REGULAR ARTICLE

blood advances

Targeting conditioned media dependencies and FLT-3 in chronic lymphocytic leukemia

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Key Points

- CCL2 is critical for stroma-dependent CLL survival and combination of anti-CCL2 and venetoclax is highly active in killing primary CLL cells.
- Signature including IGHV status can distinguish stroma-independent versus stroma-dependent CLL that can be targeted by FLT3 inhibitors.

The importance of the stromal microenvironment in chronic lymphocytic leukemia (CLL) pathogenesis and drug resistance is well established. Despite recent advances in CLL therapy, identifying novel ways to disrupt interactions between CLL and its microenvironment may identify new combination partners for the drugs currently in use. To understand the role of microenvironmental factors on primary CLL cells, we took advantage of an observation that conditioned media (CM) collected from stroma was protective of CLL cells from spontaneous cell death ex vivo. The cytokine in the CM-dependent cells that most supports CLL survival in short-term ex vivo culture was CCL2. Pretreatment of CLL cells with anti-CCL2 antibody enhanced venetoclax-mediated killing. Surprisingly, we found a group of CLL samples (9/23 cases) that are less likely to undergo cell death in the absence of CM support. Functional studies revealed that CM-independent (CMI) CLL cells are less sensitive to apoptosis than conventional stroma-dependent CLL. In addition, a majority of the CMI CLL samples (80%) harbored unmutated immunoglobulin heavy-chain variable (IGHV) region. Bulk-RNA sequence analysis revealed upregulation of the focal adhesion and RAS signaling pathways in this group, along with expression of fms-like tyrosine kinase 3 (FLT3) and CD135. Treatment with FLT3 inhibitors caused a significant reduction in cell viability among CMI samples. In summary, we were able to discriminate and target 2 biologically distinct subgroups of CLL based on CM dependence with distinct microenvironmental vulnerabilities.

Introduction

Despite the broad incorporation of highly effective targeted therapies, such as B-cell receptor (BCR) pathway inhibitors and the BCL2 antagonist venetoclax, for the treatment of CLL, the disease remains incurable. Identifying complementary therapeutic strategies is warranted.

Targeting the interactions between CLL and the microenvironment is an attractive novel strategy to treat CLL given the well-acknowledged dependency of CLL on microenviromental cues for survival and expansion. CLL cells receive survival signals via accessory cells like mesenchymal stroma cells, nurse-like cells, and T-cells residing in the microenvironment. Interaction with microenvironmental stimuli results in activation of downstream signaling pathways that promote CLL survival and increases resistance to drug-induced apoptosis.[1,2] Thus, it is not unexpected that CLL cells

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The full-text version of this article contains a data supplement.

typically undergo spontaneous apoptosis within 24 hours when cultured in vitro in simple media supplemented only with fetal bovine serum. In vitro studies have demonstrated that bone marrow–derived stromal cells protect CLL cells from apoptosis and support their survival.[3]

Here, we investigated whether the soluble factors present in the media conditioned by a bone marrow–derived stromal cell line (NKTert) could impact CLL cell survival in short-term ex vivo culture. We asked whether media conditioned by supporting cells (conditioned media [CM]) could replace the more complicated existing coculture systems. We found that CM support depended on the presence of a finite number of cytokines, mostly on CC-chemokine ligand 2 (CCL2). In particular, we identified 2 groups of primary CLL cells; one group requiring CM for their survival in vitro (CM dependent [CMD]) and another group with resistance to apoptosis even in the absence of CM support (CM independent [CMI]). These 2 distinct groups were distinguishable by a simple 24-hour functional assay. Furthermore, combined treatment with anticytokine CCL2 with venetoclax markedly reduced CMD CLL survival more effectively than monotherapy. Remarkably, the resistant CMI group showed high expression of fms-like tyrosine kinase 3 (FLT3) and treatment with FLT3 inhibitors resulted in a selective decrease in cell viability in this particular CLL group. This finding opens new therapeutic opportunities for a distinct CLL subtype.

Materials and methods

Samples from patients with CLL and cell purification

Peripheral blood from patients with untreated CLL with informed consent was obtained according to the guidelines and regulations of Dana-Farber Cancer Institute Review Board. Peripheral blood mononuclear cells (PBMCs) from blood samples were isolated using Ficoll-Paque density gradient centrifugation. Samples were viably frozen using fetal bovine serum (FBS, Sigma Aldrich) with 10% dimethyl sulfoxide (DMSO, Sigma Aldrich) and stored in liquid nitrogen until use. The clinical characteristics of these patients are provided in supplemental Table 1.

CM preparation and CLL cell culture

Stromal NKTert cell line (Riken Cell Bank) were cultured in RPMI 1640 medium with 10% FBS, 1% penicillin-streptomycin (Invitrogen), and 2.05 mM L-glutamine. Cells were kept in a humidified 5% CO_2 incubator at 37°C. After 48 hours, the supernatant (CM) was collected, filtered, and stored at −20°C.

Primary CLL cells were cultured in CM and treated with drugs for different experiments.

Cell viability testing

A total of 8000 cells per well were plated in 384-well plates, and cell viability was measured using CellTiter-Glo reagent (Promega) according to manufacturer's instructions.

Cytokine array

Cytokine array was performed using Proteome Profiler Human Cytokine Array Kit (R&D Systems) according to the manufacturer's protocol.

IL-6, IL-8, and CCL2 enzyme-linked immunosorbent assay

Protein levels of IL-6, IL-8, and CCL2 in CM were measured using the DuoSet ELISA kit for IL6, IL8, and Human CCL2/MCP1 (R&D Systems) according to the manufacturer's protocol.

BH3 profiling

Viably frozen CLL cells were thawed and stained with live/dead fixable Zombie-NIR dye (1:100, BioLegend). BH3 profiling was performed as previously described (Ryan et al[4]), in which CLL cells were exposed to synthetic BH3 peptides and 0.002% digitonin in MEB2 buffer (150 mM mannitol, 10 mM HEPES-KOH pH 7.5, 150 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% BSA [bovine serum albumin], and 5 mM succinate) for 1 hour at 25°C. After 1 hour incubation, cells were fixed with 8% formaldehyde for 15 minutes followed by neutralization with N2 buffer (1.7M tris, 1.25M glycine, pH 9.1) for 5 minutes. After neutralization, cells were stained with anticytochrome c Alexafluor-488 antibody (Biolegend) in staining buffer (10% BSA, 2% tween 20, PBS) to measure the sensitivity to BH3 peptides via flow cytometry.

Dynamic BH3 profiling (DBP)

Viably frozen CLL cells were thawed and cultured in CM ex vivo with or without drugs. After 20 hours, cells were collected and BH3 profile performed using different synthetic BH3 peptides. Fluorescence-activated cell sorter data were analyzed, and delta priming was calculated (delta priming = cytochrome C release^{drug} − Cytochrome C release^{DMSO}).

Western blot analysis

Whole-cell extract was prepared by lysing CLL PBMC samples using RIPA lysis buffer (Sigma Aldrich) with protease inhibitor (Millipore) and phosphatase inhibitor (Sigma Aldrich). Protein concentration was quantified using Coomassie Protein Assay Reagent (Pierce). Protein lysates were separated through SDS-polyacrylamide gels (4%-12%) and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were blocked with 5% milk powder in 0.1% tween 20 in 1× PBS (PBS-T) for 1 hour, followed by primary antibody incubation. Images were obtained by exposing membrane to ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc).

Antibodies

The following antibodies were used in this study: PARP (9542S, cell signaling), caspase 3 (9665S, cell signaling), Actin (58169S, cell signaling), and MCL1 (94296S, cell signaling). Neutralizing antibodies used in this study were human IL-8 MAB (CL 6217) from R&D Systems, human IL-6 MAB (CL 6708) from R&D Systems, and human CCL2/MCP-1 MAB (CL 23007) from R&D Systems.

