1. ABSTRACT

Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy characterized by a highly variable clinical course. The behavior of the disease is believed to be influenced by microenvironmental signals that regulate the proliferation and survival of the malignant B-cells. Signals transduced through Toll-like-receptor-9 (TLR9) may play a particularly important role, as they could drive the expansion of a subset of cells that express B-cell receptors reactive with DNA or DNA-containing complexes. Interestingly, leukemic cells from patients with aggressive disease respond more effectively to TLR9 stimulation than their less aggressive counterparts, suggesting that the capacity to respond to TLR9 signals can define distinct prognostic subsets in CLL. The exact mechanism(s) accounting for the variability in the response to TLR9 engagement are still unclear, although important differences have been observed between prognostic groups in terms of downstream signaling events and gene- and miRNA-expression profiles. Understanding the mechanism(s) that underlie the different TLR9 responses should provide further insight in the pathophysiology of CLL and may lead to the identification of novel targets for therapeutic intervention.

2. INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a common lymphoid malignancy characterized by the expansion and progressive accumulation of mature B lymphocytes that coexpress the T-cell antigen CD5 and B cell surface antigens CD19, CD20, and CD23. The disease has a highly heterogeneous clinical course. More than half of the patients have a relatively indolent disease and many of them can live for decades without requiring treatment. In the remaining patients the disease can take a rapidly progressive course, leading to an early fatal outcome despite treatment (1).

A number of prognostic tools have been developed in the past decades to help predict the clinical course in patients with CLL. In clinical practice the most commonly used are the staging systems of Rai and Binet, which are based on routine clinical and laboratory examinations that evaluate the extent of lymphadenopathy, organomegaly and the presence of cytopenias (2,3). In essence, these staging systems measure tumor mass and therefore are useful to identify patients with advanced disease that require immediate treatment. However, they can not predict the clinical course in patients with early-
stage disease, which represent the vast majority of patients at the time of diagnosis.

In recent years a number of molecular and biological parameters have been introduced that can predict rather accurately the risk for disease progression in patients with early-stage disease. These prognostic markers include the mutational status of the immunoglobulin heavy-chain variable (IGHV) genes, expression of ZAP-70 and CD38, and the presence of high-risk chromosomal abnormalities, such as the 17p13 and 11q22 deletions (4-14).

The observation that CLL can be divided into 2 roughly equal subsets based on the mutational status of the IGHV genes was made more than 10 years ago (15). The prognostic impact of this feature was shortly afterwards independently reported by two groups (4,5). These studies demonstrated that CLL patients with mutated immunoglobulin genes (M-CLL) have a significantly longer overall survival (OS) than patients with unmutated immunoglobulin genes (U-CLL). The ability of IGHV mutation status to stratify OS was also retained when only early-stage patients were included in the analysis (5).

Soon thereafter, gene expression profiling (GEP) studies were conducted in an attempt to identify differences that could account for the different survival of patients with M-CLL and U-CLL (16,17). Unexpectedly, these analyses revealed relatively few differences between the two CLL subsets, as opposed to the thousands of differences between normal B lymphocytes and CLL cells, suggesting that the two subsets share a common mechanism of transformation and/or cell of origin. Interestingly, approximately half of the genes that were found to be differentially expressed between the two CLL subsets were genes that are induced in normal B cells during signaling through the B cell receptor (BCR). These genes were expressed at higher levels in U-CLL samples, suggesting that the leukemic cells from this subset are more often exposed to antigen in vivo or are endowed with a greater capacity to transduce BCR signals.

Among the genes most significantly overexpressed in U-CLL was ZAP-70, a protein tyrosine kinase that plays a key role in T cell receptor signaling in T cells (16). Flow-cytometry analysis of ZAP-70 expression was quickly introduced as a potential surrogate for IGHV mutation analysis (11,12). However, standardization of this assay turned out to be rather complicated and still remains an important obstacle towards greater reliance on this prognostic factor in the routine clinical setting.

Although unmutated IGHV genes and ZAP-70 expression are strongly associated with unfavorable prognosis, it is still not clear how these two features could contribute to the greater aggressiveness of the disease. Expression of unmutated IGHV genes and/or ZAP-70 has been associated with a greater capacity to signal through the B cell receptor, indicating that the ability to respond to antigen stimulation may underlie the different disease activity in these prognostic subsets (18-24). Along these lines, more recent studies have shown that leukemic cells belonging to different prognostic subsets also differ in their capacity to respond to other microenvironmental stimuli that regulate proliferation and survival. An especially noteworthy difference was observed in the response to unmethylated CpG oligonucleotides (CpG-ODN). These oligonucleotides are used to mimic unmethylated CpG-DNA, as they can enter into B-cells by endocytosis or nonspecific fluid-phase pinocytosis and trigger the intracytoplasmically localized Toll-like receptor 9 (TLR9).

