B-cell receptor signaling in chronic lymphocytic leukemia

Freda K Stevenson¹, Sergey Krysov², Andrew J Davies², Andrew J Steele², Graham Packham²

¹Molecular Immunology Group, and ²Cancer Research UK Centre, Cancer Sciences, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, SO16 6YD, UK.

Address correspondence to Professor Freda Stevenson. Cancer Sciences, Southampton University Hospitals Trust, Southampton, SO16 6YD, UK; Tel.: +44 0 2380 796923; fax: +44 0 2380 795154; e-mail: fs@soton.ac.uk.

Running title: The BCR in CLL.
Abstract

The B-cell receptor (BCR) is a key survival molecule for normal B cells and for most B-cell malignancies. Recombinatorial and mutational patterns in the clonal Ig of chronic lymphocytic leukemia (CLL) have revealed two major IgM-expressing subsets and an isotype-switched variant, each developing from distinct B-cell populations. Tracking of conserved stereotypic features of Ig variable regions characteristic of U-CLL indicate circulating naïve B cells as the likely cells of origin. In CLL, engagement of the BCR by antigen occurs in vivo, leading to downregulated expression and to an unanticipated modulation of glycosylation of surface IgM, visible in blood cells, especially in U-CLL. Modulated glycoforms of IgM are signal competent and could bind to environmental lectins. U-CLL cases express more IgM and have increased signal competence, linking differential signaling responses to clinical behavior. Mapping of BCR signaling pathways identifies targets for blockade, aimed to deprive CLL cells of survival and proliferative signals. New inhibitors of BCR signaling appear to have clinical activity. In this Perspective, we discuss the functional significance of the BCR in CLL and we describe strategies to target BCR signaling as an emerging therapeutic approach.
Introduction

If we are to exploit biological insights for new therapies of hematologic malignancies, it is important to consider two distinct features of the target tumor: first, the developmental process, by which a normal cell becomes transformed into an established tumor; second, the susceptibility or resistance of the malignant cells to current therapies. Most investigators do not separate these two features, focusing only on the link between the particular aspect being studied and clinical outcome. For example, in B-cell lymphoma, gene expression profiles have been used to predict survival, an outcome more likely to be determined by susceptibility to treatment than by the pathogenic process. In many cases, this restricted view is inevitable, since hematologic malignancies are often treated quite quickly, making the connection between cellular features and the natural history of the tumor difficult to analyze.

Among B-cell malignancies, chronic lymphocytic leukemia (CLL) is a shining exception, since its relatively indolent nature enables detailed investigation of tumor cells, often in the absence of treatment, as well as observation of tumor behavior over time. This window of opportunity has facilitated the identification of prognostic factors which relate to pathogenesis. It is even possible now to detect minor clonal expansions, defined as monoclonal B-cell lymphocytes, in ~3% of healthy individuals, potentially revealing the very early stages of CLL. In CLL, there is the added advantage of availability of tumor cells from blood, although conclusions based on this compartment have to be tempered by the fact that critical proliferative events occur in tissue sites.

There is now strong evidence that signaling via the B-cell receptor (BCR) plays a major role in the development of CLL, and that it determines the variable clinical behavior. In this Perspective, we discuss the functional significance of the BCR in CLL and we describe strategies to target BCR signaling as a new therapeutic approach.

Insights into pathogenesis from the expressed immunoglobulin

B-cell malignancies offer a major advantage to investigators, in that the immunoglobulin (Ig) component of the BCR has unique molecular features which mark the tumor cell and reveal the nature of the B-cell of origin. Most cases of CLL express IgM and IgD and it is now clear that the disease can be divided into two main subsets, based on whether the tumor arose from a B cell prior to initiation of somatic hypermutation in Ig variable (V) region genes (unmutated (U) CLL), or after this process had taken place and then stopped (mutated (M) CLL). The rather dramatic difference in tumor behavior, with U-CLL being generally more aggressive than M-CLL, was unexpected, although perhaps it should not have been, given the clinical differences between other B-cell tumor categories.

