Chronic Lymphocytic Leukemia Cells in a Lymph Node Microenvironment Depict Molecular Signature Associated with an Aggressive Disease

Amit K. Mittal  
*University of Nebraska Medical Center*

Nagendra K. Chaturvedi  
*University of Nebraska Medical Center*

Karan J. Rai  
*University of Nebraska Medical Center*

Christine E. Gilling-Cutucache  
*University of Nebraska at Omaha, ccutucache@unomaha.edu*

Tara M. Nordgren  
*University of Nebraska Medical Center*

See next page for additional authors

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the Western world. CLL is a disease of long-lived B cells that are capable of proliferating upon appropriate stimuli and accumulate in the peripheral blood (PB), bone marrow (BM) and lymph nodes (LNs) (1–4). There is emerging evidence that the tumor microenvironment influences the survival and drug resistance of CLL cells (5) and other cancer cells (6,7), playing a critical role in the growth, invasion and progression of a variety of malignancies, including hematological malignancies. CLL cells rapidly undergo apoptosis in vitro, but survive for a longer time in vivo, thus underscoring the role of the microenvironment in the growth and survival of CLL cells (8). Several studies have used in vitro culture systems involving T cells, stromal cells, follicular dendritic cells, nurse-like cells (NLCs) and CD40 engagement to study the role of the microenvironment in CLL (9–11). The presence of stromal components in the microenvironment, such as NLCs, protects CLL cells from death and enhances the expression...
of genes related to chemokines and B-cell receptor (BCR) signaling (9,12). Furthermore, CLL cells proliferate in vitro in the presence of stroma and soluble mediators such as interleukin (IL)-2 or IL-10 (13). Although these in vitro culture systems simulate the in vivo microenvironment to a certain extent, studying CLL cells directly from in vivo sites will more accurately define the molecules associated with vital functions in vivo.

Emerging evidence suggests that clinical/biological heterogeneity in CLL is due to tissue microenvironment-influenced variation in proliferation and survival (8). Only a few studies have reported the importance of PB, BM and LN microenvironments in CLL (14–19). This study enhances the information by providing a comprehensive list of gene signatures of CLL cells from three distinct tumor microenvironments, such as PB, BM and LN not reported previously, and associates these signatures to clinical markers that predict disease progression and clinical outcome.

MATERIALS AND METHODS

CLL Patient Information and Sample Collection

PB (n = 20), BM (n = 18) and LN (n = 15) samples were collected from CLL patients with informed consent using an institutional review board–approved protocol at University of Nebraska Medical Center. Of these, seven PB and BM samples, two PB and LN, and three BM and LN paired samples were from the same patient. Only untreated patients and patients who had not received treatment in the prior 6 months were included in this study. The patient characteristics are described in Results.

Isolation and Characterization of CLL Cells

CLL cells from PB and BM were isolated and immunophenotyped by flow cytometry by using the following combinations of antibodies as described previously (20). Briefly, wherever needed, antibody cocktail for B-cell enrichment (RosetteSep; Stemcell Technologies Inc., Vancouver, BC, Canada) was used to purify the B-CLL cells from PB and BM samples. This cocktail kit binds and eliminates erythrocytes and other white blood cells except B cells by subsequent density gradient centrifugation using Lymphoprep and isolation of purified B cells (>90%) from the interface. Frozen LN samples were obtained from the University of Nebraska Medical Center tissue bank. CLL cells were identified in the 10-micron sections of these LNs by using immunohistochemical techniques to localize CD19+ and CD5+ cells. Fifteen to twenty (8–10 microns thick) sections were prepared from each LN on membrane-coated glass slides. These slides were prepared at 4°C, and these sections were immediately fixed with ice-cold acetone and stained with cresyl violet (LCM Staining Kit; Ambion/Life Technologies, Carlsbad, CA, USA) for 30 s. The corresponding CD19+ and CD5+ areas were laser microdissected by using the laser capture microdissection technique (Leica Microsystems Inc., Wetzlar, Germany), specially focusing on areas with >90% CLL cells, which were identified and microdissected. Areas containing stromal cells either were excluded or burnt out by using a laser, thus further decreasing the stromal cell components. The microdissected cells were immediately processed for isolation of RNA to avoid degradation of RNA.

