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Temporal Dynamics of Clonal Evolution in Chronic Lymphocytic Leukemia with Stereotyped *IGHV4-34/IGKV2-30* Antigen Receptors: Longitudinal Immunogenetic Evidence

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Chronic lymphocytic leukemia (CLL) patients assigned to stereotyped subset 4 possess distinctive patterns of intraclonal diversification (ID) within their immunoglobulin (IG) genes. Although highly indicative of an ongoing response to antigen(s), the critical question concerning the precise timing of antigen involvement is unresolved. Hence, we conducted a large-scale longitudinal study of eight subset 4 cases totaling 511 and 398 subcloned IG heavy and kappa sequences. Importantly, we could establish a hierarchical pattern of subclonal evolution, thus revealing which somatic hypermutations were negatively or positively selected. In addition, distinct clusters of subcloned sequences with cluster-specific mutational profiles were observed initially; however, at later time points, the minor cluster had often disappeared and hence not been selected. Despite the high intensity of ID, it was remarkable that certain residues remained essentially unaltered. These novel findings strongly support a role for persistent antigen stimulation in the clonal evolution of CLL subset 4.

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INTRODUCTION

From an immunogenetic perspective, the critical role of the B-cell receptor (BcR) in chronic lymphocytic leukemia (CLL) is underscored by the biased immunoglobulin heavy variable (*IGHV*) gene repertoire and the allocation of patients into subgroups with markedly different prognosis on the basis of *IGHV* gene mutational status of the clonotypic BcRs (1–8). Even more compelling, however, is the fact that almost 30% of CLL patients share BcRs with restricted, quasi-identical "stereotyped" immunoglobulin (IG) sequences with highly homologous IG variable heavy-chain complementarity-determining region 3 (VH CDR3), the key determinant of antigen specificity (9–19). Increasing evidence also indicates that cases expressing such stereotyped BcRs, and consequently assigned to distinct subsets, share biological and clinical features (10,16,17,19–22). These findings, along with other structurally unique features of CLL BcRs, strongly suggest that antigens, superantigens or both may play an active role in the disease.

Elucidation of the antigenic specificity of the clonogenic BcRs in CLL has previously been hindered by technical difficulties; however, more recent procedures using cell lines derived from the neoplastic CLL clone established that these cells can produce BcRs/monoclonal antibodies (mAbs) that bind autoantigens and molecular structures present on apoptotic cells and bacteria such as IgG, vimentin, filamin B, cardiolipin and DNA (23–25). In addition, by using recombinant DNA technologies, the role of antigenic reactivity and the impact of somatic hypermutation (SHM) on CLL mAb specificity have been investigated and revealed that CLL cells likely derive from B cells producing polyreactive, natural antibodies encoded by germline IG genes, which either retain or lose polyreactivity due to SHM (26–28). Although the accumulated data on antigen reactivity do not provide the definitive agent driving CLL, they do provide a framework from which comparisons between other entities, most notably autoimmune diseases, can be drawn.

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While the results from studies aimed at defining the antigenic reactivity profile of CLL cells convincingly demonstrate a role for antigen in CLL pathogenesis, the timing and duration of antigenic exposure remain largely unknown. We recently investigated intraclonal diversification (ID) within the IG genes of patients with CLL and found that most cases showed no or low levels of ID. In sharp contrast, we reported intense ID within both the heavy- and light-chain IG genes of patients assigned to subset 4 (29,30).

CLL patients assigned to stereotyped subset 4 are characterized clinically by an early age at diagnosis and an indolent disease course and molecularly by BcR IGs that exhibit a series of distinctive immunogenetic features (16). More specifically, they are IgG-switched and composed of heavy chains encoded by the *IGHV4-34* gene and light chains encoded by the *IGKV2-30* gene. Their VH CDR3 is long and enriched in positively charged residues, being particularly defined by a (K/R)RYY motif at IMGT positions 112.4-112.1 (17,19). Furthermore, the VH and VK domains demonstrate a high impact of SHM and are remarkable for carrying shared ("stereotyped") amino acid changes induced by SHM: noteworthy among these are changes leading to the introduction of negatively charged residues in both the heavy and light chains (17,31).