In CLL, the Ig expressed at the cell surface is rarely lost, indicating an essential influence on the tumor cell. Since the *IGHV*/*DHJ* and *IGLV*/*LJ* sequences, and the isotype, reflect the normal counterpart, it is relatively straightforward to determine the point of differentiation reached by the parental B cell. This allows us to probe the relationship between the subsets, and it is immediately obvious that *IGHV* and *IGLV* gene usage differs markedly between the
two main subsets, indicating no conversion of U-CLL to M-CLL. The third minor subset of CLL expressing isotype-switched Ig, although usually derived from B cells with mutated V genes, displays IGHV gene patterns distinct from M-CLL, again suggestive of a separate origin. It appears that each subset has arisen independently during B-cell differentiation, possibly from different B-cell lineages, and, although there are morphological and phenotypic similarities, in the developmental sense they should be considered as three diseases. The routes leading to the three subsets of CLL, each likely to have transformed from normal B cells stimulated by infection, are illustrated in Figure 1.

As with many indolent tumors, including follicular lymphoma, CLL cells, especially at Stage A, have undergone rather few, although significant, chromosomal changes. The nature of the initiating changes in CLL, likely to involve the loss of the microRNA 15/16 cluster, and the subsequent chromosomal changes influencing tumor growth and response to therapy, have been extensively reviewed and will not be discussed further.

Evidence for an influence of surface IgM engagement on tumor behavior

In spite of the initiating chromosomal changes, CLL cells betray a continuing need for support from the surrounding microenvironment, similar to that of normal B cells. One key component of the microenvironment is antigen. Perhaps surprisingly, both U-CLL and M-CLL cases appear to undergo engagement of surface IgM (sIgM) in vivo. While this has been inferred from restricted IGHV and IGLV gene usage and the rather conserved “stereotypic” IG V(D)J sequences, direct proof for antigen binding was only provided by the fact that CLL cells from blood can recover expression of surface IgM in vitro, consistent with down-regulation in vivo. It is likely that antigen exposure in vivo leads to sig-mediated signaling events as well as loss by endocytosis. There is evidence for constitutive activation of kinases and of NF-kB in CLL cells, which could be mediated by BCR signaling. Interestingly, sIgD, in spite of having identical antigen specificity, behaves differently from sIgM, with no evidence for down-modulation in vivo. This differential behavior is reminiscent of data from the double transgenic mouse model of anergy, where B cells expressing anti-hen egg lysozyme (HEL) are continuously confronted with HEL antigen. The result in that setting was down-modulation of sIgM with no effect on sIgD, exactly mirroring the findings in CLL. Whether this reflects functional differences or simply the level of expression remains to be determined.

The normal B-cell counterpart of U-CLL

Various bacterial and autoantigens have been proposed as candidates for binding to the sIgM of CLL cells in vivo, and there could be a range depending on the V-gene sequences involved. The question is whether the antigen which caused proliferation of the normal B cell of origin is able to stimulate the transformed cell. Knowledge of the cell of origin is therefore useful and, focusing on the dominant IGHV1-69-derived fraction of U-CLL, we were able to identify candidate precursors with similar stereotypic sequences within the naïve B cell population of normal blood. The identification of these cellular analogues of CLL in 3/3 healthy donors suggests that the cells of origin of U-CLL derive from a population...
of B cells retained in the hematopoietic repertoire by evolution. Our suggestion was that these B cells are innate-like B cells expressing natural IgM antibodies aimed to protect against common infections. However, it is possible that the antigenic drive on CLL cells is via autoantigens. Cross-reactivity between pathogens and autoantigens is common, with one example being IGHV4-34-encoded IgM which can react with both microbial lipid A and with DNA. Low level stimulation of CLL cells by autoantigens in tissue sites could be providing life-support, with additional contributions from environmental factors, provided by T cells and by cells of innate immunity.

