $CD52^+$ MVs in association with CLL disease progression, depth of clinical response, clinical complications such as infections and to determine if the elevation of the $CD52^+$ MV plasma level may be a relevant biomarker predictive of progression or relapse for both previously untreated and treated CLL patients respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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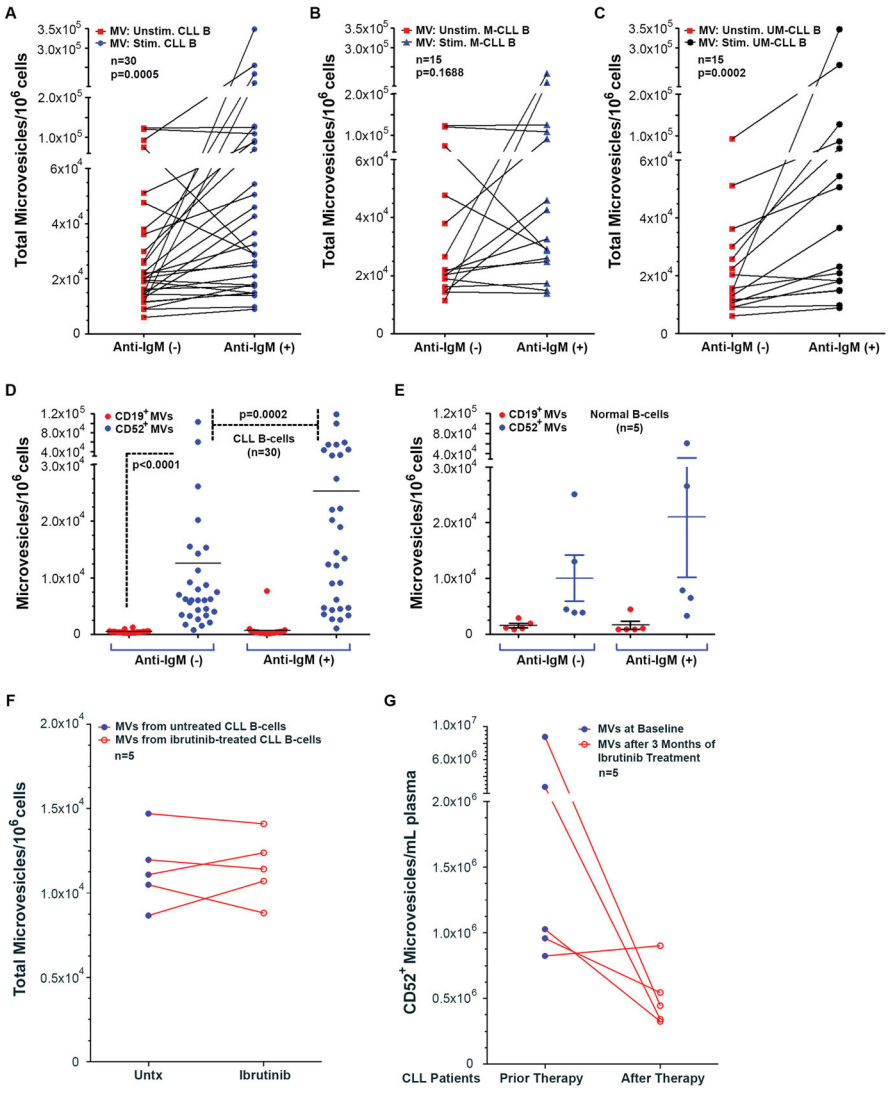

