

Celebrating 20 Years of IGHV Mutation Analysis in CLL

Nicholas Chiorazzi¹, Freda K. Stevenson²

Correspondence: Nicholas Chiorazzi, and Freda K. Stevenson (e-mail: NChizzi@Northwell.edu; fs@soton.ac.uk).

Abstract

The division of CLL into 2 broad subsets with highly significant differences in clinical behavior was reported in 2 landmark papers in Blood in 1999.^{1,2} The simple analysis of the mutational status of the IGV regions provided both a prognostic indicator and an insight into the cellular origins. Derivation from B cells with very low or no IGV mutations generally leads to a more aggressive disease course than derivation from B cells with higher levels. This finding focused attention on surface Ig (slg), the major B-cell receptor, and revealed dynamic antigen engagement in vivo as a tumor driver. It has also led to new drugs aimed at components of the intracellular activation cascades. After 20 years, the 2 senior authors of those papers have looked at the history of the observations and at the increasing understanding of the role of slg in CLL that have emanated from them. As in the past, studies of CLL have provided a link between biology and the clinic, enabling more precise targeting which attacks critical pathways but minimizes side effects.

Historical context

The story from Southampton (UK)

The Tenovus Institute was established in 1970 at the new medical school in Southampton and George and I moved there from Oxford. With the help of Tenovus funding, I established a laboratory aimed at studying B cells, while George focused on antibody therapy. The new tools of immunogenetics became available in the early 1990s, and my lab seized on the new opportunity to understand immunoglobulins. Part of the drive arose from our studies of cold agglutinin (CA) disease, where Myf Spellerberg's precious CA-secreting single B cells had been handed to other laboratories for the then novel approach of DNA sequencing. Those labs had the fun of showing that they were all derived from the IGHV4–34 gene. We immediately bought a PCR

machine and Caroline Chapman and I set up a molecular biology laboratory and taught ourselves how to sequence IGV genes, initially using gel-based methods. Protein sequencing had already been applied to clonal Igs secreted by plasma cells, but DNA sequencing added critical information on V(D)J recombination, somatic hypermutation and isotype switch. Interpretation of sequence data however totally relied on having available the libraries of human IGV, D and J genes existing in the unrearranged DNA, which were provided by the labs of Tasuku Honjo, Fred Alt and Greg Winter. For once, human genetics was ahead of the mouse, and we could map the clonal history of any B cell.

For normal B cells, the range and level of IGV gene usage was investigated by Peter Lipsky's group.³ Strangely the functional repertoire in circulating B cells did not simply reflect the available library and a similar selection was evident in different ethnic groups. Rearrangements of one, or sometimes both, alleles were mapped giving information on non-functional IGHV genes. Leaning on the revelations of somatic hypermutation from Cesar Milstein, the Lipsky lab analyzed mutational patterns and revealed differential distribution across IGV sequences. In what seems to be an evolutionary mechanism, most "hot spots" are in the complementarity-determining regions.⁴

In a fairly short time we had sequenced B cell IGHV genes in every possible situation ranging from normal B cells, IgE, EBV infection, autoimmunity to the full range of B-cell tumors. Comparison with the databases allowed insights into the point of differentiation reached by the transformed B cell, and any subsequent changes. During this time, Terry Hamblin, based in the then non-university hospital at Bournemouth, provided the important bridge from his "typical" patient clinic to our lab. One day we discussed whether we should look in detail at the most common B-cell tumor, chronic lymphocytic leukemia (CLL). I had already had a quick look at cases from David Oscier who was focused on chromosomal abnormalities, and we showed that

This study was supported by NC: National Cancer Institute, NIH; CLL Global Research Foundation; Karches Foundation; Marks Foundation; Nash Family Foundation; Jean Walton Fund for Leukemia, Lymphoma, and Myeloma Research; and Lauri Strauss Leukemia Foundation. FK: Cancer Research UK; Experimental Cancer Medicine Centre; University of Southampton and Bloodwise

The authors declare no conflicts of interest

¹Center for Oncology Research, The Feinstein Institute for Medical Research, Northwell Health, Manhasset, NY

²Cancer Research UK Centre, Cancer Sciences Unit, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, United Kingdom

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HemaSphere (2020) 4:1(e334)