The intense ID in subset 4 BcR IGs convincingly implicates antigen selection in the development and evolution of subset 4. However, our previous studies were limited to depicting what was occurring at a single time point and could not provide insight into the temporal dynamics of the CLL clones. Hence, we gathered a novel and unique dataset from serial sampling of eight subset 4 cases, and through this approach, we could trace clonal evolution over time and investigate the impact of functionally relevant mutations for subclone selection. Overall, the results reported herein provide conclusive evidence that subset 4 patients continue to acquire SHMs within their IG genes over time and that this observation is best explained by a clear role for antigen selection in the clonal evolution of CLL subset 4.

MATERIALS AND METHODS

Patient Group

Peripheral blood samples were collected at multiple time points from eight CLL patients who, based on IG gene sequence features and following previously established criteria, were assigned to subset 4 (*IGHV4-34/IGKV2-30*, 20–amino acid VH CDR3 and IgG-switched) (16,17,19). All patients displayed the typical CLL immunophenotype and met the recently revised diagnostic criteria of the National Cancer Institute Working Group (32). Patient demographics and clinical and molecular data are summarized in Supplementary Tables S1 and S2. Cases were analyzed over a 6-year period (range 7–72 months, median 20 months), and no patient received treatment during sampling (Supplementary Tables S1 and S3). The diagnostic sample was available and called "time point 1" for six of the eight patients analyzed. No diagnostic samples were available for the remaining two patients (P0103 and P2451); therefore, the initial sample (time point 1) analyzed for these patients was 81 and 63 months after diagnosis, respectively. Written informed consent was obtained in accordance with the Declaration of Helsinki, and the study was approved by the local ethics review committee.

Polymerase Chain Reaction Amplification of IG Rearrangements

Polymerase chain reaction (PCR) amplification was performed on either genomic DNA (gDNA) or complementary DNA (cDNA) by using the IGHV4 leader primer and the antisense IG heavy chain constant region (IGHC) primer for analysis of IGHV-IGHD-IGHJ rearrangements and the VK2 FR1 primer together with the antisense IG κ chain constant region (IGKC) primer for analysis of the IGKV-IGKJ rearrangements. All amplification reactions were run by using the high-fidelity Accuprime Pfx (Invitrogen/Life Sciences, Carlsbad, CA, USA), and purified PCR amplicons were subjected to direct sequencing on both strands (16,17,29–31).

Subcloning

PCR amplification products were gel-purified with the Qiagen DNA purification columns, ligated into the pCR2.1 vector (Invitrogen/Life Sciences) and subsequently transformed into *Escherichia coli*/TOP10F′ competent bacteria (Invitrogen/Life Sciences). A range of 8–39 colonies per case (median, 22 colonies) was chosen randomly and sequenced by using the 20 universal primers or M13 primers.

Sequence Data Analysis and Definitions

Sequences obtained from subcloning were analyzed by using the Antigen Receptors Research Tool/ARResT (http://bat.infspire.org/arrest), IMGT® databases and the IMGT/V-QUEST tool (http://www.imgt.org) (33,34). Codons and amino acid positions are according to the IMGT unique numbering system for the V domain. For the IGHV-IGHD-IGHJ rearrangements, the entire V region was evaluated (from VH FR1 codon 1 down to the VH CDR3). To avoid misidentification of mutations when the VK2 FR1 consensus primer was used, nucleotide changes in the obtained sequences were evaluated from codon 12 in VK FR1 down to VK CDR3.

Intraclonal Diversification Analysis

Intraclonal diversification in sets of subcloned sequences obtained from the same sample was assessed by examination of sequence variation in the V domain following definitions previously proposed by our group (29,30). In brief, all "nonubiquitous" sequence changes from the germline were evaluated in the counts and characterized as follows: (a) unconfirmed mutation (UCM): a mutation observed in only one subcloned sequence from the same specimen (unique);

and (b) confirmed mutation (CM): a mutation observed more than once among subcloned sequences from the same specimen (partially shared). Amino acid changes resulting from UCMs or CMs are designated by the abbreviations UAA or CAA, respectively.

All supplementary materials are available online at www.molmed.org.

RESULTS

Incidence, Topology and Molecular Characteristics of ID over Time in Subset 4 Immunoglobulin Heavy and Light Chains

Eight CLL subset 4 cases were investigated for ID by analyzing 511 and 398 subcloned IG heavy and kappa sequences obtained from serial sampling (Supplementary Tables S1–S3). Overall, all cases carried intraclonally diversified IG genes, whereas the level of ID observed over time could be described as increasing, decreasing or complex (that is, a mutation appears or disappears and then reemerges at a subsequent time point) (Figures 1A, B; Supplementary Figures S1, S2).