In this article we provide an overview of the microenvironmental stimuli that have been reported to induce different outcomes in CLL cells from different prognostic subsets and discuss the possibility that the course of the disease is influenced by the capacity of the leukemic cells to interact with the microenvironment. We also describe the signaling pathways that are activated in CLL B cells in response to TLR stimulation, focusing in particular on TLR9 signaling and the possible mechanisms that could account for the different responses. In this regard, we also discuss recent studies by our group that suggest a role for microRNAs in regulating the response of CLL cells to TLR9 triggering.

3. RESPONSE OF CLL B CELLS TO CpG-ODN STIMULATION IN DIFFERENT PROGNOSTIC SUBSETS OF CLL

A heterogeneous response of CLL cells to stimulation with CpG-ODN was first reported by Jahrsdorfer et al, who observed different behavior of CLL cells from patients with high and low serum thymidine kinase (s-TK) activity (25). In most samples from patients with high s-TK activity, incubation with CpG-ODN inhibited the spontaneous apoptosis of the malignant cells, whereas apoptosis was predominantly induced in samples from patients with low s-TK activity. In addition, a direct correlation was observed between s-TK levels and the capacity of the leukemic cells to proliferate in response to CpG-ODN stimulation. These correlations were the first indication that the response to CpG-ODN stimulation could differ between the different prognostic subsets in CLL, because high s-TK activity is usually found in patients with more active disease and has been associated with rapid disease progression (26).

The heterogeneous response to CpG-ODN stimulation was subsequently confirmed by the same authors in another small series, which showed that CpG-ODN induce CLL B cell proliferation in approximately half of the samples (27). In addition, in another series a highly variable effect on survival was observed, with CpG-ODN predominantly inducing apoptosis in samples with low-risk cytogenetic abnormalities, such as 13q14 deletion, whereas a heterogeneous effect was observed in CLL cells with other aberrations, including trisomy 12 and the high-risk 17p13 and 11q22 deletions (28). Interestingly, stimulation with CpG-ODN induced expression of activation markers in all investigated samples, regardless of the subsequent cellular response (27). This indicated that all CLL samples are capable of responding to CpG-ODN stimulation, but that the outcome can vary depending on the CLL subset.
TLR9 signaling in CLL

To further evaluate the association between CLL behavior and response to CpG-ODN stimulation, we more recently correlated the proliferative response induced by CpG-ODN with other established prognostic markers in a large series of well characterized CLL samples (29,30). Significant proliferation was detected by [3H] thymidine incorporation in 30 of the 91 (33%) investigated cases. The proliferating CLL cases were found to express mainly unmutated IGHV genes (73% of proliferating versus 25% of nonproliferating cases, p<0.001), and were more often ZAP-70 positive (70% of proliferating versus 37% of nonproliferating cases, p=0.01). A weaker association was observed with CD38 expression (37% of proliferating versus 15% of nonproliferating cases, p=0.035), whereas the association with high-risk cytogenetic abnormalities did not reach statistical significance (p=0.059), possibly because of the fewer cases with 17p13 and 11q22 deletions. Importantly, the proliferative response to CpG-ODN stimulation significantly correlated with shorter progression-free survival, time-to-treatment and overall survival, further suggesting that this feature can be considered a novel prognostic marker in CLL (30).

The association between the proliferative response to CpG-ODN stimulation and IGHV mutation status was independently confirmed by Tromp et al (31). These authors also showed that the difference in the proliferating response between U-CLL and M-CLL samples remains or becomes even more prominent in a combined CD40/TLR9 co-culture system, which was used to mimic the in vivo lymph node microenvironment where CLL cells are believed to receive growth and survival signals.

4. OTHER EXTERNAL STIMULI THAT INDUCE DIFFERENT RESPONSES IN DISTINCT PROGNOSTIC SUBSETS OF CLL

Several recent studies have reported associations between the clinical course of CLL and the capacity of the leukemic cells to respond to other external stimuli, in addition to CpG-ODN and anti-IgM. In particular, Plander et al showed that only a subset of CLL samples can be induced to proliferate by a combination of soluble CD40L/IL-2/IL-10 in co-culture with stromal cells; 75% of these samples expressed unmutated IGHV genes and all were derived from progressive clinical cases and were ZAP-70-positive (32). Furthermore, Hammond et al reported that only 28 of 51 investigated CLL samples responded to changes in extracellular calcium concentration as normal B cells, with a rapid release of internal calcium stores and activation of downstream signaling pathways (33, 34). These cases had unfavorable prognostic features, such as high CD38 expression and short lymphocyte doubling time, and more often required treatment than the nonresponding cases. In another recent study by the same group, leukemic cells from CLL patients with high-risk cytogenetic abnormalities were shown to exhibit an altered response to type I interferons (IFNs) (35). In these samples, type I IFNs induced sustained phosphorylation of the transcription factor STAT3 and an increase in the size and number of cycling cells. This response was in contrast to the antiproliferative effects of type I interferons in normal B cells and CLL cells with low-risk cytogenetic abnormalities. Along the same lines, Renaudineau et al reported that crosslinking of the CD5 antigen induces apoptosis in a subset of CLLs; the majority of the cases that underwent apoptosis were ZAP-70 and CD38 positive, whereas most apoptosis-resistant cases were ZAP-70 and CD38 negative (36). Finally, Coscia et al recently reported that IL-4 and CD40L more strongly prevent spontaneous apoptosis in U-CLL than M-CLL cells (37). Altogether, these studies further support an evolving concept that the capacity of the leukemic cells to respond to microenvironmental stimuli may differ between cases with good and bad prognosis and may in part account for the different disease activity in the two subsets.