**Effects of antigen exposure on CLL cells**

Normal sIg-expressing B cells respond to antigen by activation, proliferation and differentiation. In both U-CLL and M-CLL, we observe B cells which are apparently responding to antigen, presumably leading to proliferation and/or survival. The outcome of antigen engagement in U-CLL differs from that in M-CLL, in that sIgM is down-modulated to a lesser extent in U-CLL than in M-CLL. Although antigens are likely to differ, this overarching distinction could be due to intrinsic differences in the B cells of origin, with U-CLL tending to have a lower affinity for antigen than affinity-matured M-CLL. In normal B cells, affinity is a critical determinant of subsequent behavior, affecting endocytic antigen presentation and therefore interaction with helper T cells. Affinity may therefore be influencing endocytosis, being less or of a different nature in U-CLL, leading to an apparently greater retention of sIgM expression, coupled to an increased ability to respond to engagement of sIgM in vitro. In contrast, M-CLL cases tend to express less sIgM and show a reduced ability to respond, features which are described as “anergic”. The difference is not absolute, being based on the picture in peripheral blood cells which is dynamic and reversible. This is consistent with events at tissue sites being the likely drivers of CLL behavior.

**Antigen engagement leads to modification of the glycosylation status of sIgM.**

A recent finding which supports the concept of engagement of sIgM of CLL cells *in vivo* is the observation of changes in the N-glycosylation status of the μ-chain sites. This change is reversible *in vitro* and is more apparent in the sIgM of U-CLL than in M-CLL, thereby associating with poorer prognosis. The major change appears to be that expression of fully N-glycosylated μ-chains is down-regulated by antigen-induced endocytosis, leaving only sIgM expressing so-called “immature” glycans which terminate at high mannose (Figure 2). This residual sIgM may be important for the B cell, possibly providing a “tonic” survival signal, since it is able to mediate phosphorylation of downstream kinases. The retention of sIgM in U-CLL *in vivo* as compared to M-CLL appears therefore to be due to continued expression of the mannosylated glycoform. This ability to modulate the sIgM glycoform is not tumor-specific since it occurs in normal B cells, where it can be induced by engagement of the BCR, presumably acting as a prelude for antigen presentation. While the function of the modified sugars is as yet unknown, there is a possibility that the expressed mannosyl-modified sIgM is interacting with environmental lectins (Figure 2). Such an interaction can lead to stimulation of malignant B cells, as shown...
in follicular lymphoma where N-glycosylation sites frequently introduced by somatic mutation also carry highly-mannosylated glycans. In primary follicular lymphoma, mannose-binding lectin or DC-SIGN recognizing mannosylated sIg were each able to generate sIg-mediated intracellular iCa\^{2+} mobilisation offering an alternative stimulator of the BCR derived from innate immunity.

The importance for CLL is that, although expression of mannosylated sIgM is apparently transient in normal B cells following antigen engagement, it persists in CLL cells as they continue to encounter antigen. In contrast to normal B cells, malignant B cells are unable to differentiate further in vivo but are protected from the normal default pathway of apoptosis, at least partly by increased BCL2 expression associated with loss of miRNA-mediated control. Expression of mannosylated sIgM may be important for tumor cells as they receive a life-giving stimulus via the BCR during the repeated visits to microenvironmental depots (Figure 2). In fact there is growing evidence from studies of the T-cell receptor that sugar-lectin interactions influence signaling and endocytosis. If a parallel is confirmed in B cells, glycan modification, especially evident in U-CLL, could be similarly influencing sIgM-mediated events.

**Linkage to clinical behavior**

Differences in antigen-induced modulation of sIgM are likely to contribute to the variable clinical behavior of CLL (Figure 3). U-CLL appears to retain signaling capacity, leading to increased proliferation and survival, both associated with aggressive disease. By contrast, “anergy” in M-CLL is associated with indolent disease. Differential modulation of sIgM appears to be linked to alterations in sIgM glycoforms, which may directly influence signal reception and therefore tumor cell behavior.

