B-cell receptor signaling in chronic lymphocytic leukemia

Freda K. Stevenson,1 Sergey Krysov,2 Andrew J. Davies,2 Andrew J. Steele,2 and Graham Packham2

1Molecular Immunology Group and 2Cancer Research UK Centre, Cancer Sciences, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, United Kingdom

The B-cell receptor (BCR) is a key survival molecule for normal B cells and for most B-cell malignancies. Recombinatory and mutational patterns in the clonal immunoglobulin (Ig) of chronic lymphocytic leukemia (CLL) have revealed 2 major IgM-expressing subsets and an isotype-switched variant, each developing from distinct B-cell populations. Tracking of conserved stereotypic features of Ig V variable regions characteristic of U-CLL indicate circulating naive B cells as the likely cells of origin. In CLL, engagement of the BCR by antigen occurs in vivo, leading to down-regulated expression and to an unanticipated modulation of glycosylation of surface IgM, visible in blood cells, especially in U-CLL. Modulated glycoforms of slgM are signal competent and could bind to environmental lectins. U-CLL cases express more slgM 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. (Blood. 2011;118(16):4313-4320)

Introduction

If we are to exploit biologic insights for new therapies of hematologic malignancies, it is important to consider 2 distinct features of the target tumor: (1) the developmental process, by which a normal cell becomes transformed into an established tumor; and (2) the susceptibility or resistance of the malignant cells to current therapies. Most investigators do not separate these 2 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.1 In many cases, this restricted view is inevitable because 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 because 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 that relate to pathogenesis. It is even possible now to detect minor clonal expansions, defined as monoclonal B-cell lymphocytes, in ~3% of healthy persons, potentially revealing the very early stages of CLL.2 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 Ig

B-cell malignancies offer a major advantage to investigators, in that the immunoglobulin (Ig) component of the BCR has unique molecular features that mark the tumor cell and reveal the nature of the B cell of origin.3 Most cases of CLL express IgM and IgD, and it is now clear that the disease can be divided into 2 main subsets, based on whether the tumor arose from a B cell before 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,4,5 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. Because the IGHVHDHJ and IGLVLJ 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 2 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.6,7 It appears that each subset has arisen independently during B-cell differentiation, possibly from different B-cell lineages; and although there are morphologic and phenotypic similarities, in the developmental sense they should be considered as 3 diseases. The routes leading to
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.\(^9,\text{20}\) The question is whether the antigen that 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 naive B-cell population of normal blood.\(^14\) The identification of these cellular analogs of CLL in 3 of 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.\(^14\) However, it is possible that the antigenic drive on CLL cells is via autoantigens.\(^19,\text{20}\) 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.\(^22\) 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.\(^10,\text{23}\)

Effects of antigen exposure on CLL cells

Normal sIgM-expressing B cells respond to antigen by activation, proliferation, and differentiation. In both U-CLL and M-CLL, we observe B cells that are apparently responding to antigen, presumably leading to proliferation and/or survival.\(^24\) 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.\(^14\) Although antigens are likely to differ, this overarching distinction could be the result of 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.\(^25\) 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.\(^26,\text{27}\) In contrast, M-CLL cases tend to express less sIgM and show a reduced ability to respond, features that are described as “anergic.” The difference is not absolute, being based on the picture in peripheral blood cells that 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 that 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.\(^28\) 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.\(^28\) 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 “imma-
ture” glycans, which terminate at high mannose (Figure 2). This
residual sIgM may be important for the B cell, possibly
providing a “tonic” survival signal because it is able to mediate
phosphorylation of downstream kinases. The retention of sIgM
in U-CLL in vivo compared with M-CLL appears, therefore,
to be the result of continued expression of the mannosylated
glycoform.
This ability to modulate the sIgM glycoform is not tumor-
specific because it occurs in normal B cells, where it can be induced
by engagement of the BCR, presumably acting as a prelude for
antigen presentation. Although the function of the modified sugars
is as yet unknown, there is a possibility that the expressed
mannose-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 mannosy-
lated glycans. In primary follicular lymphoma, mannose-binding
lectin or DC-SIGN recognizing mannosylated sIgM was each able to
generate sIgM-mediated intracellular Ca2+ mobilization, 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
after 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). Indeed, 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, glycans modification, especially evident
in U-CLL, could be similarly influencing sIgM-mediated events.

**Linkage to clinical behavior**

Differences in antigen-induced modulation of sIgM probably
contribute to the variable clinical behavior of CLL (Figure 3).

**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. The “trigger” event in the
formation of the signalosome is phosphorylation of the
immunoreceptor tyrosine-based activation motifs in the C-terminal
tail of BCR-associated Igα (CD79A) and Igβ (CD79B) by the
SRC-family kinase LYN. Phosphorylated immunoreceptor tyrosine-
based activation motifs 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
tyrosine kinase (BTK) and phospholipase Cγ2 (PLC-γ2). LYN-
dependent phosphorylation of the cytoplasmic domain of CD19
also recruits the p85 subunit of phosphoinositide 3-kinase (PI3K).

