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IgM clones with unmutated IG receptors (U-CLL; refs. 4, 5). 

IgM populations of isotype-switched cells often exist within the great majority of chronic lymphocytic leukemia (CLL) cases express surface immunoglobulins (IG) of the mu and delta isotypes (IgM+IgD+, MD-CLL; ref. 1). However, subpopulations of isotype-switched cells often exist within IgM+IgD+ CLL clones (2, 3). Indeed, class switch recombination (CSR) seems to be dissociated from somatic hypermutation (SHM) in CLL, at least in certain cases, and, perhaps paradoxically, in vitro CSR is predominantly identified in CLL clones with unmutated IG receptors (U-CLL; refs. 4, 5).

Activation-induced cytidine deaminase (AID), which is normally required for both SHM and CSR, is expressed in a small proliferative compartment within the CLL clone, irrespective of the immunoglobulin heavy variable (IGHV) gene mutational status, and can be functionally induced in vitro under conditions that mimic T-cell help (6). Furthermore, ongoing CSR and high AID expression define a subgroup of U-CLL displaying even worse prognosis, underscoring the importance of intense (micro) environmental stimulation for shaping clonal behavior and eventual clinical outcome (7). That notwithstanding, the precise signals that induce in vivo CSR in CLL are presently not fully elucidated.

CLL cases in which the major clone is isotype-switched are relatively rare (6%–10%; ref. 8). The majority of such cases are immunoglobulin G–switched chronic lymphocytic leukemia (G-CLL); however, little published data exist about this subgroup. That said, a phenotypic, molecular, and functional characterization of 14 unselected G-CLL cases suggested a relationship with CLL bearing mutated IG receptors (M-CLL) and derivation from post-germinal B cells (9). However, the existence of U-CLL with immunoglobulin G (IgG)–switched receptors challenges this scenario and warrants the search for alternative possibilities.

Here, we sought evidence about the origin of G-CLL by comprehensively profiling the IG gene repertoires in the largest series studied to date for this purpose. Our focus on
Translational Relevance

The great majority of chronic lymphocytic leukemia (CLL) clones express surface immunoglobulin (IG) of the mu and delta isotypes (IgM+ IgD+, MD-CLL). CLL cases in which the major clone is isotype-switched are relatively rare and insufficiently characterized. We comprehensively profiled the IG gene repertoires of cases with MD versus G isotypes in the largest series by far studied for this purpose. Immunoglobulin G-switched chronic lymphocytic leukemia (G-CLL) displayed an overall distinct immunogenetic signature from MD-CLL, prompting speculations about distinct ontogenetic derivation and/or distinct immune triggering. Remarkably, two subsets with quasi-identical (stereotyped) B-cell receptor IGs included only G-CLL cases and accounted for 25% of the respective cohort. Given their markedly different somatic hypermutation status and clinical behavior, these two subsets may be considered paradigmatic for the elucidation of distinct immune pathogenetic pathways and clinical phenotypes in CLL. We argue that this endeavor may prove clinically relevant in light of the increasing use of drugs targeting immune signaling in CLL.

The immunogenetic characteristics of the clonotypic B-cell receptor (BCR) IGs is justified by both biologic and clinical evidence indicating, on the one hand, that different types of BCRs convey signals of different intensity and, on the other hand, that interfering with BCR signaling holds therapeutic promise in CLL (10–16). We demonstrate that G-CLL exhibits an overall distinct immunogenetic signature from MD-CLL, even when restricting the comparison with cases with mutated IG receptors, thus prompting speculations about distinct ontogenetic derivation and/or distinct immune triggering.

Patients and Methods

Patients

We performed detailed immunogenetic profiling of a large multinational CLL cohort (n = 1,256) from collaborating institutions in Greece, Italy, and Sweden. Cases were included in the study if information for the clonotypic heavy chain isotype was available, as determined by either peripheral blood flow cytometry (n = 1,030) or reverse transcriptase PCR (RT-PCR) for tumor-specific mu, delta, and gamma constant region transcripts (n = 226). Within this cohort, 1,087 and 169 cases expressed IG mu/delta and gamma heavy chains, respectively. A summary of available demographic and clinical data is provided in Table 1. The diagnosis of CLL was made according to the established iwCLL/National Cancer Institute (NCI) criteria (17). The study was approved by the local Ethics Review Committee of each participating institution.