Reverse transcription polymerase chain reaction

The pellets from CLL PBMC samples were resuspended in Trizol. The RNA from these samples were harvested using the Zymo Direct-zol RNA Microprep kit. Complementary DNA was prepared by Bio-rad clear gDNA iScript gDNA Clear cDNA Synthesis Kit, as per the manufacturer's protocol. Quantitative reverse

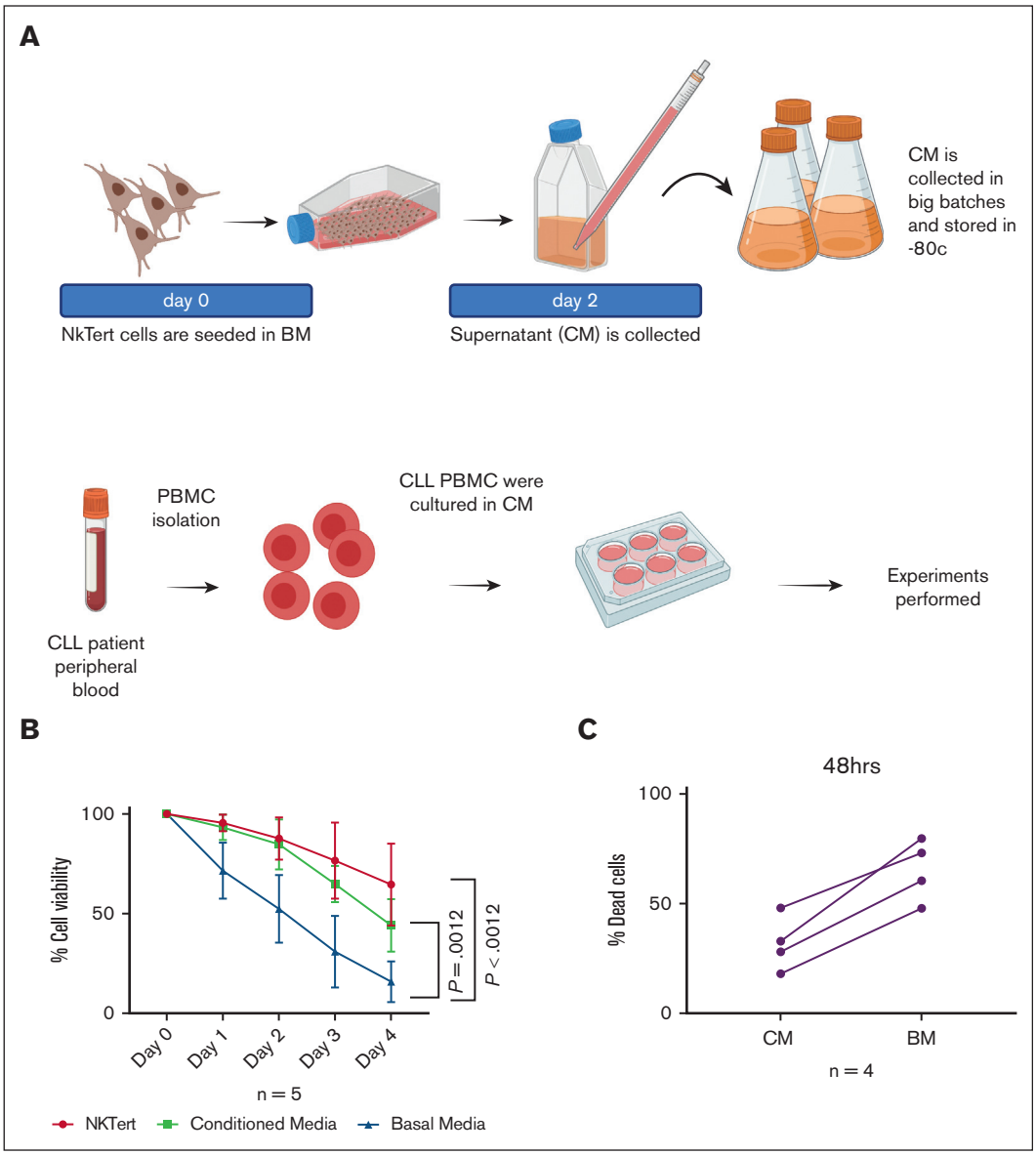

Figure 1. Establishment of CM for CLL. (A) Stromal NKTert cell line was cultured in complete media containing RPMI supplemented with 10% Fetal bovine serum (FBS) and penicillin-streptomycin (BM). Once the cells reached around 90% confluency, the supernatant was collected and filtered and stored at −80°C . Mononuclear cells, isolated from peripheral blood of samples from patients with CLL, were cultured in the presence of this CM (100%). More than 90% cells were CD19+ CD5+. (B) Relative CLL cells viability were measured when grown in presence of either CM, BM, or NKtert cells for 4 days (n = 5). CLL cells were separated from NKtert cells before measuring cell viability. (C) Annexin V staining of CLL cells after 2 days in culture with CM or BM (n = 4). Error bars represent standard error of the mean, *P* values were calculated using paired *t* test, and *P* value < .05 indicate statistical significance. Figure created with BioRender.com.

transcription polymerase chain reaction was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a QuantStudio Flex 6 System platform. Gene of interest amplification was normalized using the b-actin or Gapdh expression, and alterations among sample groups were calculated using the 2-ΔΔ cycle threshold method. The IL8 primers used were as follows: forward, 5'-CGGAAGGAACCATCTCACTG-3'; and reverse, 5'-CCAGTTTTCCTTGGGGTCCA-3'. The FLT3 primers used were as follows: forward, 5'-CTGCCGCTGCTCGTTGTT-3'; and reverse, 5'- TGATGATGACTTCCCCACTGA-3'.

RNA-seq

Libraries were prepared using Roche Kapa messenger RNA (mRNA) HyperPrep strand specific sample preparation kits from 200 ng of purified total RNA according to the manufacturer's protocol on a Beckman Coulter Biomek i7. The finished double-stranded DNA libraries were quantified by Qubit fluorometer, Agilent TapeStation 2200, and RT-qPCR using the Kapa Biosystems library quantification kit according to manufacturer's protocols. Uniquely dual indexed libraries were pooled in equimolar ratios and sequenced on an Illumina NovaSeq 6000 with paired-end 50bp

