

# The outcome of B-cell receptor signaling in chronic lymphocytic leukemia: proliferation or anergy

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## ABSTRACT

Biologists and clinicians agree that the B-cell receptor influences the behavior of chronic lymphocytic leukemia, and promising new drugs are aimed at receptor-associated kinases. Engagement of surface immunoglobulin by antigen is a key driver of malignant cells with outcome influenced by the nature of the cell, the level of stimulation and the microenvironment. Analysis of surface immunoglobulin-mediated signaling in the two major disease subsets defined by *IGHV* mutational status reveals bifurcation of responses toward proliferation or anergy. Mutated chronic lymphocytic leukemia, generally of relatively good prognosis, is mainly, but not exclusively, driven towards anergy *in vivo*. In contrast, unmutated chronic lymphocytic leukemia shows less evidence for anergy *in vivo* retaining more responsiveness to surface immunoglobulin M-mediated signaling, possibly explaining increased tumor progression. Expression and function of surface immunoglobulin M in unmutated chronic lymphocytic leukemia appear rather homogeneous, but mutated chronic lymphocytic leukemia exhibits a highly heterogeneous profile that may relate to further variable clinical behavior within this subset. Anergy should increase susceptibility to apoptosis but, in leukemic cells, this may be countered by overexpression of the B-cell lymphoma-2 survival protein. Maintained anergy spreads to chemokines and adhesion molecules, restraining homing and migration. However, anergy is not necessarily completely benign, being able to reverse and regenerate surface immunoglobulin M-mediated responses. A two-pronged attack on proliferative and anti-apoptotic pathways may succeed. Increased understanding of how chronic lymphocytic leukemia cells are driven to anergy or proliferation should reveal predictive biomarkers of progression and of likely response to kinase inhibitors, which could assist therapeutic decisions.

## Introduction

The B-cell receptor (BCR) controls the fate of normal B cells. The main component is surface immunoglobulin (sIg) that has no fixed ligand but continually senses the environment for molecules that bind with significant avidity. BCR responses vary with signal strength and are modulated by co-receptors, with outcome ranging from a low level, antigen-independent 'tonic' signal essential for survival, to strong antigen-mediated signals which drive the cell toward activation, differentiation or apoptosis.

Surface Ig (sIg) expression generally persists in mature malignant B cells, suggesting a role post-transformation.<sup>1,2</sup> As for other B-cell malignancies, the molecular nature of the sIg in chronic lymphocytic leukemia (CLL) has provided insight into the development and pathogenesis of the disease. We recently reviewed this topic<sup>3</sup> and will summarize it only briefly here. A significant finding has been the identification of two major subsets that arise at distinct points of differentiation and express unmutated or mutated *IGHV* genes: U-CLL and M-CLL, respectively. The clinical behavior of the two subsets differs substantially, with U-CLL having a poorer prognosis.<sup>4,5</sup> This is underlined by the fact that most genomic aberrations are found in U-CLL, and that transformations to Richter syndrome are mostly from this subset.<sup>6-8</sup>

Investigation of the underlying biology has indicated that growth-promoting BCR signaling is generally higher in U-

CLL,<sup>9,10</sup> offering a possibility of therapeutic inhibition. In fact, new inhibitors of BCR-associated kinases are already radically altering treatment.<sup>11</sup> Interestingly, although fewer patients with M-CLL require treatment, early data suggest that this subset responds differently from U-CLL to the BTK inhibitor ibrutinib.<sup>12</sup> It appears that, although lymph node shrinkage and clinical benefit occur in both subsets, lymphocytosis tends to persist in patients with M-CLL.<sup>13</sup> In fact, it is becoming clear that within the two broad divisions, there are further heterogeneities in both biology and clinical behavior, some of which may arise from genomic changes. Within M-CLL, there is a surprisingly wide variability in BCR-mediated signaling,<sup>9</sup> not obviously connected to chromosomal changes. It would be useful to understand the biology behind this and to probe this subset further for the importance of signaling for predicting disease progression. It would also be useful to find associated biomarkers both for prognosis and for assessing responses to kinase inhibitors.

If antigen is driving the tumor cells, the main question concerns the outcome of this interaction in terms of proliferation, which is undesirable, or anergy, which may be less dangerous. In this review, we describe the variable responses to engagement of sIg and discuss their influence on tumor cell behavior in CLL (Figure 1). We will integrate those concepts with recent findings from clinical trials of novel drugs targeted towards kinases associated with the BCR, bearing in mind that the same kinases are involved in pathways mediated by

other receptors. For all CLL, the predominant BCR response *in vivo* appears to be anergy, a mechanism of tolerance whereby autoreactive B cells are rendered non-responsive to activation via their cell surface BCRs.<sup>14</sup> This is observed at variable levels and would be expected to hold the disease in check, explaining its generally chronic nature. However, a small proportion of cells within the clone might engage whatever T-cell help is available and these would then proliferate. The balance between ‘positive’ signaling leading to proliferation/survival and anergy will determine the behavior of the tumor. It seems to be set differently in U-CLL and M-CLL, and this distinction is likely to explain differences in prognosis.

### Antigen recognition by CLL cells

Unequivocal evidence for interaction of CLL cells with antigen *in vivo* is provided by the observed downregulation of sIgM expression in circulating cells.<sup>9</sup> The fact that it can be reversed *in vitro* is consistent with “endocytosis *in vivo*” occurring following engagement in tissue sites. Antigens recognized by CLL cells are not a single entity but, from patterns of growth and persistent effects, are most likely to be autoantigens. U-CLL appears to develop from naïve, possibly CD5<sup>+</sup> B cells of the natural antibody repertoire and the relatively conserved variable region sequences reflect this origin.<sup>15,16</sup> M-CLL may derive from rare, post-germinal center CD5<sup>+</sup>CD27<sup>+</sup> cells.<sup>16</sup> There is no evidence for crossover between U-CLL and M-CLL, and *IGHV* gene usage differs markedly indicating a distinct origin.<sup>17</sup>

Candidate antigens have been identified for both U-CLL and M-CLL.<sup>18-22</sup> Antigens which influence established disease need not be those which stimulated the B cells of ori-

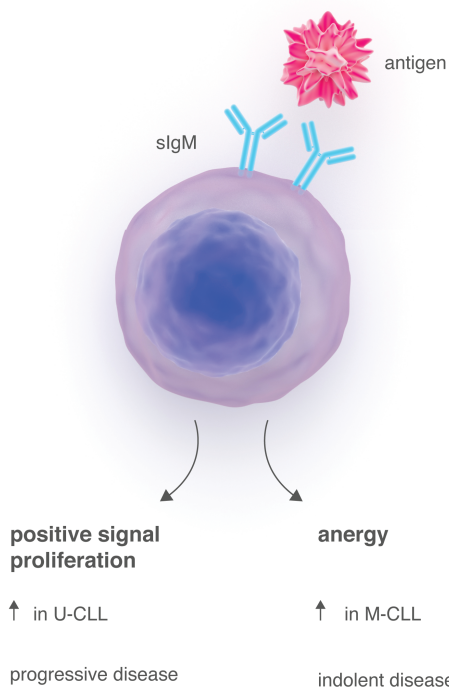
gin but may be cross-reacting substitutes of lower affinity. Some of these could be autoantigens, with auto/polyreactivity more common in U-CLL than M-CLL<sup>18</sup> possibly reflecting their normal B-cell counterparts.<sup>23</sup> However, specificity for the initiating antigen may be retained in some cases. A small number of U-CLL *IGHV1-69*-encoded BCRs react with a cytomegalovirus phosphoprotein and a proportion of M-CLL *IGHV3-7/IGKV2-24*-encoded BCRs recognize fungal  $\beta$ -(1,6)-glucan.<sup>21,22</sup> In these cases, the pathogen-derived antigen might influence malignant cell growth.

Autonomous signaling has also been described whereby BCRs derived from CLL, but not normal or other malignant B cells, induce intracellular calcium (iCa<sup>2+</sup>) mobilization in reconstituted pro-B cells without additional cross-linking.<sup>24</sup> This appears to be due to inter- or intra-molecular interactions between CLL BCRs. However, the relevance of these interactions *in vivo*, where cells are surrounded by high levels of serum Ig that would be expected to compete with these Ig/Ig interactions, even in tissues of CLL patients with hypogammaglobulinemia, is unclear.

