Immunogenetic Studies of Chronic Lymphocytic Leukemia: Revelations and Speculations about Ontogeny and Clinical Evolution

Anna Vardi1,2, Andreas Agathangelidis3, Lesley-Ann Sutton4, Paolo Ghia3,5, Richard Rosenquist4, and Kostas Stamatopoulos1,2,4

Abstract

Over the last decade, immunogenetic analysis of B-cell receptor immunoglobulins (BcR IG) has proved instrumental in dissecting chronic lymphocytic leukemia (CLL) pathogenesis. Initially, it was the finding that the level of somatic hypermutations in rearranged IG heavy-chain genes could define two CLL subtypes associated with a different clinical course that drew attention. As the years ensued, this not only continued to hold strong, but also revealed an unprecedented BcR restriction (aptly coined as “stereotypy”), thus cementing the idea that antigenic elements select the leukemic clones. With all this in mind, in the present review, we focus on the CLL BcR IG, a molecule that clearly lies at the heart of disease pathogenesis, and attempt to distil from past and emerging biologic knowledge the most relevant aspects in the context of the immunogenetics of CLL, while at the same time provoking questions that remain unanswered.

We juxtapose CLL with mutated BcR IGs against CLL with unmutated BcR IGs due to their striking clinicobiologic differences; however, when considering ontogeny, common derivation of the two mutational subtypes cannot be excluded. The issue of stereotypy is intertwined throughout and we also raise the subject of isotype-switched CLL, which, despite its rarity, contributes intriguing ontogenetic hints. Cancer Res; 74(16): 4211–6. ©2014 AACR.

Immunogenetic Analysis in Chronic Lymphocytic Leukemia: Early Evidence of Immunoglobulin Repertoire Restriction

Studies from the 1990s offered the first indications of immunoglobulin (IG) gene usage restriction in chronic lymphocytic leukemia (CLL; refs. 1–3). A turning point in CLL biology came in 1999 when Hamblin and colleagues (4) and Damle and colleagues (5) independently demonstrated that the somatic hypermutation (SHM) load of the rearranged IGHV genes defines two subgroups of patients with CLL with distinct prognosis: those carrying unmutated IGHV genes (U-CLL) experienced an aggressive clinical course with clonal evolution and resistance to therapy, which translated into a shorter overall survival when compared with patients carrying mutated IGHV genes (M-CLL). All subsequent studies have reached similar conclusions, linking clinical outcome with the molecular characteristics of the clonotypic IGs, which is in itself a strong argument for antigen selection in disease ontogeny and evolution (6, 7).

The Concept of B-cell Receptor Stereotypy

A paradigmatic exception to the prognostic value of SHM concerns IGHV3-21 gene usage, which was repeatedly shown to represent an adverse prognostic factor regardless of the IGHV mutational load (8, 9). In 2003, Tobin and colleagues demonstrated that nearly half of the IGHV3-21 CLL cases display highly distinctive B-cell receptor (BcR) IGs with quasi-identical heavy complementarity determining region 3 (VH CDR3) and restricted usage of the IGLY3-21 gene, and suggested the existence of a common antigenic epitope (10). Soon afterward, subsets of unrelated cases with highly homologous, “stereotyped” VH CDR3s/BcRs were reported among both U-CLL and M-CLL, collectively accounting for one third of all cases (11–13). BcR IG stereotypy strongly supports antigen selection at some time point in the pathway leading to CLL.

In 2007, we first showed that similarities in stereotyped CLL subsets extend from primary IG sequences to shared clinicobiologic characteristics (14). Major subsets #1, #2, #4, and #8 amply exemplify this concept. For instance, cytogenetic aberrations are differentially distributed among these subsets, with del(11q) predominating in subset #2, trisomy 12 and t(14;19) (q32q13) in subset #8, and del(13q) in subset #4 (15, 16). Furthermore, NOTCH1 mutations are preferentially found in subsets #1 and #8, whereas SF3B1 mutations are strikingly

1Institute of Applied Biosciences, Center for Research and Technology Hellas, Thessaloniki, Greece. 2Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece. 3Division of Molecular Oncology and Department of Onco-Hematology, IRCCS Istituto Scientifico San Raffaele and Fondazione Centro San Raffaele, Milan, Italy. 4Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Sweden. 5University Vita-Salute San Raffaele, Milan, Italy.