Since U-CLL tends to require therapy more than M-CLL, a focus on the differential features of the sIgM should be useful in revealing specific pathways susceptible to inhibitory drugs. Prognostic indicators will help in the selection of patients for therapy and CLL should be treatable at an early stage rather than, as in many other malignancies, when the tumor cells have escaped all restraint. The key question concerns the nature of the repetitive BCR-mediated signal and its perversion by CLL cells for survival. Understanding the details of this signal, and its modification by microenvironmental co-signals, offers an opportunity for precise targeting of inhibitory drugs.

**BCR-mediated signaling in normal B cells**

**Signalosome activation**

For a consideration of signaling in CLL, it is first useful to recap consequences of sIg engagement in normal B cells. Initial antigen binding leads to the formation of the “signalosome”, a complex of kinases and scaffold proteins tethered at the plasma membrane at sites of sIg activation (Figure 4A). The “trigger” event in the formation of the signalosome is phosphorylation of the immunoreceptor tyrosine-based activation motifs
(ITAMs) in the C-terminal tail of BCR-associated Igα (CD79A) and Igβ (CD79B) by the SRC-family kinase LYN. Phosphorylated ITAMs act as docking sites to recruit the tyrosine kinase SYK through its tandem SH2 domain, leading to SYK activation via SRC-family kinase-dependent phosphorylation and autophosphorylation. The BCR signal is further propagated by SYK via association with the adaptor molecule B-cell linker protein (BLNK) and its downstream signaling components Bruton’s tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2). LYN-dependent phosphorylation of the cytoplasmic domain of CD19 also recruits the p85 subunit of phosphoinositide 3-kinase (PI3K).

**Downstream signaling**

Following formation of the signalosome, the second phase of BCR signaling involves the activation of distal signaling molecules (Figure 4B). Activation of PLCγ2 leads to the generation of the downstream second messengers inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) which induce the release of intracellular Ca²⁺ (iCa²⁺) and activate protein kinase C (PKC), respectively. PKC subsequently induces the activation of transcription factors including nuclear factor kappa B (NF-κB) and nuclear factor of activated T cells (NFAT). Recruitment of PI3K to the plasma membrane leads to the production of phosphatidylinositol 3,4,5-triphosphate (PIP₃) which is required for optimal activation of BTK, as well as for recruitment of 3'-phosphoinositide-dependent kinase (PDK) and subsequent activation of AKT. PLCγ2 is also involved in the activation of MAP kinase pathways, including the ERK1/2, c-Jun NH2-terminal kinase (JNK) and p38 kinases (Figure 4B). The ERK/MA PK pathway can also be regulated by RAS/RAF1 signaling.

The third phase of events involves modulation of multiple downstream regulators which ultimately mediate changes in cell proliferation, survival and migration, via both transcriptional modulation and phosphorylation (Figure 4B). For example, BCR signaling leads to modulation of key regulators of cell survival (e.g., MCL1, BIM) and cell cycle (cyclin D2, MYC).

**Negative regulation**

Negative regulators of BCR signalosome signaling, such as CD22, CD5, CD72 and FcγRIIB are essential in controlling the duration and intensity of the BCR signal (Figure 4A). These receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIM) which are phosphorylated by LYN upon BCR stimulation. This leads to the recruitment of inhibitory phosphatases such as SH2 domain-containing tyrosine phosphatase-1 (SHP-1), SH2 domain-containing phosphatidyl 5-phosphatase (SHIP)-1 and -2, and protein tyrosine phosphatase non-receptor type 22 (PTPN22) which attenuate BCR signaling. Therefore LYN both positively and negatively regulates signal transduction via the BCR.

Both positive and negative signaling responses are subject to tight modulation and even in normal B cells the precise levels of activation of distinct signaling pathways and biological outcomes (proliferation, survival, apoptosis, anergy etc) are influenced by a wide range of factors. Thus, there is not a single BCR signaling response, but a variety of outcomes that are influenced by the developmental stage of the B cell, the nature of the antigen, levels of BCR
expression and the presence of cosignaling. In the next section we discuss the heterogenous responses observed in CLL cells.