Exposure of CLL cells to either foreign or autoantigen is most likely to occur in lymphoid tissues following extravasation and migration, processes mediated by adhesion molecules and chemokine gradients.<sup>25,26</sup> Unprocessed antigens may be presented by conventional dendritic cells in the perivascular area, but CLL cells appear to remain extrafollicular, with no evidence for germinal center formation. Instead, cell division occurs within looser agglomerates known as proliferation centers (PC), where interactions with antigen and other microenvironmental elements presumably coalesce to promote survival and proliferation.<sup>25</sup> Although CD4<sup>+</sup> T cells are present,<sup>27,28</sup> there is no evidence for substantial cognate T-cell help, an absence expected for autoantigens where tolerance operates. For normal B cells in this setting, anergy would be a likely outcome and it appears to occur in the majority of CLL cells. Whatever the outcome of engagement of antigen in tissues, CLL cells will exit to the circulation and these cells carry a temporary imprint of their prior stimulation (Figure 2). This then decays, or reverses, while cells are circulating, priming them for re-entry into tissues for iterative rounds of stimulation. Careful analysis of blood CLL cells can, therefore, reveal the consequences of prior tissue-based responses.

### The nature of BCR-mediated anergy in normal B cells

Anergy, as defined by a failure to respond to BCR-mediated stimuli, is a component of normal B-cell behavior.<sup>14</sup> It is a state of cellular lethargy resulting from binding of antigen by B cells (signal 1) in the absence of significant CD4<sup>+</sup> T-cell help (signal 2). In effect, the B cells are left suspended in an unresponsive state and are prone to apoptosis. To probe the mechanism, it was necessary to develop mouse models where anergy can be induced in a controlled manner by creating a chronic interaction between antigen-specific B cells and antigen.<sup>14</sup> A favorite model is the hen egg lysozyme (HEL) double transgenic mouse, where persistent co-expression of a high affinity HEL-specific BCR and soluble HEL results in anergy. In a parallel arsonate (Ars)-specific model, the transgenic BCR has modest affinity for the Ars hapten, but cross-reacts weakly with endogenous



**Figure 1.** Differential BCR signaling responses and variable clinical outcome in CLL.

antigen (most likely single-stranded DNA) with a similar anergic outcome. This may be reminiscent of the autoantigenic drive on CLL cells.

Although the models differ in detail, common features have emerged. In both cases, chronic exposure to antigen leads to a selective downmodulation of sIgM, whereas sIgD levels are relatively unaffected.<sup>29,30</sup> Anergic cells also have raised basal ERK phosphorylation and  $iCa^{2+}$ .<sup>30,32</sup> Signaling responses, including induction of activation markers such as CD80 and CD86, proliferation and antibody secretion, are reduced or absent following BCR stimulation, as compared to control B cells.<sup>14</sup> Anergy is dependent on continual antigen binding and is, therefore, reversible following removal of antigen, consistent with anergy being a variable cell state, rather than a distinct B-cell lineage.<sup>32,33</sup> Interestingly, many of these features are characteristic of CLL cells,<sup>9,24,34,35</sup> supporting earlier speculation that CLL appears to be a tumor of largely anergic B cells which are protected from death.<sup>36</sup>

Although studies of anergy have focused on transgenic models, many of the key features identified have also been observed in naturally occurring anergic B cells. For example, anergy as assessed by selective downmodulation of sIgM, but not sIgD, and raised  $iCa^{2+}$ , has been observed both in minor cell populations from non-transgenic mice and in human cells from healthy individuals.<sup>37,40</sup> In these studies, potential anergic cells were enriched for autoreactivity/polyreactivity and, consistent with the reversibility of anergy, down-modulated sIgM expression/signaling capacity recovered following culture *in vitro*.<sup>37,38</sup>

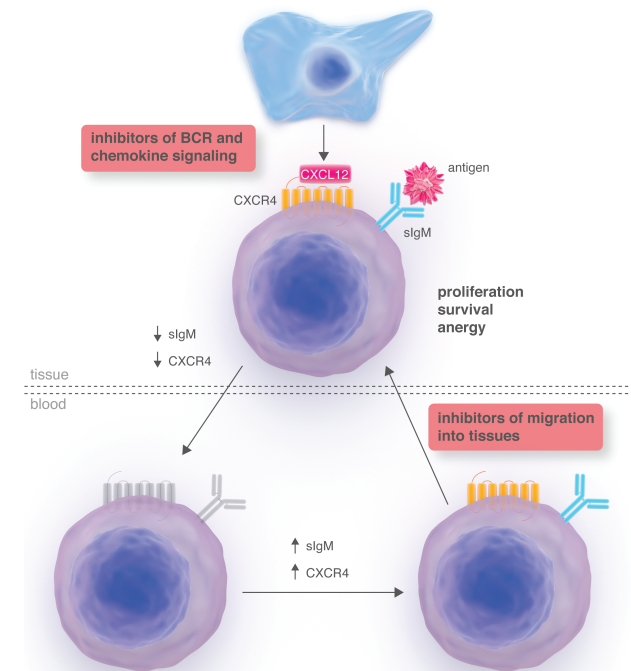
### BCR signaling in anergic cells

The pathways of positive BCR signaling in normal B cells which activate the signalosome and downstream pathways linked to proliferation, survival and migration have been described in our previous review<sup>3</sup> and are shown in Figure 3A.<sup>41</sup> Much less is known about signaling pathways operating in anergic cells. Chronic antigen signaling appears to lead to a shift in the balance between BCR-activation pathways and opposing inhibitory pathways but the means of achieving this are unclear. The role of receptor endocytosis may be important since this reduces the level of sIg. One study reported that there is an unsheathing of CD79A/B molecules from sIg, thereby curtailing signaling and facilitating endocytosis.<sup>42</sup> The same group also showed that chronic BCR signaling in anergic B cells is associated with mono-phosphorylation of CD79A/B rather than the dual-phosphorylation which underlies positive signaling (Figure 3B).<sup>43</sup> Since SYK contains dual SH2 domains, its activation is substantially decreased in anergic cells with CD79A/B mono-phosphorylation.

Like positive signaling, ITAM mono-phosphorylation appears to be catalyzed by LYN; anergic B cells have elevated levels of LYN activation<sup>31</sup> and, despite its role in positive signaling, LYN deletion in mice results in BCR hyperactivity and autoimmunity.<sup>44,45</sup> As part of feedback control, ITAM mono-phosphorylation results in inhibition of signaling via phosphatases.<sup>46</sup> SHIP1 activation appears to play a prominent role in anergy since SHIP1 is constitutively phosphorylated in anergic cells and its deletion is associated with rapid onset of autoimmunity in the HEL model.<sup>40,43,37</sup> Other phosphatases, including SHP1 and

PTEN are also likely to contribute.<sup>46</sup> What causes the partial activation of downstream signaling responses, such as increased ERK phosphorylation and activation of NFAT (without increases in other kinases and transcription factors associated with positive responses, including AKT and NF- $\kappa$ B)<sup>34</sup> is also unknown. Selective activation of a subset of responses otherwise associated with positive signaling may reflect lower thresholds for activation of these specific signaling responses<sup>14</sup> or differences in the kinetics of activation, perhaps linked to increased BCR recycling in anergic cells.<sup>48</sup>

Another important feature of anergic cells is suppression of remote receptors (trans-inhibition).<sup>14</sup> In the HEL model, antigen occupancy of less than 30% is associated with profound lack of BCR signaling so mechanisms must exist to facilitate 'spreading' of non-responsiveness to non-engaged BCRs (Figure 3B). Signaling via other receptors, such as CXCR4, which binds the chemokine CXCL12 (SDF1) and is thought to be important for migration of CLL cells into tissues, may also be suppressed in a similar way.<sup>47</sup> SHIP1 and its partner DOK1 are thought to play an important role in trans-inhibition since, once activated, SHIP1/DOK1 complexes can reduce PI3K-mediated signaling at remote receptors. ERK activity may play a role in inhibition of signaling responses via TLR9, which can also be suppressed in anergic cells.<sup>49</sup>



**Figure 2.** A model for CLL recirculation and receptor modulation. Engagement of sIgM and CXCR4 by antigen and CXCL12 (from stromal cells), respectively, leads to downmodulation of these receptors and exit of cells into the blood where they are accessible for study. These cells therefore carry a temporary "imprint" of prior tissue-based stimulation. In the absence of stimulation there is recovery of sIgM and CXCR4 expression which primes these cells for re-entry into tissues and antigen restimulation and can be modelled by incubating cells *in vitro*. New inhibitors targeted towards BCR-associated kinases are likely to act at multiple points (indicated in red boxes), potentially blocking chemokine signals required for homing and migration into tissues, as well as signaling via sIg.

## BCR-mediated anergy in CLL

There are two reasons to study anergy in CLL. First, to understand how BCR engagement bifurcates into either proliferation or anergy and second, to know why the apoptosis that awaits normal anergic B cells is avoided. A cautionary note is that, although anergy may be more desirable than proliferation, the state is reversible and anergic CLL cells cannot be assumed to be harmless since they may act as a reversible source for subsequent positive restimulation. Mouse models have been used to illuminate the process in normal B cells and this insight may be useful for understanding CLL cells.

