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Divergence in CD19-Mediated Signaling Unfolds Intraclonal Diversity in Chronic Lymphocytic Leukemia, Which Correlates with Disease Progression

Yair Herishanu,*1 Sigi Kay,*1 Nili Dezorella,*1† Shoshana Baron,* Inbal Hazan-Halevy,* Ziv Porat,‡ Svetlana Trestman,* Chava Perry,* Roni Braunstein,* Varda Deutsch,* Aaron Polliack,* Elizabeth Naparstek,*† and Ben-Zion Katz*†

Emerging data on intraclonal diversity imply that this phenomenon may play a role in the clinical outcome of patients with chronic lymphocytic leukemia (CLL), where subsets of the CLL clone responding more robustly to external stimuli may gain a growth and survival advantage. In this study, we report intraclonal diversity resolved by responses to CD19 engagement in CLL cells, which can be classified into CD19-responsive (CD19-R) and -nonresponsive subpopulations. Engagement of CD19 by anti-CD19 Ab rapidly induced cellular aggregation in the CD19-R CLL cells. The CD19-R CLL cells expressed higher surface levels of CD19 and c-myc mRNA, exhibited distinct morphological features, and were preferentially abolished in rituximab-treated patients. Both subpopulations reacted to sIgM stimulation in a similar manner and exhibited similar levels of Akt and Erk phosphorylation, pointing to functional signaling divergence within the BCR. CD19 unresponsiveness was partially reversible, where nonresponding CD19 cells spontaneously recover their signaling capacity following incubation in vitro, pointing to possible in vivo CD19–signaling attenuating mechanisms. This concept was supported by the lower CD19-R occurrence in bone marrow–derived samples compared with cells derived from the peripheral blood of the same patients. CLL patients with >15.25% of the CD19-R cell fraction had a shorter median time to treatment compared with patients with <15.25% of CD19-R cell fraction. In conclusion, divergence in CD19-mediated signaling unfolds both interpatient and intraclonal diversity in CLL. This signaling diversity is associated with physiological implications, including the location of the cells, their responses to anti-CLL therapeutics, and disease progression.

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Chronic lymphocytic leukemia (CLL) is an indolent neoplasm characterized by progressive accumulation of small mature B lymphocytes in the peripheral blood, bone marrow, and secondary lymphoid organs (1). CLL cells depend on the microenvironment for proliferation and survival (2). The response of CLL cells to the microenvironment is heterogeneous, and cells originating from more aggressive subtypes of CLL (e.g., IgVH unmutated, ZAP70+, and CD38+) tend to respond more robustly to external signals (3–5). In particular, an antigenic stimulation is considered to play a central role in the evolution and progression of CLL (6). Antigenic stimulation is channeled into the cytoplasm of CLL via the BCR, which is a multimolecular complex comprised of surface IgM and associated components within the membrane plane, including CD19, CD21 and CD81, and associated cytoplasmic accessory/signaling molecules, including Syk, and in some patients ZAP70 (3, 6, 7). Owing to the prime importance of the BCR in the physiology of malignant B cells, the expression of some of its components and associated molecules serves as prognostic factors (e.g., ZAP70) (8) or as therapeutic targets (e.g., CD20, Syk, Btk) in CLL and other lymphoproliferative disorders (LPD) (9, 10). Growing evidence supports the concept that intraclonal CLL cell diversity plays a role in the disease biology and eventual clinical outcome (11–13). Intraclonal heterogeneity is acquired through spontaneous genetic alterations or driven by microenvironmental stimuli (14, 15).

Recently, CLL cells expressing CD38 were shown to be a proliferating subset, as they strongly express Ki-67 and have a faster proliferation rate in vivo than do CD38− CLL cells of the same clone (13). Despite the established importance of extracellular stimuli in CLL and other LPD, the functional intraclonal diversity of the BCR complex components has not as yet been evaluated in CLL. Current methodologies focus on the molecular status of the receptor (e.g., mutational status) or the level of expression of its associated molecules (e.g., ZAP70), with no functional assessments. Although these assays provide important prognostic information, they are only informative of the steady-state of the disease as a uniform entity without providing any functional physiological information relating to the diverse members of the CLL clone.