Cytogenetic Analyses

The cytogenetic analyses including fluorescent in situ hybridization (FISH) were performed by the Human Genetics Institute at University of Nebraska Medical Center as described earlier (21). Chromosome 11q deletion, 17p deletion and trisomy 12 were considered as the poor outcome group, whereas normal karyotype and 13q deletion were grouped as the better outcome group (22).

In Vitro Stromal Cell Culture System

To simulate the in vivo microenvironment, an in vitro stromal culture system was used to study the survival and proliferation of CLL cells in vitro as described earlier (25). Freshly isolated primary CLL cells were cocultured on the mouse-derived OMA-AD or human-derived HMEC stromal feeder cell layer in the presence of RPMI with 10% fetal bovine serum medium for 48–72 h, and survival and proliferation of CLL cells were determined by flow cytometry.

Gene Expression Analyses

Total RNA was extracted from CLL cells by using TRIzol (Invitrogen/Life Technologies) as described earlier (20). RNAs (approximately 0.5 μg per sample) from CLL cells were used for gene expression profiling on a DNA microarray chip (MWG Biotech, Ebersberg, Germany, Human 30K oligo set B) consisting of 50-mer oligonucleotide representing 10,000 different genes. Stratagene reference RNA, labeling of cDNA, hybridization procedure and locally weighted scatterplot smoothing (LOWESS) intensity-dependent normalization were implemented using standard procedures, Gene Pix 6.0 and BRB Array Tools as described previously (20). Differential mRNA expression in PB-, BM- and LN-CLL cells was evaluated by using a random variance t test (p < 0.005), significance analysis of microarrays (false discovery rate [FDR] <10%) and gene set enrichment analysis computational program in conjunction with BRB array tools (version 4.2.0-Beta) (23,24). Cluster and TreeView programs were also used in the analyses (Eisen Laboratory, University of California, Berkeley, CA, USA).

Validation of Significant Genes Using Real-Time Quantitative Polymerase Chain Reaction

SYBR Green real-time polymerase chain reaction (PCR) was used to further confirm differential gene expression between CLL groups. Complementary cDNAs were mixed with primers and Power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies) as previously described (25).
Detection of Surface and Intracellular Markers Using Flow Cytometry

Cells were stained with CD19-FITC (fluorescein isothiocyanate) marker to specifically analyze the proportion of CLL cells. Further, surface apoptotic marker annexin V, intracellular phospho-Syk and proliferation marker Ki-67 were detected by using annexin V/PI staining, “phosphoflow” (BD Phosflow) and Ki-67 staining, respectively, following the manufacturer protocol (BD Biosciences, San Jose, CA, USA). For analyses, a BD FACS-STAR Plus flow cytometer (BD Biosciences) was used.

Identification of Key Signaling Molecules Using Eμ-TCL1 Transgenic Mouse

TCL1 transgenic (TCL1-tg, n = 3), a mouse model for CLL, and C57BL/6 control (n = 3) mice were a generous gift from our collaborator Rene Opavsky at University of Nebraska Medical Center. These mice were reared and maintained at a pathogen-free animal facility in the University of Nebraska Medical Center. LN and spleen tissues were harvested from these mice to study key molecules in the leukemic cells. All experiments were performed in accord with the approved protocol by the Institutional Animal Care and Use Committee of University of Nebraska Medical Center.

Evaluation of Expression of Key Molecules at the Protein Level Using Immunohistochemistry

To validate our findings from the gene expression analyses, we examined the expression levels of phosphor-SYK and phosphor-p-65, the major molecules associated with BCR and nuclear factor (NF)-κB signaling using immunohistochemistry. Briefly, the 5- to 10-micron histological sections of the LNs from CLL patients and lymphoid tissue from TCL1 transgenic mice were stained with p-SYK (catalog number PAB0623, 1:200 dilution) or p-P65 (catalog number AB28810, 1:50 dilution). A Leica Bond Polymer Refine Detection Kit (Leica Microsystems Inc.) was used to visualize the cells positive for these two primary antibodies, as described in the manufacturer protocols.