5. TLR EXPRESSION IN CLL B CELLS

Human naive B cells express low to undetectable levels of most TLRs, whereas memory B cells constitutively express high levels of TLR2, TLR6, TLR7, TLR9, TLR10 and the TLR-related protein RP-105 (38). The TLR expression pattern of memory B cells is highly similar to the pattern observed in freshly isolated CLL B cells, further supporting the notion that CLL cells are derived from antigen-experienced B cells (39-41). The levels of TLR9 in CLL cells are in general higher than in normal B cells, although considerable variability exists (39). This variability, however, does not account for the different proliferative response to CpG-ODN stimulation of CLL cells belonging to different prognostic subsets. In two independent studies, no association was observed between TLR9 mRNA or protein levels and the proliferative response or IGHV mutation status (29, 31).

The TLR-related adaptor protein RP105 is also variably expressed in CLL, and higher levels have been detected in leukemic cells with mutated IGHV genes (42). The significance of this association is still unclear, as the biological function of RP105 in CLL cells is not known.

6. TLR9 SIGNALING PATHWAYS IN CLL CELLS

In normal human B cells, the signaling pathways activated by all TLRs are rather similar. With the exception of TLR3, which uses the adaptor protein TRIF, all other TLRs use the adaptor protein MyD88 through its TIR domain. The TLR9-MyD88 complex then recruits the IL1 receptor-associated kinase (IRAK) family members, IRAK1 and IRAK4. IRAK4 phosphorylates and activates IRAK1, which recruits the adaptor protein TRAF-6 and the kinase TAK1. This complex then propagates the signal to several downstream effector pathways, including NF-kB, PI3K/Akt and the mitogen activated protein kinases JNK, ERK1/2 and p38MAPK (43-47), which together regulate the cellular response.

Studies performed by some of us a few years ago revealed that B cells from both U-CLL and M-CLL
TLR9 signaling in CLL

Figure 1. TLR9 signaling pathways. TLR9 is an intracytoplasmic protein that is localized in the endoplasmic reticulum and endosomal and lysosomal compartments. Binding of CpG-containing DNA to preformed endosomal TLR9 homodimers induces a conformational change that leads to MyD88 recruitment and formation of a signaling complex composed of IRAK1, IRAK4 and the adaptor TRAF6. Phosphorylation of IRAK1 by IRAK4 leads to activation of TRAF6 and recruitment of TAK1. Subsequently, TAK1 activates downstream effector pathways, such as NF-kB, PI3K/AKT, ERK, JNK and p38 MAPK. These pathways trigger various cellular responses, including B cell activation, proliferation, apoptosis and immunoglobulin production.

Respond to TLR9 triggering by activating the same downstream signaling pathways that are activated in normal B cells (29). However, the magnitude and duration of the signal was found to differ substantially between the proliferating, mainly U-CLL, and non-proliferating, mainly M-CLL cases. In the proliferating cases CpG-ODN induced greater degradation of the NF-kB inhibitor IkB and stronger and prolonged phosphorylation of the Akt, ERK, JNK and p38 MAPK kinases. Prolonged activation of these downstream signaling molecules was associated with a greater capacity of the leukemic cells to traverse the G1/S checkpoint of the cell cycle. In particular, whereas leukemic cells from all cases entered the G1 phase of the cell cycle, as evidenced by induction of cyclin D3 and downregulation of p27KIP1, only cases with a sustained downstream signal showed evidence of S phase progression, such as induction of cyclin A and [3H] thymidine incorporation. Interestingly, introduction of a constitutively active Akt kinase in CLL B cells from the non-proliferating subset was sufficient to drive their progression through the G1/S checkpoint following CpG-ODN stimulation, suggesting that this kinase is at least in part responsible for the distinct responses of the different CLL subsets.

Differences in the activation of downstream signaling pathways between U-CLL and M-CLL cells were also reported in the study of Tromp et al (31). These authors observed that combined CD40/TLR9 triggering induces a dichotomous response with respect to activation of the alternative NF-kB pathway. In U-CLL cells, CD40/TLR9-stimulation induced sustained activation of this pathway, as evidenced by expression of the NF-kB subunit p52 and the NF-kB target gene Bcl-X(L), whereas expression of both molecules declined in CD40/TLR9-stimulated M-CLL cells.

