Monoclonal B-cell lymphocytosis (MBL): right track or red herring?

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Running title: B-cell premalignant states

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Summary

Monoclonal B-cell Lymphocytosis (MBL), a newly recognized entity found in about 3% of normal individuals, precedes Chronic Lymphocytic Leukemia (CLL). However MBL progress into overt malignancy only in a very minor portion of cases thus raising the clinical concern of whether and how we can discriminate at diagnosis which rare cases will evolve into a fully fledged tumour. Understanding the molecular/biological features underlying the risk of progression may significantly modify our strategies for correctly managing B-cell premalignant states. MBL cells bear the same chromosomal abnormalities of CLL. Genome wide sequencing and animal models indicate that genetic abnormalities disrupting the control of cell growth and survival cooperate with microenvironment-triggered events, mainly represented by Antigen-mediated B-Cell-Receptor and co-receptors stimulation, to trigger and fuel clonal expansion. The initial functional activation of survival/proliferation pathways may later become subsidized by autonomous genetic abnormalities (e.g. a single mutation) affecting the same or parallel critical signalling pathway(s).
Mature B-cell tumors represent approximately 4% of new cancers arising each year in the world\(^1\). B-cell premalignant states are vastly more common but only rare cases progress into overt malignancy raising the clinical concern of whether and how we can discriminate at diagnosis which cases will evolve into a fully fledged tumour. At diagnosis we are presently unable to unequivocally state to the individual subject to which category (progressive or non-progressive) he/she belongs. This causes frustration in the doctor and anxiety in the subject both leading to controls and tests that are economically and socially costly. Understanding the molecular/biological features associated with a risk of progression would significantly impact on the strategies of clinical management of B-cell premalignancies. This would allow physicians to focus onto the rare risky subjects and to refrain from unnecessary monitoring the majority of otherwise healthy individuals.

The presence of tiny numbers of apparently indolent monoclonal B cells with a malignancy-specific phenotype is common to the very early phases of most mature B-cell tumours. The time-honoured example is Monoclonal Gammopathy of Uncertain Significance (MGUS) which has an incidence of 1% in the population over 50 years of age (and up to 10% over 75 yrs)\(^2\). At least two independent studies\(^3,4\) have demonstrated that most cases of multiple myeloma (MM) are preceded by MGUS whose transformation rate is 1-2%/year\(^5\). A more recent example of B-cell premalignant state is the detection of \textit{in situ} lymph node aggregations of cells that reproduce the immunophenotypic and molecular features of follicular lymphoma (FL)\(^6,7\) and/or of mantle cell lymphoma (MCL). These aggregations that appear to represent \textquoteleft \textit{in situ lymphomas}’’ are found incidentally, show a localization restricted to the areas usually involved by FL or MCL\(^8\) and have an uncertain clinical behaviour.

The entity defined Monoclonal B-cell Lymphocytosis (MBL)\(^9\) has given great impulse to investigate how B-cell premalignant states relate to overt malignancies.
Monoclonal B-cell lymphocytosis (MBL): *terra incognita*

MBL are found in about 3% of normal individuals and even attain higher frequency (above 10%) when more sophisticated analytical techniques are applied\textsuperscript{10-13}. Most cases are characterized by the presence of circulating monoclonal B cells which have the phenotype of chronic lymphocytic leukemia (CLL-like MBL). MBL are more common among males and in the advanced age (same gender and age predisposition as CLL) and their frequency is significantly increased (13.5%) in relatives of CLL patients in line with the well known familial character of CLL\textsuperscript{14,15}. A large study has demonstrated that, as MM is preceded by MGUS, CLL is always preceded by an MBL state\textsuperscript{16}. It has also been shown that MBL may progress into CLL with a frequency of 1-2%/year\textsuperscript{17-19}. That notwithstanding, as with MGUS, we do not have a clue as to whether individual subjects with MBL will or will not progress into fully fledged CLL nor when this event will occur.