Statistical Analyses

For the identification of differentially expressed genes, a significant analysis of microarray was used. To identify the tissue-specific gene signatures, analysis was performed using a random variance F test with a p value of 0.01 and FDR of 0.08. Most of the analyses were performed at p < 0.05 and FDR < 0.25, unless specified otherwise. The Kaplan-Meier method using the log-rank test was used to study the association of gene expression or clinical parameter with the clinical outcome as done previously (22,25). Time to treatment among CLL patients was used as an outcome and defined as the time period in months between diagnosis and initiation of the first treatment regimen.

All supplementary materials are available online at www.molmed.org.

RESULTS

Patient Characteristics

We performed GEP on 20 PB, 18 BM and 15 LN specimens from 37 consenting CLL patients. The clinical characteristics of these 37 patients are shown in Supplementary Table S1. The patients included 25 males (67.5%) with a median age of 55 years and 12 females (32.5%) with a median age of 65 years. The median age of the patients at the time of diagnosis was 59 years (range 29–85). Of the 37 patients, GEP was obtained from paired BM/PB (n = 7), BM/LN (n = 3) and LN/PB (n = 2) samples from the same patient. We also tested an additional cohort of 40 PB samples (20) from CLL patients to validate the biologic and clinical findings.

Mouse- and Human-Derived Stroma Supports CLL Cell Growth

To investigate the influence of the microenvironments on the survival and growth of CLL cells, we used an in vitro system and cocultured CLL cells with stromal cells using BM-derived (OMA-AD) and endothelial-derived (HMEC) stromal cell lines (Figure 1A). Consistent with our previous observation, cocultured CLL cells showed a tendency of higher proliferation, as measured by a 1.5- and 3.5-fold increase in Ki-67–positive CLL cells, in OMA-AD and HMEC cocultured conditions, respectively. Consistent with this observation, prolonged survival was observed with significantly (p < 0.0001, p < 0.005) reduced frequency of early apoptotic (positive for annexin V only) and late apoptotic (positive for annexin V/PI) co-cultured CLL cells under both types of stromal cell systems (Figure 1B). Interestingly, cocultured CLL cells demonstrated significant (p < 0.05) upregulation of NF-κB target genes, for example, IL-6, BCL2 and TRAF1, as measured by quantitative real-time (qRT)-PCR (Figure 1). Thus, these studies indicate the importance of a compact stromal microenvironment usually found in tissues such as LNs in providing prosurvival and pro-proliferation signals to CLL cells.

Gene Expression Signature of PB-, BM- and LN-CLL Cells

In our initial analyses using unsupervised hierarchical clustering of ~9,900 genetic probes on GEP of CLL cells from three in vivo sites, we observed that CLL cells from the three anatomic sites did not form tight clusters, indicating heterogeneity within the tissue compartments (Figure 2A). However, of the samples from the three lymphoid compartments, LN specimens (n = 15) clustered into two major nodes, whereas PB-CLL and BM-CLL samples were interspersed randomly, suggesting more homogeneity of gene expression among LN-CLL cells compared with PB-CLL and BM-CLL samples. These two groups of LNs were not distinguished from each other based on the known chromosomal abnormalities, high versus low CD38 expression, presence or absence of lymphadenopathy and/or time to treatment. Therefore, to obtain tissue-specific CLL gene signatures, supervised differential gene ex-
expression analysis was performed using a random variance F test \( (p < 0.01, \text{FDR} < 0.08) \). With this approach, we observed ~1,000 genes that were differentially expressed among PB-, BM-, and LN-CLL cells (Figure 2B). A detailed analysis showed that LN-CLL cells overexpressed various gene signatures involved in B-cell activation \((CD40, SYK, TACI, BCMA)\), chemokine receptor signaling \((CXCL13, CXCL14, CCL21)\) and induction of immune tolerance \((CAV1, BATF, TTK, INDO1, IL10, CAMLG)\) (Figure 2B, Supplementary Figures S1A, B). Further, genes associated with mitogen-activated protein kinase (MAPK)-, phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT)- and NF-κB–signaling pathways were distinctly expressed in CLL cells from PB, BM and/or LN sites. Additionally, transcripts involved in B-cell activation and differentiation \((CXCR4, FCER2, AKT2)\) were noted in the PB signature, whereas cytoskeleton and cell adhesion molecules \((KRT19, KRT18, COL10A1)\) were noted in the BM-CLL signature (Figure 2B, Supplementary Figure S1).