Received: 5 November 2019 / Received in final form: 28 November 2019 /

Accepted: 13 December 2019

Citation: Chiorazzi N, Stevenson FK. Celebrating 20 Years of IGHV Mutation Analysis in CLL. *HemaSphere*, 2020;4:1. <http://dx.doi.org/10.1097/HS9.0000000000000334>

cases with trisomy 12 tended to have unmutated IGHV genes whereas those with a sole 13q14 abnormality had higher mutational levels.⁵ This confirmed the view from others that CLL was heterogeneous, but we had only small numbers. Terry and I decided to do a blitz of VH sequencing on the large number of clinically well-documented cases he and David had stored. It meant directing the lab effort toward this, with Zaidie Davis, then in my lab, at the forefront. With RNA as the preferred source to avoid non-functional IGHVs, we used 5'-leader primers to get the full sequence in combination with 3'-primers from mixed JHs or constant regions. Because I was aware of polymorphic differences which could be wrongly counted as mutations, I decided to divide the cases into "unmutated" (U-CLL) and "mutated" (M-CLL) with unmutated being >98% homologous to germline sequence, and this was later agreed with Nick who had independently come to the same conclusion.

On matching to Terry's clinical records, it only took 84 cases to see the dramatic differences in clinical behavior between U-CLL (~40% of cases) and M-CLL (~60%).² Nick meanwhile had done his study with the same result.¹ Other insights emerged, with the over-expressed IGHV1-69 gene reported by Tom Kipps⁶ being found almost entirely in U-CLL while the IGHV4-34 gene, which is not particularly over-expressed, was mainly in M-CLL. To me, the evidence pointed to two different diseases, but it took a while to convince the hematology community of this, partly because, apart from clinical behavior, CLL looks similar in many respects. Kanti Rai asked if our findings meant that once a CLL patient was designated as U-CLL, should he/she wrap themselves in a shroud and climb into a coffin? He knew the answer which was that therapy might be more targeted toward U-CLL.

We discussed our findings with Nick and decided we would each send our manuscripts to the *New England Journal of Medicine*. Both were rejected without review, denying that journal the citations of ~5000 to date. So we sent our document to Nick, and he forwarded both to *Blood* in the same envelope since at that time submissions were made as "hard" copies. Both papers were immediately accepted there and were published with commentaries. A matter of pride is that there was never any competitive edge but only complementarity between Nick's group and ours. In spite of many efforts to replace IGHV mutational status as a prognostic factor, it stubbornly remains the best at all disease stages and allows patients and clinicians to manage CLL in a more informed way. For the biologists, the question of how the cell of origin influences clinical behavior has been paramount. It became clear that Ig is not only a passive marker of differentiation but a major receptor through which CLL is driven, leading to the application of new drugs to target this pathway.

The story from New York (USA)

After completing medical training, I spent postdoctoral periods at Harvard Medical School (Baruch Benacerraf's Department of Pathology working with David H. Katz) and The Rockefeller University (Henry G. Kunkel's Laboratory), becoming exposed to concepts of basic T-cell/B-cell interactions and immunoglobulin structure and function, very foreign ideas for a physician with no basic science experience. It was at Rockefeller that I learned the value of studying clonal populations to derive concepts applying to diverse populations, as Kunkel had done for the structure of IG using myeloma proteins. Based on this, in collaboration with Shu Man Fu, we showed that CLL B cells were not frozen at the membrane Ig-expressing stage of B-cell maturation but could differentiate to plasma cells if T-cell help

or mitogenic signals were provided.⁷ It was also at Rockefeller that I first met Kanti Rai, who provided patient samples for the CLL studies, and Manlio Ferrarini, both of whom became long-lasting collaborators.

Because the focus of the Kunkel Laboratory at that time was autoimmunity, our work for the next decade addressed autoreactive B cells in systemic autoimmune conditions. However after moving to the North Shore University Hospital to start a Division of Rheumatology & Allergy-Clinical Immunology, my experiences studying autoimmune and lymphoproliferative disorders merged. This came about upon realizing that patients with lymphoproliferative disorders could be considered the human equivalent of IG transgenic mice, since both expressed almost exclusively a single B lymphocyte with a single IGHV-IGHD-IGHJ (IGHV-D-J) rearrangement. This spurred investigating CLL B cells and the IG they produced to understand autoreactivity at the clonal level, a major advantage for the study of polyclonal autoimmunity. This also led to re-engagement with Kanti Rai, who worked at Long Island Jewish Medical Center ~1 mile away, and Drs. Steven L. Allen and Jonathan E. Kolitz at North Shore. It also led to rekindling a partnership with Manlio Ferrarini of the University of Genoa. Each of these people became dear friends and close collaborators in our work on CLL and each provided invaluable input to our observations over the years.

