LYMPHOID NEOPLASIA

MicroRNA-155 influences B-cell receptor signaling and associates with aggressive disease in chronic lymphocytic leukemia

Bing Cui,[1] Liguang Chen,[1] Suping Zhang,[1] Marek Mraz,[1,2] Jessie-F. Fecteau,[1] Jian Yu,[1] Emanuela M. Ghia,[1] Ling Zhang,[1] Lei Bao,[1] Laura Z. Rassenti,[1] Karen Messer,[1] George A. Calin,[3] Carlo M. Croce,[3] and Thomas J. Kipps[1]

[1]Moores Cancer Center, University of California, San Diego, CA; [2]Center of Molecular Medicine, Central European Institute of Technology, Masaryk University, Brno, Czech Republic; and [3]Department of Molecular Virology, Immunology, and Medical Genetics and Comprehensive Cancer Center, Ohio State University, Columbus, OH, of the CLL Research Consortium

Key Points

- High-level *miR-155* enhances BCR signaling, and is associated with poor prognosis in CLL.
- Signals within the CLL microenvironment, such as CD154 or BAFF, can induce *miR-155* and enhance BCR signaling.

High-level leukemia cell expression of *micro-RNA 155* (*miR-155*) is associated with more aggressive disease in patients with chronic lymphocytic leukemia (CLL), including those cases with a low-level expression of ζ-chain–associated protein of 70 kD. CLL with high-level *miR-155* expressed lower levels of Src homology-2 domain-containing inositol 5-phosphatase 1 and were more responsive to B-cell receptor (BCR) ligation than CLL with low-level *miR-155*. Transfection with *miR-155* enhanced responsiveness to BCR ligation, whereas transfection with a *miR-155* inhibitor had the opposite effect. CLL in lymphoid tissue expressed higher levels of *miR155HG* than CLL in the blood of the same patient. Also, isolated CD5^{bright}CXCR4^{dim} cells, representing CLL that had been newly released from the microenvironment, expressed higher levels of *miR-155* and were more responsive to BCR ligation than isolated CD5^{dim}CXCR4^{bright} cells of the same patient. Treatment of CLL or normal B cells with CD40-ligand or B-cell–activating factor upregulated *miR-155* and enhanced sensitivity to BCR ligation, effects that could be blocked by inhibitors to *miR-155*. This study demonstrates that the sensitivity to BCR ligation can be enhanced by high-level expression of *miR-155*, which in turn can be induced by crosstalk within the tissue microenvironment, potentially contributing to its association with adverse clinical outcome in patients with CLL. (*Blood*. 2014;124(4):546-554)

Introduction

Signaling via the B-cell receptor (BCR) is thought to play a role in the pathogenesis and/or progression of chronic lymphocytic leukemia (CLL).[1,2] BCR signaling is triggered by phosphorylation of immunoreceptor tyrosine-based activation motifs of CD79a/b and induces a cascade of downstream signaling pathways.[3,4] The importance of this cascade in CLL biology appears underscored by clinical trials demonstrating clinical activity of small-molecule kinase inhibitors that can block BCR signaling.[5]

The biology of CLL also can be influenced by microRNAs.[6-10] In particular, the evolutionarily conserved microRNA *miR-155* is a critical regulator of posttranscriptional gene expression in B cells.[6] It is encoded within a region known as the B-cell integration cluster (*BIC, miR155HG*), which originally was identified as being a frequent integration site for avian leucosis virus.[11-13] This microRNA is overexpressed in numerous B-cell lymphomas,[14-16] including CLL,[8,17-20] suggesting that *miR-155* contributes to lymphoma development.[19] Consistent with this notion is the observation that mice made transgenic for *miR-155* under a B-cell–specific promoter developed pre–B-cell lymphomas.[21] Specifically, high levels of *miR-155* also are present in diffuse large B-cell lymphomas with an activated B-cell phenotype, which is associated with a relatively poor clinical prognosis.[14] Moreover, relatively high-level expression of *miR-155* in CLL has been associated with expression of adverse prognostic markers, such as the ζ-chain associated protein of 70 kD (ZAP-70), unmutated immunoglobulin heavy chain variable region genes (IGHV), and/or deletions in 17p or 11q.[22-25] Overexpression of *miR-155* in transgenic mice induces polyclonal B-cell expansion, suggesting that *miR-155* could enhance B-cell proliferation.[21]

One identified target of *miR-155* is the Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1), which is encoded by *INPP5D*.[26,27] SHIP1 is a phosphatase that acts in opposition to kinases, which are integral to many signal transduction pathways.[28] Such inhibitory phosphatases may suppress surface immunoglobulin and BCR signaling.[29] Such signaling appears most prominent in lymph nodes (LNs), where CLL cells apparently become activated in response to signals from the LN microenvironment.[30] Moreover, CLL cells in the LNs have upregulated BCR gene expression signatures and higher proportions of cells undergoing proliferation than CLL cells in the blood.[30] Conceivably, the expression of *miR-155* could influence the relative expression of SHIP1 in CLL, which

Submitted March 26, 2014; accepted June 4, 2014. Prepublished online as *Blood* First Edition paper, June 9, 2014; DOI 10.1182/blood-2014-03-559690.

B.C. and L.C. contributed equally to this work.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Characteristics of the patients

Characteristic	Value Training dataset	Value Validation
No. of patients	86	181
Male sex, no. of patients (%)	55 (64)	107 (59)
Rai stage		
0-II	60 (70)	154 (85)
III-IV	9 (10)	6 (3)
No data	17 (20)	21 (12)
Age at diagnosis, y		
Median	56	56
Range	34-78	34-81
ZAP-70 expression		
ZAP-70 negative (0% to 20%)	41 (48)	110 (61)
ZAP-70 positive (>20%)	45 (52)	71 (39)
IGHV		
Unmutated (≥98% homology)	43 (50)	81 (45)
Mutated (<98%homology)	43 (50)	100 (55)

then could influence the relative activation of signaling pathways triggered by ligation of the BCR by self- or environmental antigen(s). We hypothesize that high-level expression of *miR-155* in CLL can repress expression of SHIP1 and increase the responsiveness to BCR ligation, thus possibly accounting for its association with adverse clinical outcome in patients with CLL.

Materials and methods

Cells and sample preparation

Blood samples were collected from consenting patients who satisfied diagnostic criteria for CLL and enrolled in University of California San Diego Moores Cancer Center biorepository per a protocol approved by the institutional review board (080918). At the time of sample collection, patients had not received prior therapy. IGHV mutation status and ZAP-70 expression were assessed according to established criteria.[31] We used Ficoll-Hypaque density-gradient centrifugation to obtain mononuclear cells, of which ≥95% were $CD5^+CD19^+$ cells. Descriptions of cell–cell transfection, measurement of intracellular calcium flux, real-time polymerase chain reaction (PCR), flow cytometry, and statistical analyses are provided in the supplemental methods on the *Blood* Web site. This study was conducted in accordance with the Declaration of Helsinki.

Results

High-level expression of *miR-155* is associated with adverse clinical outcome

We studied the relationship between the relative leukemia cell expression of *miR-155* and treatment-free survival (TFS), or overall survival (OS), in a cohort of 86 CLL patients (Table 1; supplemental Table 1), for which we assayed *miR-155* expression levels using absolute real-time PCR. Forty-three of the samples used mutated IGHV and the other 43 used unmutated IGHV; 41 of the samples lacked expression of ZAP-70, whereas 45 expressed ZAP-70. We used the profile-likelihood method in a Cox regression model of TFS to determine the optimal threshold level of *miR-155* that might segregate these patients into 2 subgroups with disparate progression tendencies. Thirty-one patients were stratified into a "*miR-155*-Low" subgroup and 55 patients were assigned to a "*miR-155*-High" subgroup, defining a cutoff point of 2553 copies per CLL cell. The subgroup of patients in the *miR-155*-High subgroup had a median TFS of 4.4 years, whereas those patients in the *miR-155*-Low subgroup had a median TFS of 11.4 years (Figure 1A). The median OS for patients in the *miR-155*-High subgroup (11.3 years) also was shorter than that of patients in the *miR-155*-Low subgroup (>20 years) (supplemental Figure 1). Triplicate analyses using relative real-time PCR (*miR-155* vs *RUN6B*) demonstrated that the CLL cells in the *miR-155*-High subgroup had 2.4-fold higher mean level of *miR-155* than did the CLL cells in the *miR-155*-Low subgroup (6.6 ± 0.5 vs 2.8 ± 0.3; supplemental Figure 2).