Corresponding Author: Kostas Stamatopoulos, Institute of Applied Biosciences, Center for Research and Technology Hellas, 57001 Thessaloniki, Greece. Phone: 30-2310-498271; Fax: 30-2310-498270; E-mail: kostas.stamatopoulos@gmail.com

doi: 10.1158/0008-5472.CAN-14-0630
©2014 American Association for Cancer Research.
enriched in subset #2 (17, 18). These findings imply that common genetic aberrations acquired and/or selected in the context of shared immune signaling mediated by distinctive BcR IGs could shape the natural history of a given subset. Ultimately, the unique immunogenetic profile of each of these major subsets may underlie a distinct clinical outcome, i.e., subset #4 patients experience a particularly indolent disease course, whereas subsets #1, #2, and #8 are aggressive and enriched in cases requiring treatment, with the latter also displaying the highest risk for Richter's transformation among all CLL cases (14).

**IG-Unmutated CLL**

**IG gene repertoire**

In U-CLL, the IGHV1-69, IGHV4-39, and IGHV1-2 genes are collectively used in approximately 40% of cases, with the former expressed in a quarter of all U-CLL (19). Rearrangements of these genes show a strong bias for certain IGH and IGH genes, frequently leading to the generation of VH CDR3s with shared motifs, defining characteristic stereotyped U-CLL subsets, e.g., #3, #5, #6, #7, and #8 (13, 14, 20). U-CLL also carry significantly longer VH CDR3s than M-CLL, resembling polyreactive autoantibodies (13).

**Activation-induced cytidine deaminase is highly expressed in U-CLL: interpreting an apparent paradox**

Activation-induced cytidine deaminase (AID), mediating both SHM and class-switch recombination (CSR), is expressed at the mRNA level in many CLL cases, primarily among U-CLL (21). Initially, this was thought to indicate functional inactivation of AID or defects in other yet unidentified factors critical for SHM. However, more recently, AID was shown to be expressed in both M-CLL and U-CLL at both the mRNA and protein level, and more particularly within the small proportion of cells that are undergoing or have recently undergone division (22). AID was capable of inducing SHM and CSR after protein level, and more particularly within the small proportion of cells that are undergoing or have recently undergone division (22). AID was capable of inducing SHM and CSR after in vitro stimulation with CD40 and IL4, a system, in essence, mimicking costimulatory signals provided by T cells in vivo. On these grounds, it was proposed that the lack of SHM in U-CLL clones in vivo is not due to an intrinsic AID defect, but rather due to active inhibition (22).

An alternative hypothesis favors the lack of appropriate stimuli from the microenvironment of U-CLL clones: either the U-CLL clones develop and expand in a T cell–independent manner, outside germinal centers (GC), or the SHM/CSR process is disadvantageous for the malignant clone and therefore selected against (22).

**BcR signaling capacity**

The ability of cells to respond to surface IgM (sIgM) ligation is highly variable in CLL (23). However, U-CLL cells display augmented BcR signaling compared with M-CLL cells (24). Given that the downstream signaling machinery seems to be intact in almost all CLL cases, the varying signaling capacity observed between U-CLL and M-CLL might simply reflect functional differences relating to the type of antigenic interaction through the BcR, or the cellular origins of these subtypes.

A critical determinant of IgM-mediated signaling is the level of sIgM, with U-CLL generally expressing higher levels of sIgM compared with M-CLL (24). The level of affinity for the eliciting antigen is also important, with U-CLL tending to have a lower affinity for antigen than M-CLL. This feature may be associated with decreased BcR endocytosis, thereby providing a possible mechanism for higher IgM expression levels and retained signaling capacity in U-CLL, ultimately translating into increased proliferation and survival. That said, differences in responsiveness among cases with similar sIgM expression levels suggest that additional factors are contributing to the signaling response (e.g., ZAP70 and CD38, being predominantly expressed by U-CLL; ref. 25).

The responsiveness of sIgM might also be modulated by modifications to the BcR itself. In follicular lymphoma, N-glycosylation sites are frequently introduced through SHM into the Ig variable regions and carry highly mannosylated glucans (26). It is hypothesized that mannosylation renders the neoplastic cells independent from antigen(s), as survival signals are provided by mannose interaction with stromal cells expressing mannose receptors. Interestingly, a mannosylated form of sIgM is preferentially expressed in U-CLL, suggesting that the BcR exploits microenvironmental ligands for sustained signaling (27).

**Reaction profile**

**CCL**

BcR IGs display poly/auto-reactivity, binding auto-antigens present on apoptotic cells, proteins, and lipoproteins modified by catabolic reactions; most of these antigens were found to be relevant mainly for U-CLL mAbs (28, 29). U-CLL mAbs have also been shown to react against various Gram-positive and Gram-negative bacterial strains, as well as viral proteins (29, 30). Interestingly, molecular mimicry between oxidized LDL and Streptococcus pneumoniae capsular polysaccharides has been reported as a critical link between autoreactivity and alloreactivity of U-CLL mAbs.