Figure 1. Dynamics of leukemic B-cell derived microvesicle generation *in vitro*

(A) CLL B-cells generate MVs spontaneously and upon BCR-stimulation. Freshly isolated, purified CLL B-cells from previously untreated CLL patients (n=30) were treated with anti-IgM antibody for 72 hours or left untreated. MVs were isolated from the used culture media and levels of total MVs generated in CLL B-cell culture were determined by flow cytometric analysis using BD Trucount beads after staining the MVs with annexin V-FITC. CLL B-cells spontaneously produce MVs *in vitro* however; BCR-stimulation increases the production of MVs from CLL B-cells. **(B & C) CLL B-cells with unmutated or mutated IGVH status generate increased levels of MVs upon BCR-stimulation.** MVs generated in the used culture media of CLL B-cells from the CLL patients mentioned above (**panel A**) were divided into two groups based on the IGVH mutational status of the leukemic B-cells in order to determine levels of MV generation in response to BCR-ligation. CLL B-cells with UM-IGVH (**panel C**) appear to be more sensitive to BCR-stimulation than the cells with M-IGVH (**panel B**) in terms of MV generation *in vitro*. **(D) CLL B-cells generate primarily CD52^{+} MVs *in vitro***. MVs generated in purified CLL B-cell culture *in*

vitro with or without BCR-stimulation (n=30) as described above (**panel A)** were phenotyped using chromogen-conjugated antibodies to various cell surface markers including CD19 and CD52. MV levels were determined using standard BD Trucount beads. $CD19^+/CD52^+$ MV levels are presented as number of MVs per 10^6 CLL B-cells. **(E) Normal B-cells preferentially shed $CD52^+$ MVs.** Freshly isolated, purified normal B-cells from healthy individuals (n=5) were treated with anti-IgM or left untreated, and MVs were isolated from the used culture media. MVs were phenotyped and levels of $CD19^+/CD52^+$ MVs were determined by flow cytometry. Results are presented as number of MVs per 10^6 normal B-cells. **(F) Inhibition of BCR signal pathway showed no impact on spontaneous generation of MVs.** Freshly isolated, purified CLL B-cells from previously untreated CLL patients (n=5) with UM-IGVH status were treated with 0.5μM of ibrutinib and cultured for 72 hours or left untreated. MVs were isolated from the used culture media and total MV levels were determined as described above. Values of individual CLL patients are shown. **(G) Ibrutinib-therapy reduced $CD52^+$ MV levels in CLL circulation.** MVs were isolated from plasma of CLL patients (n=5) before (baseline) and 3 months after ibrutinib-therapy. MVs were phenotyped and levels of $CD52^+$ MVs in CLL plasma were determined as described above. Values of individual CLL patients are shown. Four of five CLL patients showed substantial reduction of plasma $CD52^+$ MV levels 3 months after ibrutinib-therapy.