MBL is complex, heterogeneous and it arises in a background of several B cell subsets. First, circulating MBL can be found whose phenotype differs from CLL (non-CLL-like MBL)\textsuperscript{11}. These MBL might result from the transient activation of the immune system by agents such as those causing intercurrent infections\textsuperscript{20}. Still the possibility exists that they may be premalignant states of other mature B-cell malignancies (e.g. Splenic Marginal Zone Lymphoma) bearing similarities to “in situ lymphomas”\textsuperscript{7,8} Next, two categories of CLL-like MBL exist\textsuperscript{21}. One is represented by *clinical MBL*, as we may call the MBL which are detected in the context of lymphocytosis investigated with laboratory techniques\textsuperscript{17}. The second category is represented by the MBL discovered while screening perfectly normal individuals with the specific scientific purpose of identifying the presence of an MBL population\textsuperscript{10}. In what we might call *population-screening MBL* the absolute number of lymphocytes is not increased, rather a number of monoclonal B cells, in the range of few dozen monoclonal B cells/μl, is detectable within a vast majority of polyclonal B lymphocytes\textsuperscript{22}. Further, some phenotypically *population screening* CLL-like MBL are
Indeed polyclonal\textsuperscript{10,23}. Clearly the suggestion is that, similar to the events occurring in other cancers, the development of CLL may start from a mixture of polyclonal cells with one clone progressively taking over.

Surprisingly not only clinical MBL\textsuperscript{17,18} but also population-screening MBL bear the cytogenetic abnormalities which are the hallmark of CLL, including 13q\textsuperscript{-}, 17p\textsuperscript{-}, trisomy 12\textsuperscript{12,20}. In population-screening MBL these abnormalities are observed even when the number of circulating monoclonal CLL-like cells is extremely small and the subjects do not show any evidence of progression. This finding recalls the observation that in most MGUS monoclonal plasma cells frequently bear the same chromosomal abnormalities of MM\textsuperscript{24,25} and that cells carrying the t14;18 translocation, the cytogenetic hallmark of FL, can be found in the peripheral blood of around 50\% of healthy individuals\textsuperscript{26}. Accordingly it may be asked whether the investigation of MBL is pursuing the right track toward understanding the development of CLL.

The key question

The key question raised by these observations is whether, given enough time, all population-screening MBL will become clinical MBL and in turn every clinical MBL will become overt CLL. In other words whether we have a time-dependent pipeline of unavoidable events or whether population-screening MBL may be simply considered an example of immune senescence while clinical MBL are already CLL-committed. More realistically, the possibility has to be taken into account that clinical MBL may be an offspring of rare population-screening MBL progressing because of a random, individual (unpredictable?) event and similarly that each full-blown CLL is a rare, unfortunate descendant emerging from the large reservoir of clinical MBL.

To this end two related issues deserve mention. The first is whether the development of MBL is an inevitable fate for all human beings provided they live long
enough. This appears to be a reasonable possibility considering that the MBL frequency increases with age becoming 50-75% in individuals above 90 years of age\textsuperscript{12,27,28}. The second aspect to consider is whether given enough time every single CLL will progress from stage 0 to stage 4. Although this is the prevalent situation there are certainly some stage 0 CLL patients who are absolutely stable during a period of more than 20 years.\textsuperscript{29} Their malignant cells have an incapacitated proliferative activity but a very efficient survival, not unlike the “tumour dormancy” observed in solid tumors. We might increase the IWCLL allowable threshold guidelines to diagnose stage 0 CLL\textsuperscript{30}. This would allow some rare stage 0 patients who do not progress to be demoted into an MBL state (where they conceptually belong to)\textsuperscript{31-33}. That would perhaps allow a more proper classification of MBL and possibly reassure some rare patients. However, because clinically it would be retrospective, it would not lead to an understanding of which MBL will progress and which will not. To approach this problem we have to integrate two different perspectives.