**Differentially Expressed Genes Associated with the Seven Major Signaling Pathways**

We next analyzed gene expression of CLL cells from different sites by using gene set enrichment analysis \((24)\) to identify enriched cellular pathways/signatures. We observed >100 enriched cellular pathways/signatures among the CLL cells from LN, BM and PB. These analyses showed that LN-CLL cells were enriched in gene signatures related to tumor progression, and immunosuppression or immune tolerance, suggesting that the LN microenvironment is conducive to tumor cell growth while inhibiting host immune response against CLL (Table 1; Supplementary Figures S2A, B, D, E). Additionally, the poor prognostic IGHV3-21 gene signature \((26)\) consisting of genes associated with a worse outcome in CLL was enriched in
Figure 2. Unsupervised and supervised clustering of gene expression profiles of PB-CLL (n = 20), BM-CLL (n = 18) and LN-CLL (n = 15) cells in vivo from patients. (A) Unsupervised hierarchical clustering of genes obtained from CLL enriched lymphoid compartments. (B) Supervised clustering of genes of CLL cells isolated from different lymphoid compartments (p < 0.01, FDR < 0.08).
LN-CLL compared with PB-CLL (Table 1, Supplementary Figure S2F). BM-CLL showed enrichment of gene signatures involved with cell cycle and antiapoptotic compared with PB-CLL (Table 1, Supplementary Figure S2C).

From the several enriched pathways differentially expressed among PB-, BM- and LN-CLL cells, seven major pathways/functional signatures relevant to CLL and/or B-cell biology were identified based on gene ontology: BCR signaling, B cell–activating factor/A proliferation–inducing ligand (BAFF/APRIL) signaling, MAPK signaling, PI3K/AKT signaling, NF-κB signaling, chemokine ligands/receptors and tolerance (Figure 3A). Further, the expression of critical genes in these signatures was validated by qRT-PCR. Only genes with significant differential expression (t test, p < 0.05) are shown in Figure 3. Several transcripts involved in BCR signaling (for example, SYK, BTK and VAV2) and BAFF/APRIL signaling (for example, BAFF, BCMA and TRAF2) that were significantly expressed in LN-CLL by microarray were confirmed by qRT-PCR (Figure 3B, Supplementary Table S2), thus suggesting activated BCR and BAFF/APRIL signaling in LN compared with PB or BM. Despite the absence of probes for ZAP-70 on our microarray, we studied its expression using real-time PCR and found that it was significantly (p < 0.05) overexpressed in LN-CLL compared with PB-CLL and BM-CLL (Figure 3B). Similarly, transcripts of different genes associated with NF-κB (IL6, NF-κB1, RELB, STAT1), MAPK (MAP3K2, MAP2K5, MAP2K11, MAPK4) and PI3K/Akt (AKT1, 4E-BP1, PDK1, IGFBP6) pathways that were overexpressed in CLL cells from the different microenvironments (Figure 2, Supplementary Figure S1) were validated by qRT-PCR (Figure 3B). These pathways play an active role in the proliferation of CLL cells. It is likely that CLL cells in different microenvironments use different molecules for survival/proliferation.