PCR amplification and sequence analysis of IGHV–IGHD–IGHJ rearrangements

PCR amplification and sequence analysis of IGHV–IGHD–IGHJ rearrangements were performed as previously...
described (18). Sequence data were interpreted using our suite of purpose-built bioinformatics methods, the Antigen Receptors Research Tool (ARResT; http://bat.infspire.org/arrest). Stereotyped rearrangements were identified following our previously described criteria (19, 20).

Results

IG gene repertoire and SHM load in G-CLL versus MD-CLL

G-CLL was found to exhibit a particularly skewed IG gene repertoire. In particular, IGHV4 was the predominant subgroup (90 of 169, 53%), followed by IGHV3 (60 of 169, 35%). Thirty-two functional IGHV genes were identified, with the IGHV4-34 gene being by far the most frequent (38%), followed by IGHV4-39 (9%), IGHV3-23 (7%), and IGHV3-30 (5%). Collectively, these four genes accounted for almost 60% of the respective repertoire.

A comparison of G-CLL with MD-CLL revealed marked differences, with the IGHV4-34 and IGHV4-39 genes being significantly (P < 0.001) overrepresented in the former. This is in contrast with several other genes with significantly (P < 0.05) lower frequencies in G-CLL compared with MD-CLL, namely the IGHV1-69, IGHV3-21, IGHV1-2, and IGHV3-48 genes (Fig. 1A). The markedly reduced frequency of the IGHV1-69 gene in G-CLL versus MD-CLL is particularly noteworthy, given that this gene is known to dominate the IG gene repertoire in CLL (20–22).

We next investigated the impact of SHM in G-CLL versus MD-CLL. On the grounds that even a minimal SHM load can have profound functional implications, we subdivided the cases into three mutational subgroups, namely (i) truly unmutated, that is, no SHM/100% germline identity (GI); (ii) borderline/minimally mutated (GI = 98%–99.9%); and (iii) mutated (GI < 98%; refs. 18, 23). We noted a significant (P < 0.05) asymmetrical distribution of G-CLL versus MD-CLL cases across these SHM categories. In particular, only 11% (19 of 169) of the G-CLL rearrangements were truly unmutated compared with 32% (347 of 1,087) of MD-CLL rearrangements; a similar result was obtained for borderline/minimally mutated rearrangements (5%, 8 of 169 for G-CLL versus 12%, 126 of 1,087 for MD-CLL). In sharp contrast, 84% (142 of 169) of G-CLL rearrangements carried IGHV genes, with less than 98% GI versus only 56% (614 of 1,087) for MD-CLL (Fig. 1B).

On these grounds, we also performed IG repertoire comparisons between G-CLL and MD-CLL for the different SHM subgroups. We noted significant overrepresentation of (i) the IGHV4-34 gene among mutated rearrangements in G-CLL versus MD-CLL (63 of 142 versus 65 of 614 cases, respectively; P < 0.0001); and (ii) the IGHV4-39 gene among truly unmutated rearrangements in G-CLL versus MD-CLL (10 of 19 versus 23 of 347 cases, respectively; P < 0.0001; Fig. 2). Because of the low number of G-CLL cases (n = 8) within the borderline/minimally mutated subgroup, we did not undertake such comparisons.

Figure 1. IG gene repertoire and SHM status in G-CLL versus MD-CLL. A, IGHV gene repertoire in G-CLL versus MD-CLL. B, relative distribution of G-CLL versus MD-CLL into three subgroups defined according to SHM load, namely truly unmutated (100% IGHV identity to the germline, GI), borderline/minimally mutated (98%–99.9% GI), and mutated (<98% GI). Differences at a level of statistical significance, P < 0.05 and P < 0.001, are shown by * and **, respectively.
BCR stereotypy in G-CLL versus MD-CLL

Aiming to identify the patterns that would explain the IG gene repertoire polarization between G-CLL versus MD-CLL, we next investigated the incidence of BCR IG stereotypy in both subgroups. Cluster analysis of the heavy variable complementarity-determining region 3 (VH CDR3) sequences of our series revealed that the extreme skewing of the G-CLL repertoire was due to the fact that almost one third of all cases were represented by only three stereotyped subsets, namely (i) mutated subset #4, defined by stereotyped IGHV4-34/IGKV2-30 BCRs: 31 of 169 cases (18%); (ii) unmutated subset #8, defined by stereotyped IGHV4-39/IGKV1(D)-39 BCRs: 11 of 169 cases (7%); and (iii) mutated subset #16, defined by stereotyped IGHV4-34/IGKV3-20 BCRs: 7 of 169 cases (4%). Notably, subset #4 and #8 cases could be considered as prototypes for mutated G-CLL and truly unmutated G-CLL, respectively, because they comprised 22% and 42% of the respective categories (Fig. 3). The extreme polarization of the G-CLL IG repertoire was also underscored by the fact that all major stereotyped subsets using the IGHV1-69, IGHV3-21, IGHV1-2, and IGHV3-48 genes exclusively concerned MD-CLL.

