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Heterogeneous Functional Effects of Concomitant B Cell Receptor and TLR Stimulation in Chronic Lymphocytic Leukemia with Mutated versus Unmutated Ig Genes

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We recently reported that chronic lymphocytic leukemia (CLL) subgroups with distinct clonotypic BCRs present discrete patterns of TLR expression, function, and/or tolerance. In this study, to explore whether specific types of BCR/TLR collaboration exist in CLL, we studied the effect of single versus concomitant BCR and/or TLR stimulation on CLL cells from mutated (M-CLL) and unmutated CLL (U-CLL) cases. We stimulated negatively isolated CLL cells by using anti-IgM, imiquimod, and CpG oligodeoxynucleotide for BCR, TLR7, and TLR9, respectively, alone or in combination for different time points. After in vitro culture in the absence of stimulation, differences in p-ERK were identified at any time point, with higher p-ERK levels in U-CLL versus M-CLL. Pronounced p-ERK induction was seen by single stimulation in U-CLL, whereas BCR/TLR synergism was required in M-CLL, in which the effect was overall limited in scale. An opposite pattern was observed regarding induction of apoptosis, as studied by Western blotting for the cleaved fragment of poly(ADP-ribose) polymerase, and the active isoform of caspase-8, with M-CLL responding even to single stimulation, contrasting with U-CLL that showed minimal response. Our findings suggest that concomitant engagement of BCR and TLR leads to differential responses in CLL depending on the mutational status of the BCR. Differential intensity and duration of responses in M-CLL versus U-CLL indicates that the differences in signal transduction between the two subgroups may be primarily quantitative rather than qualitative. The Journal of Immunology, 2014, 192: 4518–4524.

Mounting evidence suggests that the development and evolution of chronic lymphocytic leukemia (CLL) are dependent upon the interplay between genetic defects and stimuli originating from the microenvironment (1), including cellular components and soluble factors (2). A critical role is played by antigenic stimulation through the BCR as evidenced by: 1) the biased IGHV gene repertoire of CLL (3, 4); 2) the subdi-

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time, thus prompting the question of how BCR/TLR concomitant collaboration may ultimately contribute to tumor proliferation and/or apoptosis. In the current study, we studied the effects of single versus dual BCR and TLR stimulation on CLL clones with different IGHV gene mutational status. We focused onto ERK and poly (ADP-ribose) polymerase (PARP) molecules as distinct molecular indicators of key signaling pathways in CLL (13). We report in this study that concomitant BCR/TLR stimulation induces similar qualitative responses in mutated CLL (M-CLL) and unmutated CLL (U-CLL), though different in terms of intensity and duration. These results, obtained in a more physiological setting, indicate that the differences in signal transduction are primarily quantitative rather than qualitative, as suggested earlier (27). These findings offer hints for understanding the immune responses of CLL cells and also for better characterizing the potential benefits from novel therapeutics targeting immune signaling pathways.

Materials and Methods

Patient group

Blood samples were collected from 21 patients with typical CLL, diagnosed according to International Workshop on Chronic Lymphocytic Leukemia/ National Cancer Institute guidelines (28). All patients were either untreated or off therapy for at least 6 mo before the study. The study was approved by the local ethics committees of the participating institutions. Demographic, clinical, and biological data for the patients included in the study are given in Table I and Supplemental Table I.

Cell enrichment

CD19+ B cells were negatively selected from whole blood using the RosetteSep B-cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) as previously described (23). The purity of all preparations was checked by flow cytometry and always exceeded 95% for CD19+ cells.

Stimulation of CD 19+ B cells

Freshly isolated CD19+ B cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mmol/lL-glutamine, and 15 μg/ml gentamicin, at a density of 3 × 10^6 cells/ml. Viability was measured with trypan blue and >95% in all cases.

Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO₂ in the presence or absence of specific BCR [goat F(ab') anti-human IglM (20 μg/ml); Thermo Scientific] and TLR ligands (imiquimod [IM] for TLR7/8 [R837; Invirogen]; 0.1 μg/ml and CpG for TLR9 [ODN2006, stimulatory; CpG-ODN type B; human specific; 2.5 μg/ml; Invirogen]) at different time points. Cells were: 1) cultured without any stimulation; 2) stimulated with anti-IgM, CpG, or IM alone; and 3) concomitantly stimulated with anti-IgM plus CpG or anti-IgM plus IM. Details on the culture conditions and ligands are given in Table II.