As mentioned previously, TLR9 triggering by CpG-ODN usually induces apoptosis in CLL cells from cases with favorable prognostic features (25, 28, 29, 31). The nature of the signaling pathway that transduces this apoptotic signal downstream of TLR9 was recently investigated by Liang et al (48). These authors observed reduction in leukemic cell viability in 18 of 23 investigated cases following a prolonged, 5-day culture period with CpG-ODN. Induction of apoptosis was associated with NF-kB-dependent production of autocrine interleukin-10 (IL-10), which provoked leukemic cell death by inducing JAK1-dependent tyrosine phosphorylation of the pro-apoptotic transcription factor STAT1. This study, unfortunately, did not investigate the signaling response in cases that did not undergo apoptosis in response to CpG-ODN, so no information is available whether activation of the JAK1/STAT1 pathway differs in CLL cells that display different responses to CpG-ODN stimulation. This question appears particularly relevant in view of the recent study by...
TLR9 signaling in CLL

Tomic et al (35), which showed differences in the activation of STAT1 and STAT3 in response to IFN signaling in CLL cells belonging to the different prognostic subsets.

The exact mechanisms accounting for the variability in the responses induced by TLR9 triggering are still unclear. As previously mentioned, the only possibility that has been excluded so far is the level of expression of TLR9, which although variable did not correlate with the capacity of the leukemic B-cells to respond to CpG-ODN stimulation (28, 29, 31). Another possible explanation for the greater proliferative capacity of U-CLL cells is increased production of reactive oxygen species (ROS). ROS are known to inhibit tyrosine phosphatases (49), which are negative regulators of many signaling pathways, and higher ROS levels have been detected in leukemic cells from patients with aggressive CLL (35, 50). Increased ROS production may therefore enhance signaling through TLR9 or other pathways activated by external stimuli. In line with this possibility, the aberrant activation of STAT3 in response to IFN signaling in CLL cells from patients with aggressive disease has been correlated with increased ROS production and inhibition of tyrosine phosphatase activity (35).

Another possible explanation for the lack of a proliferative response in M-CLL cells is that this feature reflects some type of anergy, as suggested by certain transgenic mouse models in which the anergic B cells do not proliferate in response to TLR4 or TLR9 stimulation (51, 52). It has already been postulated that U-CLL and M-CLL cells differ in the nature of the antigen recognized by the leukemic BCRs, which in the case of M-CLL could be autoantigens that have anergized the malignant cells (53-55). This possibility could also help explain why M-CLL cells behave differently from normal B-cells, which proliferate vigorously in response to CpG-ODN stimulation. Further support for this possibility comes from some of our recent experiments, which showed that CpG-ODN-induced proliferation of U-CLL cells can be inhibited by soluble but not by immobilized anti-IgM antibodies, suggesting that the nature of the antigenic stimulus can influence the subsequent response to TLR9 triggering (30).

Finally, it also remains possible that the different responses of CLL cells to CpG-ODN stimulation could be a consequence of mutations in regulatory elements of the TLR9 signaling pathway. In this respect, somatic mutations in MyD88 were identified in 2.9% of CLL cases in a recent whole-genome sequencing study (56). The most frequently detected mutation (L265P) was reported to enhance TLR signaling, at least in terms of cytokine production, but inactivating MyD88 mutations (E52DEL) were also observed. Further work is needed to determine whether other regulatory elements of this pathway are mutated in CLL and their possible impact on the TLR9-induced response.

7. TLR9 STIMULATION INDUCES DISTINCT GENE EXPRESSION PROFILES IN U-CLL AND M-CLL CELLS

The wide application of new techniques of whole genome and transcriptome analysis has increased the knowledge on the origin of different tumor types and contributed to identify new prognostic markers, as well as specific profiles associated with treatment response. As for CLL, gene expression profiling (GEP) studies have been at the origin of the identification of new prognostic markers, including ZAP-70, LPL and CLL1U (16, 17, 57-59). Moreover, GEP has also been successfully used to define the pathways activated in CLL cells by external and/or microenvironmental stimuli upon triggering of various receptors, including BCR, CD40, integrins, etc (57, 60-66).

To further define the pathways activated in CLL cells upon TLR9 triggering, we recently performed GEP analysis of U-CLL and M-CLL cells stimulated with CpG-ODN (67). The U-CLL cases showed a rather uniform GEP profile, with more than 5000 genes being differentially expressed between CpG-ODN-stimulated and unstimulated cells, utilizing an adjusted p value p<0.001 (Figure 2). When these genes were used for hierarchical clustering of the M-CLL samples, two subsets of M-CLL could be identified. In particular, while 3 of the 8 investigated M-CLL samples completely lacked any of the changes that defined the U-CLL-specific gene signature, in the remaining 5 samples the GEP signature of CpG-ODN-stimulated cells partially resembled that of CpG-ODN-stimulated U-CLL cells. However, specific groups of genes (yellow boxes in Figure 2) failed to be modulated and remained unchanged compared to unstimulated controls, causing the inclusion of these M-CLL samples in a separate branch of the cluster shown in Figure 2.