**Downstream signaling**

After formation of the signalosome, the second phase of BCR signaling
involves the activation of distal signaling molecules (Figure 4B).
Activation of PLC-γ2 leads to the generation of the downstream
second messengers inositol-1,4,5-trisphosphate and diacylglycerol, which induce
the release of intracellular Ca2+ and activate protein kinase C, respectively.
Protein kinase C subsequently induces the activation of transcription
factors, including NF-κ B and nuclear factor of activated T cells.
Recruitment of PI3K to the plasma membrane leads to the production of
phosphatidylinositol 3,4,5-trisphosphate, which is required for optimal
activation of BTK, as well as for recruitment of 3-phosphoinositide-
dependent kinase and subsequent activation of AKT. PLC-γ2 is also
involved in the activation of mitogen-activated protein kinase pathways,
including the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun
NH2-terminal kinase, and p38 kinases (Figure 4B). The ERK/mitogen-
activated protein kinase 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

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**Figure 2.** Antigen-mediated effects on surface IgM of CLL cells. Antigen
engagement in tissue sites appears to lead to endocytosis of sIgM in both U-CLL and
M-CLL. Endocytic events lead to a modulation of the glycans of the μ-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.
modulation and phosphorylation (Figure 4B). For example, BCR signaling leads to modulation of key regulators of cell survival (eg, 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, which are phosphorylated by LYN on BCR stimulation. This leads to the recruitment of inhibitory phosphatases, such as SH2 domain-containing tyrosine phosphatase-1, SH2 domain-containing phosphatidy l 5-phosphatase-1 and -2, and protein tyrosine phosphatase nonreceptor type 22, which attenuate BCR signaling. Therefore, LYN both positively and negatively regulates signal transduction via the BCR.38

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 biologic outcomes (eg, proliferation, survival, apoptosis, anergy) 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 heterogeneous 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 sIgM engagement, at least using anti-IgM as a surrogate for antigen. As indicated in Figure 4, this is most common among M-CLL and is associated with indolent disease.14 However, even in the subset of samples that do retain sIgM 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 c-Jun NH2-terminal kinase.39 The activation of nuclear factor-κB is also variable compared with ERK1/2.40

The levels of sIgM appear to be one major determinant of these heterogeneous signaling responses. sIgM levels are generally but variably reduced in CLL compared with 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 sIgM signaling responses, especially in M-CLL.14 Interestingly, lack of signaling capacity in CLL is associated with constitutive activation of ERK and nuclear factor of activated T cells consistent with the idea that ongoing antigen engagement in vivo leads to anergy.15 Moreover, constitutive activation of ERK and nuclear factor of activated T cells recapitulates features of anergic B cells, in at least some mouse models.40

Less dramatic sIgM down-modulation, as observed most commonly in U-CLL, results in retention of a degree of signaling capacity, characterized 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.14 It is possible that the heterogeneous responses of blood CLL cells reflect variation in the timing of prior antigen engagement among these recirculating cells. Finally, activation of distinct sIg isotypes may also result in variable responses. sIgM 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 transient14,27 (S.K., C.I.M., K.N.P.,
Persistent ERK1/2 activation is critical for cell cycle entry, so this may explain why sIgD signaling status does not appear to influence disease behavior; although signal competent, the receptor does not effectively couple to downstream growth-supporting biologic responses. The differential behavior of sIgM and sIgD 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 sIgG in vitro, with 9 of 14 cases generating an intracellular calcium flux. Again, there appeared to be an association with the level of expression of sIgG. 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 sIgM-mediated signaling

Although sIgM 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. 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 sIgM signaling capacity. CD38 associates with sIgM 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 sIgM signaling capacity, including the phosphatase PHLPP1, protein kinase C-βII, TCL1, CD79B, LYN, SH2 domain-containing phosphatidylinositol 5-phosphatase-1 and p66SHC. However, because lack of signaling responsiveness is associated with a failure to induce SYK phosphorylation, a very early event in signalosome formation (Figure 4A), it seems that a 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 immobilized, 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 vs 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 after BCR stimulation (Figure 4). 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 PI3K-δ, 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 produg 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 approximately 60% for PCI-32765 and FosD. 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 of 15) of SL/LCL patients achieving partial responses. Although, like SYK, LYN is activated very early after BCR engagement, it plays both positive and negative roles in signal transduction. Dasatinib induces apoptosis in CLL cells and interferes with slgM signaling in vitro. However, LYN-deficient mice demonstrate hyper-apoptosis in CLL cells and interfere with sIgM signaling in vivo. 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 PI3K-δ 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. In CLL cells, SYK phosphorylation is increased in cells stimulated via chemokine receptors and integrins and SYK inhibition reduced migration toward CXCL12 and adhesion to VCAM-1. CAL-101 also interferes with survival effects of CD40L, TNF-α, and fibronectins in CLL cells, and BTK is required for Toll-like receptor signaling. 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 mobilization 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. A reduction in lymphadenopathy was observed in 100% (32 of 32) of patients treated with CAL-101 (www.clinicaltrials.gov as NCT00710528). As discussed in the next section, 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). Homing and retention of CLL cells may also be directly influenced by antigen because anti-IgM can modulate CLL cell migration and chemokine receptor expression. FoS-D interferes with anti-IgM-induced migration. Again, to what extent these mobilization effects are mediated via inhibition of BCR signaling versus other receptor systems is unclear.

**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 probably 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 after 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 slgM, much of it as a glycosylated variant that 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 slgM 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 be aware that none of the targets for these inhibitors is specifically involved in BCR signaling. Understanding the precise therapeutic mechanisms of these agents will be challenging; and indeed, their therapeutic effects may depend 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 mobilization of malignant cells from the tissues to the periphery. 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 \(^77\) (www.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 Eμ-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 toward downstream targets of BCR signaling pathways may be advantageous because 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 with 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 actively inhibit of BCR-induced events would be a particularly attractive strategy because it probably leads to a “reprogramming” of signaling, selectively retaining prosapoptotic responses. In this way, ongoing antigen signalling could also be actively driven 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.

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Authorship

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**Correspondence:** Freda K. Stevenson, Cancer Sciences, Southamptom University Hospitals Trust, Southampton, SO16 6YD, United Kingdom; e-mail: fs@soton.ac.uk.

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