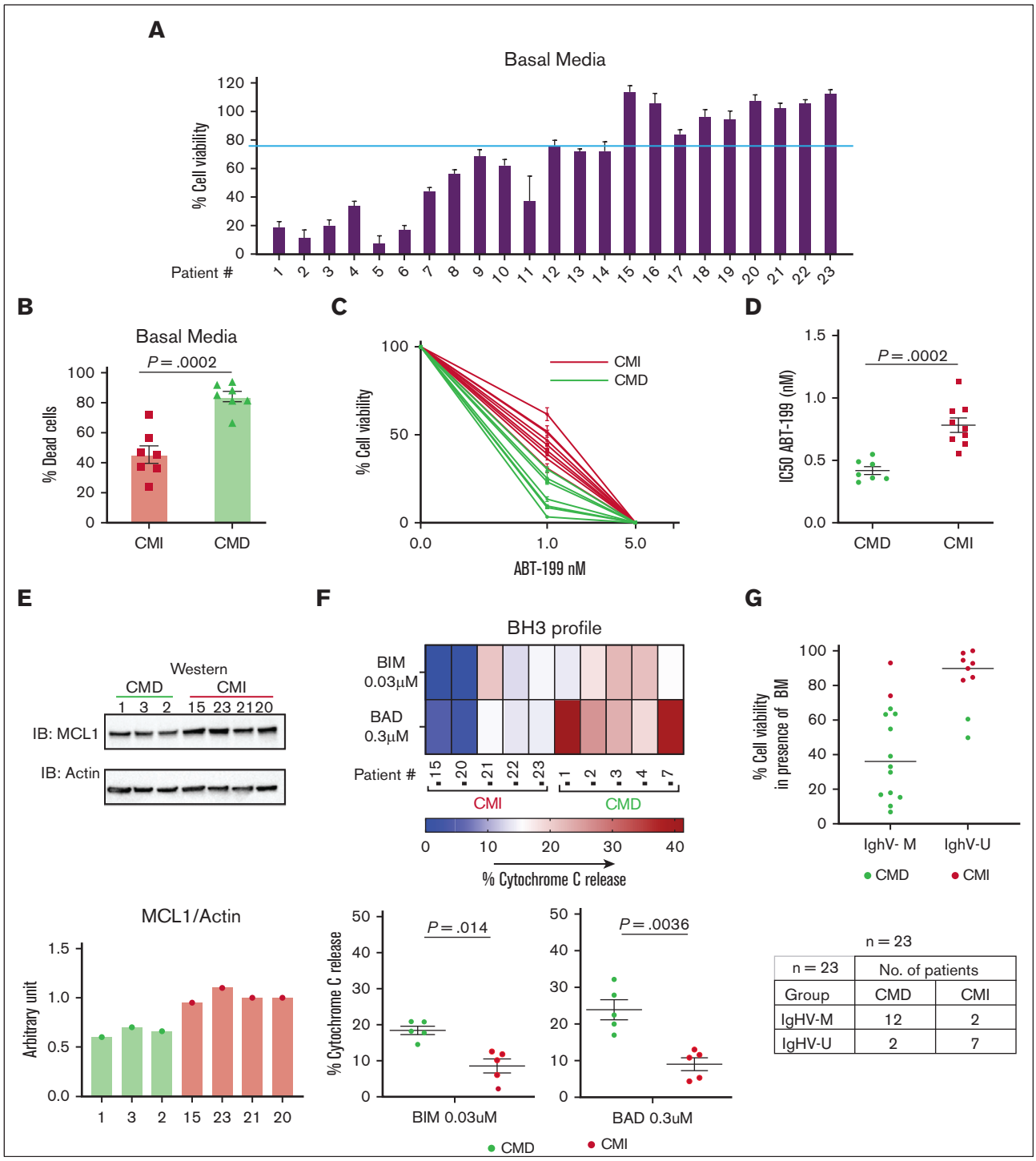

Figure 2. A subgroup of CLL does not undergo spontaneous apoptosis in absence of stroma support. (A) PBMCs were isolated from samples from 23 patients with CLL and plated in 384-well plates in BM. After 24 hours, cell viability was measured using Cell Titer-Glo. Blue line represents 75% cell viability. Percent cell viability is calculated relative to day 0 (100%). (B) Cell death was measured in 7 PBMC samples whose viability >75% (CMI) and in 5 PMBC samples whose viability <75% (CMD). PBMCs from both groups were seeded in 6-well plates in presence of BM. After 48 hours, cell death was measured by annexin V/Hoechst staining. Percent cell death is calculated as 100% live cells (viable cells [negatively stained, lower left quadrants]). Results represent the mean of 4 samples per group. Error bars represent SEM, *P* values were calculated using Welch *t* test, and *P* value < .05 indicate statistical significance. (C) PBMCs from both groups (CMD and CMI) were grown in CM and treated with increasing doses of venetoclax (0 nM, 1 nM, and 5 nM) for 48 hours. Cell viability was measured using Cell Titer-Glo (percentage relative to dimethyl sulfoxide). (D) IC50 values of venetoclax segregate based on CM dependence (CMD, n = 9 and CMI, n = 7). Results represent the mean per group. Error bars represent SEM, *P* values were calculated using Welch *t* test, and *P* value < .05 indicate statistical significance. (E) Upper, Western blot analysis of whole cell extracts using the MCL1 antibody from both CLL groups. Actin was used as loading control. Lower, densitometry analysis was performed for images shown in upper panel. *P* values were calculated between the 2 groups using Welch *t* test, *P* value = .0004

reads by the Dana-Farber Cancer Institute Molecular Biology Core Facilities. Sequenced reads were aligned to the UCSC hg19 reference genome assembly, and gene counts were quantified using STAR (v2.7.3a).[5] Differential gene expression testing was performed by DESeq2 (v1.22.1).[6] RNA-sequence (RNA-seq) analysis was performed using the VIPER Snakemake pipeline.[7]

Data analysis and statistics

Statistical analyses were mostly performed using GraphPad Prism 9, unless otherwise specified in "Methods."

Results

CM derived from NKTert stroma cells supports CLL survival

Although CLL cells undergo spontaneous cell death in ex vivo culture, coculture with bone marrow–derived NKTert stromal cells has been reported to inhibit this death.[8] The importance of stroma cells in CLL survival during drug-induced apoptosis is well characterized; however, it is unclear whether cell-cell contact is necessary for survival enhancement or whether humoral elements in the coculture supernatant is sufficient.

We first asked if CM from the supernatant of NKTert cells could substitute for NKTert coculture to maintain CLL survival. We compared survival of CLL cells maintained in CM (Figure 1A) with that of CLL cells grown in coculture with NKTert cells for 4 days and found no significant difference in cell viability between them until day 3 (Figure 1B). Similarly, we also compared CLL survival when grown in CM derived from the HS-5 stroma cell line with CLL cells that were cocultured directly with HS-5 cells and found similar results (supplemental Figure 3). CLL cells grown in CM supplement showed less cell death compared with those grown in RPMI complete media alone (basal media [BM]) (Figure 1C).

A subgroup of apoptosis-resistant CLL cells have a reduced requirement for CM support

Unexpectedly, we found ~39% (9/23 cases tested) CLL samples underwent little spontaneous cell death even when cultured in BM without CM supplementation (Figure 2A-B; supplemental Figure 1). To define whether there were any functional differences between those primary cells that survived without CM (CMI) vs the primary samples that depended on CM for ex vivo survival (CMD), we evaluated whether there was a difference in venetoclax sensitivities between these 2 groups, because venetoclax-based therapy has been reported to improve outcomes even in patient populations with high-risk CLL. CMI samples (n = 7) were less sensitive to venetoclax treatment compared with that of the CMD group (n = 9) ($P = .0002$; Figure 2C-D), even when grown in identical CM.

Previous studies showed increased MCL1 expression as one of the characteristics of venetoclax resistance.[9,10] We next asked if there is any difference in MCL1 protein expression between these 2 groups. Immunoblot analysis revealed a moderate increase in MCL1 expression in CMI samples compared with CMD samples (Figure 2E).

Because CMI cells were less sensitive to venetoclax, we performed BH3 profiling in a subset of 5 samples from patients from each group (because of the limited number of samples available, we tested 5 samples) to assess whether differences in mitochondrial apoptotic priming correlated with differences in venetoclax sensitivity. BH3 profiling measures cytochrome c release from the mitochondria as a result of mitochondrial outer membrane permeabilization in response to synthetic BH3 peptides.[11] CMI samples showed decreased mitochondrial outer membrane permeabilization in response to BIM ($P = .014$), PUMA ($P = .029$), and BAD ($P = .0036$) peptides compared with that of CMD specimens, consistent with decreased mitochondrial apoptotic priming (Figure 2F; supplemental Figure 2).

Given the influence of immunoglobulin heavy-chain variable region (IGHV) mutational status on BCR signaling, we also checked whether we could categorize CMI CLL cells vs CMD CLL samples from patients based on IGHV. We found that 7 of 9 (~78%) CMI CLL had unmutated IGHV and 12 of 14 (86%) patients with CMD CLL harbored mutated IGHV (Figure 2G).

CCL2 from CM is critical for CM-dependent CLL cell survival

We next asked what cytokines and chemokines were present in CM that might have influenced CMD CLL cell survival. By profiling of the CM against a total of 36 cytokines, we identified 3 (IL6, IL8, and CCL2) that were selectively present in CM (Figure 3A). Notably, CCL2 has been previously reported as expressed by CLL PBMCs cells but not by purified CLL cells and has been shown to facilitate CLL cell survival.[12] We hypothesized that CMI CLL PBMCs would have higher levels of IL6, CCL2, and IL8 than CMD CLL PBMCs. To test this idea, supernatants from samples of patients (n = 4) that were cultured in BM were analyzed for IL6, CCL2, and IL8 by ELISA. Indeed, a higher level of IL8 was detected from the supernatants of CMI CLL PBMCs than that of CMD CLL PBMCs ($P = .0401$; Figure 3B), whereas levels of CCL2 and IL6 in the supernatants from both groups were similar. Moreover, CMI expressed higher IL8 mRNA levels than CMD (supplemental Figure 4), suggesting that CMI PBMCs might express high levels of IL8 than CCL2 and IL6, which might contribute to CMI cell survival.