When analysis was performed on M-CLL cells only and the same adjusted p value was applied, a distinct set of genes differentially expressed between CpG-ODN-stimulated and unstimulated M-CLL cells was identified (67). Among these genes, only a small fraction was in common with the genes differentially expressed between CpG-ODN-stimulated and unstimulated U-CLL cells. Importantly, bioinformatics analysis revealed that the differentially expressed genes in M-CLL and U-CLL cells belong to distinct Gene Ontology (GO) categories, which were concordant with the distinct cellular responses of the two prognostic subsets (Table 1). In particular, the majority of the differentially expressed genes in U-CLL cells belonged to GO categories related to proliferation, cell cycle and apoptosis. Such categories were infrequent in the M-CLL gene signature, which was primarily characterized by differential expression of genes belonging to GO categories related to “regulation of transcription”. Altogether, these data provide further evidence that TLR9 triggers different pathways in U-CLL and M-CLL cells.

8. microRNAS REGULATE THE PROLIFERATIVE RESPONSE INDUCED BY TLR9 TRIGGERING

Several lines of evidence suggest a prominent role for microRNAs (miRNAs) in the pathogenesis of CLL (68, 69). miRNAs are a family of small, non-coding RNAs of typically ~18–24 nucleotides in length, discovered less than two decades ago, which are emerging as key players in the posttranscriptional regulation of intracellular protein concentrations (70). By base-pairing to the 3’-untranslated region (UTR) of the target mRNA, miRNAs are known to operate by stopping translation and/or activating
TLR9 signaling in CLL

Table 1. GO categories differentially represented in CpG-ODN stimulated versus unstimulated CLL cells

<table>
<thead>
<tr>
<th>GO category ID</th>
<th>Function</th>
<th>CLL subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006346</td>
<td>RNA processing</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0017155</td>
<td>cell adhesion</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0007243</td>
<td>protein kinase cascade</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0008333</td>
<td>RNA processing</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:005502</td>
<td>intracellular protein transmembrane transport</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:006461</td>
<td>protein complex assembly</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:009815</td>
<td>response to virus</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:006826</td>
<td>iron ion transport</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:006696</td>
<td>glucose metabolic process</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:006896</td>
<td>response to unfolded protein</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0051028</td>
<td>mRNA transport</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0042254</td>
<td>ribosome biogenesis</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0019221</td>
<td>cytokine-mediated signaling pathway</td>
<td>U-CLL</td>
</tr>
<tr>
<td>GO:0007165</td>
<td>signal transduction</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0007049</td>
<td>cell cycle</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0019941</td>
<td>modification-dependent protein catabolic process</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0015031</td>
<td>protein transport</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0044419</td>
<td>interspecies interaction between organisms</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0016192</td>
<td>vesicle-mediated transport</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:000122</td>
<td>negative regulation of transcription from RNA polymerase II promoter</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0006357</td>
<td>regulation of transcription from RNA polymerase II promoter</td>
<td>U-CLL, M-CLL</td>
</tr>
<tr>
<td>GO:0006355</td>
<td>regulation of transcription, DNA-dependent</td>
<td>M-CLL</td>
</tr>
<tr>
<td>GO:0006350</td>
<td>transcription</td>
<td>M-CLL</td>
</tr>
<tr>
<td>GO:0045944</td>
<td>positive regulation of transcription from RNA polymerase II promoter</td>
<td>M-CLL</td>
</tr>
<tr>
<td>GO:0016568</td>
<td>chromatin modification</td>
<td>M-CLL</td>
</tr>
<tr>
<td>GO:006464</td>
<td>protein modification process</td>
<td>M-CLL</td>
</tr>
<tr>
<td>GO:0007264</td>
<td>small GTPase mediated signal transduction</td>
<td>M-CLL</td>
</tr>
<tr>
<td>GO:0008886</td>
<td>intracellular protein transport</td>
<td>M-CLL</td>
</tr>
</tbody>
</table>

Adapted from Bomben et al (67).

degradation of these target miRNAs (71). miRNA expression is highly tissue-specific and is known to orchestrate various biological processes, including cell cycle, proliferation, apoptosis, differentiation, stress response, and hematopoiesis (72-75). An altered expression of specific miRNAs has been shown to promote tumorigenesis and play critical roles in cancer development (76, 77).

miRNA expression profiling of human tumors has identified signatures that have been associated with diagnosis, prognosis, and treatment efficacy (77). So far, several miRNAs and miRNA clusters have been implicated in CLL initiation, progression, dissemination, and drug resistance, including miR-34, miR-155, miR-29, miR-221, miR-222, miR-223, and particularly miR-15a and miR-16 (60, 69, 78-83). The latter microRNAs are located in a region of chromosome 13q14 that is frequently deleted in CLL. Formal proof for the involvement of miR-15a and miR-16 in the pathogenesis of CLL was recently provided by Klein et al, who demonstrated that deletion of the DLEU2/MIR15A/MIR16-1 locus in mice leads to the development of a lymphoproliferative disorder that strongly resembles human CLL (82).
TLR9 signaling in CLL

Figure 2. Gene expression profiling of unstimulated and CpG-ODN-stimulated U-CLL and M-CLL cells. Hierarchical clustering was performed on 9 U-CLL and 8 M-CLL samples using the 5186 genes that were identified by Limma analysis as differentially expressed between U-CLL cells stimulated with CpG-ODN or left unstimulated. Yellow boxes indicate groups of genes with discordant expression between M- and U-CLL cells stimulated with CpG-ODN (see text for details).