### Variable levels of anergy in U-CLL and M-CLL

Studies of anergy in CLL have so far been confined to circulating cells. These generally co-express low levels of sIgM and sIgD (although a minor subset have undergone class-switching) and, although overall sIgM-mediated responses are weak compared to naive B cells,<sup>50</sup> signaling is particularly down-modulated in a proportion of cases.<sup>9,51</sup> There is a strong tendency for the anergic profile of sIgM downmodulation and reduced signaling to be in cases with a good prognosis, especially in M-CLL and/or ZAP70-negative samples.<sup>9,10,50-55</sup> This has been confirmed by single cell network profiling<sup>56</sup> and directly linked to good clinical outcome.<sup>55</sup> The same overall clinical relevance of signaling has been found using proteomic analysis.<sup>57</sup>

The constitutive features of anergy in CLL, derived from several studies and summarized in Table 1<sup>58-63</sup> are seen to reflect those of normal anergic B cells and, in terms of the two subsets of CLL, confirm that they are more evident in M-CLL/ZAP70-negative cases. While it is difficult to prove that the constitutive changes in signaling molecules detected in circulating cells are due solely to prior BCR stimulation *in vivo*, elevated basal  $iCa^{2+}$ , ERK1/2 phosphorylation and NFAT activation in the absence of other features of BCR signaling, such as AKT activation, are consistent with anergy.<sup>24,34,35</sup> Expression and phosphorylation of SHIP1, an important mediator of anergy-associated signaling, is also mainly observed in ZAP70-negative CLL.<sup>58</sup> Taken together, these studies reveal variable levels of a

constellation of anergic features in CLL. Importantly, where tested, these features are associated with relatively good prognosis, underlining the association between more pronounced anergy and favorable outcome. LYN is also over-expressed and activated in some CLL samples, and this appears to be linked to inhibitory signaling since it is required for activation of SHP1 via effects on CD5.<sup>64,65</sup> However, the extent of LYN activation is not clearly linked to ZAP70 expression or *IGHV* mutation status; this complexity may reflect the dual role of LYN in normal B cells in both stimulation and inhibiting signaling via sIg.<sup>65</sup>

### Dichotomy in IgM- or IgD-mediated signaling in CLL cells

In CLL, there is an overall reduction of sIgM and sIgD expression as compared to normal naïve B cells. However, there is in addition a reversible downmodulation of sIgM expression, but not of sIgD, a differential now recognized as a key characteristic of anergy.<sup>9</sup> A similar dichotomous behavior of these isotypes has been reported both in mouse models of anergy and in normal human anergic B cells.<sup>14,37-40</sup> In CLL clones, it is difficult to explain given that the two isotypes carry identical Ig variable regions and will recognize the same antigen. It could reflect the different topography of the two Igs<sup>66</sup> or differences in the number of molecules expressed, which is not easy to compare. However, engagement of sIgM alone by antigen is also indicated by a change in the N-glycosylation pattern of the constant regions of the surface IgM  $\mu$  heavy chain, with no change in the  $\delta$  chain of sIgD.<sup>67</sup>

The lack of perturbation of expression by antigen means that levels of sIgD are stably maintained *in vivo* and, in contrast to sIgM, do not increase *in vitro*.<sup>9</sup> Consistent with expression, the vast majority of CLL samples retain the ability to transmit signals via sIgD.<sup>9,51</sup> However, sIgD-mediated signaling responses *in vitro* in CLL cells differ from sIgM (in responsive samples) in being more transient and in failing to effectively activate downstream responses, including MYC.<sup>68</sup> sIgD signaling responses have been relatively poorly characterized in non-malignant anergic cells so the significance of this remains unclear but could include a reduced ability to recruit signaling mediators. However, the kinetics of ERK phosphorylation and  $iCa^{2+}$

**Table 1.** Comparison of features of anergy in non-malignant B cells and CLL.

	Anergic cells <sup>a</sup>	CLL	Links to prognosis	Refs
Attenuated sIgM responses <sup>b</sup>	+	+	M-CLL > U-CLL ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(9, 50, 51)
sIgM downmodulation (>sIgD) <sup>b</sup>	+	+	M-CLL > U-CLL ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(9)
Raised basal ERK1/2 phosphorylation <sup>b</sup>	+	+	ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(34, 35)
Raised basal intracellular Ca <sup>2+</sup>	+	+	NK <sup>c</sup>	(24)
Raised basal NFAT	+	+	ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(34)
Constitutive SHIP-1 phosphorylation	+	+	ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(58)
Reduced responses to CpG-ODN	+	+	M-CLL > U-CLL	(59)
Reduced responses to CXCL12 <sup>b</sup>	+	+	ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(60), (61)
Increased basal BIM expression	+	+	M-CLL > U-CLL ZAP70 <sup>-</sup> > ZAP70 <sup>+</sup>	(62, 63) and our unpublished data
Shortened survival	+	-	-	-

<sup>a</sup>Features of B-cell anergy<sup>14,37-40</sup>; <sup>b</sup>Reversible following culture *in vitro*; <sup>c</sup>not known

responses that have been observed in CLL cells following sIgD stimulation are similar to those in anergized cells in the HEL model.<sup>69</sup> It seems, therefore, that, in CLL, sIgD may not have a substantial role in activating proliferative events. Its role, if any, in anergic normal cells or in CLL cells remains unknown.

### Effects of BCR-mediated signaling on remote receptors in CLL

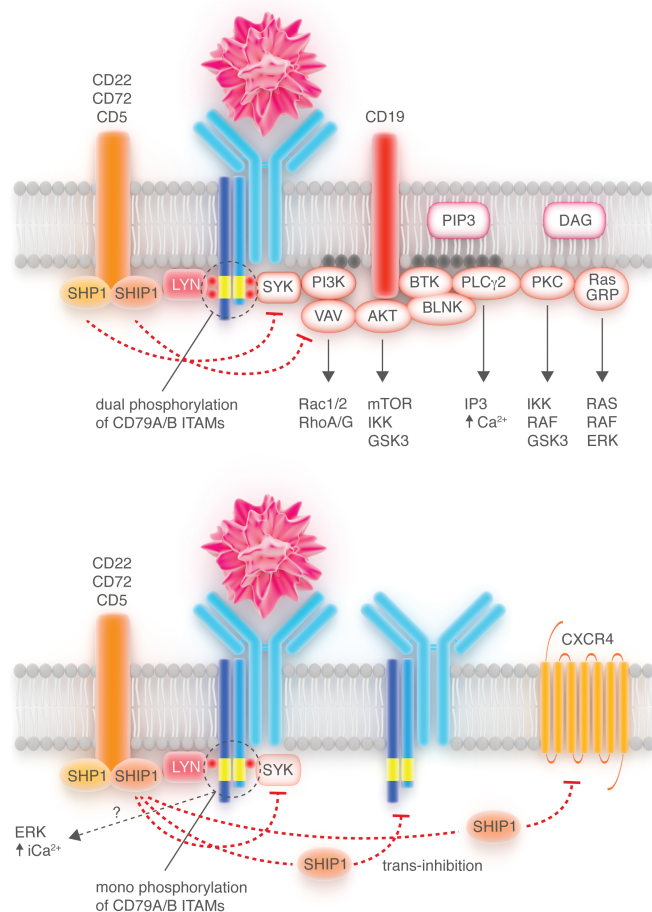
The phenomenon of trans-inhibition, whereby BCR-induced anergy can affect remote receptors (Figure 3B), described above for normal B cells, appears also to operate in CLL cells (Table 1). Thus, as in anergic B cells,<sup>14</sup> CLL cells generally have reduced proliferative responses to the TLR9 ligand CpG-ODN, especially in M-CLL and ZAP70-negative CLL.<sup>59</sup> Also, some CLL samples have reduced CXCR4 responsiveness, and, in one study, this has been linked to decreased ZAP70 expression.<sup>60,70</sup> Similar to other features of anergy, reduced CXCR4 signaling capacity reverses following culture *in vitro*.<sup>70</sup> However, since CXCR4 expression recovers during culture *in vitro*,<sup>71</sup> it is not clear whether this is a direct consequence of receptor recovery per se, or reflects reversal of BCR-mediated trans-inhibition. Mechanisms of trans-inhibition have not been explored directly in CLL, but, as for normal anergic cells, the increased ERK<sup>49</sup> or SHIP1 activity<sup>47</sup> that characterizes anergic CLL cells may play important roles. For CXCR4, it has been proposed that impaired endosomal recycling of Rap1 is important.<sup>61</sup>

In addition to these studies which potentially reveal a

trans-inhibitory effect of prior antigen engagement *in vivo*, other modes of BCR crosstalk have been demonstrated following sIgM engagement *in vitro* with clear effects on the expression and function of the chemokine receptor CXCR4 and the adhesion molecule CD49d.<sup>72-74</sup> For CXCR4, sIgM engagement has been shown to reduce expression of CXCR4 but can either enhance or decrease CXCL12-dependent migration.<sup>72,74</sup> One important determinant of these differential responses is likely to be BCR signaling strength. For example, in immature B cells, low concentrations of anti-IgM enhance, whereas high concentrations inhibit CXCR4-dependent migration towards CXCL12.<sup>47</sup> Low-level BCR engagement may enhance migration allowing CLL to 'search' for antigen-rich tissue sites, whereas subsequent high-level BCR engagement within these depots then inhibits migration allowing cells to effectively engage supporting interactions. Given the key role of the BCR and CXCR4 in determining the clinical behavior of CLL, it will be essential to understand the detailed interactions between these molecules, and others such as CD49d, and how these interactions are affected by clinical signaling inhibitors.