To test whether these cytokines support CLL survival, we added antagonistic antibodies recognizing IL6, IL8, and CCL2 to fresh CLL samples cultured in CM and survival was analyzed after

Figure 2 (continued) for the comparison of CMD vs CMI samples. (F) Upper, heatmap of BH3 profile performed on freshly isolated CLL PBMCs, 5 samples from patients were derived from each group (CMD, patient# 1, 2, 3, 4, and 7 and CMI, patient# 15, 20, 21, 22 and 23 as defined in panel A of Figure 2) using BIM and BAD peptides. Percent cytochrome C release (indicative of mitochondrial outer membrane permeabilization) is calculated for each BH3 peptide treatment relative to maximum value of negative control (dimethyl sulfoxide). Lower, graphs showing difference in cytochrome C release in response to BIM and PUMA peptides between both groups. Means are depicted as horizontal bars ± standard error bars, P values were calculated using Welch t test, and P value $< .05$ indicate statistical significance. (G) Left, PBMCs were cultured in BM for 24 hours and their cell viability was measured using Cell Titer-Glo. Percent Cell viability is calculated relative to day 0 (100%). IGVH status of the 23 samples were compared with their viability. Right, the table showing the odds ratio is ~17:1 for CMI status for IGHV-U vs IgHV-M with a Fisher exact test P value = .01. IGHV-M, Immunoglobulin heavy-chain variable region-Mutated; IGHV-U, Immunoglobulin heavy-chain variable region-Unmutated.

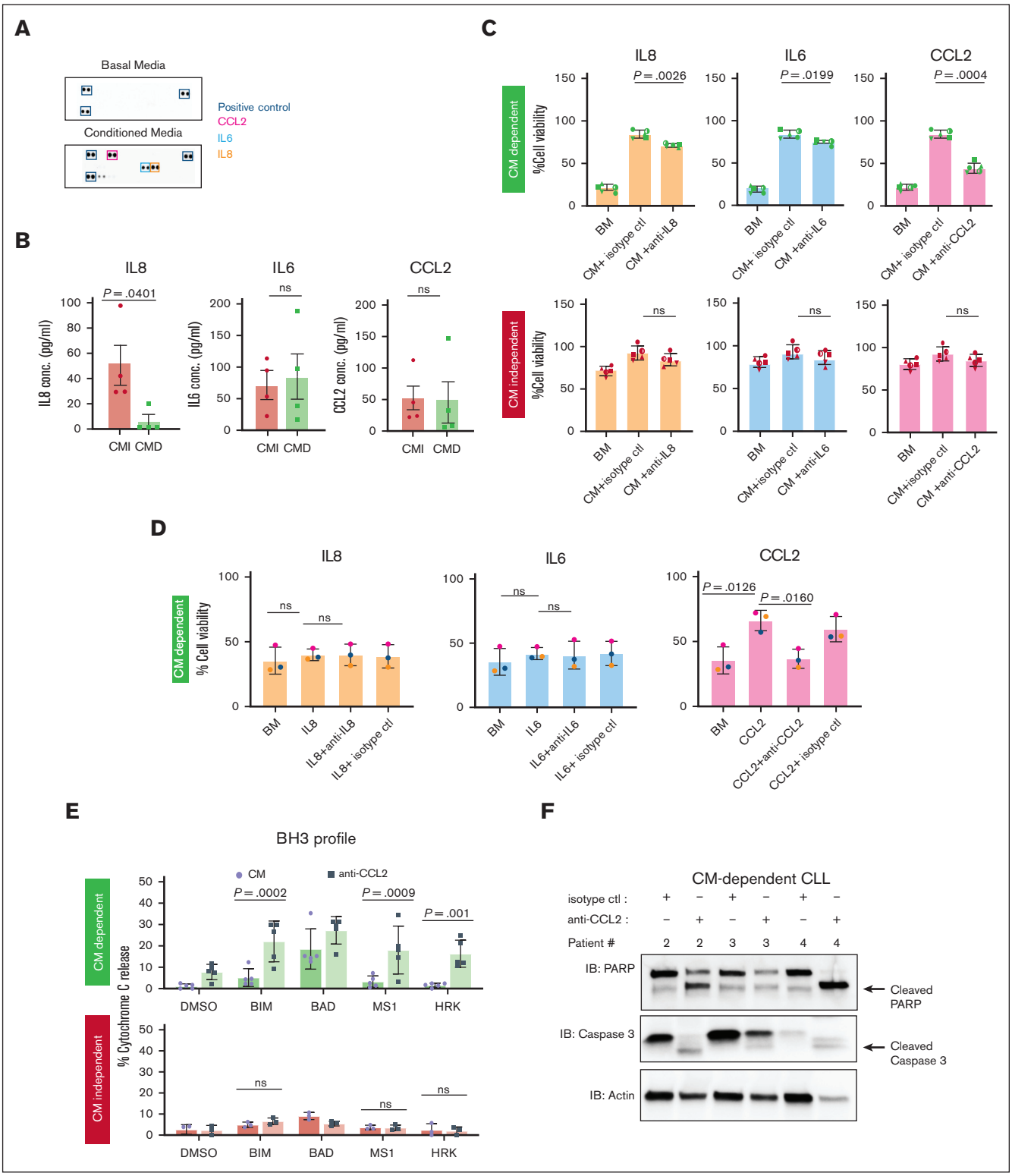

Figure 3. Identification of cytokines present in CM. (A) CM was incubated with proteome profile array membrane for cytokine detection. BM was used a negative control to detect any noise or background. Localization of duplicate dots of IL6, IL8, and CCL2 on the membrane are indicated with arrows. (B) Supernatant was collected from CMI, n = 4 and CMD, n = 4, CLL PBMC were cultured in BM for 48 hours and the concentration of IL6, IL8, and CCL2 were determined by ELISA. Means are depicted as horizontal bars ± standard error bars, *P* values were calculated using Welch *t* test, **P* < .05 indicates statistically significance. (C) CLL PBMCs (CMD and CMI) were cultured in CM either in presence of IL8-specific neutralizing antibody (left) or in presence of IL6-specific neutralizing antibody (middle) or in presence of CCL2-specific neutralizing antibody (right). Cell viabilities were measured after 48 hours (n = 5 for each group). Percent cell viability was calculated relative to day 0 (100%). Error bars represent standard error of the

2 days. In the CMD group, we observed a decrease in cell viability in the presence of each anticytokine antibody, with the largest difference observed for the CCL2 blocking antibody ($P = .0004$) (Figure 3C, top). Surprisingly, in the CMI group, blocking cytokines, including IL8, did not show any effect on CLL survival (Figure 3C, bottom), suggesting that IL8, CCL2, and IL6 do not have any role in CMI CLL survival. Upon culturing CLL PBMCs in the presence of recombinant IL6, IL8, and CCL2 proteins, at concentrations previously shown to have ex vivo activity,[12] only recombinant CCL2 increased CLL cell viability above the baseline. This effect was reversed by exposure to the CCL2-blocking antibody (Figure 3D).

To determine the effect of anti-CCL2 on the apoptotic priming of CMD cells, we applied DBP, a BH3 profiling technique that rapidly measures the drug-induced initiation of apoptotic signaling, commonly known as "delta priming." Delta priming is characterized as the difference between drug-induced apoptotic priming vs untreated apoptotic priming. Primary CLL cells from both groups (n = 5 for CMD, n = 3 for CMI samples) were cultured in anti-CCL2 antibody in presence of CM for 48 hours followed by BH3 profiling. We found that blocking CCL2 in CM showed an increase in priming in response to different peptides in CMD CLL. Anti-CCL2 treatment did not alter mitochondrial sensitivity to any BH3 peptide in the CMI group (Figure 3E).

Because the anti-CCL2 antibody increased apoptotic priming in CMD CLL, we asked if blocking CCL2 results in activation of caspase 3 in CLL. Western blot analysis of CMD CLL showed marked increase in cleaved/activated forms of caspase 3 and proteolytic cleavage of PARP-1 (Figure 3F).

Combination of anti-CCL2 and venetoclax showed an increase in dependencies on BCL-2 as well as BCL-xL in the CMD CLL subtype

Previously, venetoclax has been shown to increase the sensitivity of CLL cells to apoptosis.[13] We, therefore, aimed to see whether pretreatment of CLL cells with anti-CCL2 would increase proapoptotic signaling in venetoclax–treated CLL cells. To test this, we performed DBP after pretreatment of both CLL groups with anti-CCL2 antibody in presence of CM for 48 hours and subsequently added venetoclax (0.5 nM) for 1 hour. In CMD CLL cells, combination of anti-CCL2 and venetoclax further enhanced apoptotic priming compared with combination of either one of these agents alone (Figure 4A). In the CMI samples, the effects of apoptotic priming of combination of the 2 agents were far more muted (Figure 4A).