At the nucleotide level, 87 unique CMs were found throughout the *IGHV4-34* gene in 374 clones, whereas 137 UCMs were also detected. Of the 137 UCMs, only 73 were considered truly unconfirmed, since 42 UCMs were ubiquitous mutations and/or CMs in either another sample or at a different time point for the same sample. Similarly, the remaining 22 UCMs were confirmed by the presence of the same mutation occurring in a single sequence from either a different case or an alternative time point for the same sample. Analogous to the IGHV4-34 heavy chains of subset 4, their partner IGKV2-30 light chains were also affected by an active ID process, accumulating 53 CMs and 55 UCMs.

All observed CMs and UCMs concerned single-base changes, resulting in both silent and replacement mutations, whereas nucleotide substitution analysis revealed that transitions predominated

A

Case	TP	N	7:W	28:G	30:F	40:S	45:P	82:T	92:S
P0103	1	33	W_{30} R_3	G_2 E_{31}	F_{33}	S_2 T_{31}	P_{31} F_2	T_{33}	T_{33}
	2	28	W_{27} R_1	G_8 E_{20}	F_{28}	S_8 T_{20}	P_{20} F_8	T_{28}	T_{28}
P0907	1	33	W_{33}	D_{33}	F_{33}	T_{33}	P_{33}	T_{33}	T_{33}
	2	22	W_{22}	D_{22}	F_{21} Y_1	T_{21} I_1	P_{22}	T_{22}	S_1 T_{21}
	3	23	W_{23}	D_{23}	F_{23}	T_{23}	P_{23}	T_{23}	T_{22} A_1
P1422	1	27	W_{27}	D_6 E_{21}	F_{27}	S_{21} T_6	S_{27}	T_{27}	S_{20} T_7
	2	33	W_{33}	D_1 E_{32}	F_{32} Y_1	S_{32} T_1	S_{33}	T_{33}	S_{31} T_1 R_1
	3	25	W_{24} R_1	E_{25}	F_{25}	S_{25}	S_{25}	T_{25}	S_{25}
P1939	1	35	W_{35}	G_1 E_{34}	F_{35}	T_{35}	A_{35}	T_{34} A_1	N_{35}
	2	35	W_{35}	E_{35}	F_{34} I_1	T_{35}	A_{35}	T_{35}	N_{33} H_2
	3	21	W_{21}	E_{21}	F_{21}	T_{21}	A_{21}	T_{20} A_1	N_{21}
P2451	1	35	W_{35}	G_1 D_{33} E_1	F_{35}	S_1 T_{34}	P_1 S_{33} F_1	T_{34} A_1	T_{35}
	2	33	W_{33}	D_{33}	F_{33}	T_{33}	S_{33}	T_{33}	T_{33}
P2920	1	26	W_{25} C_1	G_{26}	F_{26}	S_{26}	P_{15} S_{11}	T_{26}	N_{26}
	2	26	W_{26}	G_{26}	F_{26}	S_{26}	P_{10} S_{16}	T_{26}	N_{26}
P3020	1	16	W_{16}	G_{16}	F_{16}	S_{16}	S_{16}	T_{16}	T_{16}
	2	21	W_{21}	G_{21}	F_{21}	S_{21}	S_{21}	T_{21}	T_{21}
P3916	1	17	W_{17}	D_{17}	F_{17}	S_{17}	S_{17}	A_{17}	S_{17}
	2	22	W_{22}	D_{22}	F_{22}	S_{22}	S_{22}	A_{22}	S_{22}

acidic (E, D) basic (K, R, H) aromatic (F, W, Y) non-polar, aliphatic (G, A, I, V, L, P, M) polar, non-charged (S, T, C, N, Q)