### BCR-mediated positive signaling in CLL

Although anergy is observed in CLL cells, especially M-CLL, there is a proliferative fraction in all CLL patients.<sup>75</sup> It is clear that this positive response to antigen engagement occurs in a minority of cells located in specific tissue sites,



**Figure 3.** BCR signaling in naive and anergic B cells. Antigen triggers signaling that differs between naive and chronically stimulated anergic cells. (A) In naive B cells, antigen engagement triggers activation of SRC-family kinases, including LYN, which catalyzes the dual phosphorylation of immunoreceptor tyrosine-based activatory motifs within CD79A and CD79B. This results in recruitment and activation of SYK and triggers activation of downstream effectors, including protein kinases (AKT, BTK) and adaptors (BLNK), as well as lipid metabolism via lipid kinases (PI3K) and lipases PLC $\gamma$ 2 leading to the production of lipid metabolites, PIP3, DAG and IP3. These pathways are naturally inhibited by LYN-dependent activation of both protein (SHIP1) and lipid (SHIP2) phosphatases. (B) In anergic cells, chronic BCR engagement favors mono-phosphorylation of CD79A/B which results in weak SYK activation but efficient LYN activation. Activation of phosphatases, especially SHIP1, further suppress SYK-dependent signaling by preventing accumulation of PIP3, and suppresses activation of BCRs and distinct receptors such as CXCR4. Anergic cells are often characterized by raised basal levels of iCa $^{2+}$  and ERK phosphorylation. Adapted from Yarkoni et al.<sup>41</sup>

particularly within PC in lymph nodes (LN).<sup>76</sup> In a powerful gene expression analysis of matched CLL cells from blood, bone marrow and LN, enhanced activation of BCR-associated signaling pathways was observed in LN,<sup>77</sup> and was higher in cases of U-CLL. Immunohistochemical analysis also showed that proliferation (Ki-67 staining) was preferentially increased in PCs.<sup>27,68,78</sup> Significant levels of phosphorylated ERK and of the growth-promoting oncoprotein MYC, known to be induced in CLL cells following sIgM stimulation,<sup>68</sup> have also been detected in PCs. In circulating cells, levels of MYC mRNA were noted to be higher in patients with progressive disease.<sup>79,80</sup>

**Heterogeneity within M-CLL**

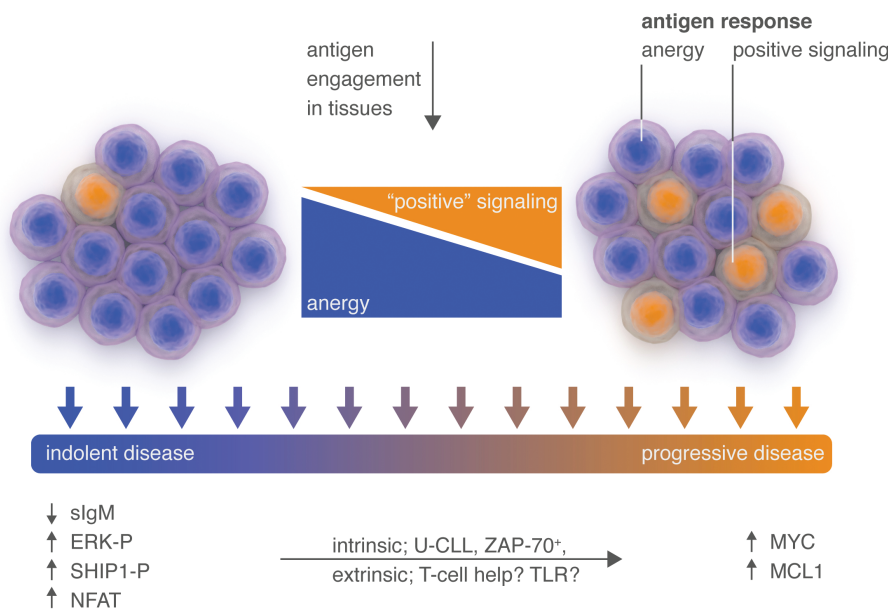
Whereas sIgM signaling is relatively homogeneous in U-CLL, sIgM expression and responses in M-CLL are more heterogeneous, with responses that range from essentially no signaling through to responses typical of U-CLL.<sup>9</sup> Heterogeneity within M-CLL was also noted in the proteomic analysis<sup>57</sup> and in the gene-expression analysis of LN samples.<sup>77</sup> Our early studies investigating anti-IgM-induced BIM phosphorylation suggest that this ability may be a useful discriminator. We reported that, overall, BCR-induced BIM phosphorylation was more evident in U-CLL than in M-CLL.<sup>81</sup> Also, although numbers were small, we noted that within the M-CLL group, activity of this signaling pathway was associated with requirement for treatment.<sup>81</sup>

Thus, IGHV-gene mutation status is not the only determinant of positive signaling. Other influences may include ZAP70, which can enhance signaling via the BCR and other receptors;<sup>60,82,83</sup> high expression may, therefore, favor positive signaling over anergy. CD38 expression may also be inversely correlated with sIgM-induced anergy since increased CD38 is associated with retained sIgM signaling.<sup>9,84</sup> There is increasing evidence that CD38 can modulate signaling responses in CLL,<sup>85</sup> although direct effects on BCR responses were not evident in our study of cases

with a bimodal expression of CD38.<sup>9</sup> In M-CLL, most, but not all, cases are negative for ZAP70, and CD38 tends to be less often expressed than in U-CLL, but a significant minority is discordant, again indicative of heterogeneity.<sup>86</sup> While intrinsic features of the cell of origin of each of the major subsets may be the predominant factor influencing positive signaling versus anergy, multiple factors, including genomic changes and environmental interactions, are likely to influence responses.

**Intraclonal heterogeneity in CLL**

If anergy and positive signaling are both evident within individual clonal populations of CLL cells, it is important to identify the features of cells capable of positive signaling, since these are more likely to proliferate and accumulate genetic abnormalities. We do not suggest that anergy and positive signaling occur simultaneously in an individual cell. There is likely to be heterogeneity amongst the tissue-based events and, therefore, in the imprint that remains in individual circulating cells (Figure 2). An approach to analysis of the variable coupling between the BCR and positive/anergic responses at single cell level is illustrated in Figure 4 where the responses of individual cells are essentially binary (i.e. anergy or positive signaling), but it is the proportion of cells that undergo these distinct responses that is linked to outcome. Heterogeneity will also arise from differences in the length of time individual cells have been in the circulation. Thus, the most recent emigrants from tissue sites will carry the strongest imprint of their prior responses compared to earlier emigrants that will have undergone variable degrees of recovery. This intraclonal heterogeneity is revealed by analysis of variation within the circulating malignant population. CD5<sup>bright</sup>CXCR4<sup>dim</sup> cells appear to be new emigrants, enriched for recently divided cells, and with a distinct gene expression profile indicative of stimulation.<sup>87</sup> We have



**Figure 4.** Variable BCR signaling responses influence clinical outcome via differential effects on anergy and positive signaling. Antigen engagement appears to be ongoing in all CLL with anergy being the predominant outcome. However, low levels of growth promoting positive signaling may tip the balance towards progressive disease. A temporary imprint of these different tissue-based responses can be detected in circulating CLL cells. Thus, markers of anergy (sIgM downmodulation, raised basal ERK phosphorylation and NFAT) are more prominent in good prognosis subsets, whereas markers of positive signaling (MYC and MCL1) are associated with a poor prognosis. The balance between anergy and positive signaling is likely to be determined by both intrinsic and extrinsic factors.

focused on the variable levels of sIgM expression within CLL clones and, by using the differential ability of cells to bind beads coated with anti-IgM we have been able to identify sub-groups.<sup>71</sup> We found that the bulk of the malignant clone was present in a sub-group with low sIgM expression and consequent weak signaling response. This subgroup had low CXCR4 expression and contained the Ki-67 positive cells.<sup>88</sup> We share the interpretation from a previous study that this subgroup includes recent emigrants with down-modulated CXCR4 resulting from binding to CXCL12 in tissues. However, we also detected low levels of sIgM, presumably down-regulated by exposure to antigen. This subgroup could contain two co-existing populations, both unable to respond significantly to BCR engagement or to chemokine. The Ki-67 expressing fraction has evidently proliferated and the remaining bulk of cells may be those most recently driven to anergy.