In light of the above, we integrated the previously described GEP of CpG-ODN-stimulated CLL cells with a concomitant miRNA profile to investigate whether miRNAs have a role in regulating the different responses of U-CLL and M-CLL cells to TLR9 triggering (67). Again, these studies highlighted a different behavior of U-CLL compared to M-CLL cells, both in terms of miRNA expression as well as expression of their specific target mRNAs. In particular, U-CLL cells showed a peculiar miRNA signature upon TLR9 triggering, characterized by the upregulation of several miRNAs and miRNA clusters (Table 2). Notably, among the miRNAs up-regulated by CpG-ODN exposure were miR-221 and miR222, two well known negative regulators of the cell cycle inhibitor CDKN1B, which have been recently reported to have a role in regulating cell proliferation in CLL (83).

The concomitant GEP was used to predict the key miRNAs allegedly responsible for the downregulated expression of specific target genes in CpG-ODN-treated U-CLL cells. Such an approach has been used to demonstrate the role of miR-223 in knock-out mice for this miRNA (84). In our study, we used both the T-REX and GSEA programs to identify TLR9-regulated miRNAs based on changes in the expression of their putative mRNA targets. This combined approach identified miRNAs belonging to the miR-17-92 cluster as important regulators of the gene expression profile of CpG-ODN stimulated U-CLL cells (67).

The miR-17-92 polycistron element (a.k.a. Oncomir-1) is one of the first reported and most well studied oncomiRs (85, 86). The cluster is located at 13q31.3 and is composed of six mature miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1), whose transcription is directly transactivated by c-MYC (87). miRNAs from this locus are able to drive a hyperproliferative phenotype, by suppressing the G1/S checkpoint of the cell cycle (88). Over-expression of the miR-17-92 locus has been identified in lung cancer, chronic myeloid leukemia, B-cell lymphomas, hepatocellular tumors, bladder cancer, and breast, colon, pancreas, prostate, and stomach solid tumors (85, 89-94). The association of miR-17-92 with a broad range of cancers not only underlines the clinical significance of this locus, but also suggests that miR-17-92 may regulate
TLR9 signaling in CLL

Table 2. miRNAs regulated by TLR9 signaling in U-CLL cells

<table>
<thead>
<tr>
<th>miRNA</th>
<th>miRNA profiling†</th>
<th>GEP/T-REX analysis‡</th>
<th>GEP/GSEA analysis†††</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-125a-3p</td>
<td>downregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-155*</td>
<td>upregulated</td>
<td>upregulated</td>
<td>-</td>
</tr>
<tr>
<td>miR-150*</td>
<td>downregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-1260</td>
<td>upregulated</td>
<td>upregulated</td>
<td>-</td>
</tr>
<tr>
<td>miR-1274a</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-1274b</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-155</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-155*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-17</td>
<td>upregulated</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>miR-17*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-18a</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-19b-1*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-20a</td>
<td>upregulated</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>miR-20b</td>
<td>upregulated</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>miR-231</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-231*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-222</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-29b-1*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-30b*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-30d*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-720</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-886-3p</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>miR-92a-1*</td>
<td>upregulated</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

† miRNAs identified as differentially expressed between unstimulated CLL cells and CLL cells stimulated with CpG-ODN by miRNA profiling. ‡ miRNAs identified as differentially expressed according to T-REX analysis on GEP data; ††† miRNAs identified as differentially expressed according to GSEA analysis on GEP data

fundamental biological processes and that its over-expression can contribute to tumorigenesis.

Among the more than 1,000 putative target genes for miR-17-92, as established by the six target prediction softwares used in T-REX, as many as 126 genes were identified as significantly down-regulated following stimulation with CpG-ODN in the GEP analysis. These down-regulated genes included several negative regulators of the cell cycle or positive regulators of apoptosis, such as ZBTB4, TP53INP1, E2F5 and TRIM8 (95-98). Over-expression of miR-17 in primary U-CLL cells resulted in significant knock-down of ZBTB4, TP53INP1, E2F5 and TRIM8, both at the mRNA and protein level, confirming that these genes are directly regulated by miR-17-92 (67). In agreement with these results, miR-17 transfection was also demonstrated to be sufficient to reduce apoptosis induced by serum deprivation in a series of primary U-CLL cells. Moreover, transfection of primary U-CLL cells with a mixture of antagoniRs targeting selected miR-17-92 microRNAs inhibited CpG-induced BrdU incorporation, a marker of DNA synthesis and S phase progression (67). Thus, lack of miR-17-92 upregulation in M-CLL may in part explain their lower capacity to traverse the G1/S checkpoint following CpG-ODN stimulation.