Western blot analysis

Whole-protein extracts from cells at the basal level as well as treated and untreated (control) cultured cells were subjected to Western blot analysis. For phospho-kinase analysis, immunoblotting was performed with phospho-p44/42 (ERK) MAPK (1:500 dilution; Cell Signaling Technology, Beverly, MA).

To ascertain equivalent loading, membranes were stripped and reprobed with anti-ERK2 (1:1000 dilution; Cell Signaling Technology) and anti-actin Ab (1:5000 dilution; Invitrogen, Paisley, U.K.).

For apoptosis studies, we used anti-PARP (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and anti–caspase-8 Abs (1:500 dilution; Santa Cruz Biotechnology). The PARP Ab used in this study detects both the intact and the cleaved product, whereas the caspase-8 detects both inactive caspase-8 and its active isoforms. Membranes were first incubated with the anti-PARP Ab and thereafter stripped and reprobed with the anti–caspase-8 and finally with anti-actin Ab.

Immunodetection was revealed by incubation with goat anti-mouse Ig (Upstate Biotechnology, Lake Placid, NY) conjugated with HRP followed by ECL reaction (Pierce, Rockford, IL) and film exposures. Ratios of specific protein band intensity relative to actin band intensity were calculated for each sample with the use of ImageJ Software (National Institutes of Health). To ascertain whether the studied samples responded or not to a given stimulation, we followed the proposed cutoff of a 1.2-fold increase (29) in the expression level of the target phosphorylated proteins compared with the corresponding unstimulated control.

Statistical analysis

Descriptive statistics were performed as described previously (23). For all comparisons, a significance level of p < 0.05 was set. All statistical analyses were performed with the use of the Statistical Package SPSS Version 18.0 (SPSS Inc, Chicago, IL) and GraphPad Prism 5 software (GraphPad, La Jolla, CA).

Results

Single BCR or TLR stimulation elicits active MAPK signaling in U-CLL, whereas BCR/TLR synergism is required in M-CLL

To determine the effects of BCR and TLR7/9 single and dual stimulation on CLL B cells, we investigated the MEK1/2-ERK1/2 downstream signaling pathway in 11 M-CLL and 10 U-CLL cases (Table I). The choice to study ERK was grounded on the fact that it is activated from both BCR and TLR triggering and also plays a critical role in B cell survival and function.

Basal level and unstimulated CLL cultures. At basal level, before any culture, ERK was found constitutively phosphorylated in all cases, albeit with great heterogeneity. U-CLL cases presented a trend of higher p-ERK levels compared with M-CLL; however, the difference was not statistically significant (p = 0.14), confirming previous reports (30, 31). Median values, average values, and range of data are presented in Supplemental Tables II and III.

When cells from all studied cases were cultured in vitro for 15 min, without any treatment, p-ERK levels differed up to 2-log in CLL cells from different cases; interestingly, some cases exhibited very low levels of p-ERK. Significantly higher p-ERK levels were seen in U-CLL versus M-CLL throughout the unstimulated cultures. Most cases showed significant (p < 0.05) reduction of p-ERK levels with time. However, the effect was more pronounced in M-CLL versus U-CLL (p < 0.05); indeed, the latter maintained sufficiently high levels of p-ERK even after 60 min of culture (Fig. 1A).

Single BCR or TLR stimulation activates MAPK mainly in U-CLL

The CLL clones included in this study uniformly expressed low levels of BCR, in keeping with the norm for CLL. Stimulation of the BCR with anti-IgM Ab induced ERK phosphorylation in 16 out of 21 (76.2%) cases, though variably. Overall, p-ERK levels were higher throughout the culture in U-CLL versus M-CLL, especially after prolonged exposure to anti-IgM (p < 0.05) (Fig. 1B).

In U-CLL, the magnitude of responses was significantly higher compared with the unstimulated cells after 50 min in culture. On the contrary, in M-CLL, the most pronounced difference compared with unstimulated cells was seen after 15 min in culture and essentially lost after 50 min in culture (Fig. 1B, Table II).