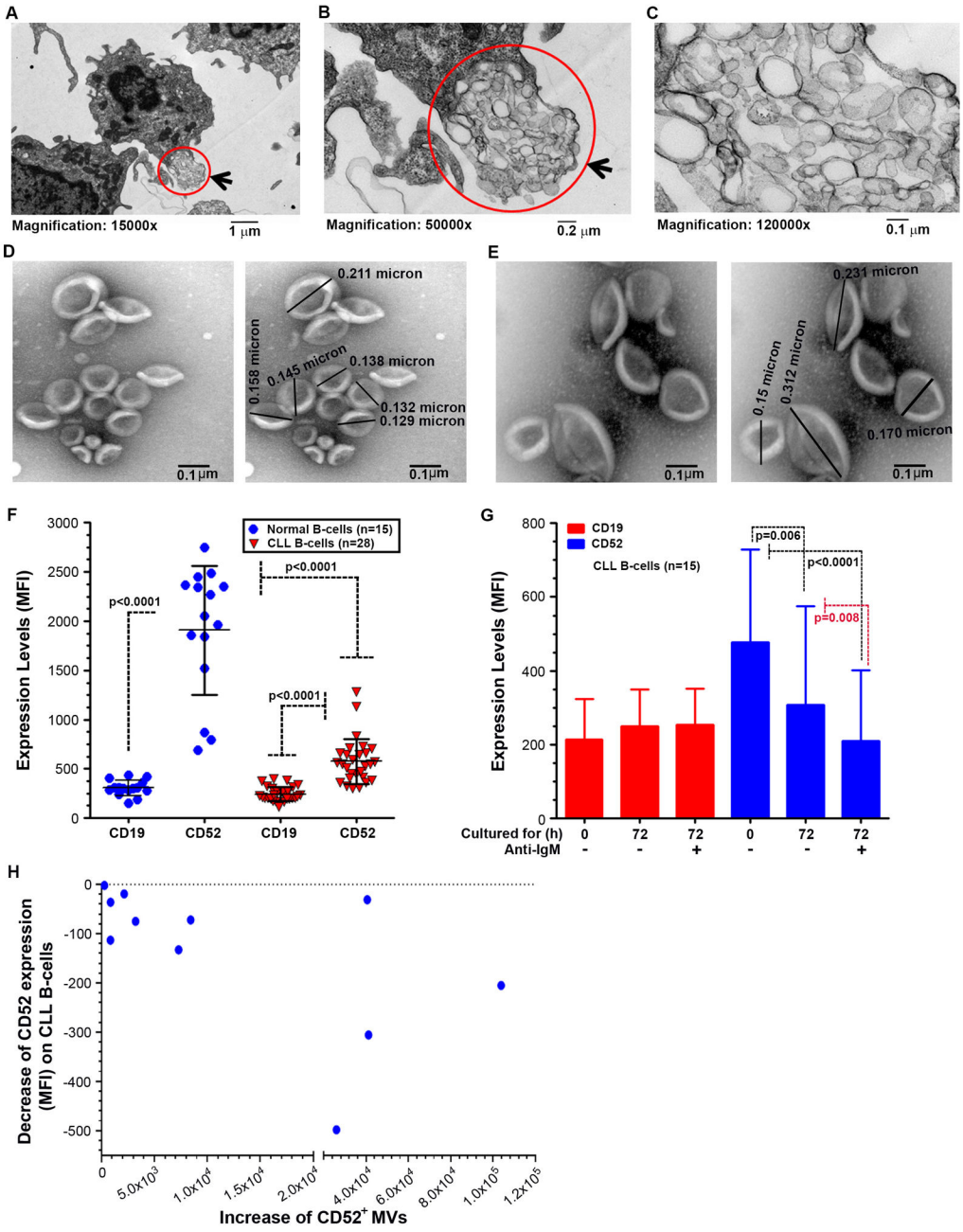

Figure 2. Microvesicle generation and expression status of CD19/CD52 on CLL B-cells *in vitro*

(A, B, C) Electron micrograph of CLL B-cells. Freshly isolated, purified CLL B-cells were cultured *in vitro* for 48 hours. Cells were harvested and processed as described in "Material and Methods" for transmission electron microscopy. The circle and arrowhead indicate accumulation of MVs from a typical CLL B-cell (**panels A & B**). **Panel C** is showing the same MV population at higher magnification (x120000) under electron microscope. Horizontal lines indicate size of the vesicles. Magnification of the respective field is also indicated. **(D & E) Electron micrograph of purified MVs.** Spontaneously generated MVs in the culture media of CLL B-cells after a 72-hour incubation (**panel D**)

were purified or MVs were isolated from CLL plasma (**panel E**) obtained from the same CLL patient and subjected to electron microscopy after staining the MVs with Uranyl acetate as described in the “Materials and Methods”. Size measurement of individual MVs is shown in separate panels. Horizontal lines indicate size of the vesicles. Magnification: 50000x. **(F) CLL B-cells and normal B-cells express higher levels of CD52 than CD19.** CLL B-cells or normal B-cells were stained with chromogen-conjugated control isotype-specific antibodies or antibody to CD19 or CD52 and analyzed on flow cytometer. Expression levels of CD19 and CD52 are presented as mean fluorescent intensity (MFI) after normalizing with the isotype controls. Comparison between the CD19 and CD52 expression levels on cell surface within each group or between the two groups was analyzed and presented. **(G) Increased shedding of $CD52^+$ MVs is associated with reduced CD52 levels on CLL B-cells.