In this study, we report an intraclonal diversity of CLL cells in response to CD19 engagement. We show that CD19 stimulation

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Abbreviations used in this article: BM, bone marrow; CD19-N, CD19-nonresponsive; CD19-R, CD19-responsive; CI, confidence interval; CLL, chronic lymphocytic leukemia; HR, hazards ratio; LPD, lymphoproliferative disorder; MJBD, methyl-β-cyclodextrin; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; TTT, time to treatment.
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rapidly induces homotypic aggregation in a subpopulation of CLL cells, designated as CD19-responsive cells (CD19-R), whereas another subpopulation is comprised of CD19-nonresponsive (CD19-N) cells. In some of the CD19-N cells the cellular response is attenuated in vivo in a reversible manner. The CD19-R subpopulation expressed higher levels of membrane CD19, c-myc mRNA, correlated with clinical disease progression, and reacted differently to anti-CLL therapeutics compared with CD19-N cells of the same clone. Our finding that CD19 responsiveness in CLL cells correlates with disease progression points to the physiological importance of this signaling response in CLL development.

Materials and Methods

Reagents and Kits

mAbs anti-human CD45-PerCP (clone 2D1), CD19-allophycocyanin (clone S25C1), CD19-FTTC (clone 4G7), CD20-FTTC (clone L27), CD22-FTTC (clone S-HCL-1), CD23-PE (clone EBVCS-5), CD38-allophycocyanin (clone H87), FMCT7-FTTC (clone FM7C), CD62L-PE (clone DRG-56), CD86-PE (clone IT2.2), DI8FTTC (β2 integrin, clone 6.7), Ki-67-FTTC (clone B56), pAKT-PE (pS473, clone M89-61), pERK1/2-PE (pT202/pY204, clone 20A), Cytofix fixation buffer, Phosflow Perm/Wash buffer I according to the manufacturer’s instructions. For ImageStream analysis, 10,000 events from each fixation buffer and Phosflow Perm/Wash buffer I according to the manufacturer’s instructions. For ImageStream analysis, 10,000 events from each

Flow cytometry and ImageStream analysis

CLL cells (1 x 10⁶) were incubated with the designated Abs for 30 min at 4°C in the dark, washed, and treated either with FACS lysing solution for cell fixation, then washed and labeled with anti-CLL cell sorting system with a 100-μm nozzle (Becton Dickinson, Franklin Lakes, NJ).

Quantitative real-time PCR

RNPACS were reanalyzed to determine intraclonal diversity in primary CLL samples. Standard FACS-based method is a standard immunophenotyping approach used in many clinical laboratories to diagnose CLL (24) and was used in this study to determine intraclonal cellular diversity in primary CLL samples. Standard FACS protocols that included an Ab combination to CD20/CD95/CD19 demonstrated the presence of an exceptionally high side scatter lymphoid population in primary CLL samples (Fig. 1A). Incubation of peripheral blood samples of CLL patients in the presence of individual Abs indicated that the high side scatter lymphoid population appeared only when anti-CD19 Ab was used (Fig. 1B) and occurred with two distinct Ab clones, irrespective of the fluorophore type, and also with unconjugated anti-CD19 Ab (data not shown). Various additional Abs to markers often used to characterize CLL cells did not induce high side scatter lymphoid population, including CD22, CD23, CD38, and FMCD (data not shown), pointing to engagement of CD19 as a critical factor in this phenomenon. Formation of high side scatter lymphoid population was completely blocked at 0°C or by cell fixation (data not shown).
shown), consistent with the involvement of intracellular signaling events.

To elucidate the disposition of the high side scatter lymphoid population events, CLL samples were observed directly by the ImageStream system, which combines flow cytometry with brightfield microscopy (25). As shown in Fig. 1C (middle panel), the high side scatter lymphoid population contains aggregates of CLL cells. High side scatter in flow cytometry is usually generated by the cytoplasmic content of granulated cells (e.g., Fig. 1C, middle panel, R1). In CD19-engaged CLL samples, high side scatter CD19+ cells (R2), and high side scatter CD19+ cells (R3) (top panel). Representative images of cells in regions I–3 are shown in the middle panel (BF, brightfield; SSC, side scatter). Bottom panel depicts the side scatter distributions of single cells versus double, triple, and multiple cell aggregates. (D) The percentage of CD19-R cells calculated out of whole CD19+ lymphocytes was quantified after CD19 engagement in peripheral blood samples from healthy individuals and from patients with CLL.