Collectively, we set out to interrogate the antigen-specificity and structural properties of CLL IGs, with the help of a series of talented postdoctoral research and clinical fellows. Zev Sthoeger found that CLL cells secreted IGs that reacted with autoantigens,⁸ consistent with findings by Peter Lydyard's laboratory⁹ and reinforcing the notion that studying CLL could serve as a model for autoimmunity. Next, because the pathogenic autoantibodies in autoimmune disorders are isotype class-switched and somatically mutated, Shiori Hashimoto studied the structure of IGs made by IgG⁺CD5⁺ CLL cells. These demonstrated that many but not all such clones bore significant numbers of somatic IGHV mutations and also that human CD5⁺ B cells could develop such mutations.¹⁰ The latter finding was different from that in mice and man and consistent with a review of IGHV-D-J sequences by Harry Schroeder and Guillaume Dighiero.¹¹ To extend these findings to the more frequent IgM⁺ CLL cells, Franco Fais carried out similar experiments with clones producing IgM, using the 2% cutoff decided upon with Harry Schroeder to avoid miscalls due to unidentified genetic polymorphisms. These studies documented that IgM⁺CD5⁺ CLL cells could but need not exhibit IGHV mutations.¹² Collectively, the studies of Hashimoto and Fais documented that CLL cases fell into two subsets defined by the presence or absence of IGHV mutations. Finally on our end, Rajendra Damle and Tarun Wasil made the now classic observation that IGHV-mutation status had major clinical inferences for CLL patients and basic implications for the disease.¹

That these discoveries have been impactful for understanding CLL as a disease, and, most importantly, clinically meaningful to patients, has been extremely gratifying to all of us involved, and continues to motivate our ongoing studies.

Prognostic power 20 years later

It is remarkable that our observation of the 2 subsets with different clinical behavior has remained so significant. One reason is that IGHV status applies to all cases and remains useful for clinicians and patients from diagnosis throughout the disease process.¹³ It is now recognized as the most reliable prognostic factor and is part of the management algorithm,¹⁴ with the recent

IWCLL guidelines recommending that IGHV mutational analysis be carried out in general practice and in clinical trials. An exciting clinical finding is that patients with M-CLL who require treatment respond better to FCR and may in fact be cured.^{15–18} In contrast, patients with U-CLL appear to benefit most from chemotherapy-free approaches, currently represented by ibrutinib. It raises the question as to whether there should be a revision of the 2016 update of the WHO classification which still considers CLL as a single homogeneous entity.

Although powerful, prognostic value of any single feature is never absolute, and, there is an intermediate category where epigenetic probing may be valuable.^{19–21} While genomic information has always been center-stage,^{22–30} chromosomal changes and mutations, at least in early disease stages, generally apply only to a minority of patients. For example, TP53 abnormalities are of obvious significance in planning drug treatment but involve ~10% of patients, mostly in the U-CLL subset.³¹ The majority of chromosomal abnormalities actually lie mainly in U-CLL.³² Those aberrations occurring in M-CLL are often distinct from U-CLL, such as MyD88 (L265P) and KLHL6 mutations, occurring in 5.6% and 4.5%, respectively.³³ Taking biology into account, there is a logic in considering the two subsets as two diseases, and then assessing sub-subsets within these categories instead of across the board.

The cell of origin (COO) of CLL

Indolent tumors, such as CLL, have often not strayed far from the normal counterpart and this allows insight into the cell of origin. Obviously there has been a transforming event but often this is only the del(13)(q14.3), which upregulates BCL2, a likely requirement for survival. The clonal V-gene sequence provides a genetic label which is largely, if not always, stable, and accumulation of somatic mutations in U-CLL such that they convert to M-CLL is extremely rare.^{34,35} Analysis of DNA methylation patterns has added support to the concept of the U-CLL and M-CLL subsets, each of which differs markedly but retains a similarity to the COO.¹⁹ A few cases lie in an intermediate zone between the two subsets, including those involving the IGHV3–21 gene belonging to stereotyped subset #2, which appear to have a relatively poor prognosis independent of mutational status. Nevertheless, the major division holds, with U-CLL and M-CLL differing according to the presence or absence of IGHV mutations. Although the specific stages of B-cell development at which they occur might be debated, here we refer to U-CLL as resembling “pre-germinal center” (pre-GC) and M-CLL as resembling “post-GC” cells.