**BCR-mediated signaling in CLL cells**

**Heterogeneity of responses**

BCR signaling responses in CLL cells are heterogeneous. At one extreme is the subset of CLL samples that appear to be essentially unresponsive to slgM engagement, at least using anti-IgM as a surrogate for antigen. As discussed above, this is most common amongst M-CLL and is associated with indolent disease. However, even in the subset of samples which do retain slgM signaling responsiveness, responses *in vitro* are variable between cases and partial, with effective activation of only some downstream responses. For example, despite activation of ERK1/2, there is weak activation of p38 and JNK. The activation of NF-κB is also variable as compared to ERK1/2.

The levels of slgM appear to be one major determinant of these heterogeneous signaling responses. slgM levels are generally but variably reduced in CLL compared to normal naïve B cells. This may be at least partly a consequence of repeated antigen engagement and receptor down-modulation. Strong down-modulation appears to contribute to lack of slgM signaling responses, especially in M-CLL. Interestingly, lack of signaling capacity in CLL is associated with constitutive activation of ERK and NFAT consistent with the idea that ongoing antigen engagement *in vivo* leads to anergy. Moreover, constitutive activation of ERK and NFAT recapitulates features of anergic B cells, in at least some mouse models. Less dramatic slgM down-modulation, as observed most commonly in U-CLL, results in retention of a degree of signaling capacity, characterised by partial activation of downstream pathways and again mirroring features of some anergic mouse B cells. However, it is the greater retention of the ability to signal in U-CLL that could be important for tumor behavior.

Heterogeneity in signaling responses also exists within the malignant clone. In our study, anti-IgM-induced intracellular Ca²⁺ responses could be detected within just 5% of the circulating malignant cells in some samples. It is possible that the heterogeneous responses of blood CLL cells reflect variation in the timing of prior antigen engagement amongst these recirculating cells. Finally, activation of distinct sig isotypes may also result in variable responses. slgM activation *in vitro* causes either no response or a relatively persistent activation of ERK1/2. By contrast, almost all CLL cells retain responsiveness to anti-IgD, however, responses are very transient. Persistent ERK1/2 activation is critical for cell cycle entry so this may explain why slgD signaling status does not appear to influence disease behaviour; although signal competent, the receptor does not effectively couple to downstream growth-supporting biological responses. The differential behavior of slgM and slgD may relate to the relative levels of expression of the isotypes, or to differences in associating signaling cofactors. The relatively rare cases of CLL which have undergone isotype switch have been less investigated. However, we found a similarly variable ability to respond to engagement of slgG *in vitro*, with 9/14 cases generating an intracellular calcium flux. Again there appeared to be an
association with the level of expression of slgG. Heterogeneity is the usual picture in CLL blood cells and it will be critical to understand its clinical significance and the mechanisms involved for effective targeting of these pathways.

**Modulation of slgM-mediated signaling**

Although slgM modulation plays an important role in determine signaling response, other factors must also contribute. Expression of the ZAP70 tyrosine kinases identifies CLL patients with a more aggressive disease and earlier time to treatment. Interestingly BCR signaling capacity in CLL correlates with ZAP70 expression and signaling can be augmented by ZAP70 overexpression. However, the effect of ZAP70 is independent of its kinase activity. ZAP70 may indirectly enhance SYK activation and/or sequester SYK inhibitors. There is also evidence that ZAP70 may modulate other signaling pathways, in particular those contributing to cell migration. Expression of CD38 expression is also a prognostic indicator and expression correlates with slgM signaling capacity. CD38 associates with slgM in CLL cells, but it is unclear whether CD38 can directly modulate signaling responses. Interestingly CD38-CD31 interactions also appear to contribute to pathways involved in migration and homing and enhance CLL survival via induction of BCL2 and BCL-XL.

Various other factors have been shown to be differentially expressed in CLL and in some cases to directly modulate slgM signaling capacity, including the phosphatase PHLP1, PKCβII, TCL1, CD79B, LYN, SHIP-1 and p66SHC. However, since lack of signaling responsiveness is associated with a failure to induce SYK phosphorylation, it seems that key regulatory step must exist at or close to the level of the receptor. Clearly one possibility is that different slgM glycoforms possess distinct signaling properties. Importantly, signaling responses can be enhanced in CLL cells by incubation with immobilised, rather than soluble anti-IgM antibodies. Thus, responses in vivo are likely to be dependent on both the features of the CLL cell and the interacting antigen (soluble versus tissue bound/macromolecular), and the potential presence of supporting cells.