Although there was a significant association between expression of high-level *miR-155* and ZAP-70 or use of unmutated IGHV (supplemental Figure 3), these associations were not absolute. Some of the CLL samples in the *miR-155*-High subgroup did not express ZAP-70 (n = 24) or used mutated IGHV (n = 17); such cases had a mean level of *miR-155* that did not differ significantly from that of cases in the *miR-155*-High subgroup that were ZAP-70 positive or used unmutated IGHV. Similarly, some of the CLL samples in the *miR-155*-Low subgroup expressed ZAP-70 (n = 14) or used unmutated IGHV (n = 10) (Figure 1B-C).

We used an independent data set of 181 additional patients, who had expression levels of the *miR-155* precursor measured by microarray (GSE13159, GSE13164).[32] The median follow-up time for this validation cohort was 7.6 years, with 64.1% (n = 116) of patients receiving therapy and 24.3% patients deceased (n = 44), similar to the original cohort. In the original collection of 86 samples, the mature *miR-155* measured by quantitative PCR was well correlated its precursor measured by microarray ($r = 0.6$, $P < .001$; supplemental Figure 4). We thus calibrated the cut-point established from the quantitative PCR assay to the microarray assay using linear regression on these original 86 samples (supplemental Figure 4). We then validated the association of high-level expression of *miR-155* (as measured by the microarray) with reduced TFS and OS in the 181 new subjects, using the optimal cut-point established through analysis on the original 86 subjects. From the analysis of these new, independent data, we observed that high-level expression of *miR-155* retained its strong predictive effect for TFS (Figure 1D), both when considered alone (hazard ratio [HR] 1.7; 95% confidence interval [CI] 1.1-2.4) or in a model that contained both IGHV mutational status and ZAP-70 status (Table 2). In addition, greater OS was observed for the *miR-155*-High group, although this did not attain statistical significance in univariate analysis (supplemental Figure 1B). In multivariate Cox models, we confirmed the independent prognostic information of *miR-155* over and above the established risk factors IGHV and ZAP-70 (Table 2), both for TFS (HR 1.52; 95% CI 1.03-2.23) and for OS (HR 2.22; 95% CI 1.08-4.54), using the validation cohort. Moreover, among patients with CLL cells that lacked expression of ZAP-70, those with high levels of *miR-155* (n = 56) had a shorter observed median TFS (6.0 years) than patients with low-level expression of *miR-155* (n = 54, 9.6 years, Figure 1E). Among patients with CLL cells that expressed mutated IGHV genes, high levels of *miR-155* (n = 48) were associated with significantly shorter median TFS (6.7 years) than those with low-level expression of *miR-155* (n = 52, 18.3 years, Figure 1F).

Relationship between high-level expression of *miR-155* and expression of SHIP1 in CLL

We examined CLL cells for expression of SHIP1 by flow cytometry. In other cell types, *miR-155* has been noted to target the gene encoding

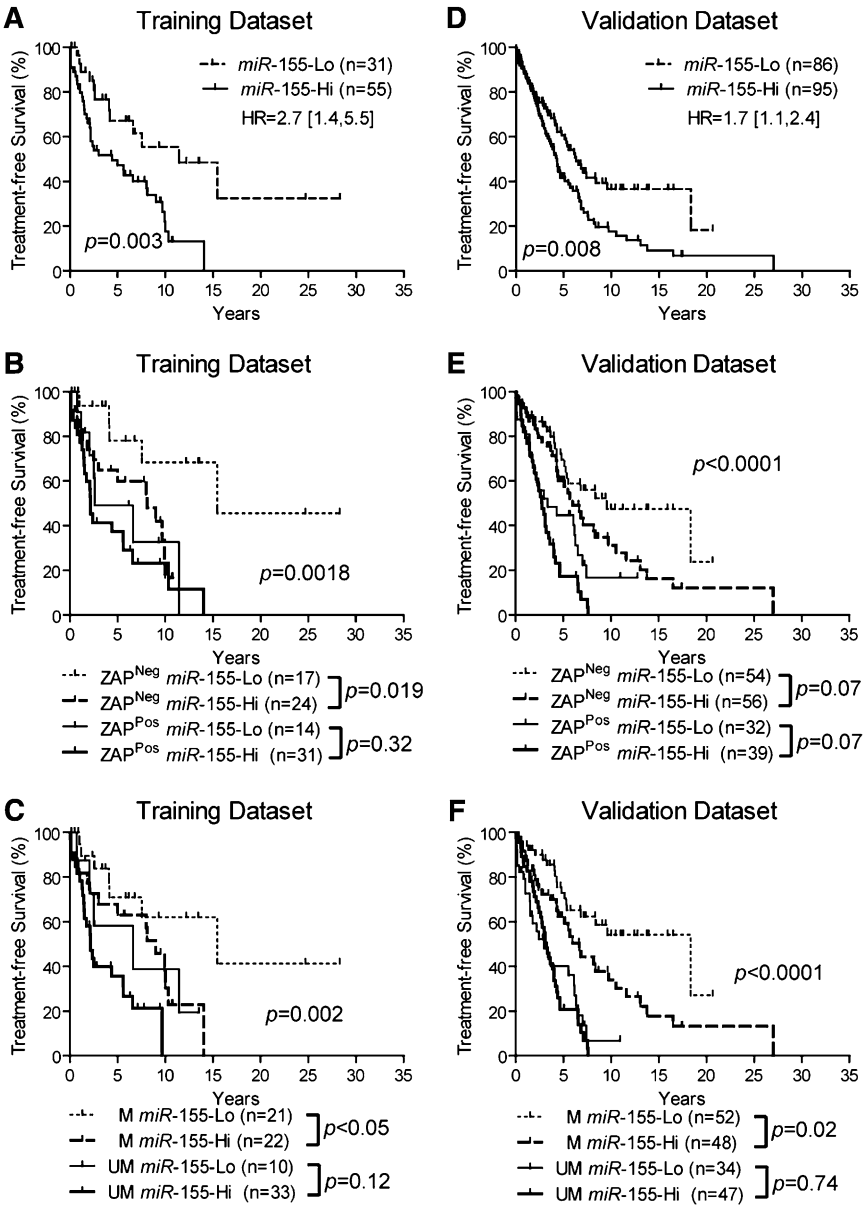

Figure 1. Relationship between expression levels of *miR-155,* ZAP-70, IGHV mutation status, and TFS. (A) In the training dataset, Kaplan-Meier curves depicting the TFS probability over time from diagnosis of patients who were segregated into 2 groups (*miR-155*-Lo or *miR-155*-Hi) according to the relative amounts of *miR-155* expressed by the blood CLL cells of each patient. (B,C) Kaplan-Meier curves depicting the TFS probability over time from diagnosis of patients segregated by *miR-155* and ZAP-70 status (B), or *miR-155* and IGHV mutation status (C). (D) In the validation dataset, Kaplan-Meier curves depicting the TFS probability over time from diagnosis for patients who were segregated into 2 groups (*miR-155*-Lo or *miR-155*-Hi) based upon whether or not the blood CLL cells expressed *miR-155* above the training set–defined threshold for high *miR-155HG*. (E,F) In the validation dataset, Kaplan-Meier curves depicting the TFS probability over time from diagnosis for patients segregated by *miR-155* and ZAP70 status (E) or *miR-155* and IGHV status (F). Statistical significance was determined by log-rank test ($P < .05$). *P* values for the comparisons between subgroups are indicated below the graph for panels B, C, E, and F.

SHIP1, namely *INPP5D*.[26,27] However, *miR-155* had not been reported to influence expression of SHIP1 in CLL cells. We found that CLL cells in the "High *miR-155*" subgroup (n = 21) had a median mean fluorescence intensity ratio (MFIR) when stained for SHIP1 of 6.4 (± 0.3), which was significantly lower than that for CLL cells in the "Low *miR*-155" subgroup (8.0 ± 0.4, n = 24, $P < .01$) (Figure 2A; supplemental Figure 5A).