The genetic and microenvironment perspectives

If CLL-associated cytogenetic abnormalities are found already in population screening MBL we have to start reconsidering the actual pathogenetic role of the “classic” (cyto)genetic abnormalities. As the genetic architecture of CLL is progressively unravelled by microRNA studies and by deep-sequencing investigations a new field of research is geared into action aimed at detecting novel genetic driving forces able to ignite the cellular proliferation that leads to clonal expansion. Are the rare cases that progress from MBL into CLL cancer-committed from the very beginning because of hitherto unknown mutations that affect critical gene function(s)? Or are they “corrupted” during their lifetime? How can we detect them? Is the risk of acquiring such (unknown) mutations maintained throughout the entire MBL life so that it will be impossible to ever stop worrying about progression?
The first attempts of genome wide sequencing are identifying new relevant genes such as MyD88\textsuperscript{34}, Notch1\textsuperscript{34} and SF3B1\textsuperscript{35,36} that are mutated in a variable though essentially small proportion of CLL cases. It is of interest that the spliceosome mutation SF3B1 occurs primarily in CLL with 11q\textsuperscript{-36} and that Notch1 mutations are associated with trisomy 12\textsuperscript{37}. In general these recurrent mutations appear to be acting in the later stages of CLL and some may underlie the Richter Syndrome transformation\textsuperscript{38}. Conceivably some mutations may be harboured by a subclone that will progressively take over. It is intriguing that, all together these abnormalities appear to be elucidating the genetic bases of CLL progression rather than of MBL evolution: the initiating genetic abnormalities remain unclear. These evidence may simply underline the necessity of different technical and experimental approaches able to unravel also the initiating lesions. However, one should also take into account the possibility that the role of genetic abnormalities may be very limited or even non-existing in the early phases of MBL development, where microenvironmental stimuli could instead play a more prominent role.

Along the same reasoning, one has to consider that also CLL cells need a continuous support from the surrounding tissue microenvironment in terms of signalling pathways that favour clonal expansion, account for intra-clonal complexity and create a situation advantageous for the development of dangerous subclones\textsuperscript{39}. A critical component of the CLL microenvironment is antigen (Ag) stimulation through the B-cell antigen receptor (BCR)\textsuperscript{40}. The striking degree of sequence similarity, observed in the BCRs of almost 30\% of unrelated CLL cases regardless of the IGHV gene mutational status of the corresponding antibodies\textsuperscript{41,42}, has been taken to indicate the promoting pressure of a limited set of structurally similar antigenic epitopes. The dissection of the relevant epitopes indicates that molecular structures normally involved in eliminating cellular debris, scavenging apoptotic cells and providing a first line of defense against pathogenic bacteria\textsuperscript{43-45} may trigger and/or facilitate the onset and evolution of at least
some CLL clones. Similarly, evidence exist that the parallel stimulation of co-receptors such as CD40 and/or TLRs is likely relevant to obtain a full activation of leukemic B cells\textsuperscript{46,47}. These observations lead us to conclude that Ag-induced activation may represent a key triggering event able to promote the development of MBL and to favour their progression into CLL by providing an ongoing proliferative stimulation.

**Can animal models help solving the conundrum?**

Recently numerous animal models of CLL have been developed. Their analysis leads to consider three elements of main interest. The first is related to the TCL1-tg mouse model (the most studied in CLL)\textsuperscript{48}. When TCL1 mice are crossed with mice knocked-out for specific genes potentially relevant for CLL (examples being BAFF\textsuperscript{49}, HS1\textsuperscript{50} and Tir8\textsuperscript{46}) the progeny of knock-out/transgenic mice always show an evident acceleration of disease development. Therefore these genes appear to actually influence the natural history of the disease but at the same time, were all these genes concomitantly operating in patients, CLL should be a very aggressive disease in all cases and this is not the case.