From the GEP data, we surmised that CLL cells use chemokine ligands/receptors for migration; for example, CXCR4 and CCR7, were significantly overexpressed in PB-CLL and their cognate ligands CXCL12 and CCL21 were overexpressed in BM and LN microenvironments (Figures 2B and 3). These data are consistent with chemokine receptor/ligand gradients governing migration of CLL cells into different microenvironments. Interestingly, genes associated with immune tolerogenesis (CAV1, MCM3, BATF) were uniquely overexpressed in LN-CLL compared with BM-CLL and PB-CLL (Figures 2B and 3, Supplementary Figure S1), indicating the LN microenvironment plays a role in inducing immunological tolerance of CLL cells. The role of this tolerogenic signature in LN is recently validated by us (38). Together, these results elucidate the genes overexpressed in LN-CLL and indicate that the microenvironment influences CLL cell behavior. To further confirm the LN microenvironment-induced effects on CLL progression, the expression of previously reported drug resistance–related genes was studied (27,28).

Among the genes we analyzed, ABCC6, HSP70 and HSP90 were significantly overexpressed in LN-CLL compared with BM-CLL and PB-CLL cells, indicating that the LN microenvironment also induces drug resistance in CLL (data not shown). We validated the expression of phosphor-SYK (BCR signaling) and p-P65 (NF-κB signaling) in TCL1 transgenic mouse and patients’ LN by using immunohistochemistry (Figure 4). LN tissue was harvested from TCL1-Tg mice at 37 wks, at which time accumulation of leukemic B-cells (B220/CDS1) occur in lymphoid organs (40). A high percentage of leukemic B-cells from LN of TCL1-Tg mice were found to be positive for p-SYK (~50%) and p-P65 (~99%) (Figure 4, panel I: A–F). The expression pattern of p-SYK and p-65 in leukemic cells from spleen tissue of TCL1-Tg mice at 37 wks were highly similar with that of LN from the mice (data not shown). Furthermore, the expression of these key signaling molecules were investigated in patient LNs. As expected, a high percentage of CLL cells were positive for p-SYK (~90%) and p-P65 (100%) signaling molecules, validat-

### Table 1. Enriched pathways observed by using gene set enrichment analysis. 

| Pathway Description                                      | LN enriched                                                                 | BM enriched                                                                 | PB enriched                                                                 |
|-----------------------------------------------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------|
| Immune suppressive (Sasaki ATL Up), Cancer progression    | B-cell differentiation (Martinelli Ifns Differentiation, Haddad HSC CD7 Up) | No comparison made                                                          | Immunlty and inflammation (Passerini_Immune, Passerini Inflammation)       |
| Cancer Neoplastic Meta Up                                 |                                                                              |                                                                              |                                                                              |
| LN versus BM CLL                                          |                                                                              |                                                                              |                                                                              |
| Immune suppressive (Sasaki ATL Up), Cancer progression    |                                                                              |                                                                              |                                                                              |
| Cancer Neoplastic Meta Up                                 |                                                                              |                                                                              |                                                                              |
| LN versus PB CLL                                         |                                                                              |                                                                              |                                                                              |
| Immune suppressive (Sasaki ATL Up), Cancer progression    |                                                                              |                                                                              |                                                                              |
| Cancer Neoplastic Meta Up                                 |                                                                              |                                                                              |                                                                              |
| B-CLL poor prognosis (IgVH3–21 Gene signature)            |                                                                              |                                                                              |                                                                              |
| BM versus PB CLL                                         |                                                                              |                                                                              |                                                                              |
| No comparison made                                        |                                                                              |                                                                              |                                                                              |
| Cell cycle regulatory (Cell Cycle pathway. Ageing Brain   |                                                                              |                                                                              |                                                                              |
| Up. G1 To S Cell Cycle Reactome)                          |                                                                              |                                                                              |                                                                              |