We also identified a small population of cells with relatively high levels of sIgM and CXCR4, and with strong inducible anti-IgM signaling responses. This subgroup presumably represents earlier emigrants that have recovered expression of both receptors. Although a minor fraction of the clone, these cells may be particularly dangerous since they are primed for re-entry into tissues and for a response to antigen.<sup>71</sup> They may be the key target for drug therapy, and ibrutinib was shown to be able to inhibit signaling. However, nothing is stable in CLL and it would be anticipated that, once in the tissues, these cells undergo the same cyclical changes on encountering antigen. Whether all CLL cells recirculate in this way or whether there are distinct sub-populations of recirculating and tissue resident cells is unclear. Regardless, careful analysis of blood CLL cells can reveal, at least partially, the consequences of previous BCR stimulation.

### Determinants of BCR signaling outcomes in CLL

Since the extent of anergy *versus* positive signaling appears to influence disease outcome, then a key question is what determines the choice between these responses. If the driver antigens are in fact autoantigens, they are likely to be widely expressed. That this is the case is indicated by the fact that almost all cases of CLL exhibit some degree of downregulation of sIgM, presumed to be mediated by antigen. However, proliferation requires more than just antigen engagement and, for normal B cells, the most important are CD40L and cytokines, both provided by CD4<sup>+</sup> T cells.

Cognate T-cell help is normally provided following recognition by CD4<sup>+</sup> T cells of peptide bound to the MHC Class II molecules of the B cells. In CLL, toleragenic pressure against autoreactivity will have eliminated the high affinity antigen-specific T cells. This means that substantial T-cell help is unlikely to be available and, in addition, T cells in CLL commonly have an inverted CD4:CD8 ratio and display markers of exhaustion.<sup>89</sup> However, activated CD4<sup>+</sup> T cells are known to be present in close proximity to dividing tumor cells in PCs<sup>28</sup> and, although technically difficult to confirm, a proportion was reported to express CD40L.<sup>27</sup> Competition among CLL cells for the low levels of competent T cells could mean that only those members of the clone, possibly those expressing the highest levels of sIgM, and, therefore, most efficient at internalizing and presenting antigen, receive effective cognate T-cell help.

The ability to receive T-cell help seems to be set differently in U-CLL and M-CLL and this may reflect CLL cell intrinsic differences, or variation in T-cell status between the two disease subsets. An alternative possibility is that of obtaining some level of innate stimulation from various cell sources which could promote survival and even isotype switch.<sup>90</sup> This includes stimulation of Toll-like receptors, and some studies have demonstrated that U-CLL appears to be more responsive to the cell survival and proliferation promoting effects of CpG-ODN.<sup>59</sup> It is unclear to what extent this might facilitate BCR signaling, or if the difference between subsets reflects trans-inhibition of TLR9 function in anergy-prone M-CLL.

The PTPN22 phosphatase may also be an important determinant of signaling in CLL.<sup>91</sup> This phosphatase is over-expressed in CLL and selectively reprograms BCR-associated signal transduction, down-modulating phosphorylation of LYN, SYK, ERK1/2 and p38 MAPK while increasing phosphorylation of AKT and enhancing survival responses. However, the relationship between PTPN22 and anergy remains unclear since its expression is not clearly associated with either *IGHV* mutation status or sIgM signaling capacity.

### Apoptosis and BCR signaling in CLL

It is clear that proliferative events occur predominantly in the LN and that this is followed by exit to the blood. Unlike normal B cells, CLL cells and their progeny are protected from death, with expression of the anti-IgM-inducible survival-promoting MCL1 protein detected in CLL blood cells and, like MYC, correlating with progressive disease.<sup>50,92</sup> Correlation with *IGHV* mutational status is evident but, as for all candidate biomarkers, a careful dissection of M-CLL is required. Induction of BIM phosphorylation downstream of sIgM may also play a role in promoting survival of stimulated cells.<sup>93</sup>

Extended survival also appears to occur in the anergic fraction, mainly M-CLL, and this runs counter to the known vulnerability of normal anergic B cells which are short-lived *in vivo*.<sup>14</sup> One of the key regulators of apoptosis in normal anergic B cells is BIM, a BH3-only apoptosis-inducing protein.<sup>94</sup> Increased expression of the two major isoforms of BIM, BIM<sub>EL</sub> and BIM<sub>ML</sub> in anergic cells<sup>95</sup> is important for the rapid turnover of anergic cells in mouse models since deletion of BIM promotes the accumulation of self-reactive B cells *in vivo*.<sup>96,97</sup> Consistent with the idea that CLL cells display multiple features of anergic cells, BIM isoforms are also over-expressed in CLL cells compared to normal human B cells.<sup>62</sup> Within CLL, BIM isoform expression positively correlates with M-CLL (G Packham *et al.*, unpublished data, 2014) and with low ZAP70 expression,<sup>63</sup> reinforcing linkage between anergy and indolent clinical behavior (Table 1).

Since CLL cells over-express BIM, a key question is how these malignant cells tolerate increased expression of this pro-apoptotic molecule. CLL cells are highly dependent on overexpression of BCL2 for their survival and 'BH3 profiling' studies have demonstrated that the principal function of BCL2 in CLL cells is to neutralize BIM.<sup>62</sup> Indeed, CLL cells are highly sensitive to BCL2 antagonists which prevent association of BIM with BCL2, and other related anti-apoptotic molecules, and appear to unmask the latent apoptosis-inducing activity of BIM in CLL cells (Figure 5).

This class of drugs includes the recently developed ABT-199 which has shown very promising results in early trials in CLL.<sup>98,99</sup> The potent pro-apoptotic effects of these compounds may, in part, reflect their ability to restore the BIM-dependent apoptosis susceptibility that is a feature of normal anergic B cells.<sup>100</sup> One route to BCL2 overexpression in CLL may be via loss of 13q14, which deletes miRNAs (miR15A/miR16) which normally act to down-modulate BCL2 expression.<sup>101</sup> This could explain why loss of 13q14 is an early genetic event in CLL since it may be required to counter anergy-associated apoptosis from an early stage in disease development.

Other molecules associated with apoptosis control in CLL may also relate to their function in modulation of survival of anergic cells (Figure 5). In particular, anergic B cells compete poorly with non-anergic B cells for limited availability of the B-cell survival factor BAFF (B-cell activating factor of the TNF family).<sup>95</sup> BAFF appears to be required for the survival of anergic B cells since it over-rides the pro-apoptotic function of BCL2-related molecules, including BIM. Indeed, in contrast to normal anergic B cells, Bim-deficient anergic B cells are able to survive in the absence of BAFF *in vitro*.<sup>97</sup> Thus, BCL2 overexpression, via its inhibitory effects on BIM, appears to be one mechanism by which anergic CLL cells mitigate requirements for BAFF. However, direct production of BAFF by CLL cells<sup>102</sup> or by cells of the microenvironment<sup>103</sup> may also provide important sources of BAFF to counter anergy-associated apoptosis. In some models, killing mediated via the cell surface death receptor FAS has been implicated in apoptosis of anergic B cells and CLL cells are frequently resistant to FAS-mediated apoptosis.<sup>104-106</sup>

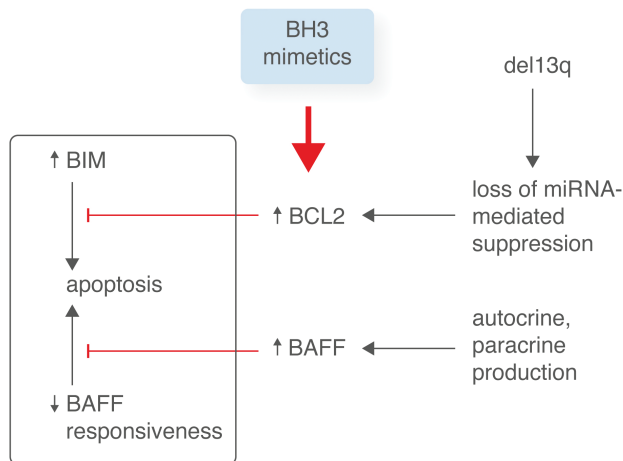
### Therapeutic implications

The concept that BCR stimulation plays a critical role in driving proliferation and survival of the malignant clone is clearly supported by recent advances in the development of inhibitors targeted towards BCR-associated signaling kinases for treatment of CLL.<sup>11</sup> New inhibitors of BCR-associated signaling kinases, including ibrutinib (BTK inhibitor), idelalisib (PI3Kδ inhibitor) and fostamatinib (SYK inhibitor), are showing particularly promising clinical results and are likely to become a key component of CLL therapy.<sup>11,107</sup> These agents induce a transient lymphocytosis followed by long-term reductions in peripheral blood cell counts, indicating an important interplay between the BCR and tissue-homing events. However, it is important to recognize that effects on antigen-independent signaling pathways (e.g. downstream of chemokine receptors and integrins) may also contribute to clinical responses.<sup>73,108-110</sup>