These findings have overtaken the many attempts to find the COO using phenotypic features, although transcript analysis of normal B cells revealed a continuing connection of each CLL subset with CD5⁺ populations in either pre- or post-GC cells.³⁶ For U-CLL, tracking of CLL-associated VDJ sequences from the over-represented 51p1 allele of IGHV1–69, in combination with IGHJ6, revealed highly similar junctional regions in normal blood B cells, some of which were CD5⁺, indicating an origin of this subset from the natural antibody repertoire, presumably selected over evolutionary time to fight infections.³⁷

However, no tumor cell is exactly the same as its COO partly because the transforming events can influence behavior. Clonal selection based on proliferative activity and exploitation of the microenvironment can occur after transformation, and for CLL this includes engagement with a range of potential (auto) antigens.^{38–43} The membrane phenotype⁴⁴ and telomere

lengths^{45,46} in circulating CLL cells have given rise to the term “antigen-experienced”. However, it is unclear whether this occurred prior to transformation, or if it is acquired after, and whether the antigens in the two settings are the same. Curiously, intracлонаl diversification of IGHV in either subset is rare but can occur. Additionally, there is a relatively rare variant of CLL that has undergone isotype switch to IgG/A. These cases have IGHV asymmetries, like IgM⁺ CLLs, but are not yet fully understood.

An important clinical feature of CLL is immunosuppression which develops early and can lead to hypogammaglobulinemia, likely due to plasma cell loss. This contrasts with most other B-cell tumors and, while occupation of the bone marrow is a factor, suggests a direct influence of CLL cells on developing and mature B cells. In this regard, CLL cells bear some resemblance to regulatory B (B10) cells,⁴⁷ which, in common with anergic B cells, arise in the setting of chronic antigen exposure, express CD5 and suppress B cell responses by producing IL-10.⁴⁸

Structural features of sIg in CLL

The asymmetric usage of IGHV genes is most evident in the over-expression of IGHV1–69 in U-CLL, and adds weight to the distinction between the subsets. Another distinguishing feature of the expressed CLL IGHV-D-J repertoire is the biased presence of discrete VH CDR3 sequences resulting from the association of specific IGHV, IGHD and IGHJ segments leading to “stereotyped” BCRs. These structural aspects have been comprehensively reviewed,⁴⁹ so here we focus primarily on new insights.

One of these is the provocative finding that CLL IGs self-associate, that is, bind each other. This was initially suggested by peptide binding studies,⁵⁰ and then documented by X-ray crystallography for 2 stereotyped BCR subsets.⁵¹ When the “antigen” on one IG interacts with the “antigen-binding site” on the adjacent membrane IG, BCR signaling can be detected *in vitro*;⁵² this has been termed “autonomous signaling”. Based on measuring the affinity of self-association in the crystallized stereotyped subsets, it has been proposed that high avidity interactions lead to more frequent autonomous signaling and consequent dampening of signal transduction and an anergic-like state and better clinical outcome.⁵¹ A so-far unanswered question is whether these IG-IG interactions will occur in the presence of serum IG.

The weight of evidence is that CLL cells are stimulated to divide during passage through tissue sites although the nature of the likely multiple (auto) antigens involved is unclear. Attempts to define these have to take into account an observation on some of the findings on polyreactivity, that is, that when sIgM is expressed as secreted IgG, a mismatch in reactivity can occur, with polyreactivity seen only in the recombinant secreted IgG.⁵³ This might reflect the misfolding that can occur in recombinant soluble Ig which renders proteins “sticky”.