** CLL B-cells were stained with a chromogen-conjugated antibody to CD52 or CD19 before *in vitro* culture (time 0) or after a 72-hour culture with or without anti-IgM treatment. Expression levels of CD19 or CD52 were determined by flow cytometric analysis and presented as MFI after normalizing with the isotype controls. Mean MFI values with standard deviations are shown. **(H) Association of increased $CD52^+$ MV levels in culture and simultaneous reduction of CD52 expression levels on CLL B-cells.** Freshly isolated, purified CLL B-cells from previously untreated CLL patients (n=11) were stimulated *in vitro* with anti-IgM treatment or left unstimulated for 72 hours in culture. Cells were harvested, stained with a chromogen-conjugated antibody to CD52 and analyzed by flow cytometry to determine CD52 expression levels. MVs were isolated from the used culture media and the $CD52^+$ MV levels in culture were determined by flow cytometric analysis as described elsewhere. Difference of CD52 expression levels (MFI) on CLL B-cells and levels of $CD52^+$ MV levels shed by the leukemic B-cells before and after BCR-stimulation was determined and we performed a Spearman correlation between these differences. The Spearman rho is −0.60 with a p-value of 0.0510 indicating an inverse relationship between the $CD52^+$ MV levels found in culture medium when compared to the expression levels of CD52 on CLL B cells.