To further evaluate the relationship between expression of SHIP1 and *miR-155* in primary CLL cells, we transfected CLL cells with a mimetic of *miR-155*. Transfection of CLL cells with the *miR-155* mimetic reduced expression of SHIP1, as assessed by immunoblot or flow cytometric analysis (n = 5, Figure 2B, top; supplemental Figure 5B,C). Moreover, the median MFIR of samples stained for SHIP1 was significantly lower in cells transfected with the *miR-155* mimetic than in control-transfected cells (4.4 ± 1.3 vs 6.5 ± 1.2, n = 3, $P < .05$) (Figure 2B, bottom). Conversely, we transfected CLL cells with inhibitors *of miR-155*. CLL cells transfected with a *miR-155* inhibitor were found to express higher levels of SHIP1, as assessed by immunoblot analysis or flow cytometry (5.7 ± 0.8 vs 4.0 ± 0.9, n = 3, $P < .05$) (Figure 2C). Collectively, these studies demonstrate that expression of SHIP1 in primary CLL cells is influenced by the relative expression of *miR-155*.

Relationship between relative expression of *miR-155* and sensitivity to BCR ligation

CLL cells were stimulated with anti-μ, which rapidly induced a calcium flux that could be assessed via flow cytometry. The median increase in fluorescence intensity of calcium flux induced by BCR ligation inversely correlated with SHIP1 expression in CLL cells (supplemental Figure 6). The median increase in calcium flux induced by anti-μ was significantly greater for CLL cells in the *miR-155*-High subgroup (3.4 ± 0.6) than in CLL cells in the *miR*-155-Low subgroup (1.2 ± 0.3, n = 10, $P < .01$) (Figure 3A). Transfection of the cells with a *miR-155* mimetic enhanced the magnitude of calcium flux induced by treatment with anti-μ (Figure 3B). The median increase in fluorescence intensity of calcium induced by treatment of CLL cells with anti-μ was significantly greater in *miR-155*-transfected

Table 2. Multivariable Cox regression analysis of TFS and OS (Cox proportional hazards regression model)

	Training dataset (quantitative PCR data)					
	TFS			OS		
Predictor	HR	95% CI	*P*	HR	95% CI	*P*
miR-155 (high vs low)	2.3	1.1-4.6	.02	3.4	1.0-11.1	.04
ZAP-70 (positive vs negative)	1.7	0.9-3.4	.11	3.2	0.9-11.0	.07
IGHV (unmutated vs mutated)	1.8	0.9-3.6	.09	7.0	1.8-27.8	.006
	Validation dataset (microarray data)					
	TFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
miR-155 (high vs low)	1.5	1.0-2.2	.03	2.2	1.1-4.5	.03
ZAP-70 (positive vs negative)	1.4	0.8-2.5	.23	1.4	0.6-3.3	.48
IGHV (unmutated vs mutated)	2.6	1.4-4.7	.002	3.0	1.1-8.0	.03

cells than in control-transfected CLL cells (Figure 3B) (3.4 ± 0.5 vs 1.9 ± 0.4, n = 7, $P < .01$). Conversely, CLL cells had reduced calcium flux in response to anti-μ after transfection with an inhibitor of *miR-155*. The median increase in fluorescence intensity of calcium flux induced by anti-μ was significantly lower in cells transfected with inhibitor of *miR-155* than in control-treated CLL cells (3.7 ± 0.4 vs 4.7 ± 0.5, n = 6, $P < .01$) (Figure 3C). Furthermore, we found that the median increase in calcium flux induced by treatment with anti-μ was significantly greater in ZAP-70–negative CLL cells with a high-level expression of *miR-155* than in ZAP-70–negative CLL cells with a low-level expression of *miR-155* (2.5 ± 0.6 vs 1.0 ± 0.4 n = 9, $P < .05$) (Figure 3D).

Intraclonal heterogeneity in CLL-cell expression of *miR-155*

We interrogated the PubMed GEO database on CLL cells from LN, bone marrow, or peripheral blood. We found that expression of the precursor to *miR-155*, namely *BIC (miR155HG)*, was significantly greater in CLL cells isolated from LN than in CLL cells isolated from the bone marrow or peripheral blood of the same patient (supplemental Figure 5). On the other hand, the expression level of *INPP5D*, encoding SHIP1, appeared higher in CLL cells isolated from the blood than in CLL from the LNs (supplemental Figure 6).

Recent studies have identified markers that can distinguish CLL cells that recently have exited from the LNs from those that have been in the circulation longer and that presumably are poised to reenter the LN tissue compartments.[33] The former CLL cells express low levels of CXCR4 ($CXCR4^{dim}$), but higher levels of CD5 ($CD5^{bright}$), relative to the latter, which are instead $CXCR4^{bright}CD5^{dim}$ CLL cells.[33] Prior studies demonstrated that $CXCR4^{dim}CD5^{bright}$ CLL cells have higher proportions of cells that stain for the proliferation marker Ki-67 than $CXCR4^{bright}CD5^{dim}$ CLL cells. Furthermore, gene expression analyses of isolated CLL subpopulations have revealed that $CXCR4^{dim}CD5^{bright}$ CLL cells have higher levels of pro-proliferation and antiapoptotic genes than $CXCR4^{bright}CD5^{dim}$ CLL cells of the same patient.[33]

We isolated blood $CXCR4^{dim}CD5^{bright}$ CLL cells and $CXCR4^{bright}CD5^{dim}$ CLL cells from the same patient and examined each subset for expression of *miR-155* and SHIP1 and their capacity to respond to BCR ligation (Figure 4A). Expression of *miR-155* was significantly higher in $CXCR4^{dim}CD5^{bright}$ CLL cells than that in $CXCR4^{bright}CD5^{dim}$ CLL cells (8.0 ± 4.5 vs 1.0 ± 0, n = 4, $P < .05$) (Figure 4B). On the other hand, expression of SHIP1 was significantly lower in $CXCR4^{dim}CD5^{bright}$ CLL cells than that in $CXCR4^{bright}CD5^{dim}$ CLL cells (Figure 4C). Furthermore, the median increase of calcium fluorescence intensity induced by anti-μ was significantly greater in $CXCR4^{dim}CD5^{bright}$ CLL cells than that in $CXCR4^{bright}CD5^{dim}$ CLL cells (2.4 ± 0.4 vs 0.4 ± 0.1, n = 10, $P < .01$) (Figure 4D).