From a clinical perspective, BcR IG polyreactivity significantly correlates with clinical aggressiveness. "Multireactivity" (i.e., when the clonal BcR IG recognizes ≥5 epitopes) translates to an even more dismal prognosis, suggesting that "promiscuous" BcRs may be more prone to sustained signaling (20). This observation provides a biologic interpretation for the poor prognosis typical of U-CLL.

**IG-Mutated CLL**

**IGHV gene repertoire**

The IGHV gene repertoire of M-CLL is markedly different than that of U-CLL, indicating no interconversion of one mutational subtype to the other. IGHV3 subgroup genes collectively account for almost 60% of all M-CLL; however, with the striking exception of IGHV3-21, none of the IGHV3 subgroup genes frequently observed in M-CLL, namely IGHV3-7, IGHV3-23, and IGHV3-30, contribute to a major CLL stereotyped subset. In contrast, major M-CLL subsets mainly use genes of the IGHV4 subgroup and more specifically the IGHV4-34, IGHV4-4, and IGHV4-59 genes (13, 14).
SHM patterns of M-CLL BcR IGs provide evidence of antigen selection, given the accumulation of replacement mutations within the VH CDRs and the relative paucity of such mutations in the VH framework regions (FR; ref. 19). Although SHM across the VH domain can refine antigen-binding specificity and confer affinity maturation, in the case of inherently autoreactive IGs, it may also serve as an editing mechanism by altering critical positions and, thus, changing antigen specificity. In line with this concept, it has been shown that nonpolyreactive M-CLL IG may acquire polyreactivity when reverted to the germline configuration (28). Interestingly, shared replacement mutations at particular codon positions within the VH domain have been described for M-CLL cases belonging to certain stereotyped subsets, thus raising the intriguing possibility that the leukemic progenitor cells may have responded in a similar fashion to the selecting antigen(s) (19).

BcR signaling capacity

Most M-CLL cases fail to respond to in vitro sIgM engagement. This is associated with lower levels of sIgM expression, together with an overall lower proportion of mannosylated sIgM compared with U-CLL (24, 25). Such features are reminiscent of B cells that have undergone receptor desensitization, following chronic antigenic stimulation. Indeed, lack of signaling capacity in M-CLL is associated with constitutive activation of MEK1/2, ERK1/2, and nuclear factor of activated T cells (NF-AT) in the absence of Akt phosphorylation, which represents a biochemical signature of anergy in mouse models (23). Interestingly, M-CLL cells may upregulate sIgM expression and reverse the anergic status when transferred in vivo, thereby being deprived of a putative anergy-inducing signal operating in vivo (24).

IgG⁺ CLL

Most CLL cases carry IgM⁺ IgD⁻ clones (MD-CLL; ref. 31). However, subpopulations of isotype class-switched cells often exist within the IgM⁺ IgD⁺ clone (32). CSR seems to occur independently of SHM, given that class-switch events predominate in U-CLL (33). CLL cases where the major clone is isotype-switched are relatively rare (6–10%) and mostly express IgG (G-CLL; refs. 34, 35). A recent study of 169 IgG⁺ CLL cases from our joint cohort revealed that G-CLL exhibits an overall different immunogenetic signature from MD-CLL, even when restricting the comparison to cases with mutated BcR IGs (36). A significant proportion (~18%) of this rare subgroup was found to consist of just two major CLL subsets, namely subsets #4 and #8. Interestingly, the BcR IGs of subset #4 are heavily mutated, whereas those of subset #8 are minimally or not mutated, further suggesting that CSR and SHM may occur as independent processes at least in certain CLL cases (13, 14). CLL subset #4 (IGHV4-34/IGKV230) is considered to bear an inherently autoreactive BcR IG, which is effectively edited by the SHMs in critical positions (19). This may well be in line with the particularly indolent clinical course followed by these patients with CLL (14, 36). At the other end of the spectrum, unmutated subset #8 (IGHV4-39/IGKV1(D)-39) carries a BcR IG with a conspicuously broad antigen reactivity profile, perhaps underlying clinical aggressiveness and risk of transformation to high-grade lymphoma (18). It is conceivable that this particularly poly/autoreactive clone actively evades SHM to retain the unmutated configuration, which provides ample opportunity for a multitude of antigenic interactions, translating into proliferation and antiapoptotic signals. Given that CSR is traditionally thought to occur within GCs, this could argue in favor of a GC origin for not only subset #8 clones, but also isotype-switched, U-CLL clones. However, one cannot exclude the possibility that subset #8 CLL clones correspond to IgG-switched memory cells generated through a GC-independent pathway early after antigen encounter (37, 38).