With the exception of subsets #4, #8, and #16, G-CLL BCR IGs were very heterogeneous in terms of IGHV gene usage and VH CDR3 composition. Interestingly, a group of cases were found to carry coexisting trisomies of chromosomes 12, 18, and 19 (n = 8/169; 5% of the G-CLL cohort). These cases carried somatically mutated BCR IGs yet with different IGHV genes and VH CDR3 features, which is in keeping with a previous study from our group (24).

Subsets #4 and #8 display distinct clinicobiologic characteristics

The emergence of subsets #4 and #8 as the most prominent representatives of G-CLL strikes as odd, given that these two subsets are known to be opposites in terms of clinical presentation and outcome. We therefore looked into the clinicobiologic data of cases of the present series assigned to these subsets (summarized in Table 2).

In line with previous studies (25, 26), subset #4 patients were significantly younger at diagnosis compared with subset #8 patients and presented more frequently with early-stage disease; in addition, with similar follow-up times, patients assigned to subset #4 required treatment significantly less frequently than subset #8. All subset #4 BCR IGs were mutated, whereas subset #8 BCR IGs were unmutated. In addition, subsets #4 and #8 exhibited differential expression of CD38 and ZAP70 (infrequent in subset #4, pronounced in subset #8). Furthermore, they displayed distinct genomic aberration profiles, especially about the incidence of trisomy 12 (0% in subset #4 versus 60% in subset #8; P < 0.001).

Discussion

We performed a detailed immunogenetic analysis of the largest series of IgG-switched CLL cases studied to date, combined with a search for clinicobiologic associations. We demonstrated that this rare CLL subgroup exhibits an immunogenetic signature clearly distinct from non-switched CLL and is remarkably skewed toward the representation of two major CLL stereotyped subsets, namely subsets #4 and #8.
Subset #4 clones are “born” to be autoreactive, because the expression of the IGHV4-34 gene per se conveys autoreactive potential. In fact, IGHV4-34-expressing normal B cells represent an in vivo model of autoreactivity, given that they are endowed with the ability to recognize, in a superantigenic fashion, the N-acetyllactosamine antigenic epitope present in both self and exogenous antigens (27). This recognition is mediated through a germline-encoded motif in the heavy variable framework region 1 (VH FR1) of the IGHV4-34 gene. Importantly, as in previous studies (18), all subset #4 cases of the present series carried an intact VH FR1 motif.

Further molecular hints linking subset #4 with autoreactivity, also evident in the present series, are (i) the predicted high electropositivity of their VH CDR3s, which are enriched in basic and aromatic amino acid residues, reminiscent of pathogenic anti-DNA antibodies; and (ii) the presence of recurrent SHMs in both the IG variable heavy and kappa (VK) domains, typified by the frequent introduction of acidic residues, similar to edited anti-DNA antibodies (18, 28). Relevant to the latter observation, despite intense ongoing SHM leading to subclone formation and pronounced intraclonal diversification, the IG heavy and light chain sequences of subset #4 cases have been shown to retain the somatically introduced acidic residues, likely due to functional constraints in a context of maintaining tolerance (29–31). This is in line with the previous finding that recombinant monoclonal antibodies from CLL subset #4 cases do not recognize DNA, whereas their germline revertants regain this capacity (32).
We sought for additional immunogenetic hints into the ontogeny of CLL subset #4, taking advantage of a recent high-throughput sequencing study of the paired IG heavy and light chains of peripheral IgG-switched B cells in healthy individuals (33). By careful meta-analysis of the respective sequence datasets, we found only one IGHV4-34/IGKV2-30 combination in a total of 1.4 × 10^5 IGH/IGK pairs, albeit with VH and VK CDR3s drastically distinct from those characterizing subset #4.