B

Case	TP	N	21:I	25:S	28:S	66:N	86:D	88:T	99:V
P0103	1	38	I_{38}	S_{38}	S_{32} G_6	N_{38}	D_{32} Y_6	T_{38}	V_{37} A_1
	2	33	I_{33}	S_{33}	S_{31} G_2	N_{33}	D_{31} Y_2	T_{33}	V_{33}
	3	8	I_8	S_8	S_8	N_8	D_8	T_8	V_8
P0907	1	16	V_{16}	S_{16}	S_{16}	D_{16}	D_{16}	T_{16}	V_{16}
	2	17	V_{17}	S_{17}	S_{17}	D_{17}	D_{17}	T_{17}	V_{17}
P1422	1	14	V_{14}	S_{14}	N_{14}	D_{14}	A_{14}	T_{14}	V_{14}
	2	26	V_{26}	S_{26}	N_{26}	D_{26}	A_{26}	T_{25} A_1	V_{26}
	3	11	V_{11}	S_{10} F_1	N_{11}	D_{11}	A_{11}	T_{11}	V_{11}
P1939	1	19	I_{17} V_2	S_2 C_{17}	S_{19}	N_{17} D_2	D_{17} G_2	T_{19}	V_{19}
	2	19	I_{19}	S_1 C_{18}	S_{19}	N_{19}	D_{19}	T_{19}	V_{19}
	3	13	I_{13}	C_{13}	S_{13}	N_{13}	D_{13}	T_{13}	V_{13}
P2451	1	39	I_{36} V_3	S_{39}	S_{37} N_2	N_{37} D_2	D_{37} A_2	T_{38} A_1	V_{39}
	2	24	I_{24}	S_{24}	S_{24}	N_{24}	D_{24}	T_{24}	V_{24}
P2920	1	18	I_5 V_{13}	S_{18}	S_{18}	D_{18}	D_{18}	T_{18}	V_6 I_{12}
	2	11	V_{11}	S_{11}	S_{11}	D_{11}	D_{11}	T_{11}	V_1 I_{10}
P3020	1	21	V_{21}	S_{21}	S_{21}	D_{21}	T_{21}	T_{21}	I_{21}
	2	21	V_{21}	S_{21}	S_{21}	D_{21}	T_{21}	T_{21}	I_{21}
	3	22	V_{22}	S_{22}	S_{22}	D_{22}	T_{22}	T_{22}	I_{22}
P3916	1	15	V_{15}	S_{15}	S_{15}	T_{15}	D_{15}	T_{15}	V_{15}
	2	13	V_{13}	S_{12} C_1	S_{13}	N_1 T_{12}	D_{13}	T_{13}	V_{13}

acidic (E, D) basic (K, R, H) aromatic (F, W, Y) non-polar, aliphatic (G, A, I, V, L, P, M) polar, non-charged (S, T, C, N, Q)

Figure 1. Restricted patterns and hotspots of ID over time. (A) Certain mutations observed within the *IGHV4-34* gene of subset 4 cases. (B) Mutations within the *IGKV2-30* gene of subset 4 cases. Precise targeting of ID infers strong functional constraints for preservation of critical physicochemical properties. This idea is further underscored by the observation that many UCMs (that is, mutations present in a single subcloned sequence, either at a single-case or time-point level) were present as ubiquitous mutations, CMs (that is, mutations present in more than one but less than all subcloned sequences) or UCMs in other samples or at another time point of the same sample and thus may be considered as imprints of the ID process. The substitution of isoleucine (I) with valine (V) at codon 21 (VK CDR1) together with the asparagine (N) to aspartic acid (D) change at codon 66 (VK FR3) provides an example of patterns of SHM that could perhaps be interpreted within the context of "concerted mutagenesis." Amino acids are represented by a single-letter code. The germline amino acid is indicated in the header with the number referring to the codon position according to the IMGT unique numbering for V-DOMAIN (V-QUEST, version 2.1.2). Within the figures, the number of subcloned sequences containing a certain amino acid is indicated after the single-letter code, whereas amino acids highlighted in white exemplify the phenomenon of UCMs' being confirmed by either a different case or another time point of the same case. A comprehensive list of amino acid changes observed in all cases analyzed is provided in Supplementary Tables S6–S8. N, number of subcloned sequences analyzed; TP, time point.

over transversions, in keeping with a canonical SHM process. Detailed information on the type (replacement versus silent) and distribution of CMs/UCMs throughout the V region is provided in Supplementary Tables S4 and S5. At the amino acid level, 48 confirmed amino acid changes (CAA) were identified among sets of subcloned IGHV4-34 rearrangements, resulting in either a conservative (n = 19) or nonconservative (n = 29) amino acid substitution. Such amino acid changes were also evidenced throughout the IGKV2-30 rearrangements analyzed, and of the 30 CAAs detected, 40% (n = 12) resulted in conservative changes (Supplementary Tables S6–S8; Supplementary Figure S3).