GEP analysis on CpG-ODN stimulated U-CLL cells also provided evidence for an important role of MYC in regulating the TLR9 response. Pathologically activated expression of MYC is one of the most common oncogenic events in human cancers (99). A major consequence of MYC activation is extensive reprogramming of the miRNA expression pattern of tumor cells. Although the predominant influence of MYC on miRNA expression is widespread down-regulation (100), the pro-tumorigenic miR-17-92 cluster is known to be directly up-regulated by MYC (87). We confirmed this finding by showing that MYC was among the genes up-regulated after stimulation with CpG-ODN and that its transcriptional activity, defined by the expression of three independent target genes (CAD, TFAM, and PGK1), was significantly higher in U-CLL cells stimulated with CpG-ODN compared to their unstimulated counterparts. This suggests that the miR-17-92 cluster is regulated by MYC also in CLL cells, and that induction of these miRNAs is a physiologic response to proliferative stimuli supplied by CpG-ODN.

With respect to M-CLL, we could not identify any difference in the miRNA expression profile by supervised analysis of CpG-ODN-stimulated and unstimulated cells. However, in a hierarchical cluster driven by the miRNA signature of U-CLL cells stimulated or not with CpG-ODN, we were able to demonstrate that a fraction of M-CLL cases (4/8 cases tested) actually expressed a miRNA profile similar to that found in U-CLL, including an increase in the expression of miRNAs belonging to the miR-17-92 cluster (67). These “responsive” cases belonged to the same subset of previously described M-CLL cases, whose GEP signature partially resembled the GEP signature of CpG-ODN stimulated U-CLL cells. Thus, the combined GEP and miRNA profiling data suggest that two types of responses to CpG-ODN stimulation exist in M-CLL, one that is completely different from the response of U-CLL cells and one that is an intermediate between the two extremes. Given the significant differences in disease outcome between CLL cases stratified according to CpG-ODN-induced proliferative response, analysis of the clinical behavior of these two M-CLL subsets may help further refine prognosis in this group of patients.
TLR9 signaling in CLL

9. ROLE OF TLR9 SIGNALING IN CLL PATHOGENESIS

CLL has long been considered an antigen-driven disease, ever since the initial observations that the leukemic cells express a highly restricted IGHV gene repertoire (101, 102). This hypothesis was subsequently reinforced by the identification of CLL subsets with nearly identical (stereotyped) B-cell receptors, further suggesting that the malignant clones expand, at least initially, as a consequence of antigen stimulation (103-107). In addition to being considered an important initiating event, antigen stimulation is also believed to contribute to disease progression. Evidence in favour of this possibility is the strong association between the clinical course of CLL and certain BCR-related features, such as IGHV mutation status and ZAP-70 expression (4, 5, 11, 12). In addition, immunophenotyping and gene expression profiling studies have shown that CLL cells display features of B lymphocytes that have been in recent contact with antigen, suggesting that the malignant cells are continuously exposed to antigen in vivo (16, 108).

Despite all the evidence that suggests an important role for antigen stimulation in the pathogenesis of CLL, the nature of the antigens that drive CLL has remained a mystery. It has been known for many years that the leukemic immunoglobulins are frequently polyreactive and can bind with low affinity to various common self-antigens (109, 110). However, it has been difficult to imagine how reactivity with autoantigens could contribute to the expansion of the malignant clones, as the expected outcome would be induction of anergy rather than proliferation or increased survival. A possible explanation has emerged from recent studies by the groups of Rosen and Chiorazzi, who investigated the reactivity of two large panels of soluble CLL immunoglobulins against a range of foreign and self antigens. Many of the antibodies in their panels were found to react with autoantigens on apoptotic cells, which were either translocated to the apoptotic cell surface or were generated by oxidation during the apoptotic process (111-113). Such reactivity was particularly frequent among CLL immunoglobulins encoded by unmuted IGHV genes and was significantly correlated with poor patient outcome (114).

The autoantigens that were identified included non-muscle myosin heavy chain IIA, vimentin, filamin B and cofilin-1, as well as several autoantigens involved in systemic autoimmunity, such as Sm, Ku, snRNP A, BB', and C, and CENP-B (112, 115). It is interesting that most of these antigens are either directly associated with DNA or translocate to apoptotic blebs together with DNA- and RNA-containing protein complexes. This, in turn, implies that CLL cells reactive with these antigens could also receive signals through TLR9, following BCR-mediated internalization of the DNA-containing complexes to the endosomal compartment (Figure 3) (116, 117). Activation of the TLR9 pathway could therefore provide the second stimulus that is required to induce the proliferation of the malignant cells. Consistent with this possibility, recent gene expression profiling of CLL cells obtained from peripheral blood and lymph nodes showed that the latter are enriched in BCR and TLR target genes, indicating that these two pathway are co-activated in vivo (118).

A similar mechanism has been reported to activate autoreactive B cells in lupus-prone MRL/lpr mice, leading to the development of autoantibodies against double-stranded (ds) DNA and the RNA-containing Smith antigen (Sm) (119). In addition, studies in transgenic mice have shown that DNA-containing chromatin–IgG complexes can activate autoreactive B cells to produce antibodies against self-IgG (120). Altogether, these studies suggest that TLR signals could be driving both the autoantibody production in certain systemic autoimmune diseases as well as the expansion of the malignant clones in CLL. Such a mechanism could be particularly plausible for CLL clones with poor prognostic features, given the more frequent reactivity of this subset with apoptosis-related antigens and their greater capacity to respond to TLR9 stimulation.