Characterization of the CD19-responsive subpopulation of CLL

Because CD19 appears to trigger the cellular aggregation of CLL cells, we examined its surface level of expression on the CD19-R versus the CD19-N fractions of the disease. Levels of CD19 expression per cell in the CD19-R fraction were estab-
lished by dividing fluorescence intensity by the area of the cells in the aggregates. As shown in Fig. 2A, lower levels of CD19 per cell were found in single CLL cells (CD19-N), with a considerable increase of its expression in cells comprising doublets and triplets (Fig. 2A). The highest CD19 levels were observed in CLL cells incorporated into high-order aggregates (Fig. 2A, top panel). However, accurate CD19 measurements per cell cannot be performed in the high-order aggregates, as not all the cells that comprise them are presented to the ImageStream in a single plane for accurate area measurements (Fig. 2A, top panel).

To further characterize the differences between the CD19-R and CD19-N CLL cells, we sorted these two subpopulations (Fig. 2B), which were then applied on slides by cytospin, stained by Giemsa, and viewed with a light microscope. The CD19-N cells were typically small, mature-appearing lymphocytes with a dense nucleus and without discernible nucleoli (Fig. 2C). In contrast, cells sorted from the CD19-R fraction were enriched with cells larger in diameter, with large, reticulated nuclear chromatin and in some of the cells also visible nucleoli (Fig. 2C).

To better characterize the CD19-R cells and to find a possible link for clinical significance, we studied the mRNA levels of genes previously shown to be involved in CLL cell proliferation and antiapoptosis in the sorted CD19-N and CD19-R CLL subpopulations (26). We found that the latter significantly overexpressed c-myc (mean fold change of control, 1.45; \( p = 0.01 \)), whereas c-fos, BCL-2, and aurora kinase were similarly expressed in both subpopulations (Fig. 2D). As expected, the levels of actively proliferating CLL cells were very low in single cells as well as in aggregates as evaluated by Ki-67 staining (Supplemental Fig. 1D).

**Cholesterol-rich plasma membrane rafts are important for CD19-mediated aggregation**

Key biochemical elements involved in the CD19-mediated CLL cell aggregation were studied using compounds interfering with lipid raft integrity, inhibitors of major intracellular signaling cascades, as well as blocking Abs directed to adhesion molecules. First, we assessed the role of plasma membrane cholesterol on CLL cell aggregation. Pretreatment of CLL cells with MβCD, which extracts membrane-associated cholesterol, resulted in nearly complete blocking of CD19 induction of aggregates (Fig. 3A). Alkyl-lysophospholipid analogs (e.g., edelfosine and, to a lesser extent, perifosine) incorporate into lipid rafts, modify their organization, and can selectively induce programmed cell death in CLL and lymphoma cells (17, 18). We found that edelfosine (and, to a much lesser extent, perifosine) significantly reduced aggregation upon prolonged incubation with CLL cells (Fig. 3B). We further assessed downstream major intracellular signaling effectors reported to be activated in normal B cells after CD19 engagement. In these experiments, general inhibitors of tyrosine phosphorylation (genistein or herbimycin A) had a limited effect (∼25%; Fig. 3C), and no inhibitory effect was observed in CLL cells treated with Syk inhibitor II (data not shown) or with the Src family kinase inhibitor PP2 (data not shown). As shown in Fig. 3C, cytochalasin B, which blocks actin polymerization, caused