Table I. Clinical and biological data of the patient cohort

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binet stage at diagnosis</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>18</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>CD38 expression</td>
<td></td>
</tr>
<tr>
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<td>7</td>
</tr>
<tr>
<td>Negative</td>
<td>14</td>
</tr>
<tr>
<td>IGHV gene mutational status</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>11</td>
</tr>
<tr>
<td>Unmutated</td>
<td>10</td>
</tr>
<tr>
<td>Disease progression</td>
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</tr>
<tr>
<td>Progressive</td>
<td>7</td>
</tr>
<tr>
<td>Stable</td>
<td>14</td>
</tr>
</tbody>
</table>
For TLR triggering, overall, responses were essentially restricted to U-CLL for both TLR7 and TLR9 stimulation with minimal if any effects on M-CLL (Fig. 1C). In U-CLL, both TLR7 triggering with IM and TLR9 triggering with CpG for 50 min upregulated p-ERK levels compared with unstimulated cells, with CpG inducing more pronounced phosphorylation compared with IM (Fig. 1C). For both TLRs, the effect waned with prolonged (60 min) exposure to the respective ligand.

Concomitant BCR and TLR stimulation can activate MAPK in M-CLL. Concomitant stimulation of CLL cells with anti-IgM plus IM or anti-IgM plus CpG upregulated p-ERK levels in comparison with the unstimulated control in both M-CLL and U-CLL. Yet, in both groups, only anti-IgM plus TLR9 stimulation induced significantly different responses compared with the unstimulated control (p < 0.05; Fig. 2).

In contrast, comparison of concomitantly versus single-stimulated cells through either the BCR or the TLRs revealed great heterogeneity. In particular, certain cases exhibited augmented responses after concomitant stimulation, contrasting other cases in which this treatment attenuated the responses obtained with single stimulation.

When we focused our analysis on subgroups of patients based on their IGHV gene mutational status, distinct patterns of responses became evident compared with single stimulation. In particular, concomitant stimulation, especially with anti-IgM plus CpG, was able to synergistically induce ERK phosphorylation in M-CLL, whereas minimal if any effects regarding p-ERK levels were observed in U-CLL (Fig. 2).

Apoptosis resistance of U-CLL to single stimulation is not reverted by concomitant BCR/TLR stimulation

We then investigated apoptosis induction in nine U-CLL and nine M-CLL cases after 48 h of culture with anti-human IgM Abs, IM, and CpG ODN for BCR, TLR7, and TLR9 stimulation, respectively. For each experimental condition, the untreated CLL cells from the same case were used as control (Table II).

We assessed apoptosis induction by determining the cleavage of the DNA repair enzyme PARP as an indicator of the activity of executive caspase-3. Western blot analysis was conducted using an Ab for PARP that detects both the intact enzyme and its cleaved product.

To better define the apoptotic molecules involved, we also studied the patterns of proteolytic processing of initiator caspase-8, as it was recently shown that Fas-independent activation of caspase-8 may play a crucial role in the regulation of apoptosis in CLL cells (32).

**Table II. Details about the culture conditions in the present series**

<table>
<thead>
<tr>
<th>Type of Stimulator</th>
<th>15 min</th>
<th>50 min</th>
<th>60 min</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without stimulator</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CpG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Anti-IgM+CpG</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IgM+IM</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

* denotes stimulation with a given ligand at the particular time point.

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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Anti-IgM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CpG</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>IM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Anti-IgM+CpG</td>
<td>+</td>
<td>+</td>
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senting with significantly lower percentage of active caspase-8 isoform compared with M-CLL ($p = 0.05$).

**Single TLR stimulation activates apoptosis only in M-CLL.** Stimulation with anti-IgM for 48 h led to higher cleaved PARP levels in U-CLL versus M-CLL (10.7 versus 3.1%); however, this was not significant compared with the results obtained in unstimulated CLL cells, and the levels of the active caspase-8 isoforms also did not change significantly by IgM cross-linking (Figs. 3, 4, Supplemental Table IV).