**Therapeutic targeting of BCR signaling pathways – lesson from signalosome inhibitors**

Increased awareness of the importance of slgM signaling in CLL has raised new opportunities for targeted intervention. Most progress has been made with agents that directly target the signalosome. Here we consider some of the clinical results obtained with these agents (Figure 4A).

One key question is where best to target the complex network of signaling pathways activated following BCR stimulation (Figure 4A,B). Promising clinical responses have been observed with fostamatinib disodium (FosD) and PCI-32765, inhibitors of SYK and BTK, respectively, as well as CAL-101, a selective inhibitor of PI3Kδ, the isoform most closely associated with BCR signaling. In preclinical studies, signalosome inhibitors have been shown to block slgM-mediated signaling and to interfere with the survival-promoting effects of anti-IgM. (Note that FosD is a prodrug and studies in vitro were performed with its therapeutic product R406.) Objective response rates in CLL/small lymphocytic lymphoma...
(SLL) patients ranged from 33% (CAL-101) to ~60% for PCI-32765 and FosD 63 64 65 (www.clinicaltrials.gov as #NCT01105247, #NCT00710528). Responses were frequently sustained and are promising considering the advanced/ heavily pretreated state of the patients studied.

In contrast, clinical outcomes with the LYN inhibitor dasatinib have been less dramatic, with only 20% (3/15) of SLL/CLL patients achieving partial responses 66. Although, like SYK, LYN is activated very early following BCR engagement, it plays both positive and negative roles in signal transduction. Dasatinib induces apoptosis in CLL cells and interferes with 
\[ \text{sgM} \] signaling \textit{in vitro} 67 68. However, LYN-deficient mice demonstrate hyper-responsiveness to stimulation of 
\[ \text{sgM} \] indicating that LYN’s negative function predominates \textit{in vivo} 69 70. Thus, limited responses to dasatinib may reflect the dual-functional nature of this kinase. Dasatinib inhibits other Src-family kinase and the positive responses that have been observed may stem from these LYN-independent activities.

Although promising, it may be premature to ascribe positive clinical responses of SYK, BTK and PI3Kδ inhibitors to specific inhibition of BCR-mediated signaling. In particular, these kinases contribute to other signaling pathways independent of the BCR. SYK is also involved in signaling transduction from Fc receptors, C-type lectins and integrins 71. In CLL cells, SYK phosphorylation is increased in cells stimulated via chemokine receptors and integrins and SYK inhibition reduced migration towards CXCL12 and adhesion to VCAM-1 72. CAL-101 also interferes with survival effects of CD40L, TNFα and fibronectins in CLL cells 73, and BTK is required for toll-like receptor signaling 74. Thus, the contribution, if any, of specific BCR signaling inhibition to the clinical efficacy of these agents is unclear. Indeed, it is possible that their clinical effects stem from simultaneous inhibition of multiple signaling responses.

One of the key observations from these studies with FosD and CAL-101 was a mobilisation of malignant cells from the tissues to the periphery. All CLL/SLL patients treated with FosD demonstrated increased lymphocytosis during the first course of treatment 65. A reduction in lymphadenopathy was observed in 100% (32/32) of patients treated with CAL-101 64 (www.clinicaltrials.gov as #NCT00710528). As discussed below, this effect might be exploited in novel drug combinations but the underlying mechanisms are not known. Homing to tissue sites is undoubtedly important in CLL, for access to antigen and supportive cell microenvironments, and is under the influence of chemokines and their receptors (including CXCR4 on CLL cells and CXCL12 provided by cell within the CLL microenvironment) 75. Homing and retention of CLL cells may also be directly influenced by antigen, since anti-IgM can modulate CLL cell migration and chemokine receptor expression 60 76. FosD interferes with anti-IgM induced migration 60. Again, to what extent these mobilisation effects are mediated via inhibition of BCR signaling versus other receptor systems is unclear.