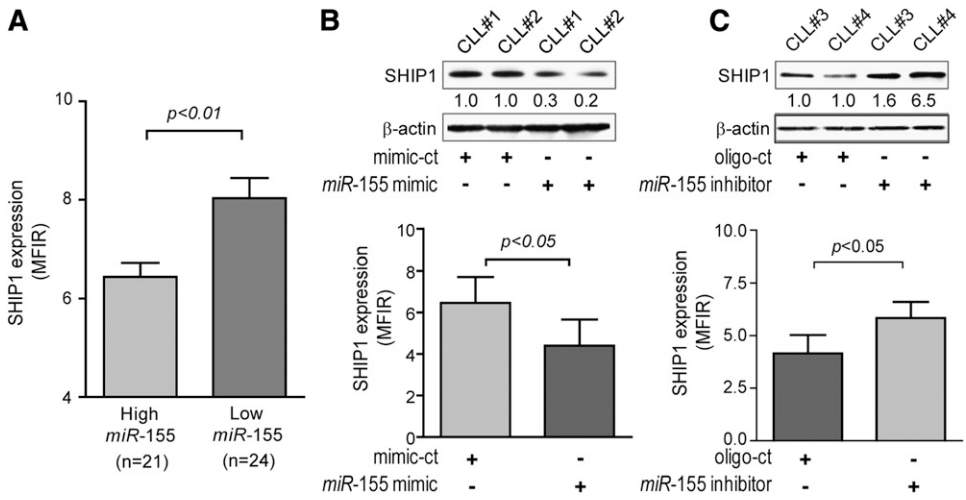

Figure 2. Expression of SHIP1 protein in CLL cells that expressed high vs low levels of *miR-155*. (A) Expression of SHIP1 in CLL cells that expressed high (n = 21) or low (n = 24) levels of miR-155, as indicated at the bottom of the histogram. The height of each column corresponds to the mean MFIR of cells stained for SHIP1 in each subgroup. Error bars indicate the standard deviation of the mean. Statistical significance was determined by unpaired Student *t* test ($P < .05$). (B) Expression of SHIP1 in representative CLL samples, CLL#1 (Rai stage 1 at diagnosis, mutated IGHV, ZAP-70–negative, Δ peak anti-μ–induced MFIR = 7) or CLL#2 (Rai stage 2 at diagnosis, mutated IGHV, ZAP-70–positive, Δ peak anti-μ–induced MFIR = 32), after transfection with mimic-ct (control microRNA) or *miR-155* microRNA, as indicated at the bottom of each panel. (C) Expression of SHIP1 in representative CLL samples, CLL#3 (Rai stage 2 at diagnosis, unmutated IGHV, ZAP-70–positive, Δ peak anti-μ–induced MFIR = 4.2) or CLL#4 (Rai stage 0 at diagnosis, mutated IGHV, ZAP-70–negative, Δ peak anti-μ–induced MFIR = 1.4), after transfection with oligo-ct (control microRNA) or *miR-155* inhibitor, as indicated at the bottom of each panel. In (B) and (C) data are presented from immunoblot analyses (top panels) or flow cytometry (bottom panels), respectively. After the immunoblots were probed with anti-SHIP1, they were stripped and probed with anti–β-actin to monitor the uniformity of protein loading. The numbers between the immunoblot panels provide density ratios of the SHIP1 band relative to that of the β-actin band, normalized with respect to the ratio observed in control-treated samples. The histograms in the bottom panel depict the mean MFIR of CLL cells stained for SHIP1 after transfection with (B) mimic-ct or *miR-155* mimic or (C) oligo-ct or *miR-155* inhibitor, as indicated at the bottom of each histogram. Statistical significance was determined by paired Student *t* test ($P < .05$).

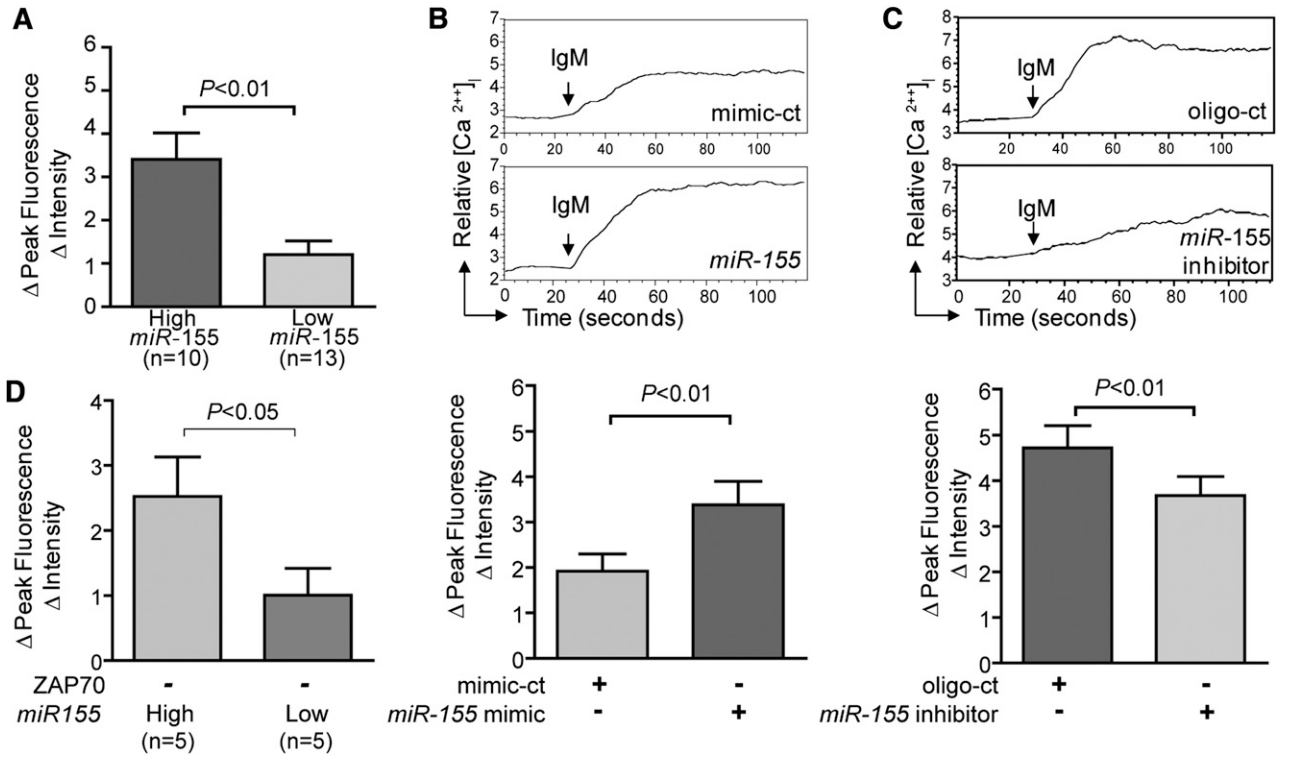

Figure 3. Anti-μ–induced calcium flux in CLL cells that express high vs low levels of *miR-155*. (A) The height of each column in the histogram describes the increase in fluorescence intensity after anti-μ stimulation of CLL cells that expressed high *miR-155* vs low *miR-155*, as indicated at the bottom of the histogram. (B) Anti-μ–induced calcium mobilization in CLL cells after transfection with mimic-ct (top graph) or *miR-155* (lower graph). The relative mean fluorescence intensity in intracellular calcium is plotted as a function of time. The arrow labeled "IgM" indicates the time at which the anti-μ was added to the cells. In the histogram at the bottom, the height of each column corresponds to the mean increase of fluorescence intensity after anti-μ stimulation for samples transfected with either mimic-ct or *miR-155* microRNA, as indicated at the bottom by a "+". (C) Anti-μ–induced calcium mobilization in CLL cells after transfection with oligo-ct or *miR-155* inhibitor. The relative mean fluorescence intensity in intracellular calcium is plotted as in panel B. The height of each column in the bottom histogram corresponds to the increase of fluorescence intensity after anti-μ stimulation for samples respectively transfected with either control-ct or *miR-155* inhibitor, as indicated at the bottom by a "+". (D) The height of each column in the histogram describes the increase in fluorescence intensity after anti-μ stimulation of ZAP-70–negative CLL cells with high *miR-155* or low *miR-155*, as indicated at the bottom of the histogram. Statistical significance was determined by the unpaired Student *t* test ($P < .05$). IgM, immunoglobulin M.