*The words in parentheses in this table are names of signatures, which pop up through GSEA analyses on the GSEA website. Each name indicates a specific gene set at the GSEA website.*
Figure 3. Mean expression of genes associated with seven major signaling pathways and validation of expression of key genes using real-time PCR. (A) Supervised cluster analyses of differentially expressed genes (p < 0.05) associated with seven major signaling pathways. The mean expressions of these significant genes are shown among PB, BM and LN cells. (B) Confirmation of the differentially expressed genes among PB-CLL, BM-CLL and LN-CLL using real-time PCR. Expression of the selected genes from each major pathway was studied by using real-time PCR. Significant differences in expression levels between PB-CLL, BM-CLL and LN-CLL were determined by using the Student t test (p values when comparing with LN cells: *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.001; p values for comparison with PB is denoted with *p and comparison with BM is denoted as *p). First row: BCR signaling: SYK, BTK, ZAP70; second row: BAFF/APRIL signaling: BAFF, BCMA, TRAF2; third row: MAPK signaling: MAP2K6, CALMG, STAT1; fourth row: PI3K/Akt pathway: PDK1, IGFBP6, AKT1; fifth row: NF-κB pathway: NF-κB1, FCER2, CCND2; sixth row: chemokine ligands/receptors: CXCR4, CCL21, CCR7; seventh row: tolerogenic signature: CAV1, MCM3, IDH; microarray analyses had shown all genes were differentially expressed among PB-CLL (n = 20), BM-CLL (n = 18) and LN-CLL (n = 15).

Differential Expression of Key Genes in Prognostic Groups in PB-CLL, BM-CLL and LN-CLL

We correlated gene expression profiles with three clinical parameters including chromosomal abnormalities, bulky lymphadenopathy (BLA) and time to treatment in CLL cells from each tissue compartment to identify gene signatures that may be responsible for adverse clinical outcome.

Lymphadenopathy. GEPs derived from PB samples were compared in patients with (n = 9) or without (n = 11) BLA. Pa-
patients with BLA had a significantly higher number (n > 400) of upregulated genes (Figure 5A), and among these BCR signaling (CD79b, CD72, SYK, BTK, BLNK) was the dominant signature in this subset of cases. To confirm BCR activation, phosphorylated-SYK (p-SYK) was measured by using flow cytometry in four samples from CLL patients with BLA and five from patients without BLA. Expression of p-SYK was significantly (p < 0.05) higher in the CLL samples with BLA compared with those without it (Figure 5B).

Chromosomal abnormalities. We performed differential expression between PB samples of patients with high-risk cytogenetic abnormalities [del(11q), del(17p) and trisomy12, n = 7] versus cases without these abnormalities (n = 12). Significantly higher expression of genes associated with BCR signaling and cell activation (BTK, AKT2, CD83, LAG3) correlated with the higher risk of genetic abnormalities (Supplementary Figure S3A). A similar observation was observed in BM-CLL GEP analysis (Supplementary Figure S3B). In addition, we observed that the transcripts of chemokine ligand CCL3 significantly correlated with higher risk of cytogenetic abnormalities in both BM-CLL (p < 0.05) and LN-CLL (p < 0.05) specimens (Figure 5C). These observations are consistent with our validation cohort of PB-CLL samples (n = 40), where high CCL3 mRNA expression was also significantly (p = 0.02) associated with a shorter time to treatment (Figure 5D).

Time to treatment. Transcripts encoding genes associated with B-cell activation (CD69) were significantly associated with short time to treatment (<12 months; n = 9) compared with the group with a longer time to treatment (>12 months; n = 10) (Supplementary Figure S3C), consistent with the above observation. A subset of genes related to apoptosis (CASP6, BID, and CASP4) was upregulated in LN cases with shorter interval of treatment, suggesting that antiapoptotic functions dominate in this group (Supplementary Figure S3D).