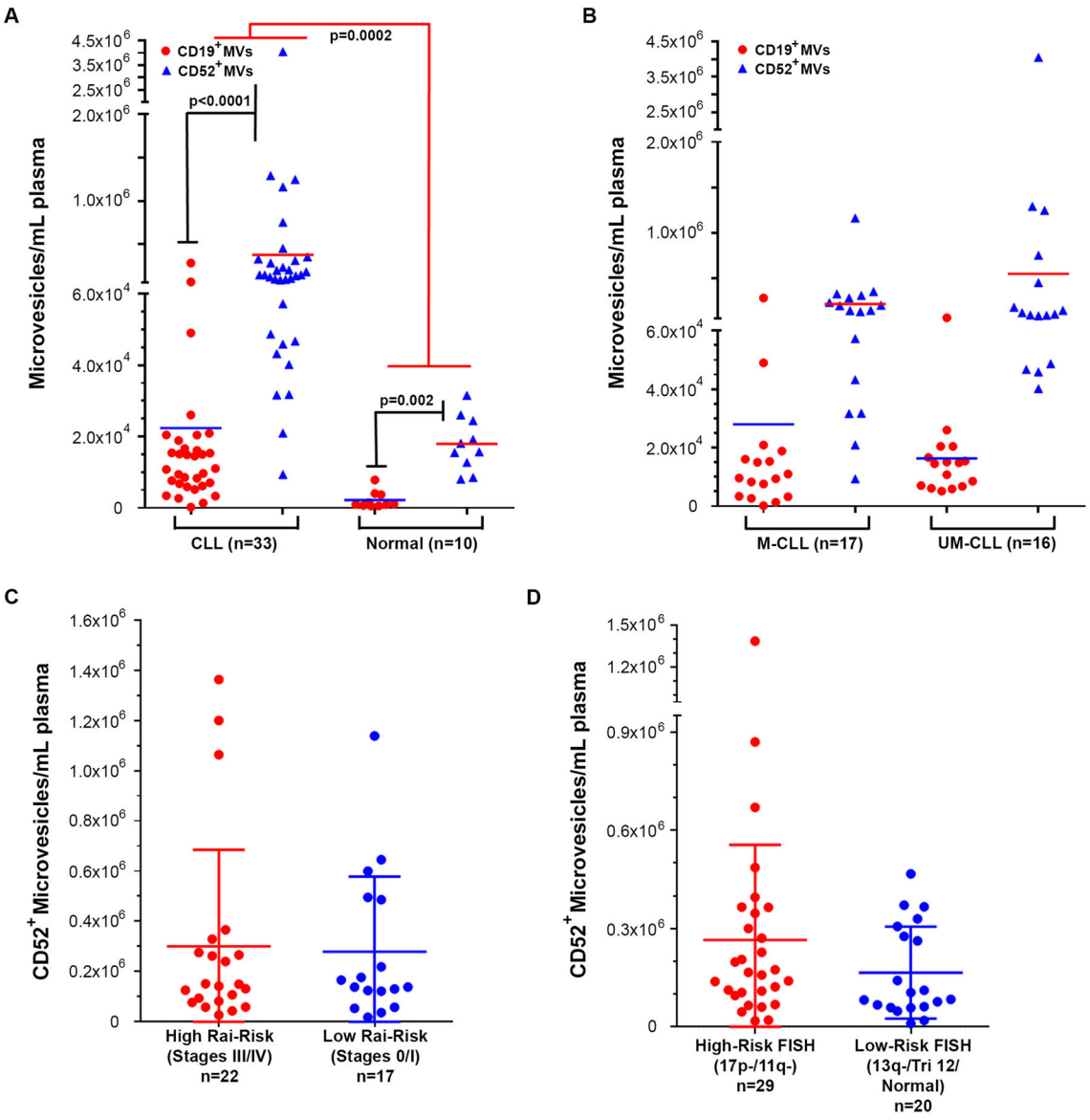

Figure 3. ***In vivo*** **features of MVs generated in CLL circulation**

(A) CLL plasma contain elevated levels of CD52$^+$ MVs. Plasma samples were collected from previously untreated CLL patients (n=33) or age-matched normal, healthy individuals (n=10) for MV isolation. Phenotypes and levels of MVs were determined by flow cytometry using antibodies to various cell surface markers as described in “Materials and Methods”. Levels of CD19$^+$ and CD52$^+$ MVs are presented as number of MVs per mL of plasma. Mann-Whitney unpaired t-tests were performed to compare the levels of CD19$^+$ or CD52$^+$ MV levels between the groups or within the group as specified. **(B) Accumulation of CD52$^+$ MVs in CLL circulation does not correlate with the IGVH mutational status.** The above CLL patients cohort (n=33) was grouped based on their IGVH mutational status and the levels of plasma CD52$^+$ MVs between the groups, M-IGVH (n=17) vs. UM-IGVH (n=16) were compared. **(C) Levels of plasma CD52$^+$ MVs in CLL patients are not associated with Rai-risk.** MVs were isolated from the plasma of CLL patients with high

Rai-risk (Rai-stages III/IV; n=22) and low Rai-risk (Rai-stages 0/I; n=17). Levels of $CD52^+$ MVs were determined and compared between the groups as described above. **(D) Relation of plasma $CD52^+$ MV levels in CLL patients with low versus high risk FISH categories.** MVs were isolated from the plasma of CLL patients with high-risk FISH (17p13.1-/11q22.3-; n=29) and low-risk FISH (13q14-/trisomy 12/normal; n=20). Levels of $CD52^+$ MVs were determined and compared. While the difference of $CD52^+$ MV levels between the groups was not statistically significant (p=0.1635), a trend for increased accumulation of $CD52^+$ MVs was evident in high-risk FISH CLL.