Active B-cell receptor (BCR) engagement of CLL cells occurs *in vivo*

There has been much speculation about why CLL cells express so little sIg. Although sIg is virtually always present, levels are lower than any suggested normal B-cell counterpart, even after recovery of expression *in vitro*. One possibility relates to the recent findings that B cells need a “Goldilocks” level of signaling for maintenance, that is, not too much, which, in the absence of T-cell help, would induce death via mitochondrial dysfunction and accumulation of reactive oxygen species; and not too little,

which would fail to activate the low level signal required to meet the metabolic demands of tumor cells.⁵⁴ CLL cells under constant stimulation have to calibrate their responses, and one way is to express a minimal level of sIg. Additionally, CLL cells can increase numbers of mitochondria and over-express BCR-associated inhibitory phosphatases.⁵⁵ Evidence consistent with CLL cells being driven by encounter with “antigen” in tissue sites arises from phosphorylation of BCR-associated kinases in lymph nodes.⁵⁶ Since antigen exposure generally leads to endocytosis and downregulation of expression of sIg, circulating CLL cells were probed for this imprint.⁵⁷ This showed that blood CLL cells of U-CLL and M-CLL subsets can increase expression of IgM following culture *in vitro*. It provides compelling evidence for downregulation of expression *in vivo*, supporting the concept that it occurred during transient exposure to lymphoid tissue antigen. Attempts to find the antigens involved have pointed to pathogens and autoantigens in U-CLL.⁴³ Although it is more difficult to find candidate autoantigens for the possibly higher affinity BCRs in M-CLL, evidence for microbial reactivity exists.^{58–60} However even in this subset, reactivity with autoantigens can be detected with one example free of potential artifacts being the IGHV4–34-encoded cases which largely retain the amino acids in FR1 which confer low affinity reactivity with the red cell I/i autoantigen.⁶¹

The dynamic nature of CLL cells after stimulation which occurs before exiting from and upon entering antigen-expressing tissue leads to circulating cells that are heterogeneous in basal sIgM levels both within and between patients. There is also an apparent overall difference between the two subsets with U-CLL generally expressing higher levels of sIgM than M-CLL.⁵⁷ This is functional as shown using anti-IgM-induced Ca^{++} flux and phosphorylation of kinases as readouts, with, in general, U-CLL cells responding more than M-CLL.⁵⁷ In fact, higher expression alone can be used as a partial surrogate for IGHV mutational status in predicting clinical behaviour.⁶² Although levels of sIgM appear stable in patients, they can be modulated by cytokines, particularly IL-4, which increases expression, mainly in U-CLL, via upregulating CD79B.^{63,64} This has clinical relevance since it is more difficult to inhibit sIgM-mediated signaling with ibrutinib after exposure to IL-4.⁶³ Curiously, response to engagement of sIgD does not show variability between subsets, and, in contrast to sIgM, there is no evidence for downregulation of expression *in vivo* or of a response to IL-4. Although difficult to explain, this differential effect on the two isotypes mirrors that of anergic normal B cells in mouse models and in human B cells.⁶⁵

The reciprocal densities of membrane CXCR4 and CD5 have allowed subsetting of CLL clones in the blood into fractions that recently left tissue sites after birth,^{66,67} the latter defined by the incorporation of deuterium into replicating DNA of CLL cells.⁶⁸ This approach indicated that the most recently born cells (CXCR4^{Dim}CD5^{Bright}) express genes associated with vitality, survival and growth and the “oldest” cells (CXCR4^{Bright}CD5^{Dim}) express genes associated with frailty and cell death. Moreover the CXCR4^{Dim}CD5^{Bright} and CXCR4^{Bright}CD5^{Dim} fractions differ in sIgM levels with the former displaying more than the latter.^{66,69} The gene expression panel and surface phenotype are consistent with the older, sIgM lower cells needing to re-enter the tissue microenvironment to survive and for IL-4 to provide rescue signals and lead to increased sIgM expression.

Other cells in the clone appear to be driven into anergy⁵⁷ with downregulation of sIgM and CXCR4 expression, and expression of both can recover during transit in the blood before returning to tissue. This recovery can be mirrored by incubating cells *in vitro*.

The various pressures on tumor cells in the tissues clearly lead to a variety of outcomes with downregulation of CXCR4 being common to all, but effects on sIgM varying, likely due to whether the cell has responded to antigen by dividing or, if the stimulus is inadequate, by becoming anergic. This dichotomy is reflected in the variable effects on sIgM, as summarized in Figure 1.