Overall, these observations indicate that chronic BCR signaling is a dominant pathway in CLL, as indicated by higher expression of BTK, AKT2, CD69, and phosphorylated SYK (p-SYK) in the chro-

Figure 4. Confirmation of expression of pSyk and p-P65 in lymphoid tissue of a TCL-1 transgenic mouse model and human patients using immunohistochemistry. Panel I: A representative immunohistochemistry of LN tissue from TCL-1 transgenic mouse. Lymphoid tissue were collected at wk 37 from the mouse and stained for B220 (A), CD5 (B), p-P65 (C), pSYK (D) and control antibody (E). Average of percent positive B220/CD5+ cells for pSYK and p-P65 from the transgenic mice (n = 3) is shown (F). Panel II: A representative immunohistochemistry of LN from CLL patient. LN tissue from CLL patient is stained with CD5 (A), CD19 (B), p-P65 (C), p-SYK (D) and control antibody (E). Average of percent positive CD19/CD5+ cells for p-SYK and p-P65 from patient LNs (n = 3) is shown (F).
mosomal abnormalities, time to treatment and BLA prognostic groups, respectively, and therefore may be a valuable prognostic and therapeutic target in CLL.

**DISCUSSION**

We and others have studied the role of microenvironments in survival/proliferation and migration of CLL cells using *in vitro* culture systems (25,29). However, *in vitro* culture systems have limitations and, therefore, we analyzed gene expression in CLL cells directly from these *in vivo* mi-
microenvironments. Although the GEP of CLL cells from PB, BM and LN did not cluster in a tissue-specific manner, LN-CLL cells are far less heterogeneous than GEP of PB-CLL and BM-CLL, as evidenced by the number of clusters in Figure 2A. Our findings are consistent with the LN microenvironment providing chronic stimulation via the BCR and BAFF/APRIL, which are critical for the growth and survival of CLL cells. Our observation is consistent with the recent findings by Herishanu et al. (18) showing that the BCR signaling pathway is activated at LN sites and is likely a major pathogenetic mechanism of CLL progression (29,30,41,42). We validated this observation by measuring levels of the kinase SYK, which is a key in BCR signaling using qRT-PCR and immunohistochemistry. Unlike Herishanu et al., our study analyzes PB, BM and LN cells from different patients, uses TCL1 transgenic mice and correlates GEP with prognostic groups. Despite using CLL cells from different sites from different patients, we observed similar findings that BCR signaling molecules are overexpressed in LN-CLL, indicating that LN tissue sites play a prominent role in influencing the gene signature. Additionally, our studies also observed that BAFF/APRIL-related genes were also enriched in LN-CLL cells. These genes have been shown to promote cell survival and adverse prognosis in CLL (31,43). These observations suggest that LN sites provide signals for chronic activation of CLL cells. Expression of p-SYK (key player of BCR signaling pathway) and p-P65 (key molecule of NF-κB pathway) in the lymphoid compartment was validated by using LN samples of patients and the Eu-TCL1 mouse model.

Although certain interactions were specifically observed in LN, molecules of other major pathways, for example, NF-κB and MAPK pathways (44), were constitutively active in all tissue sites, indicating that they may be responsible for survival/proliferation of CLL cells regardless of anatomic location. Chemokine ligands/receptors are being investigated extensively to better understand the migration of CLL cells. We observed that CCR7, CXCR5 and CXCR4 were overexpressed by PB-CLL, and their respective cognate ligands CCL21 and CXCL13 were expressed by LN and CXCL12 in BM sites. Upregulation of CCR7 was shown to facilitate the migration of CLL cells (32) and, similarly, it is also facilitated by the interaction of the CXCR4-CXCL12 axis in CLL (33,34). These observations indicate that distinct microenvironments express different ligands for interaction with tumor cells. Interestingly, CCL3 was expressed by all three types of CLL cells. CCL3 is known to express at high levels in CLL cells when cocultured on stroma (9,35) and does attract T cells and follicular dendritic cells, thus indicating that CCL3 is crucial for CLL cells in different microenvironments, probably to form clusters with other cell types. We observed that high expression of CCL3 was associated with the poor outcome groups in BM and LN, suggesting its role in disease progression. Consistent with these findings, higher CCL3 expression was also associated with a shorter time to treatment in PB cases. Furthermore, higher expression of CCL21 and CXCL13 was observed in the LN-CLL. These chemokine ligands are known to be expressed by CLL as well as other stromal cells, and thus a very high expression may be partially contributed because of some (<10%) contaminating stromal cells in our LN-CLL preparation.