The second element of interest is provided by a recent model where the deletion of the entire 13q14-minimal deleted region (MDR), which encodes the DLEU2/miR-15a/16-1 cluster\textsuperscript{51,52} leads to the development of low penetrance indolent B-cell clonal lymphoproliferative disorders that appear to recapitulate the whole spectrum of human CLL-associated phenotypes, from MBL to Richter’s syndrome\textsuperscript{53}. Interestingly, a naturally-occurring animal model, the New Zealand black (NZB) mouse, has been shown to carry a point mutation in the same region of synteny, in particular in the 3' flanking sequence of the identical microRNA, mir-16-1\textsuperscript{54}. Also in this model, the animals develop a monoclonal lymphoproliferative expansion characterized by increased numbers of CD5\textsuperscript{+} B220\textsuperscript{dull} B cells and the full disease appears only later during lifetime. The functional dissection of the 13q14 tumor suppressor locus provided by these models underlines the critical importance
of the deleted region which appears to harbour the gene(s) involved also in the first steps
that lead to CLL. At the same time, it also indicates the importance of antigenic stimulation
considering both the stereotypic gene usage by clonal cells\(^{55}\) and the long time of onset of
each condition. The conclusion is that genetic abnormalities disrupting the control of cell
growth and survival cooperate with antigenic stimulation to trigger and fuel clonal
expansion. This conclusion is supported by xenograft models based on the transplantation
of immunodeficient mice with human cells. A novel adoptive transfer model of human CLL
in NOD/Sci-scid- IL2rg\(^{-/-}\) (NSG) mice gives strength to the idea already apparent from \textit{in vitro}
studies\(^{56}\) that autologous CD4\(^+\) T cells activated \textit{in vivo} by alloantigens have a
relevant role in promoting CLL cell survival and expansion.\(^{57}\)

The third interesting aspect is the recent demonstration in a xenogeneic mouse
model\(^{58}\) that hematopoietic stem cells (HSC) from CLL patients have the propensity to
generate clonal B cells (though the VDJ genes are always unrelated to those of the
original CLL cells) suggesting that CLL-HSC have intrinsic abnormalities that cause their
skewing toward the B-cell lineage.

Taken together animal models corroborate the scenario emerging in humans and
suggest that critical genes are the instigators that pave the way toward the development of
MBL/CLL and that ongoing antigenic stimulation through the BCR together with accessory
cells operating within specific microenvironmental niches are the final executors (figure 1).

\textbf{A plausible working hypothesis}

It is reasonable to postulate that the evolution of MBL follows a pattern reminiscent
of human polygenic non-malignant diseases where tilting the balance toward a more or
less aggressive disease depends on which “passenger” or influential genes are turned on
and also on which environmental elements are coming into action\(^{59}\). We have therefore to
start asking whether the concept of driver and passenger genes applies to CLL and how it may relate to the disease development.

Genome wide sequencing\textsuperscript{34,36,38} and animal models indicate that likely we have not to concentrate upon mutations of individual driver genes but rather upon the activation of different critical pathways which may be geared into action because of both stimuli originating from the microenvironment surrounding the leukemic cells and/or a number of different genetic abnormalities. Microenvironment-triggered events, represented by Ag-mediated BCR ligation complemented by the stimulation of co-receptors including CD40\textsuperscript{47} and Toll-like receptors (TLR)\textsuperscript{60}, are more likely to occur initially and may be maintained throughout the whole natural history providing a multiplier effect. One may speculate that the repeated/prolonged activation of target cells by daily apoptosis byproducts and/or bacteria elimination may favor cell-cycle entry and increased proliferation explaining the unusually dynamic kinetic behaviour of CLL cells\textsuperscript{61}.