Because our DBP data showed that blocking CCL2 increased mitochondrial priming to venetoclax in CMD CLL cells, we tested if pretreatment with anti-CCL2 enhanced venetoclax-mediated killing of CMD CLL cells. CLL cells were, therefore, exposed to anti-CCL2 for 48 hours and then treated with venetoclax (0.5 nM) for 24 hours. As expected, based on the BH3 profiling experiments, pretreatment with anti-CCL2 enhanced venetoclax killing of CLL cells, and it did so more in the CMD than the CMI subset ($P < .0001$; n = 4 for each case) (Figure 4B).

Previous in vitro studies have shown that CD14+ cells from patient with CLL help protect CLL cells from apoptosis.[14] We, therefore, tested if CD14+ cells in primary CLL PBMC samples were increased in number and required for survival of the CMI samples. On the contrary, we found slightly fewer CD14+ monocytes and CD3+ T-cells in CMI CLL cells than in CMD CLL cells (Figure 5A). To evaluate if the presence of non-CLL accessory cells in the PBMC samples supported survival in BM, we isolated CD19+ CLL cells from both groups' PBMCs and compared the viability of the leukemia cells with that of total CLL PBMCs when cultured in BM for 2 days. The viability of CLL cells grown in presence of accessory cells (including CD14 and CD3 cells) was similar to that of the purified CD19+ CLL cells alone (Figure 5B), suggesting that CMI is a feature intrinsic to CLL cells.

To compare gene expression between CMD and CMI CLLs, we performed RNA-seq analysis (n = 5 for each group). Unsupervised hierarchical clustering revealed clear partition of the 2 groups into different clusters, and CMI CLLs were more likely to be IGHV unmutated (4/5; Figure 5C). Principal component analysis further revealed a tight clustering of CMD CLLs, whereas CMI CLLs were more dispersed, indicating higher levels of heterogeneity among those samples. Altogether, we identified 97 upregulated and 55 downregulated genes in CMI compared with CMD samples (False Discovery Rate (FDR) < 0.05, Fold Change (FC) > 2; Figure 5D). Differentially expressed genes were highly enriched for focal adhesion and RAS signaling pathways in CMI compared with CMD samples (Figure 5E). Interestingly, 5 of the 6 significantly altered genes (*KSR2, PDGFA, RASAL2, FLT3, and INSR*) involved in RAS signaling were upregulated (Figure 5F). Moreover, we also observed upregulation of FLT3 receptor expression in CMI samples compared with CMD, indicating the activation of the RAS signaling pathway (supplemental Figure 5).

CMI CLL showed high FLT3 receptor expression and were preferentially sensitive to FLT3 inhibition

Because our RNA-seq analysis revealed upregulation of FLT3 in CMI group, we aimed to further validate if FLT3 was differentially expressed between these groups. CMI samples (N = 5) demonstrated much higher levels of FLT3 mRNA ($P = .043$) and surface receptor tyrosine kinase protein than that of CMD samples ($P = .0049$) (N = 5; Figure 6A). Next, we tested if we could target the CMI group with different selective FLT3 inhibitors (quizartinib and gilteritinib). Compared with CMD samples, CMI samples showed increased sensitivity to both FLT3 inhibitors. This suggests that FLT3 inhibitors may be effective in the CMI subset of CLL (Figure 6B).

Figure 3 (continued) mean. *P* values were calculated using paired *t* test, and *P* value < .05 indicate statistical significance. (D) Cell viability of CM-dependent CLL measured PBMCs were cultured in BM with recombinant human cytokines (IL6, IL8, and CCL2) at 10 ng/mL or in combination with cytokine specific neutralizing antibody and isotype control (1 μg/mL) for 48 hours. Percent cell viability was relative to day 0 (100%) (E) Bh3 profile of primary CLL PBMCs from both group in response to BH3 peptides (BIM = 0.01μM; BAD = 0.03μM; MS1 = 2.5μM; HRK = 1μM) after exposing the cells to CM alone or CM with anti-CCL2 neutralizing antibody for 48 hours. Means are depicted as horizontal bars and error bars represent SEM. *P* values were calculated using 2-way analysis of variance, and *P* value < .05 indicate statistical significance. (F) Western blots from CMD CLL PBMCs treated with anti-CCL2 for 48 hours using the indicated antibodies.

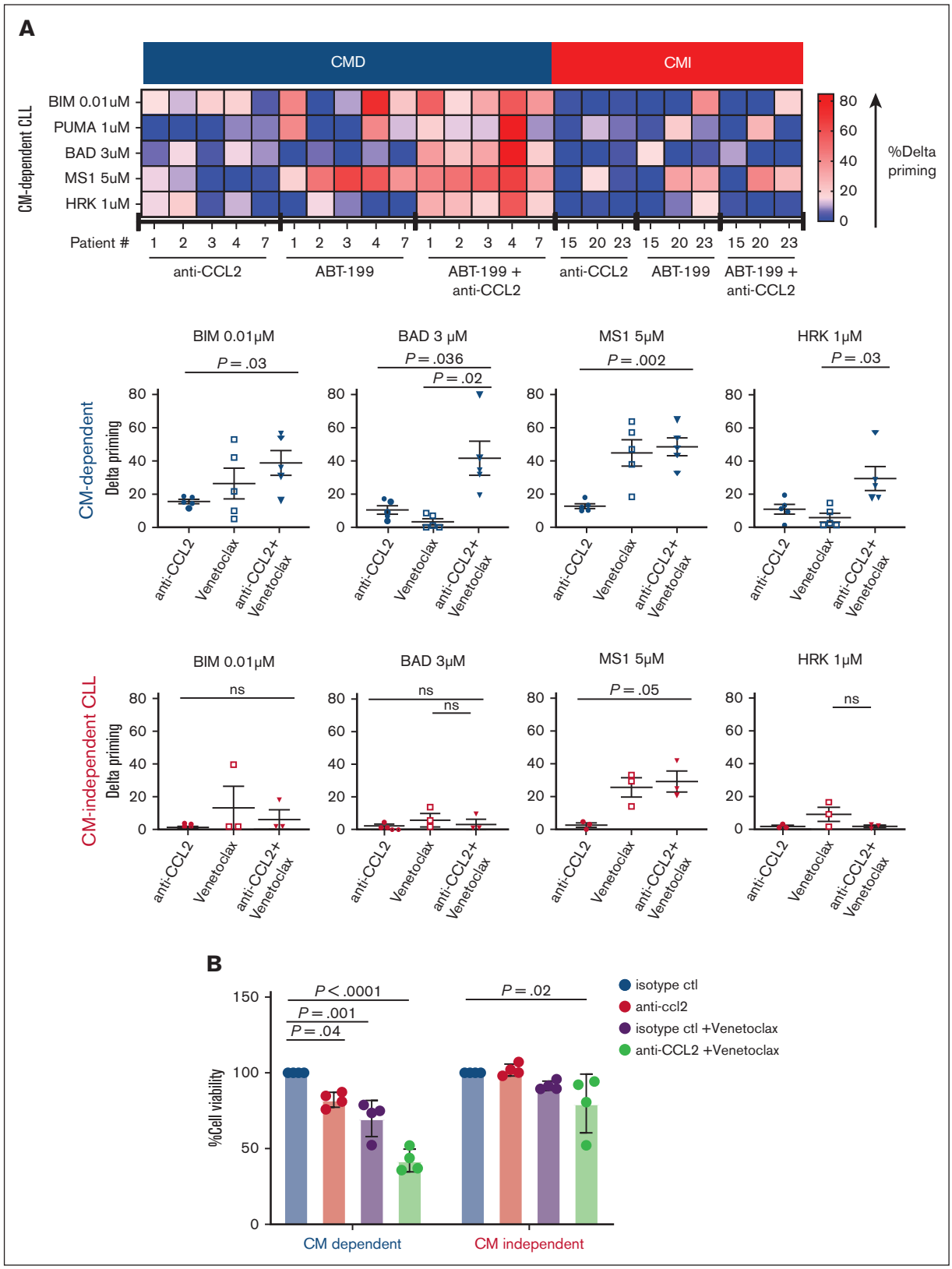

Figure 4. Combination of anti-CCL2 and venetoclax increased sensitivity of CMD CLL toward apoptosis. (A) Upper, delta priming measurement of CLL samples treated with either anti-CCL2 (1 μg/mL) alone for 48 hours, venetoclax (0.5 nM) alone for 1 hour, or with venetoclax + anti-CCL2 cultured in presence of CM. Delta priming was calculated from before and after treatment in response to BIM, BAD, and HRK peptides. (CMD, n = 5; CMI, n = 3). Lower, Comparison of delta priming among treatments. Each point

Discussion

One reason for increased resistance to drug-induced apoptosis is signaling between leukemia cells and their microenvironment. CLL cells demonstrate much lower rates of apoptosis in vivo than in vitro culture, indicating a key role of the microenvironment in CLL protection and survival. Therefore, targeting the dependence of CLL on microenvironmental factors has been a growing area of interest. In this study, we identified 2 different types of CLL based on their survival in media lacking CM supplementation. Across these types, we could identify distinct dependences on microenvironmental cues that could be targeted therapeutically. CMD cells were susceptible to blockade of CCL2, whereas CM independent cells were susceptible to inhibition of FLT3.