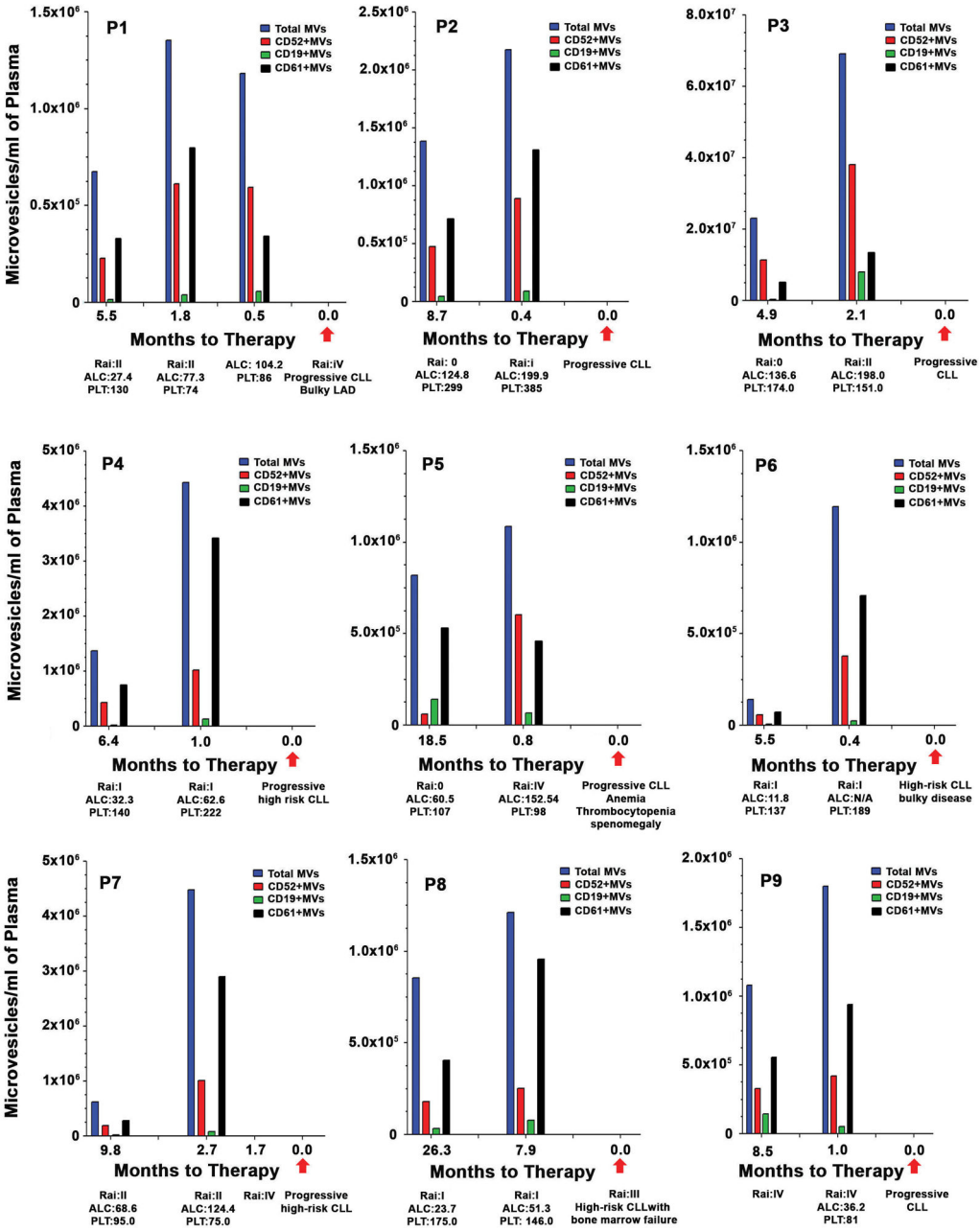

Figure 4. Dynamics of leukemic B-cell derived MVs to CLL disease progression

Total MVs were isolated from plasma of CLL patients (n=9) sequentially as indicated until time to therapy. Levels of total (annexin V^+), $CD19^+$, $CD52^+$ and $CD61^+$ MVs were determined by flow cytometric analysis as described elsewhere. Upward arrow indicates initiation of therapy. Relevant clinical information of the CLL patients including Rai-stage, absolute lymphocyte count (ALC) and platelet count (PLT) at each time point are provided at the bottom of each panel (P1–P9). LAD refers to lymphadenopathy and “high-risk” refers to those patients with 17p13.1/11q22.3 chromosomal deletion. Individual CLL patients are indicated by assigning arbitrary numbers (P1–P9).