Origin of CLL and the MBL Conundrum

The obvious conclusion about U-CLL is that it likely derives from B cells at a point of differentiation before the accumulation of high levels of SHM, which generally takes place within the GC after antigen encounter, whereas M-CLL derives from B cells stimulated in a classic T cell–dependent manner. However, the immunogenetic data argue against naiveté of U-CLL cells. B-cell activation can proceed outside GCs, within the marginal zones (MZ) around lymphoid follicles, most often in response to carbohydrates on encapsulated bacteria or viruses. These innate-like immune responses can be triggered by autoantigens or elicited by lipopolysaccharide (type I) or polysaccharide (type II) T cell–independent antigens, generating low-affinity B cells without induction of SHM (39). Recently, a subset of MZ dendritic cells (DIRC2⁺ MZ DCs) was found capable of initiating extrafollicular B-cell responses to T cell–dependent antigens, which may involve CSR without induction of GC formation and SHM if TLR7/TLR9 signaling is lacking (40).

In essence, U-CLL cells could be viewed as antigen experienced, "memory-like" cells that at one of several stages in B-cell maturation would or could not alter their antigen-binding sites despite repeated stimulation. Besides MZ B cells, potential precursors that comply with this scenario are B-1 cells (41). These cells are self-renewing CD5⁺CD21⁻ B cells, abundant in the peritoneal cavities of mice, carry unmutated IGHV genes, and produce a finite repertoire of auto/polyreactive, "natural" antibodies, capable of interacting with multiple types of antigens (e.g., carbohydrates, nucleic acids, and phospholipids) in a T cell–independent manner. Such BcRs may bind autoantigens with an affinity too low to trigger an autoimmune response, but may bind invading pathogens strongly enough to provide a first line of humoral defense and/or be involved in clearance of apoptotic cells and metabolic byproducts.

In that sense, B-1 cells are distinct from follicular (B-2) cells that mount high-affinity, isotype-switched responses to invading pathogens. It remains unclear whether a distinct B-1 lineage with unique genetic programming exists in humans, or whether such cells are induced by microenvironmental interactions (41). The recent report that a small population of human CD20⁺CD27⁺CD43⁻ cells exist, displaying fundamental functional properties of B-1 cells, has led to speculations that U-CLL may originate from such cells (42). However, a recent transcriptome study reported that U-CLL likely derives...
from mature CD5+ B cells, whereas M-CLL stems from a small population of mutated CD5+CD27+ post-GC B cells; hence, this issue is still contested (43).

If U-CLL clones derive from evolutionarily conserved memory cells, one would expect to identify CLL-specific, unmaturated stereotyped rearrangements in the immune repertoire of healthy individuals. Indeed, Forconi and colleagues identified IGHV1-69-encoded U-CLL stereotyped rearrangements in healthy donors and suggested that the precursor cells of U-CLL may derive from a population of innate-like B cells retained in the normal immune repertoire that express natural IgM antibodies and serve as a first line of defense against common infections (44). If one of these cells acquired a genetic abnormality that would allow it to resist restraint on clonal size, it would be primed for leukemic transformation. Foreign antigens and autoantigens could then serve as important stimuli for the promotion of tumor growth as repetitive exposure to these antigenic types could lead to cell expansion and/or transformation. Alternatively, these inherently autoreactive B cells, which are normally subject to peripheral tolerance mechanisms, may have developed ways to evade tolerance and respond to (auto)antigenic stimulation. Low-level stimulation of CLL cells by autoantigens could be providing life support, perhaps with additional cosignals from T cells and innate immunity cells.

All these hypotheses have to be reconciled with the existence of CLL-like monoclonal B cell lymphocytosis (MBL), present at high frequency in the peripheral blood of otherwise healthy individuals (45). Although MBL may provide an interesting perspective into CLL ontogeny and evolution, it also poses a series of questions because of the great similarity with CLL, at least in terms of phenotype (CD5\textsuperscript{bright}CD20\textsuperscript{dim}) that clearly distinguishes MBL cells from normal B cells. Are these cells functionally or ontogenetically different from the remaining B lymphocytes? If they correspond to CLL progenitors, why do not all MBL cases progress to CLL?