The effect of CD154 or BAFF on CLL-cell expression of *miR-155*

In LNs, CLL cells interact with supportive cells, such as activated T cells or Nurse-like cells, which can provide survival- or proliferation-inducing signals via the expression of members of the tumor necrosis factor family of proteins (eg, CD40-ligand or CD154), B-cell–activating factor (BAFF or CD257), or a proliferation inducing ligand (APRIL or CD256).[34,35] We examined whether CD154 or BAFF could upregulate CLL cell expression of *miR-155* and thereby influence the expression of SHIP1 and sensitivity to BCR ligation. CLL cells were stimulated with CD154 or BAFF for 24 or 48 hours in vitro and then examined for *miR-155*, SHIP1, and responsiveness to anti-μ. Treatment with CD154 or BAFF significantly upregulated expression of *miR-155,* namely 2.7 ± 0.6 vs 1.0 ± 0 (n = 3, $P < .05$) (Figure 5A) or 1.5 ± 0.1 vs 1.0 ± 0 (n = 3, $P < .05$) (supplemental Figure 7A), respectively. Conversely, expression of SHIP1 was significantly lower in CLL cells treated with CD154 than in control cells (4.6 ± 0.7 vs 8.8 ± 1.2, n = 6, $P < .05$) (Figure 5B) or in CLL cells treated with BAFF than in control cells (5.9 ± 1.0 vs 8.8 ± 1.2, n = 6, $P < .05$) (supplemental Figure 7B). Furthermore, CD154 or BAFF enhanced the responsiveness of CLL cells to BCR ligation (Figure 5C; supplemental Figure 7C); the median increase of calcium fluorescence intensity induced by anti-μ was significantly greater in CLL cells treated with CD154 than in untreated cells (5.0 ± 1.1 vs 2.4 ± 0.4, n = 9, $P < .05$) (Figure 5C) or in CLL cells treated with BAFF than in untreated cells (4.1 ± 2.2 vs 2.5 ± 1.9, n = 4, $P < .05$) (supplemental Figure 7C). To examine whether the enhanced responsiveness to BCR ligation induced by CD154 was dependent upon induced expression of *miR-155*, CLL cells were transfected with *miR-155* inhibitor before treatment with CD154. We found that the median increase of anti-μ calcium fluorescence intensity induced by treatment with CD154 was significantly lower in CLL cells transfected with the *miR-155* inhibitor than in cells transfected with control miRNAs (2.9 ± 1.3 vs 4.3 ± 1.2, n = 4, $P < .05$) (Figure 5D).

We evaluated whether stimulation with CD154 could upregulate expression of *miR-155* and thereby influence the expression of SHIP1 and responsiveness to BCR ligation in normal B cells. Blood B cells were isolated from normal donors (n = 3) and stimulated with CD154. After 24 or 48 hours of stimulation, we examined for changes in expression levels of *miR-155* and SHIP1, and in responsiveness to anti-μ. As noted for CLL cells, treatment with CD154 upregulated expression of *miR-155*, downregulated expression of SHIP1, and enhanced their responsiveness to BCR ligation (supplemental Figure 8).

Discussion

Although prior studies identified an association between high-level expression of *miR-155* and expression of ZAP-70 or use of unmutated *IGHV*,[8,22,23,36,37] we found that this association was not absolute and that high-level expression of *miR-155* in CLL has additional independent prognostic value. We determined the optimal threshold

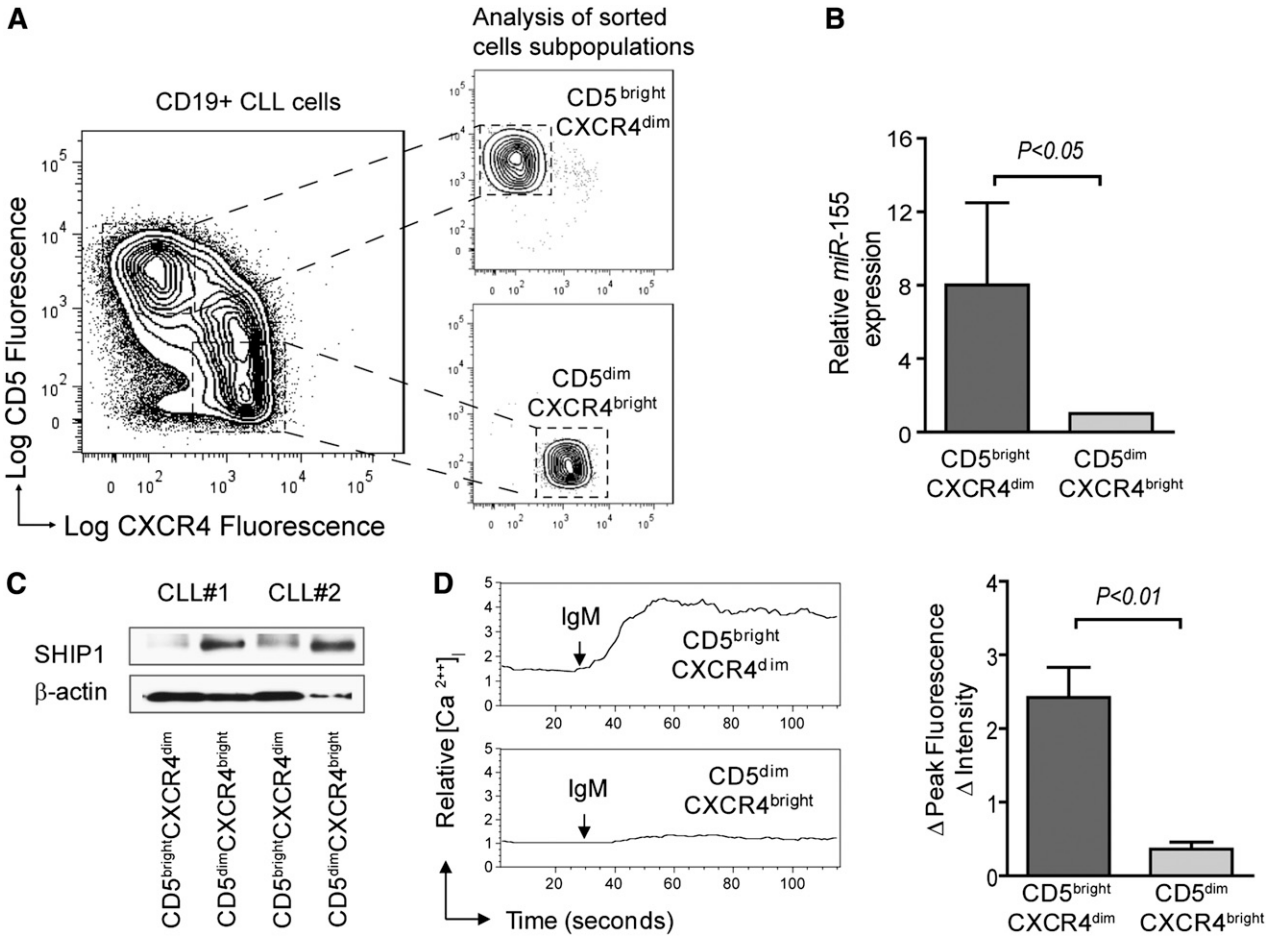

Figure 4. Expression of *miR-155*, SHIP1, and sensitivity to anti-μ in subpopulations of CLL cells isolated from the blood mononuclear cells of the same patient. (A) Flow cytometric analyses of representative unsorted CLL cells (left), isolated $CD5^{bright}CXCR4^{dim}$ CLL cells (upper right), or isolated $CD5^{dim}CXCR4^{bright}$ CLL cells (lower right). Contour plots (10% probability) depict the fluorescence of CLL cells stained with fluorochrome-conjugated mononuclear antibodies specific for human CD5 (y-axis) or human CXCR4 (x-axis). (B) Expression of *miR-155* in CLL cells that expressed high levels of CD5 and low levels of CXCR4, or low levels of CD5 and high levels of CXCR4. The height of each column in the histogram indicates the fold increase of *miR-155* copy number of sorted $CD5^{bright}CXCR4^{dim}$ cells relative to that of sorted $CD5^{dim}CXCR4^{bright}$ cells, as indicated at the bottom. Statistical significance was determined by paired Student *t* test ($P < .05$). (C) Expression of SHIP1 in sorted $CD5^{bright}CXCR4^{dim}$ or $CD5^{dim}CXCR4^{bright}$ CLL cells of representative samples (CLL1 [left 2 lanes] or CLL2 [right 2 lanes]), as indicated at the bottom. Probing for β-actin was used to monitor for equal loading of sample lysates. (D) Anti-μ–induced calcium mobilization in CLL cells that expressed high levels of CD5 and low levels of CXCR4, or low levels of CD5 and high levels of CXCR4. The relative mean fluorescence intensity in intracellular calcium is plotted in Figure 3B,C for each cell subset. The height of each column in the histogram on the right corresponds to the increase of fluorescence intensity after anti-μ stimulation for sorted $CD5^{bright}CXCR4^{dim}$ or $CD5^{dim}CXCR4^{bright}$ cells, as indicated at the bottom. Statistical significance was determined by unpaired Student *t* test ($P < .05$).

for using *miR-155* (~2550 copies CLL cell) to stratify patients in a training cohort of 86 patients into 2 subgroups with disparate progression tendencies by performing profile-likelihood analysis.[38] We observed independent prognostic value of high-level *miR-155*, over and above ZAP-70 and IGHV status. This observation was confirmed in an independent validation dataset of 181 patients. Although our threshold for defining high-level expression of *miR-155* does not take into account intraclonal variation, it nonetheless could be useful for stratifying samples from patients for whom clinical outcome data might not be available. Importantly, defining high-level expression of *miR-155* in this manner allowed us to interrogate CLL cells with high vs low levels of *miR-155* for phenotypic and functional differences that may account in part for differences in clinical outcome.