In our experimental approach, we found that soluble factors secreted by human bone marrow–derived stroma cells, NKtert cells, provide survival signaling to CLL cells in short-term ex vivo cultures. In a recent publication, Schulz et al[15] used CM derived from human bone marrow–derived stromal cell line HS-5 and showed similar findings, confirming that soluble factors secreted by stroma cells were enough to support CLL survival in vitro.

Our data obtained from the stroma-derived CM indicate that cytokines from NkTert cells protect CLL cells from apoptosis. We identified 3 major cytokines (IL6, IL8, and CCL2) from the CM. Functional studies implicated CCL2 as the most important of these to promote CLL survival in ex vivo culture. The physiological function of the chemokine CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), is to recruit and activate mononuclear cells. CCL2 signaling plays several biological roles including cell survival and inhibiting apoptosis.[16-18] It is believed that inflammatory conditions have a significant role in CLL development and progression.[19] More recently, CCL2 has been demonstrated to be involved in many cancers and inflammatory disorders.[17,20] It is notable that others have found that stromal cell support required cell-cell contact, as they found separation across micropore filters reduced the protective effect. It is possible, however, that the stromal cell density and, hence, the generated cytokine concentrations were lower under these conditions than the conditions in which we harvested CM from NkTERT cells grown in monoculture.[21]

In our functional studies, we found that blocking CCL2 decreased CLL survival in ex vivo culture. Studies from Burgess et al[12] also found that CCL2 enhances CLL survival. However, in their study CCL2 was only produced by CLL when cultured in the presence of accessory cells. Schulz et al[15] showed that CCL2 is expressed by monocytes in the presence of primary CLL cells in vitro. However, CCL2 did not show any direct role in CLL survival or migration but showed a chemoattractant role for monocytes. We did not observe CCL2 expression in our unsorted CLL samples. Moreover, unsorted CLL and CD19+ CLL did not show any difference in CLL survival, suggesting that accessory cells like CD3+ and T-cells may be less important for CLL cell survival. Unlike our experimental conditions, Burger et al[8] and Schulz et al[15] used completely different culture conditions in which very high density of unsorted CLL cells were cultured ex vivo, which might have increased the interaction of CLL cells with their accessory cells that triggered CCL2 expression. This could explain the discrepancy in the results.

Although highly effective for patients with CLL, venetoclax resistance has recently emerged, with several mechanisms thus far identified.[10,22,23,24] We found that the combination of venetoclax and anti-CCL2 increased apoptotic signaling and CLL cell death to a significantly greater extent than either single agent alone. Because BCL-2 antagonists and anti-CCL2 antibodies work in different pathways, this combination might decrease the likelihood of CLL cells acquiring resistance to both. Bindarit, a CCL2 synthesis inhibitor, is a small anti-inflammatory molecule has been reported to suppress inflammation in in vivo in a number of mouse models of inflammatory diseases.[25] In xenograft mouse models of breast and prostate cancers, this drug has been shown to impair tumor formation and metastasis by inhibiting CCL2 expression.[26] Importantly, bindarit has been shown to be safe in phase 2 clinical trials in patients with type 2 diabetic nephropathy.[27] In addition to this, in a phase 1 trial, carlumab, a human monoclonal antibody against CCL2, has been used in patients with advanced solid malignancies and found that this antibody is well tolerated,[28] suggesting that there are multiple clinical drugs that can target this pathway. These drugs could be good candidates to explore in combination with venetoclax in preclinical studies for patients with CLL.

Interestingly, we identified 2 functionally distinct CLL groups that differ in their microenvironmental dependence (CMD CLL vs CMI CLL). We found a subset of CLL PBMCs that grow independently without any support from the microenvironment (CMI CLL). This group is less sensitive to apoptosis and express more MCL1 protein than CMD CLL. Moreover, in this study, BH3 profiling confirmed that even at doses of venetoclax that induce mitochondrial apoptotic priming in CLL, CMI CLL showed less apoptotic priming. These 2 groups are strongly associated with IGHV status. Samples from patients with CMI CLL are enriched for unmutated IGHV, whereas most of the samples from patients with CLL in CMD group have mutated IGHV. It has been previously shown that patients with unmutated IGHV express low affinity BCR and can exhibit ligand independent tonic BCR pathway signaling, which could lead to increased BCR signaling compared with mutated IGHV CLL cells that expand at a slower rate via high affinity binding of specific/restricted antigens.[29] This biological difference of IGHV status in CLL might explain why unmutated-IGHV CLLs are CMI. In addition to this, we could segregate both CLL groups based on IGHV status via principal component analysis suggesting additional factors are associated with this phenotype. A comparison of gene expression between the 2 groups showed that genes involved in focal adhesion and RAS signaling pathways were upregulated in the CMI group compared with the CMD group. Genes involved in both focal adhesion and RAS pathway activate factors that execute functions, including oncogenic transcription, cell growth, metabolism, and migration, which promote cancer-cell survival. This

Figure 4 (continued) represents a CLL sample. Data are means ± standard error of the mean. *P* values were calculated using 1-sided *t* test followed by a Bonferroni correction, and *P* value < .05 indicates statistically significance. (B) Cell viability of primary CLL samples was measured 48 hours after treatment with anti-CCL2, venetoclax, or anti-CCL2 + venetoclax cultured in CM (n = 4) independent samples for each group (CMD = patient #1, 2, 3, and 7 and CMI = patient #20, 21, 22, and 23), Error bars represent standard error of the mean. *P* values were calculated using 2-way analysis of variance, and *P* value < .05 indicates statistical significance.