**Figure 3.** Percentage of cleaved PARP fragment in M-CLL (M) versus U-CLL (U) after single or concomitant stimulation with BCR or TLR7/TLR9. TLR stimulation seems to increase cleaved PARP fragment only in M. Left, Immunoblotting analysis of two representative cases (top left, M; bottom left, U). Right, Densitometric analysis of the percentage of cleaved PARP fragment in M versus U. The graphs represent the mean values in M versus U. Asterisks above bars highlight statistically significant differences compared with the unstimulated control after 48 h of stimulation, whereas horizontal lines with asterisks overhead highlight statistically significant differences between the M and U subgroups. Statistical analysis was performed with Student $t$ test for comparisons among subgroups and paired $t$ test for intrasubset comparisons. In each comparison, $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.005$. 48, duration of stimulation in minutes with each ligand. CpG, ODN 2006; CTL, unstimulated control; IgM, anti-IgM.

Single TLR stimulation induced apoptosis, however, with significantly more pronounced effects in M-CLL versus U-CLL. In particular, exposure to either IM or CpG ODN was accompanied by a significant increase of the cleaved PARP levels in M-CLL (36.2 and 37.9%, respectively, compared with 2.7% in the unstimulated control), whereas minimal or no differences were seen in U-CLL; caspase-8 active isoform seems to be less affected by single TLR stimulation in both M-CLL and U-CLL subgroups (Figs. 3, 4, Supplemental Table IV).
**Concomitant BCR and TLR stimulation does not activate apoptosis in U-CLL.** Concomitant stimulation with anti-IgM plus CpG or anti-IgM plus IM had synergistic effects compared with single stimulation or the unstimulated control, albeit with great heterogeneity among different CLL cases, depending on IGHV gene mutational status. In particular, the effect was obvious in M-CLL versus U-CLL for both active caspase-8 isoforms and cleaved PARP levels compared with single stimulation through BCR or TLRs, mainly TLR7 (Figs. 3, 4, Supplemental Table IV).

**Discussion**

Several lines of evidence suggest that specific modalities of BCR/TLR collaboration and/or regulation may exist in CLL, eventually impacting on the biological behavior of the malignant clones (21, 23, 33, 34). Against this background, all studies thus far have examined the effects of immune stimulation only after isolated, single BCR or TLR triggering. In the current study, to explore potential synergism, we performed single as well as concomitant BCR and TLR stimulation or the unstimulated control, albeit with significant heterogeneity among different CLL cases, depending on IGHV gene mutational status. In particular, the effect was obvious in M-CLL versus U-CLL for all active caspase-8 isoforms and cleaved PARP levels compared with single stimulation through BCR or TLRs, mainly TLR7 (Figs. 3, 4, Supplemental Table IV).

In vitro cross-linking of the BCR with anti-IgM in CLL cells induces ERK phosphorylation (20). Interestingly, this is significantly more pronounced in CLL with poor prognostic markers and associated with a shorter clinical progression, thus suggesting a prominent role of ERK activation induced by BCR modulation in the pathophysiology of CLL (27). Relevant to the above, ERK activation of CLL cells cultured without any stimulation, and it was detected in all analyzed cases, albeit with significant heterogeneity (from very low to high p-ERK levels).

Our previous work has indicated that constitutive activation of the MAPK signaling pathway in the absence of AKT activation is seen in a proportion of CLL patients whose cells are unresponsive to in vitro BCR cross linking, recalling the situation of mouse anergic B cells (19). In the current study, constitutively p-ERK was analyzed at different time points in CLL cells cultured without any stimulation, and we used a cutoff value of 10% for positivity. With this procedure, cases expressing low p-ERK levels were considered as negative (19). In contrast, in the present series, we measured the ratio of p-ERK band intensity in relation to total ERK and/or actin and considered any kind of positivity over time. All that notwithstanding, considering the fact that CLL cells engage in microenvironmental interactions mainly in solid-tissue compartments (35), p-ERK–positive circulating CLL cells may represent the most recently released CLL cells from solid tissues. Thus, their proportion may not be stable but rather differ between different cases or at different time points in the same case.