We found that CLL cells with high levels of *miR-155* expressed significantly lower levels of SHIP1 protein. SHIP1 is a phosphatase that is encoded by *INPP5D*, which had been found to be a target for *miR-155* in cell types other than CLL.[26] Although *INPP5D* encoding SHIP-1 is a known target gene for *miR-155*, it has never been demonstrated that *INPP5D* levels inversely correlate with expression of *miR-155* in CLL cells. This is important because each miRNA can have multiple different targets, which vary depending upon the cell type in which a given miRNA is expressed. High-level expression of an irrelevant gene that also was targeted by *miR-155,* for example, could compete with *INPP5D* for its ability to be regulated by *miR-155*. Indeed, prior studies by Loosner and colleagues identified a number of genes that could be targeted by *miR-155* in HEK293T cells.[39] However, when they compared their results with previously identified *miR-155* target proteins in other cell lines, they found major differences, demonstrating the cell-line specificity of microRNAs. Consistent with the notion that *miR-155* regulates expression of *INPP5D* in CLL cells, we demonstrated through transfection studies that enhanced expression of *miR-155* induces lower expression of *INPP5D* and that inhibition of *miR-155* induced higher expression of *INPP5D*.

We also found that CLL cells with high levels of *miR-155* also were more responsive to surface-μ ligation than CLL cells with low levels of *miR-155*. Prior studies found that CLL cells that lacked ZAP-70 generally experienced lower levels of activation after treatment with anti-μ.[8,17,18] However, there were atypical cases

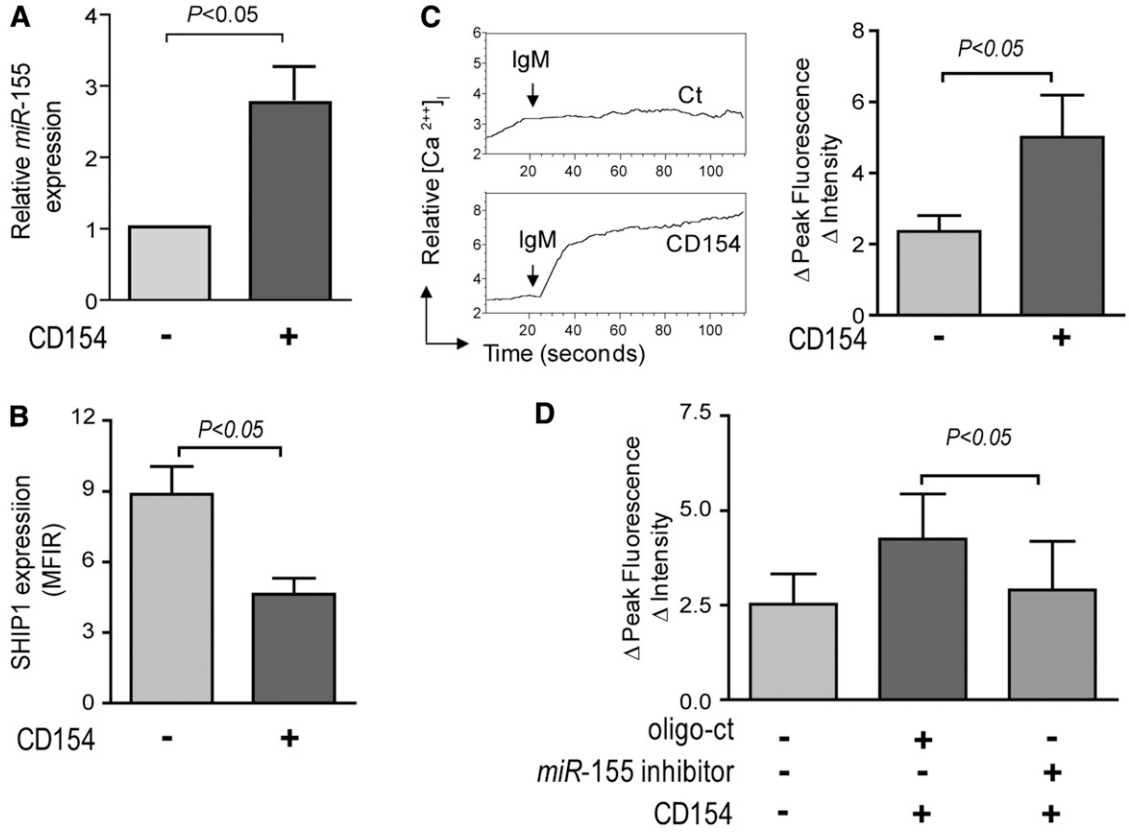

Figure 5. Stimulation of CLL cells with CD154 can induce expression *miR-155*, downregulation of SHIP1, and enhanced BCR signaling. (A) Expression of *miR-155* in CLL cells without (−) or with (+) CD154 stimulation. The height of each column in the histogram corresponds to the fold increase in expression of *miR-155* in CLL cells stimulated with CD154 (+) relative to that in CLL cells that had not been so stimulated (−), as indicated at the bottom of the histogram. We determined statistical significance using the paired Student *t* test ($P < .05$). (B) Expression of SHIP1 in CLL cells without (−) or with (+) CD154 stimulation, as indicated at the bottom of the histogram. The height of each column corresponds to the mean MFIR for SHIP1. (C) Anti-μ–induced calcium mobilization in CLL cells without (Ct, top panel) or with (CD154, lower panel) CD154 stimulation. The relative mean fluorescence intensity in intracellular calcium is plotted as a function of time. The arrow labeled "IgM" indicates the time at which the anti-μ was added to the cells (left panel). The histogram to the right depicts the mean increase of fluorescence intensity of CLL cells following stimulation with anti-μ without (−) or with (+) prior stimulation with CD154, as indicated at the bottom of the histogram. (D) Anti-μ–induced calcium mobilization in CLL cells with (+) or without (−) CD154 stimulation after the cells were transfected with a control oligonucleotide (oligo-ct) or *miR-155* inhibitor. The height of each column in the histogram corresponds to the mean increase of fluorescence intensity after treatment with anti-μ of CLL cells. We determined statistical significance using 1-way analyses of variance ($P < .05$).

that lacked expression of ZAP-70, but could be well-stimulated by treatment with anti-μ, suggesting that other factors could contribute to differences in sensitivity to BCR ligation. In the present study, we examined samples that did not express ZAP-70, but that had high vs low expression levels of *miR-155*. In such cases, the BCR signaling appeared to be associated with the expression level of *miR-155,* suggesting that the relative expression of this microRNA could influence the relative proficiency of BCR signaling in primary CLL cells. Transfecting CLL cells with *miR-155* reduced expression of SHIP1 and enhanced the cells' sensitivity to surface-μ ligation. Conversely, transfection of CLL cells with a *miR-155* inhibitor had the opposite effects. Because enhanced signaling via the BCR has been implicated to contribute to disease progression in CLL,[30] the capacity of *miR-155* to influence the responsiveness to BCR ligation may explain the noted association between high levels of *miR-155* and adverse outcome of patients with this disease.

The microRNA *miR-155* also can regulate expression of other mRNAs encoding other proteins, such as the suppressor of cytokine signaling (SOCS)1,[40,41] transforming growth factor (TGF) β-1, TGF β-2, or TGF β-receptor type 2, which may play a role in tumor invasion and/or metastasis.[42] However, other than *INPP5D*, none of the known targets of *miR-155* encode proteins that can directly influence BCR signaling. Similarly, SHIP1 expressed in hematopoietic cells could be repressed by overexpression of *miR-155*, which was associated with increased activation of the kinase AKT during the cellular response to lipopolysaccharide.[26] In any case, our results here indicate that the relative levels of *miR-155* could influence the proficiency of BCR signaling in CLL.