Detailed analysis of the mutations revealed precise targeting of ID, inferring strong selective pressure for preservation of critical physicochemical properties. In particular, we noted the following: (a) restricted ID patterns occurred in the form of identical mutations at certain VH/VK positions among subclones of different cases (for example, despite the fact that glycine at codon 28 [VH CDR1] was mutated in 410/511 [80%] sequences, the only permissible option for substitution was an acidic residue); (b) over time, ID hotspots (that is, codons that frequently carried mutations) were repeatedly observed during clonal evolution, for example, codon 40 (VH FR2) and codon 66 (VK FR3); and (c) patterns of coincident mutations occurred; as an example, the substitution of isoleucine (I) with valine (V) at codon 21 (VK CDR1) together with the asparagine (N) to aspartic acid (D) change at codon 66 (VK FR3) constitutes a pattern of SHM that could perhaps be interpreted within the context of "concerted mutagenesis" (Figures 1A, B; Supplementary Tables S7, S8).

Although, the extent of ID varied between cases with, for example, P3020 and P3916 undergoing limited diversification in comparison to several other cases analyzed (most notably, P1422 and P2920), overall, the incidence and topology of ID in subset 4 over time are suggestive of selection events governed by structural constraints for optimal antigen recognition. This idea is further underscored by the observation that many UCMs were present as ubiquitous mutations, CMs or UCMs in other samples or at another time point of the same sample and thus may be considered as imprints of a highly targeted and selected ID process.

Patterns of Over-Time Clonal Branching in CLL Subset 4

The analysis of consecutive samples enabled us to trace diversification within the subset 4 CLL antibody over time, thus revealing which mutations were negatively or positively selected (Figure 2A; Supplementary Figures S1, S2, S4). Consequently, a stepwise accumulation of mutations could be observed, with several mutations present in only a fraction of subclones at an early time point becoming ubiquitous mutations—that is, present in all subcloned sequences of the same case at subsequent time points. For example, although a serine to cysteine change at codon 25 (VK FR1) was abundant in P1939 at both time points 1 and 2, the germline serine was retained in a few subclones. However, this scenario changed by time point 3 where cysteine was present in all subcloned sequences analyzed for this case. A similar finding was observed in P2451 at both codons 66 and 86 (VK FR3). The presence of distinct clusters of subcloned sequences with "cluster-specific" mutational profiles shed light on both their evolutionary relationships, with, for instance, minor clusters appearing or, more often, disappearing over time (for example, in cases P0103 and especially P1422) (Figures 2A, B; Supplementary Figures S1, S2, S4). For instance, although a major and minor clone were observed initially within P1422, a dramatic shift in subclonal composition occurred at later time points, and the minor clone became undetectable. The majority of subcloned sequences at later time points retained the mutational pattern of the predominant clone, whereas individual clones acquired additional mutations (Supplementary Figures S1, S2). A further interesting finding was provided by case P2920 (Figure 2A), which exhibited a pattern of "clonal equilibrium," that is, two major subclones were present at diagnosis and the relative size of these subclones was maintained over the time points analyzed. Admittedly, the second time point available for this case was only 7 months after diagnosis; however, it is plausible that in time one of these clones may predominate because of positive selection.

DISCUSSION

Ontogenetic Implications

Examination of the IG sequences of CLL subset 4 antibodies highlighted their resemblance to pathogenic anti-DNA antibodies, since both carry IgG-switched BcRs with long positively charged VH CDR3s enriched with arginine residues (35–37). It is therefore conceivable that autoreactive precursors to the subset 4 CLL clones could arise early in B-cell development and subsequently undergo positive selection by DNA, nucleosomes or surface structures of apoptotic cells (38,39). Thereafter, to avoid intense self-reactivity to apoptotic stimulatory material, modifications introduced by SHM could render these clones in a state of diminished responsiveness, perhaps through the introduction of acidic residues in both the heavy- and light-chain genes (Figure 1; Supplementary Tables S6–S8) (40–42). Reinforcing the above theory, studies looking at antigen reactivity profiles in which mutated IGHV4-34 antibodies are reverted to their germline configuration demonstrated increased HEp-2 reactivity and/or acquired polyreactivity (26).