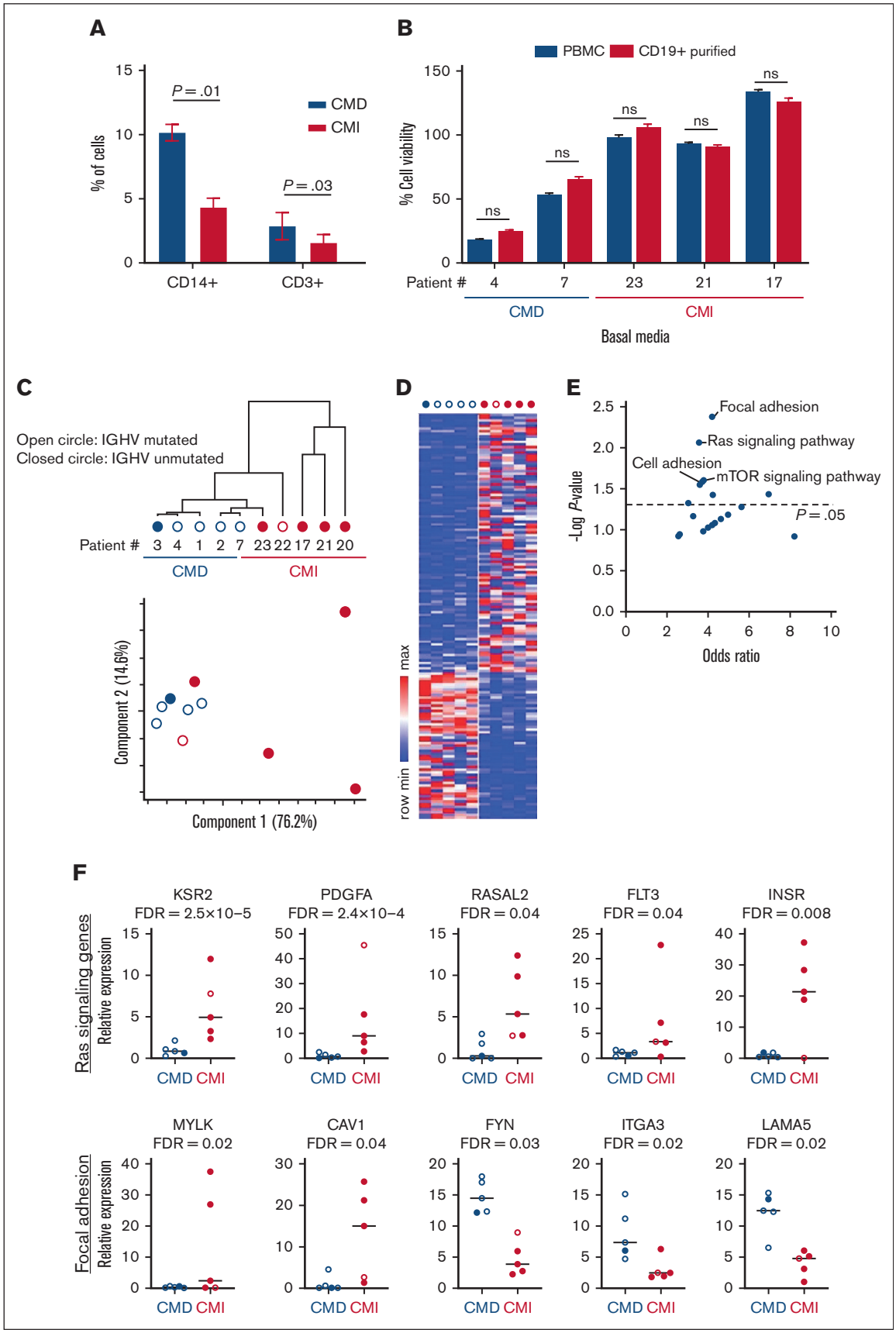
A
% of cells
P = .01
P = .03
CMD
CMI
CD14+
CD3+
B
PBMC
CD19+ purified
% Cell viability
ns
Patient # 4 7 23 21 17
CMD
CMI
Basal media
C
Open circle: IGHV mutated
Closed circle: IGHV unmutated
Patient # 3 4 1 2 7 23 22 17 21 20
CMD
CMI
Component 2 (14.6%)
Component 1 (76.2%)
D
max
row min
E
-Log P-value
Odds ratio
Focal adhesion
Ras signaling pathway
Cell adhesion
mTOR signaling pathway
P = .05
F
Ras signaling genes
Relative expression
KSR2
FDR = 2.5×10−5
PDGFA
FDR = 2.4×10−4
RASAL2
FDR = 0.04
FLT3
FDR = 0.04
INSR
FDR = 0.008
Focal adhesion
MYLK
FDR = 0.02
CAV1
FDR = 0.04
FYN
FDR = 0.03
ITGA3
FDR = 0.02
LAMA5
FDR = 0.02
CMD CMI

Figure 5.

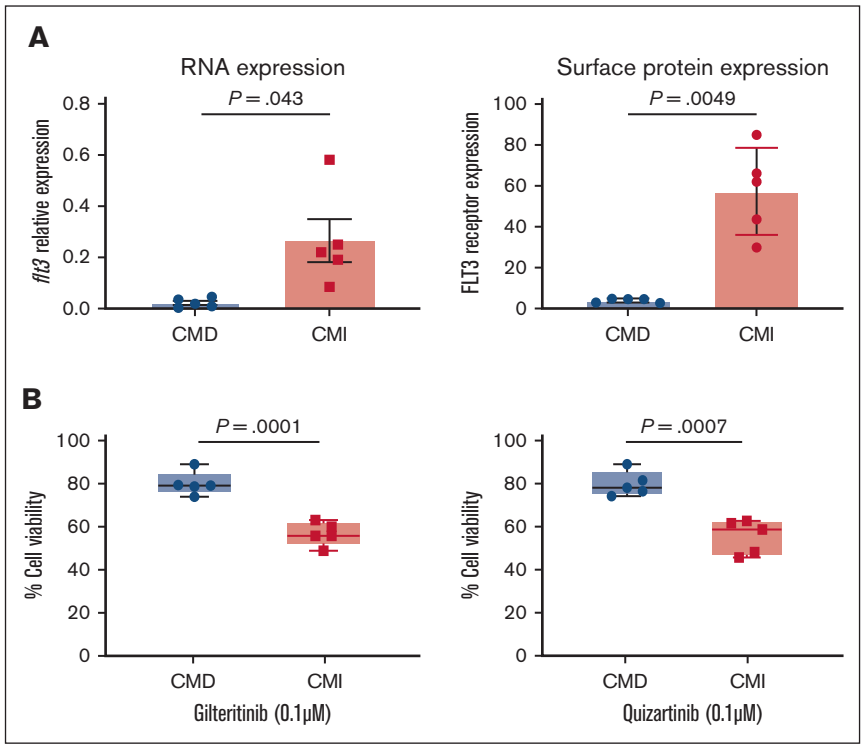

Figure 6. CMI CLL showed high FLT3 receptor expression and were preferentially sensitive to FLT3 inhibition. (A) Left, *FLT3* gene expression assessed by RT-qPCR. Data represent mean ± SEM for analysis of PBMCs of patients with CLL from both group (n = 5). *P* values were calculated using Welch *t* test, and $P < .05$ indicates statistically significance. Right, surface expression of FLT3 was determined by flow cytometry analysis. PBMCs from each CLL group (n = 5) was stained for FLT3. *P* values were calculated using Welch *t* test, $P < .05$ indicates statistical significance. (B) Cell viability of primary CLL samples (n = 5, each group) after 72 hours of FLT3 inhibitors treatment. CLL PBMCs were cultured in BM and treated with either Gilteritinib or Quizartinib for 72 hours. Cell viability was measured using Cell Titer-Glo. Percent Cell viability is calculated relative to untreated (100%). *P* value determined by 2-tailed *t* test.

differential expression pattern may, in part, explain the relative resistance to apoptosis in the CMI cells.

FLT3 is one of the RAS signaling genes that was upregulated in CMI samples. Much effort has been put on understanding and targeting FLT3 gene in acute myelogenous leukemia (AML), which is characterized as the most frequent genetic alteration, detected in 30% of patients with AML and confer poor outcome. FLT3 is a type 3 receptor tyrokine kinase that is expressed in early hematopoietic cells in bone marrow and has a role in regulating the proliferation, differentiation, and survival of immune cells.[30] FLT3 is expressed in early lymphoid progenitors and has an important role during early B-cell development; however, the expression of FLT3 is lost in mature B cells.[30,31] Recently, studies have shown that FLT3 is re-expressed in activated germinal center B cells and has a role in peripheral B-cell maturation and class switch recombination to immunoglobulin (Ig) G1.[32] The overlap of unmutated IgHV, FLT3 expression, and CM independence in a subset of samples is therefore consistent with the identification of a subset of CLL cases deriving from a less mature, more apoptosis–resistant lymphoid precursor. Second-generation FLT3 inhibitors, gilteritinib and quizartinib, are highly selective and potent FLT3 inhibitors that are used clinically to target AML. The role of FLT3 in CLL pathogenesis is not known; however, there is 1 study reported to express FLT3 in 2 of 3 patients with CLL.[33] Here, in this study, we showed that FLT3 is selectively expressed in the CMI group and can be targeted by 2 different FLT3 inhibitors. To the best of our knowledge, this is the first time that targeting FLT3 in a specific subgroup of CLL has been demonstrated.

Expression of Cav1, Mylk, and PDGFa from focal adhesion were also upregulated in CMI CLL compared with CMD samples. In previous studies, it has been shown that high expression of Cav1 and Mylk in patients with CLL were associated with poor prognosis,[34] suggesting that the focal adhesion influence in CLL survival and might provide new insights in understanding mechanisms of disease progression.