![FIGURE 2](http://www.jimmunol.org/) Distinct characterization of the high scatter lymphoid population of CLL. (A) CLL cells were incubated with monoclonal anti-human CD19-allophycocyanin and analyzed using the ImageStreamX system. Cells were gated for single, double, triple, and multiple cell aggregates using the size and aspect ratio features. Shown are representative cells from each population (top panel). BF, brightfield. The relative fluorescence intensity was calculated by dividing the total intensity by the total brightfield area of the cell or cells in each aggregate (bottom panel). (B) CLL cells were incubated with monoclonal anti-human CD19-allophycocyanin and sorted into CD19low side scatterlow cells (CD19-N, P2) and CD19high side scatterhigh (CD19-R, P3) using a flow cytometry cell sorting system as detailed in Materials and Methods. (C) The sorted two subpopulations were applied on slides by cytospin, stained by Giemsa, and viewed under light microscope (P2 for CD19-N and P3 for CD19-R). (D) mRNA levels of genes of interest in the two subpopulations of CD19-N and CD19-R quantified by real-time PCR. Levels of c-myc, c-fos, BCL-2, and AURORA were normalized to the housekeeping gene GAPDH and are shown as ratio expression between CD19-N and CD19-R CLL cells. \( n = 6 \), paired \( t \) test (NS, nonsignificant).
were treated with the indicated concentration of for treated versus control cells. (B) with M cells in paired samples untreated or pretreated in independent experiments measuring the per-

centrage of CD19-R cells out of the whole CLL cells in paired samples untreated or pretreated with MβCD. Right, Summary of six independent experiments measuring the per-

centage of CD19-R cells out of the whole CLL cells. Paired t test, p < 0.02 for treated versus control cells. (B) CLL cells were treated with the indicated concentration of 

perifosine (left) or edelfosine (right) for 1 or 24 h. Then, the CD19-R subpopulation was measured in treated versus control cells. (C) Effects of various 

chemical inhibitors on CD19 responsiveness of CLL cells. The cells were incubated with each indicated inhibitor as described in Materials and Methods. Then, the CD19-R subpopulation was measured and compared with controls. Seven to nine individual CLL samples are shown with their averages (thick lines). (D) Anti-β2 integrin inhibits CD19 responsiveness in CLL cells. The cells were preincubated with anti-β2 integrin as described in Materials and Methods. Then, the CD19-R subpopulation was measured and compared with control untreated cells. Eight indi-

vidual CLL samples are shown with their average (thick line). Paired t test.

FIGURE 3. Cholesterol-rich plasma membrane rafts are important for CD19 responsive-

ness. (A) CLL cells were pretreated with 10 mM MβCD for 30 min, then incubated with anti-human CD19-allophycocyanin and analyzed by flow cytometry. Left, Representative dot plots showing the changes in CLL cells (CD19+ cells) in an untreated sample compared with a sample preincubated with MβCD. Right, Summary of six independent experiments measuring the percentage of CD19-R cells out of the whole CLL cells in paired samples untreated or pretreated with MβCD for 30 min. Paired t test, p < 0.02 for treated versus control cells. (B) CLL cells were treated with the indicated concentration of 

perifosine (left) or edelfosine (right) for 1 or 24 h. Then, the CD19-R subpopulation was measured in treated versus control cells. (C) Effects of various 

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vidual CLL samples are shown with their average (thick line). Paired t test.

only a minor reduction (not significant) of aggregation. Pre-

incubation with EDTA, wortmannin (100 nM), or PD98509 (50 

µM), which are known to inhibit Ca2+ influx, PI3K/AKT or MEK/ 

ERK pathways, respectively, failed to inhibit CD19-mediated in-

duction of aggregation (data not shown).

Previous studies identified several molecules that may mediate 

CD19-induced homotypic aggregation in B cells, including CD21, 

CD23, CD62L, and β2 integrin (27–29). We found surface ex-

pression of all of these molecules on CLL cells, as previously 

reported (data not shown). Anti-β2 integrin inhibitory Ab reduced significantly CLL cell aggregation in response to anti-CD19 Ab stimulation (Fig. 3D). In contrast, CLL cell aggregation was neither affected by inhibitory Abs to CD21, CD23, or CD62L nor by heparin, which is a known inhibitor of selectin activities (data not shown). Overall, these data suggest that CD19 induction of aggregation in CLL cells is primarily dependent on horizontal signaling events confined to transmembrane components, probably those occurring within the lipid rafts.