In the light of the likely high costs of these agents, especially associated with treatment of chronic disease, the key emerging challenge is to develop markers to predict which patients would benefit most from these drugs. The recent observation that normalization of blood counts after lymphocytosis is more pronounced in U-CLL provides some evidence that responses may be linked to BCR status.<sup>12</sup> One potential strategy to identify appropriate patients is to characterize the signaling features of each individual patient's circulating cells. The balance between markers of positive signaling and anergy might reveal patients most suited for treatment, and potentially aid selection between different BCR-targeted agents. This could be most useful in M-CLL, where signaling responses appear particularly heterogeneous.<sup>9</sup> Analyses will ideally capture single-cell information on multiple parameters to gain a comprehensive picture of signaling status and intracellular heterogeneity. Potential profiling would include slgM expression, markers of anergy *versus* positive signaling (e.g. SHIP1 phosphorylation *vs.* MCL1), and/or assessment of anti-IgM responses *in vitro*. Multi-parametric, single cell flow cytometry linking phenotype and signaling responses, combined with systems level data analysis, could be a particularly useful approach to integrate multiple responses to strengthen predictive power.<sup>56</sup> Given the reversibility of at least some features of BCR signaling in blood CLL cells, rapid delivery and processing of samples in the laboratory would be essential to capture a representative picture of signaling *in vivo*.

Another exciting therapeutic approach that may be influenced by the anergic status of CLL cells is BH3 mimetics. As described above, BCL2 is commonly overexpressed in CLL and its principal function may be to counter the pro-apoptotic function of anergy-associated BIM. One of the earlier BH3 mimetics, navitoclax has shown promising clinical responses, but its efficacy is limited by thrombocytopenia. The more recent compound ABT-199<sup>98</sup> may offer particular promise since this drug has a modified selectivity profile and reduced platelet toxicity. However, tumor lysis syndrome has emerged as a complication that will require addressing.

It is not yet clear whether specific subsets of disease will differ in their clinical response to BH3 mimetics. Studies of BCL2-family expression, potentially linked to anergic phenotypes, using either expression or functional 'BH3 profiling' of CLL-derived mitochondria, could be a valuable



**Figure 5.** Potential mechanisms of suppression of anergy-associated apoptosis in CLL. In normal B cells, anergy is associated with increased expression of BIM, and reduced BAFF responsiveness (boxed). In CLL, overexpression of BCL2, commonly associated with loss of suppressive miRNAs on chromosome 13, appears to sequester BIM to suppress apoptosis. Autocrine or paracrine production of BAFF may also play an important role promoting additional survival signals. BH3 mimetics, such as ABT-199 and navitoclax, reverse sequestration of BIM by survival molecules such as BCL2, potentially revealing the pro-apoptotic potential of anergic CLL cells.



approach to select patients. Indeed, initial studies have shown that patients with high BIM/BCL2 ratios may be particularly responsive.<sup>100</sup> As discussed above, elevated BIM may be a feature of anergy in these cells. Although the anergic fraction could be viewed as a rather 'benign' component of the malignant clone, it is important to bear in mind that this state is reversible. Targeted depletion of these cells using BH3 mimetics could deliver a 'pre-emptive' strike prior to reversal and positive responses. Indeed, combinations of kinase inhibitors, to prevent positive signaling, and BH3 mimetics, to unmask inherent apoptosis susceptibility, may be particularly effective. Navitoclax does not bind MCL1 and high-level expression of this protein can mediate drug resistance. Thus, an added benefit of such a combination approach would be that kinase inhibitors would prevent BCR-mediated induction of MCL1 and therefore circumvent this important mechanism of BH3-mimetic resistance. Finally, direct targeting of anergic-associated signaling molecules may offer an approach to selectively ablate anergic CLL cells. For example, recent studies have shown that inhibition of the ERK pathway, using the MEK1/2 inhibitors UO126 or CI1040 or the ERK1/2 inhibitor NMS6E, or VIVIT, a peptide that blocks NFAT nuclear translocation, promotes apoptosis preferentially in strongly anergized samples.<sup>55</sup>

## Concluding remarks

Over the last ten years or so, laboratory studies have

propelled the BCR to center stage in CLL. The role of the receptor is complex, as befits such an adaptive and flexible signaling molecule. The balance between anergy and positive signaling appears to be critical for outcome, but the regulation of these responses largely reflects the checks and balances that act on normal B cells. The key influence of the BCR opens avenues for therapeutic attack and new kinase inhibitors which can deprive CLL cells of growth-supporting antigen signaling are set to revolutionize treatment. Continued discussion between the biologist and the clinician, which has become a tradition in the CLL community, will be essential to understand more fully the function of the BCR in CLL and to provide new markers which can assist the clinician in decisions about therapy.

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## References

- Kuppers R. Mechanisms of B-cell lymphoma pathogenesis. *Nat Rev Cancer*. 2005; 5(4):251-62.
- Stevenson FK, Stevenson GT. Follicular lymphoma and the immune system: from pathogenesis to antibody therapy. *Blood*. 2012;119(16):3659-67.
- Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2011; 118(16):4313-20.
- Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood*. 1999;94(6):1848-54.
- Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. *Blood*. 1999;94(6):1840-7.
- Jain P, O'Brien S. Richter's transformation in chronic lymphocytic leukemia. *Oncology*. 2012;26(12):1146-52.
- Rossi D, Rasi S, Spina V, Brusca A, Monti S, Ciardullo C, et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. *Blood*. 2013;121(8):1403-12.
- Rossi D, Spina V, Bomben R, Rasi S, Dal-Bò M, Brusca A, et al. Association between molecular lesions and specific B-cell receptor subsets in chronic lymphocytic leukemia. *Blood*. 2013;121(24):4902-5.
- Mockridge CI, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK. Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status. *Blood*. 2007; 109(10):4424-31.
- Guarini A, Chiaretti S, Tavolaro S, Maggio R, Peragine N, Citarella F, et al. BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV H unmutated chronic lymphocytic leukemia (CLL) cells. *Blood*. 2008; 112(3):782-92.
- Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood*. 2012;120(6):1175-84.
- Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013; 369(1):32-42.
- Woyach JA, Bojnik E, Ruppert AS, Stefanovski MR, Goettl VM, Smucker KA, et al. Bruton's tyrosine kinase (BTK) function is important to the development and expansion of chronic lymphocytic leukemia (CLL). *Blood*. 2014;123(8):1207-13.
- Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol*. 2007;7(8):633-43.
- Forconi F, Potter KN, Wheatley I, Darzentas N, Sozzi E, Stamatopoulos K, et al. The normal IGHV1-69-derived B-cell repertoire contains stereotypic patterns characteristic of unmutated CLL. *Blood*. 2010;115(1):71-7.
- Seifert M, Sellmann L, Bloehdorn J, Wein F, Stilgenbauer S, Durig J, et al. Cellular origin and pathophysiology of chronic lymphocytic leukemia. *J Exp Med*. 2012;209(12):2183-98.
- Stevenson FK, Sahota SS, Ottensmeier CH, Zhu D, Forconi F, Hamblin TJ. The occurrence and significance of V gene mutations in B cell-derived human malignancy. *Adv Cancer Res*. 2001;33:81-116.
- Herve M, Xu K, Ng YS, Wardemann H, Albesiano E, Messmer BT, et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. *J Clin Invest*. 2005;115(6):1636-43.
- Lanemo Myhrinder A, Hellqvist E, Sidorova E, Soderberg A, Baxendale H, Dahle C, et al. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood*. 2008;111(7):3838-48.
- Zwick C, Fadle N, Regitz E, Kemele M, Stilgenbauer S, Buhler A, et al. Autoantigenic targets of B-cell receptors derived from chronic lymphocytic leukemias bind to and induce proliferation of leukemic cells. *Blood*. 2013;121(23):4708-17.
- Hoogeboom R, van Kessel KP, Hochstenbach F, Wormhoudt TA, Reinten RJ, Wagner K, et al. A mutated B cell chronic lymphocytic leukemia subset that recognizes and responds to fungi. *J Exp Med*. 2013;210(1):59-70.
- Steininger C, Widhopf GF 2nd, Ghia EM, Morello CS, Vanura K, Sanders R, et al. Recombinant antibodies encoded by IGHV1-69 react with pUL32, a phosphoprotein of cytomegalovirus and B-cell superantigen. *Blood*. 2012;119(10):2293-301.
- Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science*. 2003; 301(5638):1374-7.
- Duhren-von Minden M, Ubelhart R,