In the periphery, these CLL precursors may exist in a quasi-anergic state, demonstrating attenuating responses to selecting antigenic elements while retaining the ability to engage in superantigenlike interactions (43,44). Along these lines, it is remarkable that despite the high level of ID, certain critical positions of the VH domain were essentially left unaltered by the ID process. For instance, the W residue at codon 7 in the VH FR1 of *IGHV4-34*,

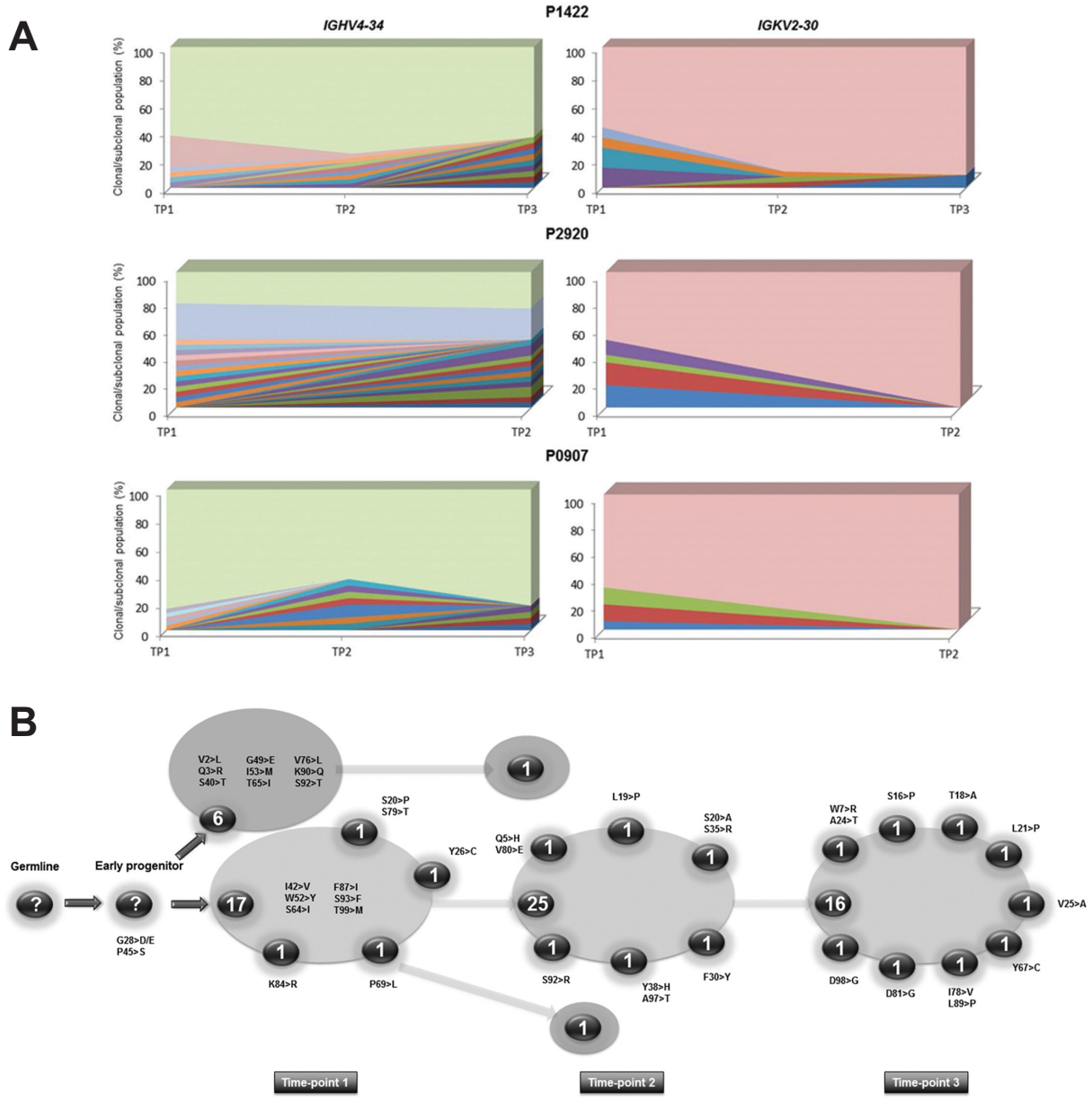

Figure 2. Temporal intraclonal dynamics of CLL subset 4. The level of ID observed over time could be described as increasing, decreasing or complex (that is, a mutation appears or disappears and then reemerges at a subsequent time point). (A) Graphs illustrate the patterns of clonal branching over time in both the *IGHV4-34* and *IGKV2-30* genes of subset 4 CLL cases. The relative size of each subclone is plotted against all time points analyzed, and within each case, a cluster of identical subcloned sequences or a unique subcloned sequence is represented by a different colored segment. TP, time point. (B) Schematic representation of the dynamic patterns of ID observed within the *IGHV4-34* gene of case P1422. Although major and minor clones were observed initially within P1422, a dramatic shift in subclonal composition occurs at later time points, and the minor clone becomes undetectable. The majority of subcloned sequences at later time points retain the mutational pattern of the predominant clone, while individual clones that acquire additional mutations are indicated by the black circles. Additional graphs and sequence information are available in Supplementary Figures S1–S4 and Supplementary Tables S6–S8.

which plays a critical role in recognition of the *N*-acetyllactosamine antigenic determinant present on both self-antigens and microbial pathogens (*Mycoplasma pneumoniae*, cytomegalovirus [CMV], Epstein-Barr virus [EBV]) (45,46), carried alterations in only 6 of 511 sequences. It is plausible that selective pressure resulted in maintenance of this autoantigenic binding motif, and consequently the IGHV4-34 clones of subset 4 retained the ability to engage with an autoantigen and/or a superantigen, as

also implied by our previous findings linking subset 4 with persistent, subclinical infection by CMV and EBV (47).

In attempting to reconcile the evidence for the continuous stimulation and selection of fully transformed CLL cells, it is worth mentioning another fascinating CLL subset (subset 8), since emerging evidence suggests that the distinct clinical profiles of certain subsets may be linked to their precise functional and genetic make-up (10,16,17,19). While both subset 4 and subset 8 express IgG, differences exist between these subsets, with subset 8 (*IGHV4-39/IGKV1-39*) patients harboring unmutated IG genes, following an aggressive disease course and also having a significantly increased risk for Richter transformation (48). In addition, subset 8 cases exhibit a high frequency of trisomy 