CD19-responsive CLL cells are preferentially eliminated 
in vivo by rituximab

Previous studies pointed to the possible involvement of CD20 in BCR signaling responses, albeit the in vivo significance of these findings is unclear (30). To further examine the physio-

logical differences between the CD19-R and CD19-N sub-

populations, we tested the effects of the anti-CD20 therapeutic 

Ab rituximab on these cells, both in vitro and in vivo. As shown in 

Fig. 4A, rituximab had no significant effect on the CLL cell aggre-

gation following 16 h incubation in vitro. In contrast, a dramatic decrease in the CD19-R subpopulation was observed in blood samples 16 h following in vivo rituximab administra-

tion (Fig. 4).

CD19 level of expression and responsiveness are regulated by sIgM

CD19 is part of the B cell coreceptor complex, which acts to 
potentiate signaling through the BCR (31). Therefore, we hypothesized that sIgM, the main signaling component of the BCR, may affect CD19 responsiveness. CLL cells were incubated with soluble anti-sIgM Ab for 18 h and then followed by CD19 en-

gagement. sIgM “priming” resulted in nearly 50% increase in the CD19-R fraction compared with the control, concomitant with a profound increase in surface CD86, an activation marker of BCR stimulation (32) (Fig. 5A). The increase in the CD19-R fraction was associated with a small but significant increase in surface CD19 levels, with no parallel upregulation of surface CD20 (Fig. 5A). Interestingly, CD86 levels were upregulated in a similar manner on both CD19-R and CD19-N subpopulations following sIgM stimulation (Fig. 5B), indicating that both subpopulations have similar potential of intracellular response to sIgM stimulation. As expected, ZAP70+ cells were more responsive to sIgM stimulation than were ZAP70− cells (Fig. 5B). These data 

emphasize the complexity of the crosstalk between the sIgM and 

CD19, in which BCR activation is capable of increasing CD19 surface expression and signaling. One of the major signaling responses triggered by the BCR is the phosphorylation of Akt (33). As shown in Fig. 5C, a relatively small increase in the levels of pAkt were observed in CLL cells following CD19 engagement, compared with significantly higher levels obtained with sIgM stimulation. These low levels of pAkt were observed in aggregates as well as in single CLL cells (Fig. 5D). In a similar manner, low levels of ERK phosphorylation were observed in both sub-

populations, compared with high levels of pERK following sIgM stimulation (data not shown). Higher levels of Akt phosphoryla-

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tion in CLL cells were obtained only following high-order clustering of CD19 by crosslinking, as previously described (34) (Fig. 5C). These data, which were confirmed by Western blotting analysis (data not shown), indicate that the threshold for CD19-mediated aggregation is considerably lower compared with CD19-mediated Akt phosphorylation.

**Spontaneous recovery of CD19 signaling capacity following in vitro incubation**

The differences between the CD19 responsiveness of different patients may represent the activation status of this molecule in vivo. With analogy to this notion, it has been previously shown that low responsiveness to sIgM stimulation may stem from an anergic status of this receptor in vivo, which can be reversed upon ex vivo propagation of the cells (35). We found that CLL cells derived from patients that respond poorly to CD19 engagement (≤ 10% CD19-R cells) exhibit significantly higher CD19 responsiveness (5.2 mean fold change of control) upon their ex vivo propagation for 3 d (n = 8 patients; Fig. 5E, top panel). Highly responsive disease (> 10% CD19-R cells) retains its responsiveness (only 1.6 mean fold change of control) during ex vivo propagation (n = 7 patients; Fig. 5E, bottom panel). The differences in the ex vivo recovery rates between these groups was significant (p < 0.004). These data indicate that in vivo operating mechanisms, such as tolerance to engagement, may attenuate the CD19 responsiveness in some patients. Previous studies attributed the low responsiveness of CLL to sIgM stimulation to an anergic state of the BCR owing to sustained in vivo activation of the cells. This putative mechanism was supported by the recovery of cell responsiveness following their in vitro propagation (35). This concept advocates for lower responsiveness of the cells within the lymphoid tissue compartment compared with cells obtained from the PB. As shown in Fig. 5F, most BM samples contain significantly lower CD19-R portion of the disease compared with PB samples obtained from the same patients in the same date.