The initial functional activation of survival/proliferation pathways in target cells may later become subsidized by autonomous genetic abnormalities (e.g. a single mutation) affecting the same or parallel critical pathway(s). As an example MyD88 mutations\textsuperscript{34} may have the same consequences of an unabated cellular stimulation due to infectious/inflammatory agents that trigger TLR\textsuperscript{46}. As highlighted by the identification of different recurrent mutations\textsuperscript{34,36,38}, multiple identical or parallel pathways can be involved. This would explain why to date the task of identifying single recurrent and common mutations in CLL remains unaccomplished). It is nevertheless conceivable that all the stimuli coming from multiple identical or parallel pathways may converge to a final common pathway. An important candidate is Nfkb\textsuperscript{62,63} considering that its abnormalities may be caused by MyD88 and Notch1 mutations as well as by the activation induced by microenvironment stimuli, deriving from the activation of the BCR, TLRs, CD40 and CXCR4\textsuperscript{64}. 
The conclusion is that we may have different ways to reach the final result of MBL progressing into CLL. These possibilities span from critical gene mutations to microenvironmental stimulation (including Ag) the latter being perhaps the most frequent causative event especially if superimposed onto a CLL-prone genetic background (Fig. 1). If we accept this reasoning, attempts to prevent Ag stimulation either by antibiotic treatment or preventive vaccination might be helpful in hindering deleterious cellular activation. Attractive though still speculative evidence exist in the literature for a link between respiratory infections and increased risk to develop CLL. It remains to be demonstrated whether this association may simply reflect underlying immune disturbances present prior to CLL diagnosis.

The clinical problem

In clinical terms our biological ignorance translates into a simple question. What should we do when we encounter a clinical MBL? And how should we behave when we scout a population screening MBL? The most sensible conclusion is that for clinical MBL we have to consider a follow up MGUS-style, first reassuring the affected individuals that their risk of progression toward a full-blown leukemia is in the range of 1-2%/year and then suggesting a yearly hematologic consultation with a complete blood cell count and whenever deemed useful abdominal US/chest RX, with no invasive investigations whatsoever. For population-screening MBL, based on the most recent evidences the risk of progressing and developing CLL is very low if any and likely not different from that of the general population. Therefore, the most reasonable attitude is to continue investigating these subjects in order to establish the genetic architecture of MBL as this would allow defining which genes are critically involved in evolution, which critical pathways are involved and how they relate to microenvironmental influences. These investigations will solve the question whether the investigation of MBL is pursuing a right
track or whether at least some MBL are irrelevant. In any case defining which elements are meaningful in the transition of MBL into overt CLL and therefore pinpointing which MBL should alert the physician would represent a major achievement. If the current concept that inflammatory/infectious agents may play a role in the appearance of CLL-like cells will turn to have solid ground, pilot trials investigating either vaccination strategies or antibiotics/anti-inflammatory profilaxis could be proposed.
Acknowledgements
The work of the Authors was supported by: Program Molecular Clinical Oncology-5 per mille number 9965 and Investigator Grant from Associazione Italiana per la Ricerca sul Cancro (AIRC - Italy); U.S./European Alliance for the Therapy of CLL, CLL Global Research Foundation (Texas, US); Cariplo Foundation (Italy); PRIN, MIUR (Italy), Ricerca Finalizzata Ministry of Health (Italy).

Authors’ Contributors
PG searched the literature, wrote the manuscript, and drew the figure; FCC discussed the current literature, wrote the manuscript and drew the figure

Conflicts of interest
The authors declare no conflicts of interests.
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FIGURE LEGENDS

Figure 1: The progressive evolution from antigen (Ag) stimulation to molecular abnormalities in the natural history of CLL.

MBL are vastly more common than CLL and only a tiny fraction of CLL progress into more advanced stages of the disease or evolve into Richter’s syndrome or undergoes prolymphocytoid transformation (shaded triangle indicating the progressively decreasing size of individual situations).

Hematopoietic stem cells (HSC) of CLL patients have been suggested to carry intrinsic abnormalities and to be skewed toward B-cell lineage (top left corner). The encounter of such a B cell with an appropriate external stimulus (here exemplified with the antigen:BCR interaction) triggers the clonal development of MBL likely enhancing the cell stimulation. The possibility exists (bottom left corner) that external stimulation may precede the appearance of genetic anomalies. Over time the pressure of (e.g. antigenic) stimulation leading to enhanced proliferation favours the acquisition of (initial or additional) genetic abnormalities and some MBL may progress into overt CLL. If and when the acquired genetic abnormalities (e.g. recurrent mutations) are able to substitute the necessity of external stimulation by impinging upon the same final pathway(s), a more aggressive version of CLL is acquired (either in the form refractory disease or of Richter’s syndrome or of prolymphocytoid transformation).
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