12, t(14;19)(q32;13) and *NOTCH1* aberrations, whereas subset 4 is conspicuous for a remarkably stable genomic profile acquiring few, if any, aberrations [namely the more favorable prognostic marker del(13q)] (49–53). These subsets are further distinguishable because of their distinct immune signaling profiles; subset 4 clones have a particular functional response to stimulation through toll-like receptors (TLRs), exhibiting a TLR7-tolerant profile, whereas subset 8 clones display an unrestricted and intense response to multiple TLR ligands (54,55).

If one considers the broad reactivity profile of subset 8, it is conceivable that the particularly polyreactive and/or autoreactive clones do not engage in SHM because their unmutated clonotypic IG rearrangements confer these cells with ample opportunities to engage in interactions with numerous antigens. In this regard, data generated by Colombo *et al.* (56), indicating a process of antigenic stimulation/selection leading to expansion of the subset 8 IgG-bearing subclone, perhaps accounts for the progressive nature of cases within this subset. However, subset 4, the focus of this study, is extremely enigmatic, with the lack of clinical aggressiveness being juxtaposed with extensive ID. Thus, this lack of clinical progression observed in most subset 4 cases, despite the presence of clonal evolution, may be linked to the proposed anergic state of the malignant cells.

CONCLUSION

Studying the intricacies of the IG sequence enabled us to trace diversification within the subset 4 CLL antibody. From this, a conceptually satisfying model of clonal evolution within subset 4 could be proposed, whereby, periodically, upon contact with microbial or viral pathogens or during bouts of excessive apoptosis induced by various triggers, low affinity autoreactive B cells may be reactivated by engagement of the BcRs and/or other immune receptors (47,55,57,58). Hence, clonal evolution may (at least in part) result from the functional interplay between CLL cells, the microenvironment and extrinsic pathogens.

Taken together, this extended immunogenetic analysis advances our understanding of the evolution of CLL subset 4 clones. Admittedly, the advent of high throughput sequencing promises even more in this direction; however, limitations of the existing protocols and platforms currently preclude its usage for reliable assessment of SHM patterns. In addition, biological questions remain concerning (a) the precise anatomical location where ongoing SHM occurs and whether T-cell help is required and (b) what the relevance of the observed molecular changes (if any) is to the clinical course and outcome. Nevertheless, the intense ID within CLL subset 4 supports the argument that self-antigens and/or neoantigens acting in synergy with extrinsic pathogens may drive CLL progenitors or even the malignant cells themselves by continuously triggering BcRs with distinctive structural features.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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