Although the average leukemia cell expression levels of *miR-155* can be used to segregate patients at different relative risk for disease progression, the levels of *miR-155* can vary between CLL cells of the same patient. Prior studies by Wang and colleagues observed high-level expression of *miR155HG* (*BIC*), the precursor to *miR-155*, in prolymphocytes and presumably CLL cells within the proliferation centers (PCs), which had high proportions of cells staining with the proliferation marker Ki-67.[19] They noted that the cells within the PCs expressed high levels of *BIC*/pre-*miR-155* relative to the lymphocytes outside the PC via RNA in situ hybridization. The relative number *BIC*/*pre-miR-155*–positive cells varied from patient to patient, depending on the relative size and number of PCs present in the LN. We interrogated the GEO database (GSE21029) and noted that CLL cells in lymphoid tissues expressed higher levels of *miR155HG*, and apparently lower levels of *INPP5D*, than CLL cells in the peripheral blood. These data suggest that the levels of *miR-155* can vary depending on where the CLL cells reside.

However, CLL cells are not fixed in their anatomic distribution, but rather recirculate between blood, lymphoid, and marrow compartments.[43] Prior studies indicated that a small proportion

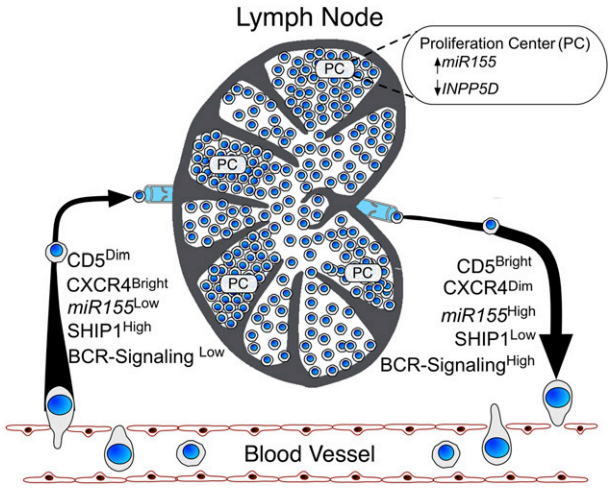

Figure 6. Model of the phenotype of CLL cells exiting the lymph node into the blood or exiting the blood into the LN. Within the PCs of the lymph node, CLL cells are stimulated via factors in the microenvironment (eg, CD154 or BAFF/APRIL), where there is upregulation of *miR-155* and downregulation of *INPP5D*. The CLL cells that recently have exited the LN express relatively low levels of CXCR4 ($CXCR4^{Dim}$), high levels of CD5 ($CD5^{Bright}$), high levels of *miR-155* (*miR-155*^{High}), and low levels of SHIP1 ($SHIP1^{Low}$), and have high responsiveness to BCR ligation with anti-μ ($BCR\text{-}signaling^{High}$). Conversely, the cells that may exit the blood for the LN are $CXCR4^{Bright}CD5^{Dim}$ and have relatively low levels of miR-155 ($miR\text{-}155^{Low}$), high levels of SHIP1 ($SHIP1^{High}$), and relatively low responsiveness to BCR ligation with anti-μ ($BCR\text{-}signaling^{Low}$). The arrows indicate the direction of trafficking from the blood to the lymph node and then back to the blood.

of CLL cells in the blood likely represent cells that have reentered the circulation after exiting the lymphoid tissues.[33] In particular, $CD5^{bright}CXCR4^{dim}$ CLL cells in the blood represent newly released cells from the tissue microenvironment, whereas the $CD5^{dim}CXCR4^{bright}$ CLL cells represent cells that may be about to reenter the tissue microenvironment.[33] In the present study, we found that the subgroup of blood $CD5^{bright}CXCR4^{dim}$ CLL cells expressed higher levels of *miR-155* and lower levels of SHIP1 protein, and were more sensitive to surface-μ ligation than the "resting" subgroup of blood $CD5^{dim}CXCR4^{bright}$ CLL cells (Figure 6). As such, this is the first study to define differences in the expression of *miR-155* by CLL cells of the same patient and to find that this can result in functional differences in the leukemia cells' responsiveness to BCR ligation.

We hypothesized that survival/stimulatory signals from accessory cells present in the leukemia cell microenvironment, such as in the PCs of the LNs, might contribute to the upregulation of *miR-155* (Figure 6). To test this hypothesis, we stimulated CLL cells with CD154 or BAFF, each of which could serve to activate nuclear factor κB (NF-κB).[44,45] Prior studies found activation of NF-κB could induce expression of *BIC*, the precursor to *miR-155*.[46] However, in some B-cell lymphomas or B-cell lines (eg, Ramos), NF-κB–induced expression of *BIC* did not necessarily cause increased expression of *miR-155*, indicating that there might be 2 levels of regulation for generating mature *miR-155*: 1 at the transcriptional level involving NF-κB and 1 at the processing level.[46] The studies reported here demonstrate that exposure of CLL cells to factors that can activate NF-κB significantly upregulated expression of mature *miR-155*, suggesting that in CLL the control of *miR-155* may predominately be at the level of *BIC* transcription.

In any case, we found that the changes induced in the expression levels of *miR-155* upon treatment with CD154 or BAFF had functional significance; such treatment significantly reduced expression of SHIP1 and enhanced the responsiveness to BCR ligation, effects that could be mitigated by transfection of CLL cells with an inhibitor to *miR-155*. Similar effects of *miR-155* also were noted for normal B cells that were stimulated via CD40 ligation with CD154, indicating that *miR-155* might play a physiologic role in regulating the B-cell response to BCR ligation. Within the lymphoid tissue microenvironment, T-cells or accessory cells may express factors, such as CD154 or BAFF/APRIL, which can enhance expression of *miR-155*. This in turn may serve as a rheostat that can enhance the signaling derived through BCR ligation (Figure 6). Conversely, attenuation in expression of *miR-155* in CLL or normal B cells when they circulate in the blood may allow for increased expression of SHIP1 and decreased responsiveness to BCR ligation. This could mitigate the risk for inadvertent stimulation by self- or foreign antigen(s) of blood B cells outside of lymphoid tissue compartments, where they would not have the microenvironment and accessory cells necessary for antigen-stimulated B cells to differentiate in an immune response to antigen. As such, *miR-155* might play an important role in the physiology or normal B cells and/or the pathophysiology of neoplastic B cells.

These studies also have implications for development of new therapies that disrupt the capacity of CLL cells to home to tissue microenvironments or that inhibit the signaling from accessory cells within the microenvironment, thereby potentially interfering with the capacity of the microenvironment to upregulate *miR-155*. Agents that block the capacity of CLL cells to home or to engage with accessory cells in PCs might be expected to decrease the levels of *miR-155* and increase expression of *INPP5D*.[16] Monitoring for these changes in patients who are treated with such agents might become a useful surrogate marker for the capacity of the drug to cause diminished leukemia cell stimulation in the lymphoid compartment. Also, agents that reduce expression of *miR-155* or that upregulate expression of SHIP1 might have therapeutic value in patients with CLL, particularly for those patients who have CLL cells with high levels of *miR-155*. Moreover, agents that inhibit leukemia cell expression of *miR-155* may have clinical activity in patients with this disease.

Acknowledgments

This work was supported in part by the National Institutes of Health, National Cancer Institute grants (R37-CA049870) (T.J.K.) and (PO1-CA81534 of the CLL Research Consortium) (L.Z.R., C.M.C., and T.J.K.), the Blood Cancer Research Fund, the South Moravian Programme for Distinguished Researchers (M.M) cofinanced by the EU and the South-Moravian Region, and the EHA Research Fellowship award (granted by the European Hematology Association).

Authorship

Contribution: B.C. designed research, performed research, analyzed data, and wrote the paper; L.C. designed research, performed research, analyzed data, and wrote the paper; S.Z., M.M., J.-F.F., J. Y., E.M.G., L.Z., and L.Z.R. performed research; L.B. and K.M. analyzed data and contributed to the manuscript; G.A.C. and C.M.C. made scientific contributions; and T.J.K. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Thomas J. Kipps, 3855 Health Sciences Dr, Rm 4307, San Diego, CA 92093-0820; e-mail: tkipps@ucsd.edu.