The proportions of CD19-R subpopulations predict time to treatment

The percentage of the CD19-R and CD19-N subsets is variable in CLL patients. Therefore, we determined whether the CD19-R fraction correlates with clinical parameters and biological markers. The percentage of CD19-R fraction was not statistically different between CLL patients with early (Rai stage 0), intermediate (Rai stages 1 and 2), or advanced (Rai stages 3 and 4) stages (Fig. 6A). No differences in the percentage of the CD19-R fraction were found between ZAP70+ and ZAP70− CLL patients (Fig. 6A).

To determine whether the CD19-R fraction correlates with the clinical course of the disease, CLL patients (n = 78) were classified into two subgroups according to the median percentage of the CD19-R fraction present (median, 15.25%). The CD19-R fraction of CLL cells predicted the time to treatment (TTT), measured as the time from diagnosis of CLL to the onset of active disease and initiation of treatment (Fig. 6B). During a median follow-up of 51 mo, patients with ≥ 15.25% of the CD19-R fraction had a median TTT of 57.7 mo, whereas this median had not been reached in patients with a lower CD19-R fraction (p = 0.026; Fig. 6B, left panel). Additionally, the CD19-R fraction also correlated with TTT as measured from the day that this fraction was determined. Patients with > 15.25% CD19-R fraction had a shorter median TTT from measurement (37.7 mo) compared with patients with a lower CD19-R fraction (median not reached, p = 0.012; Fig. 6B, right panel). A multivariate Cox regression analysis was further carried out to determine whether CD19 responsiveness, ZAP70
and CD86 levels in this fraction, cells were not fixed. (human CD45-PerCP, CD19-allophycocyanin, CD20-FITC, and CD86-PE as percentage of untreated control. Paired fraction, CD19, and CD20 levels following sIgM activation are presented whole CLL cells measured in paired BM/PB samples is presented. Paired CD45-PerCP, CD19-allophycocyanin, CD20-FITC, and CD5-PE Abs then the same day for each designated patient) were labeled with anti-human test (* from high responding patients. (R fraction measurements in fresh versus cultured cells. Immediately after PB collection, and after 3 d in culture. Presented are CD19-

**FIGURE 5.** Regulation of distinct signaling responses by BCR components. PBMCs of CLL patients were incubated with goat F(ab')2 anti-human IgM (50 μg/ml) at 37°C for 18 h, fixed, and labeled with anti-human CD45-PerCP, CD19-allophycocyanin, CD20-FITC, and CD86-PE Abs then analyzed by flow cytometry. For CD19-R fraction measurements and CD86 levels in this fraction, cells were not fixed. (A) CD86, CD19-R fraction, CD19, and CD20 levels following slgM activation are presented as percentage of untreated control. Paired t test (*p < 0.05). (B) CD86 levels in CD19-R and CD19-N fraction are presented as ratio to untreated controls. ○, ZAP70−; ●, ZAP70+ (n = 9). (C) Phospho-Akt levels were analyzed by flow cytometry in CLL cells following slgM stimulation, CD19 engagement, CD19 high-order clustering, or control unstimulated cells. C.L., crosslinking. (D) Averages ± SD of pAkt levels in three independent CLL samples. Cells were gated for single, double, and triple cells using the size and aspect ratio features. The relative fluorescence intensity was calculated by dividing the total pAkt intensity by the total brightfield area of the cell or cells in each aggregate. (E) PMNCs from 15 CLL patients were analyzed by flow cytometry at two time points: immediately after PB collection, and after 3 d in culture. Presented are CD19-R fraction measurements in fresh versus cultured cells. Top panel, Samples obtained from low responding patients; bottom panel, samples obtained from high responding patients. (F) Either BM or PB samples (collected in the same day for each designated patient) were labeled with anti-human CD45-PerCP, CD19-allophycocyanin, CD20-FITC, and CD5-PE Abs then analyzed by flow cytometry. The percentage of CD19-R subpopulation of whole CLL cells measured in paired BM/PB samples is presented. Paired t test (*p < 0.05).