\textbf{Future therapy-related questions}

The surface Ig receptor of B cells, so clearly influential in disease progression, should be a target for therapeutic intervention. However signaling via the BCR is not a single event mediated via a defined antigen, but represents the integration of multiple intracellular
pathways dependent on signal strength. Autoantigens, possibly cross-reactive with pathogens, may be involved and are likely to exist as a tissue-based array of repeated determinants, with some restriction to stereotypes but also variation among cases.

The main question concerns the fate of the CLL cell following BCR engagement. Normal cells would have to interact with antigen-specific T cells and then differentiate to plasma cells or to memory B cells. The default pathway awaiting unselected B cells would be death. CLL cells are protected to some degree but they have the task of surviving and proliferating without differentiation. Significantly, U-CLL maintains a higher expression of sigM, much of it as a glycosylated variant which is slower to endocytose. If this mediates the “tonic” signal required by all B cells for survival it offers a tempting target for therapy. We need to understand the nature of this signal and whether the mannosylated sigM receives a supporting signal from lectin-bearing cells in tissue sites.

In terms of drug treatments, the results of recent trials raise important issues. Clearly the encouraging results obtained with FosD, PCI-32765 and CAL-101 support the idea that therapeutic targeting of BCR signaling pathways is an effective strategy for treatment of CLL. However, we need to bear in mind that none of the targets for these inhibitors are specifically involved in BCR signaling. Understanding the precise therapeutic mechanisms of these agents will be challenging, and indeed, their therapeutic effects may dependent on simultaneous inhibition of multiple signaling pathways.

Clearly drugs or antibodies have to penetrate to tissue sites where BCR engagement and proliferation are occurring. One very exciting observation in clinical trials of both FosD and CAL-101 is a transient mobilisation of malignant cells from the tissues to the periphery (www.clinicaltrials.gov as #NCT00710528). Not only will this allow more access to previously sequestered tumor cell niches, but it will remove cells from the protective microenvironment where antigen is located. Several trials are underway to investigate the effects of combination of targeted agents such as FosD, PCI-32647 and CAL-101 with cytotoxic and antibody therapies (http://clinicaltrials.gov as #NCT01088048). However, it will be equally important to probe the mechanisms involved using mouse models, and to address issues such as optimal sequencing in a preclinical setting. FosD causes a similar redistribution of malignant cells in the E\textsubscript{\mu}-TCL1 mice indicating that this model may be suitable for these studies (78).

The initial clinical focus has understandably been on inhibitors of the signalosome. However, it will be important to consider the merit of targeting other molecules within the BCR signal transduction cascade. Moving towards downstream targets of BCR signaling pathways may be advantageous since it will allow direct modulation of the cell survival and proliferation machinery, whereas the impact of upstream inhibition may be tempered by parallel signaling and feedback modulation. A perceived drawback of this approach is that such targets are seen as less selective compared to signalosome components. However, it is becoming increasingly clear that molecules such as SYK also play important roles outside of BCR signaling. sigM clearly has the capacity to engage both cell death promoting and inhibiting pathways. Selective inhibition of BCR-induced events would be a particularly attractive strategy since it is likely to lead to a “reprogramming” of signaling, selectively retaining pro-apoptotic responses. In this way, on-going antigen signaling could actually be
used to actively drive apoptosis in the malignant clone. Selective targeting of survival pathways will require a more detailed understanding of the molecular pathways that couple sigM to cell death, survival and proliferation, and the effect of microenvironment on these responses.

Acknowledgements

We thank our colleagues, particularly Kathleen N Potter, C lan Mockridge, Jemimah Adams and Vania Coelho, for their contributions to our on-going research on CLL. This work is supported by Tenovus Solentside, the Experimental Cancer Medicine Center network, the Kay Kendall Leukemia Fund and Cancer Research UK.