References

1. Efremov DG, Gobessi S, Longo PG. Signaling pathways activated by antigen-receptor engagement in chronic lymphocytic leukemia B-cells. *Autoimmun Rev.* 2007;7(2):102-108.
2. Kil LP, Yuvaraj S, Langerak AW, Hendriks RW. The role of B cell receptor stimulation in CLL pathogenesis. *Curr Pharm Des.* 2012;18(23): 3335-3355.
3. Chen L, Widhopf G, Huynh L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood.* 2002;100(13):4609-4614.
4. Chen L, Huynh L, Apgar J, et al. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. *Blood.* 2008;111(5):2685-2692.
5. Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood.* 2012;120(6):1175-1184.
6. Vigorito E, Perks KL, Abreu-Goodger C, et al. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity.* 2007;27(6):847-859.
7. Koralov SB, Muljo SA, Galler GR, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell.* 2008;132(5): 860-874.
8. Calin GA, Ferracin M, Cimmino A, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med.* 2005;353(17):1793-1801.
9. Mraz M, Pospisilova S, Malinova K, Slapak I, Mayer J. MicroRNAs in chronic lymphocytic leukemia pathogenesis and disease subtypes. *Leuk Lymphoma.* 2009;50(3):506-509.
10. Mraz M, Dolezalova D, Plevova K, et al. MicroRNA-650 expression is influenced by immunoglobulin gene rearrangement and affects the biology of chronic lymphocytic leukemia. *Blood.* 2012;119(9):2110-2113.
11. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol.* 2002; 12(9):735-739.
12. Tam W, Ben-Yehuda D, Hayward WS. bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA. *Mol Cell Biol.* 1997;17(3):1490-1502.
13. Leng RX, Pan HF, Qin WZ, Chen GM, Ye DQ. Role of microRNA-155 in autoimmunity. *Cytokine Growth Factor Rev.* 2011;22(3):141-147.
14. Eis PS, Tam W, Sun L, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci USA.* 2005; 102(10):3627-3632.
15. Kluiver J, Poppema S, de Jong D, et al. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J Pathol.* 2005;207(2):243-249.
16. Zhang Y, Roccaro AM, Rombaoa C, et al. LNA-mediated anti-miR-155 silencing in low-grade B-cell lymphomas. *Blood.* 2012;120(8): 1678-1686.
17. Calin GA, Liu CG, Sevignani C, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. *Proc Natl Acad Sci USA.* 2004;101(32):11755-11760.
18. Marton S, Garcia MR, Robello C, et al. Small RNAs analysis in CLL reveals a deregulation of miRNA expression and novel miRNA candidates of putative relevance in CLL pathogenesis. *Leukemia.* 2008;22(2):330-338.
19. Wang M, Tan LP, Dijkstra MK, et al. miRNA analysis in B-cell chronic lymphocytic leukaemia: proliferation centres characterized by low miR-150 and high BIC/miR-155 expression. *J Pathol.* 2008;215(1):13-20.
20. Nana-Sinkam SP, Croce CM. MicroRNA in chronic lymphocytic leukemia: transitioning from laboratory-based investigation to clinical application. *Cancer Genet Cytogenet.* 2010; 203(2):127-133.
21. Costinean S, Zanesi N, Pekarsky Y, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci USA.* 2006; 103(18):7024-7029.
22. Visone R, Rassenti LZ, Veronese A, et al. Karyotype-specific microRNA signature in chronic lymphocytic leukemia. *Blood.* 2009;114(18): 3872-3879.
23. Rossi S, Shimizu M, Barbarotto E, et al. microRNA fingerprinting of CLL patients with chromosome 17p deletion identify a miR-21 score that stratifies early survival. *Blood.* 2010;116(6): 945-952.
24. Vargova K, Curik N, Burda P, et al. MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. *Blood.* 2011; 117(14):3816-3825.
25. Zhu DX, Zhu W, Fang C, et al. miR-181a/b significantly enhances drug sensitivity in chronic lymphocytic leukemia cells via targeting multiple anti-apoptosis genes. *Carcinogenesis.* 2012;33(7):1294-1301.
26. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci USA.* 2009; 106(17):7113-7118.
27. Pedersen IM, Otero D, Kao E, et al. Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. *EMBO Mol Med.* 2009;1(5):288-295.
28. Zolnierowicz S, Bollen M. Protein phosphorylation and protein phosphatases. De Panne, Belgium, September 19-24, 1999. *EMBO J.* 2000;19(4): 483-488.
29. Nakamura K, Brauweiler A, Cambier JC. Effects of Src homology domain 2 (SH2)-containing inositol phosphatase (SHIP), SH2-containing phosphotyrosine phosphatase (SHP)-1, and SHP-2 SH2 decoy proteins on Fc gamma RIIB1-effector interactions and inhibitory functions. *J Immunol.* 2000;164(2):631-638.
30. Herishanu Y, Pérez-Galán P, Liu D, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood.* 2011;117(2):563-574.
31. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N Engl J Med.* 2004;351(9):893-901.
32. Haferlach T, Kohlmann A, Wieczorek L, et al. Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J Clin Oncol.* 2010;28(15): 2529-2537.
33. Calissano C, Damle RN, Marsilio S, et al. Intraclonal complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med.* 2011; 17(11-12):1374-1382.
34. Ghia P, Strola G, Granziero L, et al. Chronic lymphocytic leukemia B cells are endowed with the capacity to attract CD4+, CD40L+ T cells by producing CCL22. *Eur J Immunol.* 2002;32(5): 1403-1413.
35. Nishio M, Endo T, Tsukada N, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood.* 2005;106(3):1012-1020.
36. Papakonstantinou N, Ntoufa S, Chartomatsidou E, et al. Differential microRNA profiles and their functional implications in different immunogenetic subsets of chronic lymphocytic leukemia. *Mol Med.* 2013;19:115-123.
37. Ferrajoli A, Shanafelt TD, Ivan C, et al. Prognostic value of miR-155 in individuals with monoclonal B-cell lymphocytosis and patients with B chronic lymphocytic leukemia. *Blood.* 2013;122(11): 1891-1899.
38. Tableman M, Kim JS. Survival Analysis Using S: Analysis of Time-to-Event Data. Boca Raton, FL: Chapman & Hall/CRC; 2004.
39. Lössner C, Meier J, Warnken U, et al. Quantitative proteomics identify novel miR-155 target proteins. *PLoS ONE.* 2011;6(7):e22146.
40. Chang CC, Zhang QY, Liu Z, Clynes RA, Suciu-Foca N, Vlad G. Downregulation of inflammatory microRNAs by Ig-like transcript 3 is essential for the differentiation of human CD8(+) T suppressor cells. *J Immunol.* 2012;188(7): 3042-3052.
41. di Iasio MG, Norcio A, Melloni E, Zauli G. SOCS1 is significantly up-regulated in Nutlin-3-treated p53(wild-type) B chronic lymphocytic leukemia (B-CLL) samples and shows an inverse correlation with miR-155. *Invest New Drugs.* 2012;30(6): 2403-2406.
42. Neilsen PM, Noll JE, Mattiske S, et al. Mutant p53 drives invasion in breast tumors through up-regulation of miR-155. *Oncogene.* 2013;32(24): 2992-3000.
43. Gaidano G, Foà R, Dalla-Favera R. Molecular pathogenesis of chronic lymphocytic leukemia. *J Clin Invest.* 2012;122(10):3432-3438.
44. Endo T, Nishio M, Enzler T, et al. BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. *Blood.* 2007;109(2): 703-710.
45. Zarnegar B, He JQ, Oganesyan G, Hoffmann A, Baltimore D, Cheng G. Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappaB activation pathways. *Proc Natl Acad Sci USA.* 2004; 101(21):8108-8113.
46. Kluiver J, van den Berg A, de Jong D, et al. Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. *Oncogene.* 2007; 26(26):3769-3776.