protein expression (positive versus negative subgroups), and chromosomal aberrations detected by FISH (low- to intermediate-versus high-risk subgroups) are independent predictors for TTT. In the analysis for TTT that was determined from the day of diagnosis, both FISH and CD19-R were significant (p = 0.001; hazard ratio [HR], 5.9; 95% confidence interval [CI], 2.0, 17.5 and p = 0.012; HR, 3.5; 95% CI, 1.3, 9.5, respectively) and ZAP70 had a borderline significance (p = 0.053; HR, 2.6; 95% CI, 0.99, 7.0). When TTT was calculated from the day of the flow cytometry measurement, all three factors were found to be statistically significant (for FISH, p = 0.007; HR, 3.9; 95% CI, 1.4, 10.5; for CD19 responsiveness, p = 0.005, HR, 4.3; 95% CI, 1.6, 12.6; and for ZAP70, p = 0.023, HR, 3.0; 95% CI, 1.2, 7.9).

**Discussion**

Recently, it is becoming apparent that intraclonal diversity plays a role in the clinical outcome of patients with CLL. Subsets of the CLL clone that respond more robustly to external stimuli may well gain a growth and survival advantage and possibly promote clonal evolution. Identification of these CLL subpopulations is therefore of prime importance, as these cells may be preferred targets for future therapeutics. In this study, we found that after stimulating the CD19 component of the BCR complex, CLL cells rapidly formed cellular aggregates. However, among the CLL samples, the response to CD19 was diverse and this heterogeneity was evident both as differences between the patients as well as intraclonal diversity.

CD19 is a transmembrane protein and a member of the Ig superfamily restricted to B cells (36), which has been shown to transmit pleiotropic intracellular signals, including the activation of associated cellular protein tyrosine kinases (37, 38). In our study, formation of CLL cell aggregates by CD19 was only reduced by 25% after using protein tyrosine kinase inhibitors, whereas inhibition of F-actin assembly also resulted in a minor inhibitory effect, which is in agreement with previously published results (30). In contrast, the aggregation was completely abrogated by disruption of the cholesterol-rich plasma membrane rafts. The above findings showing that blocking of the CD19 cytoplasmic domain signaling only had a minor effect on the CLL cell aggregation strongly suggest that this response is mainly attributed to CD19 association with other transmembrane components, probably those occurring within the lipid rafts. In agreement with this concept, it was previously shown that the induction of homotypic aggregation was shown to be critically dependent on the CD19 ectodomain, whereas its cytoplasmic domain is not required for this activity (39, 40). In this study, we also show that CLL cell–cell adhesiveness induced by CD19 is partially mediated through β2 integrin, in agreement with previous studies (27, 41, 42). These data, and our clinical correlations, imply that there may be two nonequivalent signaling dimensions within the BCR complex in CLL cells, as depicted in Fig. 6C. Whereas the vertical signaling events (Fig. 6C, pathway A), through the membrane into the cytoplasm, involve activation of cytoplasmic events and are affected by in the presence of ZAP70, horizontal signaling events may be limited to the membrane plane, resulting in cellular aggregation (Fig. 6C, pathway B). In keeping with this distinction, we found no correlation between the proportion of CD19-R cells and the levels of ZAP70 expression in these patients with CLL. Further support for the existence of two modes of signaling via the BCR can be found in the observation that the levels of the activation marker CD86 were elevated in a similar manner both in the CD19-N and CD19-R cells in response to slgM stimulation, but only the latter were able to aggregate in response to CD19 engagement. Interestingly, dissociated signaling between CD19 and other components of the BCR is supported by a recent study in B cell lymphomas (43). CD19 is able to provoke both Akt phosphorylation and cellular aggregation. However, the threshold for Akt phosphorylation is significantly higher and requires high-order clustering of CD19 (Fig. 6D). In a similar manner, only weak ERK phosphorylation is triggered by CD19 engagement. The concept of biochemical signaling hierarchies of receptor complexes has previously been shown for integrins (44). The
complex structure of the BCR allows signaling divergence via different components of the receptor, which may be expressed in clinical variations as we show in this study.