- Schneider D, Wossning T, Bach MP, Buchner M, et al. Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling. *Nature*. 2012;489(7415):309-12.
25. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood*. 2009;114(16):3367-75.
  26. Burger JA. Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting. *Semin Cancer Biol*. 2010;20(6):424-30.
  27. Granziero L, Ghia P, Circosta P, Gottardi D, Strota G, Geuna M, et al. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. *Blood*. 2001;97(9):2777-83.
  28. Patten PE, Buggins AG, Richards J, Wotherspoon A, Salisbury J, Mufti GJ, et al. CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. *Blood*. 2008;111(10):5173-81.
  29. Goodnow CC, Crosbie J, Adelstein S, Lavoie TB, Smith-Gill SJ, Brink RA, et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 1988;334(6184):676-82.
  30. Benschop RJ, Aviszus K, Zhang X, Manser T, Cambier JC, Wysocki LJ. Activation and anergy in bone marrow B cells of a novel immunoglobulin transgenic mouse that is both hapten specific and autoreactive. *Immunity*. 2001;14(1):33-43.
  31. Healy JJ, Dolmetsch RE, Timmerman LA, Cyster JG, Thomas ML, Crabtree GR, et al. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity*. 1997;6(4):419-28.
  32. Gauld SB, Benschop RJ, Merrell KT, Cambier JC. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol*. 2005;6(11):1160-7.
  33. Goodnow CC, Brink R, Adams E. Breakdown of self-tolerance in anergic B lymphocytes. *Nature*. 1991;352(6335):532-6.
  34. Muzio M, Apollonio B, Scielzo C, Frenquelli M, Vandoni I, Boussiotis V, et al. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. *Blood*. 2008;112(1):188-95.
  35. Apollonio B, Scielzo C, Bertilaccio MT, Ten Hacken E, Scarfo L, Ranghetti P, et al. Targeting B-cell anergy in chronic lymphocytic leukemia. *Blood*. 2013;121(19):3781-3.
  36. Stevenson FK, Caligaris-Cappio F. Chronic lymphocytic leukemia: revelations from the B-cell receptor. *Blood*. 2004;103(12):4389-95.
  37. Duty JA, Szodoray P, Zheng NY, Koelsch KA, Zhang Q, Swiatkowski M, et al. Functional anergy in a subpopulation of naive B cells from healthy humans that express autoreactive immunoglobulin receptors. *J Exp Med*. 2009;206(1):139-51.
  38. Quach TD, Manjarez-Orduno N, Adlowitz DG, Silver L, Yang H, Wei C, et al. Anergic responses characterize a large fraction of human autoreactive naive B cells expressing low levels of surface IgM. *J Immunol*. 2011;186(8):4640-8.
  39. Zikherman J, Parameswaran R, Weiss A. Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature*. 2012;489(7414):160-4.
  40. Merrell KT, Benschop RJ, Gauld SB, Aviszus K, Decote-Ricardo D, Wysocki LJ, et al. Identification of anergic B cells within a wild-type repertoire. *Immunity*. 2006;25(6):953-62.
  41. Yarkoni Y, Getahun A, Cambier JC. Molecular underpinning of B-cell anergy. *Immunol Rev*. 2010;237(1):249-63.
  42. Vilen BJ, Nakamura T, Cambier JC. Antigen-stimulated dissociation of BCR mIg from Ig-alpha/Ig-beta: implications for receptor desensitization. *Immunity*. 1999;10(2):239-48.
  43. O'Neill SK, Getahun A, Gauld SB, Merrell KT, Tamir I, Smith MJ, et al. Monophosphorylation of CD79a and CD79b ITAM motifs initiates a SHIP-1 phosphatase-mediated inhibitory signaling cascade required for B cell anergy. *Immunity*. 2011;35(5):746-56.
  44. Hibbs ML, Tarlinton DM, Armes J, Grahl D, Hodgson G, Maglitto R, et al. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell*. 1995;83(2):301-11.
  45. Nishizumi H, Taniuchi I, Yamanashi Y, Kitamura D, Ilic D, Mori S, et al. Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. *Immunity*. 1995;3(5):549-60.
  46. Waterman PM, Cambier JC. The conundrum of inhibitory signaling by ITAM-containing immunoreceptors: potential molecular mechanisms. *FEBS Lett*. 2010;584(24):4878-82.
  47. Brauweiler A, Merrell K, Gauld SB, Cambier JC. Cutting Edge: Acute and chronic exposure of immature B cells to antigen leads to impaired homing and SHIP1-dependent reduction in stromal cell-derived factor-1 responsiveness. *J Immunol*. 2007;178(6):3353-7.
  48. Blyer M, Tze L, Miosge LA, Jun JE, Goodnow CC. Essential role of membrane cholesterol in accelerated BCR internalization and uncoupling from NF-kappa B in B cell clonal anergy. *J Exp Med*. 2006;203(7):1773-83.
  49. Rui L, Vinuesa CG, Blasioli J, Goodnow CC. Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. *Nat Immunol*. 2003;4(6):594-600.
  50. Petlickovski A, Laurenti L, Li X, Marietti S, Chiusolo P, Sica S, et al. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. *Blood*. 2005;105(12):4820-7.
  51. Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson F, Packham G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. *Blood*. 2003;101(3):1087-93.
  52. Chen L, Widhopf G, Huynh L, Rassisti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. *Blood*. 2002;100(13):4609-14.
  53. Deglesne PA, Chevallier N, Letestu R, Baran-Marszak F, Beitar T, Salanoubat C, et al. Survival response to B-cell receptor ligation is restricted to progressive chronic lymphocytic leukemia cells irrespective of Zap70 expression. *Cancer Res*. 2006;66(14):7158-66.
  54. Nedellec S, Renaudineau Y, Bordron A, Berthou C, Porakishvili N, Lydyard PM, et al. B cell response to surface IgM cross-linking identifies different prognostic groups of B-chronic lymphocytic leukemia patients. *J Immunol*. 2005;174(6):3749-56.
  55. Allsup DJ, Kamiguti AS, Lin K, Sherrington PD, Matrai Z, Slupsky JR, et al. B-cell receptor translocation to lipid rafts and associated signaling differ between prognostically important subgroups of chronic lymphocytic leukemia. *Cancer Res*. 2005;65(16):7328-37.
  56. Cesano A, Perbellini O, Evensen E, Chu CC, Cioffi F, Ptacek J, et al. Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. *Haematologica*. 2013;98(4):626-34.
  57. Perrot A, Pionneau C, Nadaud S, Davi F, Leblond V, Jacob F, et al. A unique proteomic profile on surface IgM ligation in unmutated chronic lymphocytic leukemia. *Blood*. 2011;118(4):e1-15.
  58. Gabelloni ML, Borge M, Galletti J, Canones C, Calotti PF, Bezares RF, et al. SHIP-1 protein level and phosphorylation status differs between CLL cells segregated by ZAP-70 expression. *Br J Haematol*. 2008;140(1):117-9.
  59. Tarnani M, Laurenti L, Longo PG, Piccirillo N, Gobessi S, Mannocci A, et al. The proliferative response to CpG-ODN stimulation predicts PFS, TTT and OS in patients with chronic lymphocytic leukemia. *Leuk Res*. 2010;34(9):1189-94.
  60. Richardson SJ, Matthews C, Catherwood MA, Alexander HD, Carey BS, Farrugia J, 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):3584-92.
  61. Pye DS, Rubio I, Pusch R, Lin K, Pettitt AR, Till KJ. Chemokine unresponsiveness of chronic lymphocytic leukemia cells results from impaired endosomal recycling of Rap1 and is associated with a distinctive type of immunological anergy. *J Immunol*. 2013;191(3):1496-504.
  62. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A. Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *J Clin Invest*. 2007;117(1):112-21.
  63. zum Buschenfelde CM, Wagner M, Lutzny G, Oelsner M, Feuerstacke Y, Decker T, et al. Recruitment of PKC-betaII to lipid rafts mediates apoptosis-resistance in chronic lymphocytic leukemia expressing ZAP-70. *Leukemia*. 2010;24(1):141-52.
  64. Tibaldi E, Brunati AM, Zonta F, Frezzato F, Gattazzo C, Zambello R, et al. Lyn-mediated SHP-1 recruitment to CD5 contributes to resistance to apoptosis of B-cell chronic lymphocytic leukemia cells. *Leukemia*. 2011;25(11):1768-81.
  65. Contri A, Brunati AM, Trentin L, Cabrelle A, Miorin M, Cesaro L, et al. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to defective apoptosis. *J Clin Invest*. 2005;115(2):369-78.
  66. Pogue SL, Goodnow CC. Ig heavy chain extracellular spacer confers unique glycosylation of the Mb-1 component of the B cell antigen receptor complex. *J Immunol*. 1994;152(8):3925-34.
  67. Krysov S, Potter KN, Mockridge CI, Coelho V, Wheatley I, Packham G, et al. Surface IgM of CLL cells displays unusual glycans indicative of engagement of antigen in vivo. *Blood*. 2010;115(21):4198-205.
  68. Krysov S, Dias S, Paterson A, Mockridge CI, Potter KN, Smith KA, et al. Surface IgM stimulation induces MEK1/2-dependent MYC expression in chronic lymphocytic