Although CLL-like MBL is perceived as the premalignant state of CLL, we are beginning to realize that this entity may be more heterogeneous than originally thought. Indeed, CLL-like MBL can be discriminated into two categories with distinct immunogenetic and clinical characteristics, adding further perplexity as to its ontogenetic relationship with CLL. Recently, we showed that such CLL-like clonal expansions may not transit from a low-count (LC) to a high-count (HC) phase or, more relevant from a clinical standpoint, to CLL, despite carrying cytogenetic abnormalities that are considered characteristic of CLL [e.g., del(13q), trisomy 12, or even del(17p); ref. 45]. LC-MBL increases with age, being present in 75% of individuals older than 90 years of age, seems to remain stable over time, and may represent a pre-CLL state. LC-MBL quickly transits to HC-MBL, which represents a pre-CLL state.
with the acquisition of the CLL phenotype rather than a true malignant transformation potential. In contrast, HC-MBL exhibits a BcR IG repertoire that is highly similar to that of CLL Rai stage 0 and progresses to CLL requiring treatment at appreciable rates (1%–4% per year), very close to the respective rate of CLL Rai stage 0 (5% per year), indicating that the distinction from CLL Rai 0 is more likely artificial rather than truly biologic (46). However, not even all HC-MBL/CLL cases have the same propensity for progression, similar to CLL Rai stage 0.

Thus, the continuum of events leading to full-blown CLL is far from being clear: genetic predisposition and/or additional genetic/epigenetic changes induced by microenvironmental stimuli acting upon specific functional properties of the clonal BcR IG may underlie the transition from a well-contained clonal expansion to a malignant condition (Fig. 1).

Concluding Remarks

Vital gaps in our knowledge exist about the pathway(s) to CLL, and pertain to issues such as the cellular origin of CLL, the exact molecular mechanisms governing the maturation of CLL B cells, and the inciting agents that may be responsible for immunoproliferation. The pursuit for answers to these questions may now be aided by advancements in technology: in particular, next-generation IG gene sequencing holds the potential to precisely define B-cell ontogenies and allow us to reconstruct the sequence of events from LC-MBL/HC-MBL to early-stage CLL and beyond. High-throughput single-cell analysis should also prove beneficial, perhaps to address questions relating to CSR, such as whether both IgM and IgG antibodies with an identical V domain could be found in the same cell.

Thus, immunogenetics still has much to offer to this endeavor, as it has done previously, and more has to be expected in the near future, especially in light of the new therapeutic strategies that have successfully been used to target the BcR intracellular signals (47–49). The immunogenetic quest continues!

Disclosure of Potential Conflicts of Interest

K. Stamatopoulos reports receiving a commercial research grant from Roche SA. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

The authors thank the present and past members of their groups for their commitment and enthusiasm. The authors also thank their friends and fellow members of the IgCLL. Diagnosis (www.igclld.org), Drs. Belesis, Darzentas, and Davi, for many years of stimulating and fruitful collaboration, and wish to acknowledge the enormous support of Prof. Marie-Paule Lefranc and Dr. Veronique Giudicelli, Laboratoire d’Immunogenetique Moleculaire, LIGM, Universite Montpellier II, Montpellier, France, and IMGT, the international ImMunoGeneTics information system. Finally, the authors thank the Swedish Cancer Society and the Swedish Research Council for support.

Grant Support

This work was supported in part by the ENSoA project (code 09SYN-13-880) cofunded by the European Union, the Hellenic General Secretariat for Research and Technology, Associazione Italiana per la Ricerca sul Cancro AIRC (Investigator Grant and Special Program Molecular Clinical Oncology – 5 per mille #9965), and Ricerca Finalizzata 2010 – Ministero della Salute, Roma. A. Agathangelidis is the recipient of a fellowship by Associazione Italiana per la Ricerca sul Cancro (AIRC) (Tromfell fellowship ’Igghelma Lucaletto e Giorno Maeszae’).

Received March 1, 2014; revised June 4, 2014; accepted June 5, 2014; published OnlineFirst July 29, 2014.

References


Immunogenetic Studies of Chronic Lymphocytic Leukemia: Revelations and Speculations about Ontogeny and Clinical Evolution

Anna Vardi, Andreas Agathangelidis, Lesley-Ann Sutton, et al.


Updated version Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-0630

Cited articles This article cites 49 articles, 35 of which you can access for free at: http://cancerres.aacrjournals.org/content/74/16/4211.full.html#ref-list-1

Citing articles This article has been cited by 4 HighWire-hosted articles. Access the articles at: /content/74/16/4211.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.