Overall, it can be hypothesized that the progenitor of CLL subset #4 might be a very particular rare type of autoreactive cell that undergoes editing by SHM, thereby alleviating intense self-reactivity and preventing clonal deletion. Questions about when, where, and under what influence(s) this cell undergoes CSR to become IgG-switched currently remain unresolved and require further study.

At the other end of the G-CLL spectrum, unmutated and clinically aggressive subset #8 exhibits an intriguingly broad antigen reactivity profile (34). It is conceivable that this particularly poly/autoreactive clone does not engage in SHM because the unmutated configuration endows clonal cells with ample opportunities to engage in interactions with a wide range of antigens, thus receiving almost unrestricted prosurvival signals. Altogether, subset #8 can be considered as a paradigmatic example of CLL clones in which SHM is dissociated from CSR. What does this tell us about its ontogeny?

Given that CSR is traditionally considered to take place within the germinal centers (GC) of secondary lymphoid organs, this could imply derivation from GC cells and would be plausible for not only subset #8 but also for all CLL clones carrying isotype-switched, unmutated BCR IGs. However, one cannot exclude the possibility that subset #8 CLL clones originate from IgG-switched memory cells generated by a GC-independent pathway early after antigen encounter (35, 36).

In both subsets, #4 and #8, the available immunogenetic and functional evidence implies that antigenic triggering has been or is still relevant for tumor development and evolution, including CSR induction, through either BCR-dependent or BCR-independent mechanisms (37). Apropos of the latter, data suggest that anergic self-reactive B cells may be induced to undergo CSR and produce autoantibodies in vitro through Toll-like receptor (TLR) stimulation or in conditions that mimic T-cell help (38). Interestingly, our previous studies documented that subset #4 and #8 clones have distinct functional responses to TLR stimulation, with the former exhibiting a TLR7-tolerant profile and the latter displaying an unrestricted and intense response to multiple TLR ligands, thus differing significantly from other subsets that are far more selective (34, 39). How these functional profiles are linked to the physiology and clonal behavior of subsets #4 and #8, especially with regard to CSR, is currently unknown.

In conclusion, G-CLL exhibits an overall distinct immunogenetic signature from MD-CLL, prompting speculations

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<th>Table 2. Comparison of the demographic, clinical, and biologic characteristics of subset #4 versus subset #8</th>
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<td><strong>Cohort characteristics</strong></td>
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<td>Age (y, median)</td>
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<td>Gender</td>
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<td>Male</td>
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<td>Female</td>
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<td>Binet stage B–C</td>
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<td>Surface CD38 &gt;7%</td>
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<td>Cytoplasmic ZAP-70 &gt;20%</td>
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<td>Mutated IGHV (&lt;98% GI)</td>
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<td>Complex karyotypea</td>
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<td>Multiple trisomies (+12, +18, +19)b</td>
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<td>Trisomy 12 (FISH)</td>
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<td>Need for treatment</td>
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<td>Time to first treatment (months)</td>
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<td>Richter transformation</td>
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aThree or more numerical and/or structural aberrations.
bCases with multiple coexisting trisomies of chromosomes 12, 18, and 19 were analyzed separately from cases with complex karyotype on the basis of previous reports that they exhibit a particularly indolent clinical course, thus distinguishing them from complex karyotype cases (24).
about distinct ontogenetic derivation and/or immune triggering. Further studies are warranted to better understand the differential regulation of SHM among G-CLL cases and how it may affect clonal behavior and eventual patient outcome.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support

This work was supported, in part, by the ENoAL project (code 09SYN-13-880) funded by the EU and the Hellenic General Secretariat for Research and Technology, and the Swedish Cancer Society and the Swedish Medical Research Council. A. Agathanggelidis is the recipient of a fellowship by Associazione Italiana per la Ricerca sul Cancro (AIRC; Triennial fellowship “Guglielmina Lucatello e Gino Magzza”). N. Darzentas is currently supported by CEITEC M111 (CZ.1.05/1.1.00/02.0068) and project SuPReMMIE (CZ.1.07/2.3.00/20.0045). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 20, 2013, revised October 13, 2013; accepted November 3, 2013; published OnlineFirst November 15, 2013.

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IgG-Switched CLL Has a Distinct Immunogenetic Signature from the Common MD Variant: Ontogenetic Implications

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Clin Cancer Res  Published OnlineFirst November 15, 2013.

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