- leukemia cells. *Blood*. 2012;119(1):170-9.
69. Cooke MP, Heath AW, Shokat KM, Zeng Y, Finkelman FD, Linsley PS, et al. Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. *J Exp Med*. 1994;179(2):425-38.
  70. Till KJ, Harris RJ, Linford A, Spiller DG, Zuzel M, Cawley JC. Cell motility in chronic lymphocytic leukemia: defective Rap1 and alphaLbeta2 activation by chemokine. *Cancer Res*. 2008;68(20):8429-36.
  71. Coelho V, Krysov S, Steele A, Sanchez Hidalgo M, Johnson PW, Chana PS, et al. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood*. 2013;122(15):2664-72.
  72. Quiroga MP, Burger JA. BCR-mediated decrease of CXCR4 and CD62L in CLL. *Cancer Res*. 2010;70(12):5194; author reply 5.
  73. de Rooij MF, Kuil A, Geest CR, Eldering E, Chang BY, Buggy JJ, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood*. 2012;119(11):2590-4.
  74. Vlad A, Deglesne PA, Letestu R, Saint-Georges S, Chevallier N, Baran-Marszak F, et al. Down-regulation of CXCR4 and CD62L in chronic lymphocytic leukemia cells is triggered by B-cell receptor ligation and associated with progressive disease. *Cancer Res*. 2009;69(16):6387-95.
  75. Messmer BT, Messmer D, Allen SL, Kolitz JE, Kudalkar P, Cesar D, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. *J Clin Invest*. 2005;115(3):755-64.
  76. Soma LA, Craig FE, Swerdlow SH. The proliferation center microenvironment and prognostic markers in chronic lymphocytic leukemia/small lymphocytic lymphoma. *Hum Pathol*. 2006;37(2):152-9.
  77. Herishanu Y, Perez-Galan P, Liu D, Biancotto A, Pittaluga S, Vire B, et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. *Blood*. 2011;117(2):563-74.
  78. Igawa T, Sato Y, Takata K, Fushimi S, Tamura M, Nakamura N, et al. Cyclin D2 is overexpressed in proliferation centers of chronic lymphocytic leukemia/small lymphocytic lymphoma. *Cancer Sci*. 2011;102(11):2103-7.
  79. Zhang W, Kater AP, Widhopf GF 2nd, Chuang HY, Enzler T, James DF, et al. B-cell activating factor and v-Myc myelocytomatosis viral oncogene homolog (c-Myc) influence progression of chronic lymphocytic leukemia. *Proc Natl Acad Sci USA*. 2010;107(44):18956-60.
  80. Halina A, Artur P, Barbara MK, Joanna S, Anna D. Alterations in TP53, cyclin D2, c-Myc, p21WAF1/CIP1 and p27KIP1 expression associated with progression in B-CLL. *Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society*. 2010;48(4):534-41.
  81. Patterson HC, Kraus M, Kim YM, Ploegh H, Rajewsky K. The B cell receptor promotes B cell activation and proliferation through a non-ITAM tyrosine in the Ig alpha cytoplasmic domain. *Immunity*. 2006;25(1):55-65.
  82. Chen L, Huynh L, Appar J, Tang L, Rassenti L, Weiss A, et al. ZAP-70 enhances IgM signaling independent of its kinase activity in chronic lymphocytic leukemia. *Blood*. 2008;111(5):2685-92.
  83. Gobessi S, Laurenti L, Longo PG, Sica S, Leone G, Efremov DG. ZAP-70 enhances B-cell-receptor signaling despite absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B cells. *Blood*. 2007;109(5):2032-9.
  84. Zupo S, Isnardi L, Megna M, Massara R, Malavasi F, Dono M, et al. CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. *Blood*. 1996;88(4):1365-74.
  85. Deaglio S, Vaisitti T, Zucchetto A, Gattei V, Malavasi F. CD38 as a molecular compass guiding topographical decisions of chronic lymphocytic leukemia cells. *Semin Cancer Biol*. 2010;20(6):416-23.
  86. Zenz T, Frohling S, Mertens D, Dohner H, Stilgenbauer S. Moving from prognostic to predictive factors in chronic lymphocytic leukaemia (CLL). *Best Pract Res Clin Haematol*. 2010;23(1):71-84.
  87. Calissano C, Damle RN, Marsilio S, Yan XJ, Yancopoulos S, Hayes G, et al. Intracлонаl complexity in chronic lymphocytic leukemia: fractions enriched in recently born/divided and older/quiescent cells. *Mol Med*. 2011;17(11-12):1374-82.
  88. Coelho V, Krysov S, Steele A, Sanchez Hidalgo M, Johnson PW, Chana PS, et al. Identification in CLL of circulating intraclonal subgroups with varying B-cell receptor expression and function. *Blood*. 2013;122(15):2664-72.
  89. Riches JC, Ramsay AG, Gribben JG. Immune dysfunction in chronic lymphocytic leukemia: the role for immunotherapy. *Curr Pharm Des*. 2012;18(23):3389-98.
  90. Vinuesa CG, Chang PP. Innate B cell helpers reveal novel types of antibody responses. *Nat Immunol*. 2013;14(2):119-26.
  91. Negro R, Gobessi S, Longo PG, He Y, Zhang ZY, Laurenti L, et al. Overexpression of the autoimmunity-associated phosphatase PTPN22 promotes survival of antigen-stimulated CLL cells by selectively activating AKT. *Blood*. 2012;119(26):6278-87.
  92. Pepper C, Lin TT, Pratt G, Hewamana S, Brennan P, Hiller L, et al. Mcl-1 expression has in vitro and in vivo significance in chronic lymphocytic leukemia and is associated with other poor prognostic markers. *Blood*. 2008;112(9):3807-17.
  93. Paterson A, Mockridge CI, Adams JE, Krysov S, Potter KN, Duncombe AS, et al. Mechanisms and clinical significance of BIM phosphorylation in chronic lymphocytic leukaemia. *Blood*. 2012;119(7):1726-36.
  94. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*. 2007;26(9):1324-37.
  95. Lesley R, Xu Y, Kalled SL, Hess DM, Schwab SR, Shu HB, et al. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity*. 2004;20(4):441-53.
  96. Enders A, Bouillet P, Puthalakath H, Xu Y, Tarlinton DM, Strasser A. Loss of the proapoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation-induced apoptosis and deletion of autoreactive B cells. *J Exp Med*. 2003;198(7):1119-26.
  97. Oliver PM, Vass T, Kappler J, Marrack P. Loss of the proapoptotic protein, Bim, breaks B cell anergy. *J Exp Med*. 2006;203(3):731-41.
  98. Souers AJ, Levenson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med*. 2013;19(2):202-8.
  99. BCL-2 Inhibitor Yields High Response in CLL and SLL. *Cancer Discovery*. 2014;4(2):OF5.
  100. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol*. 2012;30(5):488-96.
  101. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA*. 2005;102(39):13944-9.
  102. Kern C, Cornuel JF, Billard C, Tang R, Rouillard D, Stenou V, et al. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. *Blood*. 2004;103(2):679-88.
  103. Nishio M, Endo T, Tsukada N, Ohata J, Kitada S, Reed JC, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood*. 2005;106(3):1012-20.
  104. Wang D, Freeman GJ, Levine H, Ritz J, Robertson MJ. Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. *Br J Haematol*. 1997;97(2):409-17.
  105. Kitada S, Zapata JM, Andreeff M, Reed JC. Bryostatins and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. *Br J Haematol*. 1999;106(4):995-1004.
  106. Dicker F, Kater AP, Fukuda T, Kipps TJ. Fas-ligand (CD178) and TRAIL synergistically induce apoptosis of CD40-activated chronic lymphocytic leukemia B cells. *Blood*. 2005;105(8):3193-8.
  107. Young RM, Staudt LM. Targeting pathological B cell receptor signalling in lymphoid malignancies. *Nat Rev Drug Discov*. 2013;12(3):229-43.
  108. Buchner M, Baer C, Prinz G, Dierks C, Burger M, Zenz T, et al. Spleen tyrosine kinase inhibition prevents chemokine- and integrin-mediated stromal protective effects in chronic lymphocytic leukemia. *Blood*. 2010;115(22):4497-506.
  109. Ponader S, Chen SS, Buggy JJ, Balakrishnan K, Gandhi V, Wierda WG, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood*. 2012;119(5):1182-9.
  110. Hoellenriegel J, Meadows SA, Sivina M, Wierda WG, Kantarjian H, Keating MJ, et al. The phosphoinositide 3'-kinase delta inhibitor, CAL-101, inhibits B-cell receptor signaling and chemokine networks in chronic lymphocytic leukemia. *Blood*. 2011;118(13):3603-12.