In addition to the effects of specific ligands on receptors, there is apparent cross-talk between the induced responses, perhaps best described for sIgM-induced anergy which can affect other receptors.⁷⁰ The influence of sIgM engagement is clearly evident for CXCR4^{71,72} and could be mediated via SHIP1/DOK1 axis, since complexes of these proteins can reduce PI3K-mediated signaling at remote receptors.⁷⁰

Anergy and CLL

If tissue drivers are autoantigens, there will be few, if any, cognate CD4⁺ T cells available to provide help. Again it is useful to consider the differences between U-CLL and M-CLL. U-CLL most closely resembles B cells derived from the natural antibody repertoire. Responses of the normal B cell counterparts to antigen, which is often microbial and multivalent, are usually considered to be T-cell independent. Although not absolute, there is a greater reliance on CD40L-like cytokines such as BAFF and APRIL derived from a multitude of innate immune cells.⁷³ In contrast, the normal counterpart of M-CLL is closer to a memory B cell which will be vulnerable to apoptosis on encountering high affinity antigen and more dependent on cognate TFH cells.⁷⁴ However for both pre-GC and post-GC normal B cells, low affinity antigens, including most autoantigens, can induce anergy, and this is evident in both U-CLL and M-CLL, especially the latter.⁷⁰ Anergy can be defined as a mechanism of tolerance whereby autoreactive B cells are rendered non-responsive to activation via the BCR. Normal anergic B cells are susceptible to apoptosis, but CLL cells may be protected by over-expression of BCL-2. Persistent anergic tumor cells may be relatively harmless, but there is the danger of reverting to responsiveness. The real question for each subset of CLL is what happens in tissue sites where (auto)antigen is encountered and where the limited availability of T-cell cytokines or those from innate cells, allows some cells to proliferate while others are anergized.

Targeting the sIg-mediated pathways

Therapeutic targeting of ongoing BCR activation by inhibiting essential enzymes in the signaling process have led to dramatic clinical results in CLL. These include ibrutinib,⁷⁵ which targets Bruton’s tyrosine kinase (BTK), and idelalisib,⁷⁶ which inhibits PI3K δ , and next generation derivatives of the two.^{77,78} The most efficacious to date has been ibrutinib,⁷⁹ which is now approved for the treatment, alone or in combination, of patients at all stages of the disease. BTK inhibition by ibrutinib occurs very rapidly leading to inhibition of CLL cell proliferation *in vitro* and *in vivo*.^{80–82} Moreover, BTK inhibition affects the CXCR4-CXCL12 axis directly and integrin signaling indirectly,^{81,83–85} both of which prevent returning to and might promote exiting from solid tissue niches. This leads eventually to death because of the lack of survival signals delivered by interaction with tissue-fixed antigen and IL-4 in the tumor microenvironment. Interestingly, although the cells change shape and size, sIgM expression increases, mimicking that seen *in vitro*,⁸⁶ possibly reflecting that CLL cells blocked from entering tissue sites are no longer able to engage antigen. Worryingly, the cells remain