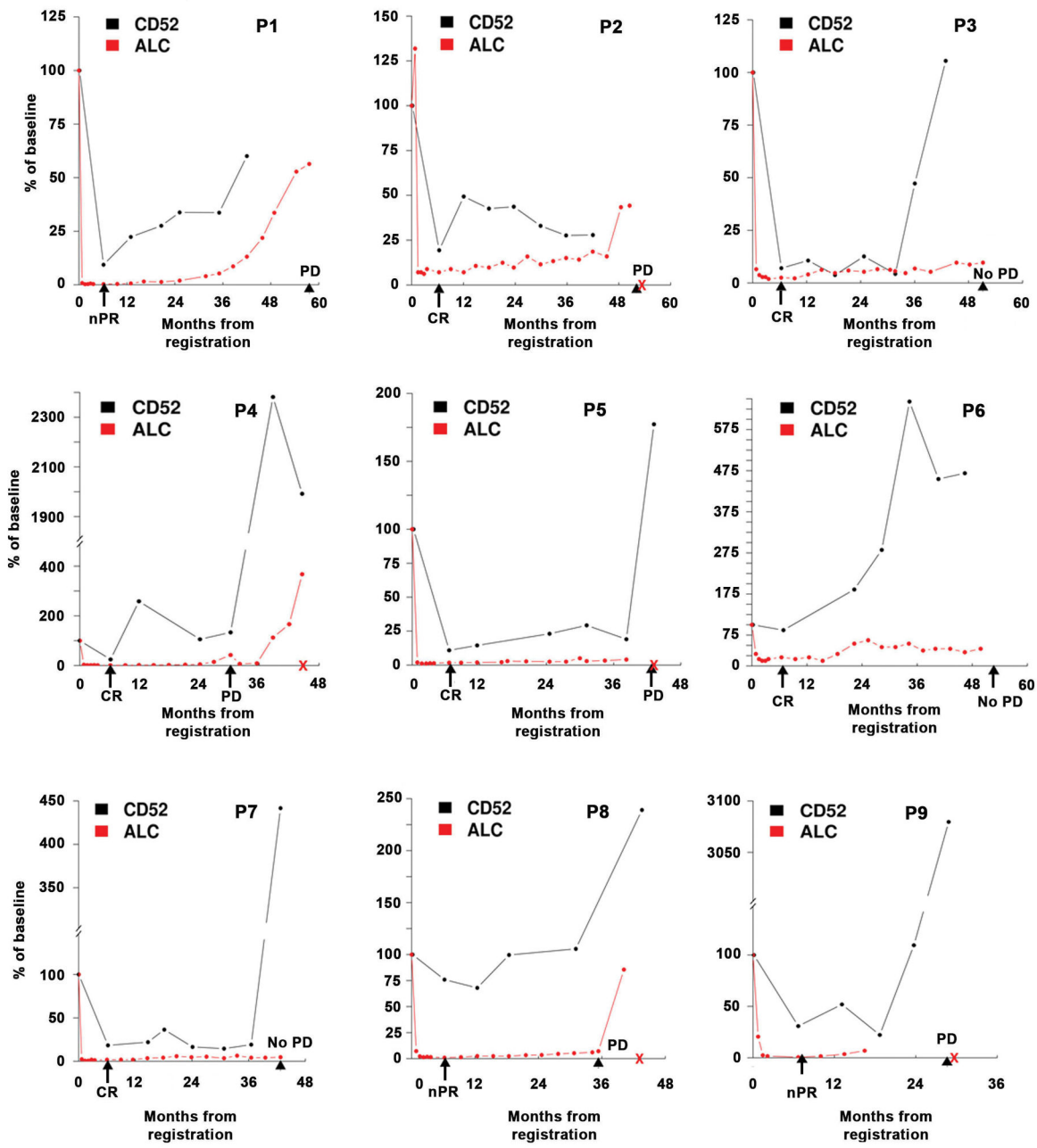

Figure 5. Dynamics of MV generation in pre- and post-therapy CLL plasma

MVs were isolated from plasma of CLL patients at baseline (before therapy) and then, sequentially at every 6 months after therapy up to a maximum observation period of 4 years. CLL patients were treated with the PCR-B (pentostatin, cyclophosphamide and rituximab with bevacizumab) regimen. Levels of $CD52^+$ MVs in pre- and post-therapy CLL plasma were plotted against the ALC values as indicated. Results are presented as percent of baseline. Therapeutic evaluation of the patients and post-therapy clinical progression of the disease (PD) are indicated. “X” indicates initiation of therapy after disease progression. Complete remission, partial remission and nodular partial remission are indicated by CR, PR

and nPR, respectively. Individual CLL patients are indicated by assigning arbitrary numbers (P1–P9).