A strong correlation was established in this study between CD19-induced aggregation and its level of cell surface expression. This link was strengthened by the observation that sIgM stimulation enhanced both CD19 surface expression and responsiveness. These data are in line with a recent study that established a linkage between the levels of CD19 expression and lymphoma progression (43). Ag stimulation is considered to be a key mediator in the development and progression of LPD (45). Apparently, the ability of sIgM (35) and the CD19 (as shown in this study) components of the BCR to provoke a cellular response to their engagement depends, to a large extent, on their surface level of expression or localized clustering (Fig. 6D).

The CD19-R component of CLL is comprised of two distinct types of cells. The first one includes cells whose ability to respond to CD19 engagement is suppressed in vivo in a reversible manner. The second type includes cells that cannot respond at all to this stimulus. These data point to in vivo operating mechanisms that may reversibly attenuate CD19-mediated signaling. A previous study indicated that the sIgM component of the BCR may become anergic in vivo in some CLL patients (35). Whether these are microenvironmental effectors (e.g., cytokines, other cells) or intrinsic regulatory mechanisms that restrain CD19 responsiveness of CLL cells remains to be established. Interestingly, we found that the level of CD19 responsiveness of CLL cells within the tissue (BM) is considerably lower compared with that of CLL cells in the PB of the same patient. This observation supports the existence of CD19 tolerance, because BCR stimulation as well as mechanisms of tolerance induction are expected to operate primarily in the microenvironment of lymphoid tissues. The finding that CD19-R cells are more resistant to such mechanisms may explain the association of more rapid disease progression in patients with higher portions of this subpopulation.

The intraclonal components of CLL are apparently sustainable owing to their distinctive properties and their long-term presence in a given patient. CD19-R CLL cells appeared to be larger cells with more dispersed nuclear chromatin with readily visible nucleoli and overexpression of c-myc, compatible with cells with a higher
proliferative potential. However, we did not find higher frequency of Ki-67+ cells in the CD19-R fraction, indicating that this subpopulation is not necessarily an actively proliferating subclone of the disease. Accordingly, Chung et al. (43) recently showed that CD19 levels correlate with c-myc–activated genes, and that CD19 is a major regulator of c-myc expression independent of BCR signaling, in a manner that is negatively correlated with the survival of human lymphoma patients. In this study, we show a similar correlation between the levels of CD19 expression, the levels of c-myc, and also with the ability of the cells to respond to CD19 engagement. These characteristics are negatively correlated with CLL progression. Support for the distinctive existence of the CD19-R subpopulation comes from the fact that this subpopulation is preferentially eliminated in rituximab-treated patients. Interestingly, we found that in vitro treatment of CLL cells with rituximab did not alter the CD19-R subpopulation. Hence, rituximab does not affect directly the CD19-mediated signaling response associated with CLL cell aggregation. The elimination of CD19-R cells by rituximab in vivo may be the result of specific eradication of this subpopulation by the Ab within the patient or by “shaving” the CD20 component of the BCR, as have been suggested previously (47, 48), thus affecting BCR-mediated signaling.

Regarding interpatient heterogeneity, we show in this study a single dimension of diversity in CLL, whose resolution is determined by CD19 responsiveness. Other dimensions of interpatient heterogeneity are determined by other components of the BCR (e.g., sIgM) (35) or its associated molecules (e.g., ZAP70) (8, 10), representing a single dimension of diversity in CLL, whose resolution is determined by CD19 responsiveness. Other dimensions of interpatient heterogeneity are determined by other components of the BCR (e.g., sIgM) (35) or its associated molecules (e.g., ZAP70) (8, 10), representing a single dimension of diversity in CLL, whose resolution is determined by CD19 responsiveness. Other dimensions of interpatient heterogeneity are determined by other components of the BCR (e.g., sIgM) (35) or its associated molecules (e.g., ZAP70) (8, 10), representing a single dimension of diversity in CLL, whose resolution is determined by CD19 responsiveness.

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Disclosures

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