We conclude that combined anticytokine and venetoclax therapy may be a useful strategy in the treatment of CMD CLL. An in vivo model to test the strategy of targeting CCL2 and BCL2 in CLL might be beneficial to understand the complexities of microenvironmental factors as seen in our ex vivo model. However, because primary CLL lack a good in vivo mouse model that could mimic the indolent nature of human CLL, it limits our ability to explore the role of this aspect of microenvironment preclinically in vivo.

Furthermore, targeting FLT3 in the subset of CLL that is CMI support a potential new avenue for CLL therapy. Future studies are needed to determine how we can effectively target these pathways

Figure 5. Monocytes and T-cells do not influence CLL survival. (A) PBMCs from patients with CLL n = 5 from both groups were stained for either CD3+ or CD14+ and their representation compared. (B) CD19+ cells were isolated from CLL PBMCs from both groups, n = 3. PBMCs and CD19+ B-CLL cells were grown in RPMI for 48 hours. Cell viability was compared between PBMCs and purified CD19+ B-CLL cells. (C) Upper, hierarchical clustering of gene expression data of CMD vs CMI CLL (n = 5 each group) using Gene Cluster 3.0. Lower, principal component analysis of different CMD and CMI samples. (D) Heatmap showing differentially expressed genes between CMD vs CMI CLL (False Discovery Rate (FDR) < 0.05; Fold Change (FC) > 2). (E) Signaling pathways enriched for the differentially expressed genes in (B) using Enrichr and the Molecular Signatures Database. (F) Relative expression of genes in RAS signaling pathway and focal adhesion.

to improve survival for patients with CLL, particularly those who develop venetoclax resistance.

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Authorship

Contributions: S.P. and A.L. designed the study and wrote the manuscript; S.P. performed most experiments and analyzed data; A.A. performed Western blot analysis; S.Y. performed RNA-sequence analyses; G.G.F. performed statistical analysis; M.S.D. provided patient samples; M.S.D. and C.J.W. assisted with study design and data analysis; and A.L provided overall supervision to the study.

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References

1. Davids MS, Deng J, Wiestner A, et al. Decreased mitochondrial apoptotic priming underlies stroma-mediated treatment resistance in chronic lymphocytic leukemia. *Blood*. 2012;120(17):3501-3509.
2. Vogler M, Butterworth M, Majid A, et al. Concurrent up-regulation of BCL-XL and BCL2A1 induces approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia. *Blood*. 2009;113(18):4403-4413.
3. Panayiotidis P, Jones D, Ganeshaguru K, Foroni L, Hoffbrand AV. Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *Br J Haematol*. 1996;92(1):97-103.
4. Ryan J, Montero J, Rocco J, Letai A. iBH3: simple, fixable BH3 profiling to determine apoptotic priming in primary tissue by flow cytometry. *Biol Chem*. 2016;397(7):671-678.
5. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29(1):15-21.
6. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550.
7. Cornwell M, Vangala M, Taing L, et al. VIPER: visualization pipeline for RNA-seq, a Snakemake workflow for efficient and complete RNA-seq analysis. *BMC Bioinformatics*. 2018;19(1):135.
8. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. *Blood*. 2000;96(8):2655-2663.
9. Thijssen R, Tian L, Anderson MA, et al. Single-cell multiomics reveal the scale of multilayered adaptations enabling CLL relapse during venetoclax therapy. *Blood*. 2022;140(20):2127-2141.
10. Guieze R, Liu VM, Rosebrock D, et al. Mitochondrial reprogramming underlies resistance to BCL-2 inhibition in lymphoid malignancies. *Cancer Cell*. 2019;36(4):369-384.e13.
11. Certo M, Del Gaizo Moore V, Nishino M, et al. Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*. 2006;9(5):351-365.
12. Burgess M, Cheung C, Chambers L, et al. CCL2 and CXCL2 enhance survival of primary chronic lymphocytic leukemia cells in vitro. *Leuk Lymphoma*. 2012;53(10):1988-1998.
13. Anderson MA, Deng J, Seymour JF, et al. The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism. *Blood*. 2016;127(25):3215-3224.
14. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ. Distinctive features of "nurselike" cells that differentiate in the context of chronic lymphocytic leukemia. *Blood*. 2002;99(3):1030-1037.
15. Schulz A, Toedt G, Zenz T, Stilgenbauer S, Lichter P, Seiffert M. Inflammatory cytokines and signaling pathways are associated with survival of primary chronic lymphocytic leukemia cells in vitro: a dominant role of CCL2. *Haematologica*. 2011;96(3):408-416.
16. Bomstein Y, Yuklea M, Radnay J, et al. The antiapoptotic effects of blood constituents in patients with chronic lymphocytic leukemia. *Eur J Haematol*. 2003;70(5):290-295.

17. Raman D, Baugher PJ, Thu YM, Richmond A. Role of chemokines in tumor growth. *Cancer Lett*. 2007;256(2):137-165.
18. Diaz-Guerra E, Vernal R, del Prete MJ, Silva A, Garcia-Sanz JA. CCL2 inhibits the apoptosis program induced by growth factor deprivation, rescuing functional T cells. *J Immunol*. 2007;179(11):7352-7357.
19. Muzio M, Fonte E, Caligaris-Cappio F. Toll-like receptors in chronic lymphocytic leukemia. *Mediterr J Hematol Infect Dis*. 2012;4(1):e2012055.
20. Conti I, Rollins BJ. CCL2 (monocyte chemoattractant protein-1) and cancer. *Semin Cancer Biol*. 2004;14(3):149-154.
21. Lagneaux L, Delforge A, de Bruyn C, Bernier M, Bron D. Adhesion to bone marrow stroma inhibits apoptosis of chronic lymphocytic leukemia cells. *Leuk Lymphoma*. 1999;35(5-6):445-453.
22. Blombery P, Thompson ER, Nguyen T, et al. Multiple BCL2 mutations cooccurring with Gly101Val emerge in chronic lymphocytic leukemia progression on venetoclax. *Blood*. 2020;135(10):773-777.
23. Roberts AW, Ma S, Kipps TJ, et al. Efficacy of venetoclax in relapsed chronic lymphocytic leukemia is influenced by disease and response variables. *Blood*. 2019;134(2):111-122.
24. Chong SJF, Davids MS. Breaking through BCL-2 inhibition in CLL. *Blood*. 2020;135(10):709-711.
25. Mirolo M, Fabbri M, Sironi M, et al. Impact of the anti-inflammatory agent bindarit on the chemokinome: selective inhibition of the monocyte chemotactic proteins. *Eur Cytokine Netw*. 2008;19(3):119-122.
26. Zollo M, Di Dato V, Spano D, et al. Targeting monocyte chemotactic protein-1 synthesis with bindarit induces tumor regression in prostate and breast cancer animal models. *Clin Exp Metastasis*. 2012;29(6):585-601.
27. Ble A, Mosca M, Di Loreto G, et al. Antiproteinuric effect of chemokine C-C motif ligand 2 inhibition in subjects with acute proliferative lupus nephritis. *Am J Nephrol*. 2011;34(4):367-372.
28. Sandhu SK, Papadopoulos K, Fong PC, et al. A first-in-human, first-in-class, phase I study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 in patients with solid tumors. *Cancer Chemother Pharmacol*. 2013;71(4):1041-1050.
29. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol*. 2013;34(12):592-601.
30. Parcells BW, Ikeda AK, Simms-Waldrip T, Moore TB, Sakamoto KM. FMS-like tyrosine kinase 3 in normal hematopoiesis and acute myeloid leukemia. *Stem Cells*. 2006;24(5):1174-1184.
31. Luc S, Luis TC, Boukarabila H, et al. The earliest thymic T cell progenitors sustain B cell and myeloid lineage potential. *Nat Immunol*. 2012;13(4):412-419.
32. Svensson MN, Andersson KM, Wasen C, et al. Murine germinal center B cells require functional fms-like tyrosine kinase 3 signaling for IgG1 class-switch recombination. *Proc Natl Acad Sci U S A*. 2015;112(48):E6644-6653.
33. Catherine E, Carow ML, Kaufmann Scott H, Chen Joseph, et al. Expression of the hematopoietic growth factor receptor FLT3 (STK-UFlk2) in human leukemias. *Blood*. 1996;87(3):1089-1096.
34. Shukla A, Chaturvedi N, Ahrens AK, et al. Stromal tumor microenvironment in chronic lymphocytic leukemia: regulation of leukemic progression. *J Leuk*. 2013;1(2):1000113.