10. CONCLUSIONS

The capacity of the malignant cells to respond to microenvironmental stimuli has recently emerged as an important determinant of the clinical course in CLL. The leukemic cells from patients with aggressive CLL typically respond with increased survival and/or proliferation when stimulated with anti-IgM, CpG-ODN, CpG-ODN/CD40L, IL-4/CD40L, CD40L/IL-2/IL-10/stromal cell co-culture or type I IFNs, whereas CLL B cells from patients with indolent disease usually do not respond or may even respond by undergoing apoptosis. These heterogeneous responses have been particularly well documented in the case of TLR9 signaling, where significant differences between prognostic subsets have been observed in terms of leukemic cell proliferation, survival, signal transduction and, more recently, gene-expression and miRNA-expression profiles.

An important feature of the GEP and miRNA signatures of CpG-ODN-stimulated U-CLL cells is induction of MYC, which appears to be responsible for the induced expression of the miR-17-92 polycistronic element. The latter, in turn, can regulate B-cell proliferation and survival by down-regulating a series of genes with anti-proliferative and/or pro-apoptotic activity. A similar sequence of inter-chained events seems to occur in a subset of M-CLL, although incomplete and/or of a lesser extent, for yet unknown reason(s). Up-regulation of the miR-17-92 cluster in U-CLL and in a subset of M-CLL might therefore mark those cases endowed with a greater capacity to respond to TLR9 ligands and possibly other microenvironmental stimuli.

A role for the TLR9 pathway in the pathogenesis of aggressive CLL is further supported by recent studies showing that the leukemic immunoglobulins, particularly those encoded by unmuted IGHV genes, often bind to autoantigens that are generated or exposed on apoptotic cells. Such autoantigens are usually associated or co-localized with DNA-containing complexes, which can be
Figure 3. Possible mechanism for activation of autoreactive CLL B-cells by autoantigen/DNA complexes generated or exposed on apoptotic cells. CpG-DNA containing complexes are internalized into early endosomes by autoreactive BCRs that bind to autoantigens present in apoptotic blebs, such as MYHIIA (non-muscle myosin heavy chain IIA), vimentin, filamin B, coflin-1, Sm, Ku, snRNPs and CENP-B. These immunocomplexes are subsequently sorted into late endosomes where they encounter and activate TLR9 derived from the endoplasmic reticulum (ER)/Golgi apparatus. Activation of the TLR9 pathway provides the second stimulus that induces the proliferation of the leukemic cells.

delivered to TLR9-containing endosomes by BCR-mediated internalization and in this manner induce the proliferation and expansion of the malignant clones. Further support for this hypothesis comes from recent GEP analysis showing that CLL cells located in lymph nodes overexpress BCR- and TLR-target genes, indicating co-activation of these pathways in vivo. Together, these studies suggest that the TLR9 pathway could be an important link between the microenvironment and the leukemic cells and, as such, could represent a novel target for therapeutic intervention in CLL.

11. ACKNOWLEDGMENTS

Drs D.G.E. and V.G are both senior authors and contributed equally to this article. This work was supported in part by grants from The Leukemia and Lymphoma Society, White Plains, NY (grant no. R6170-10 to DGE), the Italian Association for Cancer Research (A.I.R.C.), Milan, Italy (grant no. 5917 to DGE, IG-8701 to VG and MFAG-10327 to RB), “Ministero della Salute” (Ricerca Finalizzata I.R.C.C.S. “Alleanza Contro il Cancro” and “Rete Nazionale di Bio-Informatica Oncologica”), Rome, Italy; Associazione Italiana contro le Leucemie, linfomi e mielomi (A.I.L.), Venezia Section, Pramaggiore Group, Italy; Ricerca Scientifica Applicata, Regione Friuli Venezia Giulia, Trieste (“Linfoner”), Italy. RB is fellow of the “Fondazione Internazionale di Ricerca in Medicina Sperimentale” in the context of the “Giovani Ricercatori” project of the “Ministero della Salute”, Rome, Italy (grant GR-2008-1138053).

12. REFERENCES


TLR9 signaling in CLL


TLR9 signaling in CLL


65. A. Zucchetto, D. Benedetti, C. Tripodo, R. Bomben, M. Dal Bo, D. Marconi, F. Bossi, D. Lorenzon, M. Degani,
TLR9 signaling in CLL


TLR9 signaling in CLL

Grimmond: The miR-17-5p microRNA is a key regulator of the G1/S phase cell cycle transition. *Genome Biol*, 9(8), R127 (2008)


TLR9 signaling in CLL


Key Words: Chronic Lymphocytic Leukemia, Toll-like receptor 9, Signal Transduction, Prognosis, microRNA

Send correspondence to: Dimitar G. Efremov, Molecular Hematology, International Centre for Genetic Engineering and Biotechnology (ICGEB), Campus A. Buzzati-Traverso, Via E. Ramarini 32, I-00016 Monterotondo Scalo, Rome, Italy, Tel: 39-06-90091300, Fax: 39-06-90091260, E-mail: efremov@icgeb.org