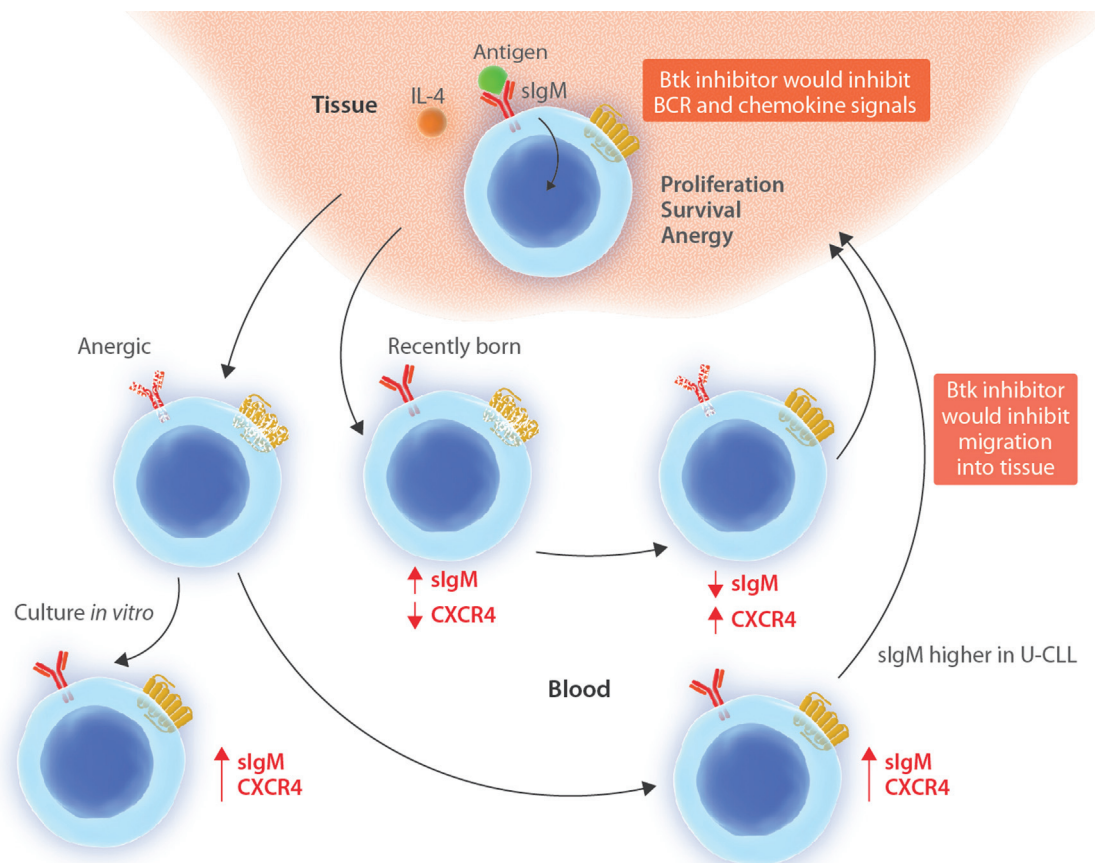


Figure 1. Dynamic events affecting the B-cell receptor of CLL cells in vivo. CLL cells circulate through lymphoid tissues and are exposed to “antigen” and to microenvironmental influences including IL-4. The outcome of these interactions is proliferation or anergy, generating heterogeneity in expression of sIgM. The recently born cells and the anergic cells that emigrate from the tissues undergo a dynamic change in membrane phenotype involving sIgM and CXCR4. Changes in both markers occur in vivo during vascular passage and in vitro. The passage of tumor cells through tissue sites and the nurturing engagement of “antigen” are inhibited by ibrutinib and by other BCR- and chemokine receptor-targeting drugs.

capable of responding to sIgM engagement by phosphorylation of upstream Syk. Should ibrutinib levels decrease because of discontinuation due to side effects or lack of patient compliance, the brakes on CLL-cell growth and migratory capacity are released, and the cells quickly return to full function. This may also occur with the outgrowth of clones containing enabling mutations in the BTK pathway.

The continuing role of the BCR

For the indolent B-cell tumors, it is quite extraordinary to see how the BCR is exploited for its ability to control cell responses and environmental interactions to maintain growth and survival. CLL appears to use lowered sIg expression to interact in just the right way with tissue autoantigen. Upregulation of BCL-2 is likely to be a necessity, as it is for follicular lymphoma, although the mechanism of upregulation is strikingly different between the two. There are likely to be many attempts by expanded clones to get further, but they might fail and remain as monoclonal B-cell lymphocytosis (MBL). To progress, there may be a need for modulation by CD40L and local cytokines, likely from innate cell populations or from a low number of available cognate T cells. The differential grading of these influences which reflects the COO is probably the key to the difference between U-CLL and M-CLL. There will be overlap, with some cases of M-CLL

avoiding the strong anergic signal which seems to operate on this subset, possibly due to variability in the antigen involved. This sub-subset deserves more study, especially as it apparently is responsive to FCR. Stimulation of the BCR leads to changes in expression of chemokine receptors and adhesion molecules,³⁷ all of which are required for tumor expansion. Both subsets of CLL have found a way to avoid the homeostatic control of cell numbers in the blood, possibly because they are cheating the system by low level engagement of antigen. Whatever the mechanism, inhibition of the BCR or anti-apoptotic pathways makes biological sense, and it is working for patients.

Acknowledgments

The authors thank all their colleagues who have contributed to the CLL story over the past 20 years. It is impossible to refer to all but FKS would like particularly to thank Dr. Francesco Forconi and the Southampton team for clinical and scientific insights and NC thanks Drs. Manlio Ferrarini and Kanti Rai and his entire group, past and present, for the same reasons. FKS also acknowledges the support of Cancer Research UK; the Experimental Cancer Medicine Centre; University of Southampton and Bloodwise. NC is grateful for the support of the National Cancer Institute, NIH; the Karches Foundation; the Marks Foundation; The Nash Family Foundation; the Jean

Walton Fund for Leukemia, Lymphoma, and Myeloma Research; and the Lauri Strauss Leukemia Foundation. Both authors thank the CLL Global Research Foundation.

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