Interestingly, an immune suppressive signature was also observed in CLL cells from LN sites as previously reported by Sasaki et al. (36) in acute-type adult T-cell leukemia. Among the key tolerogenic genes, CAV1, IFI16 and IDO1 were significantly overexpressed in LN-CLL compared with PB-CLL or BM-CLL. Although LN is an appropriate place for immune surveillance and generation of immune response, it seems to provide CLL cells with a supportive home. One explanation for this phenomenon is that the immune-suppressive gene signature expressed by CLL cells creates immune dysfunction in the LN site and, thus, may contribute to immune evasion of CLL cells in this site. These genes may play a crucial role in modulating T-cell behavior and immune synapse formation for the benefit of CLL cells (38,39). Because BLA is used as a marker of aggressive disease (21), we correlated GEPs with the presence and absence of BLA in PB. Interestingly, BCR signaling genes including SYK, BTK, CD79b and BLNK were overexpressed in PB-CLL from patients with BLA. Furthermore, genes related to hedgehog signaling, chemokines, BAFF/APRIL and MAPK signaling were also overexpressed in PB-CLL from BLA patients (Figure 5A). Thus, the gene expression signature of PB-CLL cells from patients with BLA resembles that of LN-CLL, suggesting that these PB-CLL cells share a close and expected relationship with the LN-CLL cells. This result is in accord with the recent reports that quiescent PB-CLL cells are similar to proliferating B cells of LN pseudofollicles (19), and a dynamic relationship exists between quiescent PB-CLL cells with the lymphoid microenvironment (37). Similarly, overexpression of BCR signaling molecules was also observed in LN and BM samples from poor prognostic groups, although the number of samples in each prognostic group was smaller in these sites.

The importance of the microenvironment has also been demonstrated by in vitro systems wherein CLL cells survive/proliferate in the presence of stromal cells (25). In concordance with other studies, our in vitro stromal cultures promoted survival and proliferation of CLL cells. Because the microenvironment regulates the growth of CLL cells by regulating expression of key genes (45), we studied expression of a few NF-κB–associated genes in CLL cells cocultured on stromal cells. In this study, CLL cells cultured on stroma upregulated NF-κB–associated genes IL6, BCL2 and TRAF1, thus reinforcing the importance of the microenvironment in regulating gene expression.

CONCLUSION

We have summarized our results and their possible interpretation in a hypothetical model of survival/proliferation and migration among PB-CLL, BM-CLL and LN-CLL (Figure 6), where it is shown that
PB-CLL cells use various MAPK and NF-κB target molecules for survival/proliferation in PB and use CCR7 and CXCR5 chemokine receptors for their migration toward the LN microenvironment, mediated by their cognate ligands CCL21 and CXCL13, which are expressed by LN-CLL. In the LN, CLL cells secrete the chemokine ligand CCL3 to attract T cells and stromal cells. The interactions of CLL cells and stroma lead to the activation of BCR and BAFF/APRIL signaling, which in turn activates MAPK- and NF-κB–associated molecules. PB-CLL cells express chemokine receptor CXCR4, and its cognate ligand CXCL12/SDF-1 is secreted by BM stroma, thereby influencing the migration of PB-CLL cells to the BM. In the BM, CLL cells express CCL3 to attract T cells and stromal cells; this interaction activates expression of NF-κB and MAPK molecules in BM-CLL cells, including v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NF-κBIB) and BCL2-related protein A1 (BCL2A1), for survival/proliferation. Together our results suggest that interactions between the LN microenvironment and CLL cells lead to the survival, proliferation and migration of CLL cells while inducing the host immune suppression that results in CLL progression. Overall, our study facilitates better understanding of the pathobiology of CLL and also lays the foundation for additional studies to eventually develop improved therapy for CLL by targeting Btk, Syk, Bcl2 and other identified key molecules. Importantly, these molecules are overexpressed in LN-CLL cells, which suggests that targeting these molecules/LN-CLL cells residing in LN will improve better chances of disease remission.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.
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