Authorship

Contribution: FKS, AJS, SK, AJD and GP wrote the paper, contributed comments and edited the paper.
Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


44. Richardson SJ, Matthews C, Catherwood MA, et al. ZAP-70 expression is associated with enhanced ability to respond to migratory and survival signals in B-cell chronic lymphocytic leukemia (B-CLL). *Blood.* 2006;107(9):3584-3592.


Figure legends

**Figure 1. Pathogenesis of CLL subsets.**
The two major subsets (U-CLL and M-CLL) and the isotype switched variant derive from three different normal B-cell populations with no interconversion. Infection is the likely initial drive on all, with transformation to U-CLL occurring prior to initiation of somatic mutation. M-CLL and isotype-switched CLL develop from more mature B cells presumed to have undergone antigen selection in the germinal center.

**Figure 2. Antigen-mediated effects on surface IgM of CLL cells.**
Antigen engagement in tissue sites appears to lead to endocytosis of slgM in both U-CLL and M-CLL. Endocytic events lead to a modulation of the glycans of the \( \mu \) chains with loss of the fully glycosylated form and relative retention of a mannosylated immature form. This retention is more evident in U-CLL where it could mediate new microenvironmental interactions.

**Figure 3. A working model linking differential responses to slgM engagement to clinical behaviour.**
slgM expression is strongly down-modulated in M-CLL, associated with a tendency for loss of signaling capacity. In U-CLL, slgM down-modulation is less dramatic and there is a tendency to retain signaling capacity. “Conversion” of the remaining slgM to a signal competent, high mannose, form of slgM, most strongly in U-CLL, is a second indicator of antigen engagement. Both subsets of CLL have features of anergic cells but there is a tendency for retained slgM signaling in U-CLL which, in the context of appropriate microenvironmental signals, leads to enhanced survival and proliferation, and disease progression. In M-CLL, slgM responses are strongly down-modulated, leading to indolent disease. slgD expression does not appear to be down modulated in response to antigen engagement. Thus, antigen engagement, potentially repetitive, leads to differences in slgM responses in U-CLL and M-CLL, linked to clinical behaviour. Adapted from 52. PC: proliferation center.

**Figure 4. B-cell receptor signaling in CLL and targeted inhibition.**
The diagram shows the major signaling pathways activated after BCR activation. (A) The signalosome is comprised of LYN, SYK, BLNK, BTK, PLC\( \gamma \)2 and PI3K. Chemical inhibition of LYN, SYK BTK and PI3K by dasatinib, fostamatinib disodium (Fos D), PCI-32765 or CAL-101, respectively, block BCR signaling in CLL cells *in vitro*. The BCR signal can be further enhanced or inhibited by positive and negative coreceptor signaling to control the duration and intensity of the signal. (B) Principal downstream signaling pathways linking the BCR to biological responses. See text for abbreviations and details.
Figure 1

Infection

Somatic mutation
Antigen selection
Isotype switch

IgMD

U-CLL

M-CLL

Isotype-switched
CLL

IgG
Figure 2

- Immature glycan
- U-CLL > M-CLL
- ? new properties
- ? lectin binding
- ? tonic signal
- antigen
- persistent endocytosis
- Fully glycosylated "mature" form
- High-mannose "immature" form
Figure 3

U-CLL
- slgM recovery/conversion to mature form
- Exit to circulation/extra-PC tissues

M-CLL
- slgM recovery/conversion to mature form
- Exit to circulation/extra-PC tissues

Antigen engagement in PCs

slgM downregulation (U-CLL < M-CLL)
Conversion to high-mannose slgM (U-CLL > M-CLL)
Retained slgM signaling capacity (U-CLL > M-CLL)

Retained slgM signaling → proliferation and survival PROGRESSIVE DISEASE

Downmodulated slgM signaling → strong “anergy” INDOLENT DISEASE

mature slgM
high-mannose slgM
Figure 4a
Figure 4b
B-cell receptor signaling in chronic lymphocytic leukemia

Freda K Stevenson, Sergey Krysov, Andrew J Davies, Andrew J Steele and Graham Packham