BCR stimulation with anti-IgM Ab increased p-ERK levels in almost all cases, though variably. It is known that sustained activation of ERK kinases can promote CLL cell survival by upregulating several antiapoptotic proteins (20). We show for the first time, to our knowledge, that in M-CLL p-ERK levels are rapidly upregulated upon BCR ligation but the signal is lost very soon, whereas in U-CLL ERK activation is more prolonged. Altogether, the finding of enhanced intensity and duration of p-ERK activation in U-CLL corroborates the notion that U-CLL cells may be better equipped to transmit BCR-derived signals and thus more
responsive to BCR cross linking compared with CLL cells from M-CLL cases that appear in a weaker fashion (11, 35).

Regarding TLR stimulation, we found that both TLR7 and TLR9 ligands upregulated p-ERK levels compared with unstimulated cells, but CpG induced more pronounced phosphorylation compared with IM; the observed effects declined with prolonged exposure to each ligand. These results are in accordance to previous studies showing that TLR9 stimulation elicits stronger responses in CLL cells compared with TLR7 (36). Overall, responses were significantly more pronounced in U-CLL versus M-CLL for both TLR7 and TLR9 triggering, in agreement with published studies (23, 25, 26).

Concomitant BCR and TLR stimulation with anti–IgM plus TLR ligands significantly increased p-ERK levels compared with the untreated control in both M-CLL and U-CLL. However, comparison of concomitantly versus single-stimulated cells revealed great heterogeneity, with certain cases presenting higher and others lower p-ERK levels compared with single BCR or TLR stimulation. Indeed, we report for the first time, to our knowledge, that whereas single BCR or TLR stimulation alone can elicit sustained active MAPK signaling in U-CLL evidenced by high p-ERK levels, to reach comparable p-ERK levels in M-CLL concomitant BCR plus TLR stimulation is required. Of note, synergism between BCR and TLRs seems to be relevant only in M-CLL, whereas in U-CLL, concomitant BCR and TLR (especially TLR9) triggering may even downregulate p-ERK levels, alluding to potential antagonistic effects.

Synergism between TLR-mediated and Ag-specific signals is a well-known phenomenon in both normal and autoreactive cells (37–42). In normal cells, the functional outcome may differ among different subpopulations and/or depending on the developmental stage (38). It remains to be elucidated how relevant these observations are for CLL; however, our present findings indicate the possibility of distinct synergistic and/or antagonistic effects in M-CLL versus U-CLL clones, with potential implications for shaping their biological and clinical behavior.

We also investigated the effects of single versus concomitant BCR and/or TLR triggering on apoptosis induction. Although the identity of the initiator caspase(s) involved in BCR-mediated apoptosis is still a matter of controversy, caspase-8 has been reported to be activated in a Fas-independent manner by BCR ligation (32) and also shown to play a crucial role in the regulation of apoptosis in CLL cells (43). Furthermore, a recent study supported an important role for caspase-8 in the maintenance of genomic integrity and highlighted its lymphoma-suppressive function (44). For these reasons, we investigated caspase-8 and the cleavage of the PARP, a downstream target of caspase-3, as indicators of apoptosis induction.

We found that M-CLL responded even to single stimulation, contrasting U-CLL that showed minimal responses even after concomitant stimulation, at least in the present setting; of note, similar results were obtained by the analysis of caspase-3 in a subset of cases of the current study (Fig. 5). Therefore, our novel findings confirm that BCR and/or TLR-stimulated U-CLL cells are more resistant to apoptosis (23, 25, 45) and, hence, more functionally dependent on microenvironmental signals than M-CLL B cells. Interestingly, our present results are in accordance with our previous study, at least concerning TLR stimulation and its impact on CLL cell viability, in which we showed that M-CLL cells indeed undergo apoptosis after TLR7 and TLR9 stimulation in sharp contrast to U-CLL.

In conclusion, our study documents for the first time, to our knowledge, that concomitant engagement of BCR and TLR leads to differential responses in CLL depending on the mutational status of the BCR. Differential intensity and duration of responses in M-CLL versus U-CLL indicates that the differences in signal transduction between the two subgroups may be primarily quantitative rather than qualitative. Better understanding of the interaction between the BCR and the TLRs and the regulation of their responses in CLL might prove relevant for both identifying potential therapeutic targets and also for defining subgroups of patients who might benefit from treatment with specific TLR ligands.

Disclosures
The authors have no financial conflicts of interest.

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