Biology of Human Tumors

Antigen Selection Shapes the T-cell Repertoire in Chronic Lymphocytic Leukemia

Anna Vardi[1], Andreas Agathangelidis[2], Evangelia Stalika[1], Maria Karypidou[1,3], Alexandra Siorenta[4], Achilles Anagnostopoulos[3], Richard Rosenquist[5], Anastasia Hadzidimitriou[1], Paolo Ghia[2], Lesley-Ann Sutton[5], and Kostas Stamatopoulos[1,3,5]

Abstract

Purpose: The role of antigen(s) in shaping the T-cell repertoire in chronic lymphocytic leukemia, although relevant for understanding malignant cell interactions with cognate T cells, is largely unexplored.

Experimental Design: Here we profiled the T-cell receptor β chain gene repertoire in 58 chronic lymphocytic leukemia patients, focusing on cases assigned to well-characterized subsets with stereotyped clonotypic B-cell receptor immunoglobulins, therefore those cases most evidently selected by antigen (subsets #1, #2, and #4).

Results: Remarkable repertoire skewing and oligoclonality were observed, and differences between subsets were noted regarding both T-cell receptor β chain gene usage and the extent of clonality, with subset #2 being the least oligoclonal. Longitudinal analysis of

subset #4 cases revealed that although the repertoire may fluctuate over time, certain clonotypes persist, thus alluding to persistent antigenic stimulation. Shared ("stereotyped") clonotypes were found between different patients, reflecting selection by common antigenic elements. Cross-comparison of our dataset with public databases showed that some T-cell clonotypes may have expanded secondary to common viral infections; however, the majority of clonotypes proved to be disease-specific.

Conclusions: Overall, the T-cell receptor β chain repertoire in chronic lymphocytic leukemia is likely shaped by antigen selection and the implicated antigenic elements may concern epitopes that also select the malignant B-cell progenitors or, more intriguingly, chronic lymphocytic leukemia–derived epitopes. *Clin Cancer Res; 22(1); 167–74. ©2015 AACR.*

Introduction

A pivotal role for microenvironmental interactions in the natural history of chronic lymphocytic leukemia (CLL) is strongly supported by (i) the remarkably skewed repertoire of the antigen-specific immunoglobulins (IG) expressed by the B-cell receptors (BcR) of the CLL clones (1, 2); (ii) the division of CLL cases into those with unmutated or minimally mutated IGs ("unmutated" U-CLL) who generally experience an aggressive disease course and those with a significant load of somatic hypermutation within their IG genes ("mutated" M-CLL) who display a considerably more indolent disease (3–5); (iii) the existence of subsets of patients with quasi-identical, "stereotyped" IGs within their BcRs,

pointing to clonal selection by a restricted set of antigens (6–10); and (iv) the clinical efficacy of drugs interfering with signaling through the BcR (11–13). However, it is not yet clear at which stage of clonal evolution the putative antigen(s) may act or whether this stimulation is persistent. Furthermore, their possible role in the selection and activation of cognate T lymphocytes remains obscure, yet highly relevant given accumulating evidence about the intricate cross-talk of T cells with CLL B cells (14–20).

A number of T-cell defects have previously been characterized in patients with CLL, including elevated absolute counts (21), inversion of the CD4$^+$/CD8$^+$ ratio (22), altered cytokine production and chemokine receptor expression profile (23), downregulation of co-stimulatory molecules (24), and oligoclonal T-cell receptor β chain (TRB) gene repertoire (25–28); however, the mechanisms underlying such defects are not fully elucidated. Gene expression profiling studies showed that the CLL B cells are able to induce gene expression changes in healthy CD4$^+$ and CD8$^+$ T cells following direct contact, potentially accounting for the changes observed in T cells from patients with CLL (14). Furthermore, animal model studies have demonstrated that autologous CD4$^+$ T cells may actually provide trophic signals to the CLL clone, which are critical for its survival, expansion, and CD38 expression (16).

Taking all the above into consideration, here we sought to obtain immunogenetic insight into the role of antigen selection in shaping the repertoire of T cells in CLL. To this end, we performed a large-scale molecular study of the TRB gene repertoire in patients with CLL, focusing on those cases where the malignant clone expresses stereotyped BcR IGs and thus is most evidently selected

[1]Institute of Applied Biosciences, Center for Research and Technology Hellas, Thessaloniki, Greece. [2]Division of Molecular Oncology and Department of Onco-Hematology, Istituto Scientifico San Raffaele e Fondazione Centro San Raffaele, Milan, Italy. [3]Hematology Department and HCT Unit, G. Papanicolaou Hospital, Thessaloniki, Greece. [4]Immunology and National Tissue Typing Center, General Hospital of Athens "G. Gennimatas," Athens, Greece. [5]Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden.

Corresponding Author: Lesley-Ann Sutton, Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, Uppsala SE-751-85, Sweden. Phone: 46-18-4714852; E-mail: lesley.sutton@igp.uu.se

AACR

Translational Relevance

Extracellular cues vital for chronic lymphocytic leukemia (CLL) cell survival and proliferation are provided by accessory cells that, together with cytokines and chemokines, form the tumor microenvironment. Despite accumulating evidence of an intricate cross-talk between T cells and CLL B cells, little is known regarding the role of antigens in shaping the CLL T-cell repertoire. Here, we investigated the T-cell receptor β chain gene repertoire in a large CLL cohort, with a strong focus on patients expressing stereotyped B-cell receptor immunoglobulins, therefore cases most evidently selected by antigen, including prototypic subsets of both clinically aggressive and indolent disease. We found repertoire skewing and oligoclonality in the majority of patients, strongly indicative of antigen selection in shaping the CLL T-cell repertoire. We maintain that a better understanding of CLL cells interaction with their surroundings will prove clinically relevant in light of the increasing use of novel therapies that target the microenvironment.

by antigen(s). Overall, our findings suggest that the T cell driving antigens could include epitopes implicated in the selection of the malignant B cells and, perhaps more intriguingly, CLL cell (tumor)–derived epitopes.

Materials and Methods

Patient group

We analyzed 73 peripheral blood samples from 58 untreated patients with CLL (median age, 68; range, 45–85 years) and 2 control samples from age-matched (ages, 70- and 80-year-old) healthy individuals. Fifty-two of the 58 CLL cases were selected on the basis of their expression of stereotyped BcR IGs leading to their assignment to well-annotated CLL subsets (10). Detailed information regarding the immunogenetic characteristics of these subsets is provided in the Supplementary Data. More specifically, our CLL series was composed of cases belonging to subset #4 ($n = 20$), subset #1 ($n = 16$), and subset #2 ($n = 16$), as well as cases carrying BcR IGs not assigned to subsets ($n = 6$). Five subset #4 cases were analyzed over time (19 samples in total, sampling period ranged from 18–42 months; median 28 months—the patients remained untreated throughout the sampling period). In one subset #1 case, in addition to peripheral blood, a lymph node specimen from the same time point was also analyzed. No case had evidence of infection at sampling. The local Ethics Review Committees of the participating institutions approved the study.

PCR amplification of TRBV–TRBD–TRBJ gene rearrangements

Total cellular RNA was isolated from peripheral blood mononuclear cells (or, in one case, fresh lymph node tissue) and reverse transcribed to cDNA using the SuperScript II RT Kit (Invitrogen Life technologies). RT-PCR and heteroduplex analysis of TRBV–TRBD–TRBJ gene rearrangements was performed according to the BIOMED-2 collaborative effort (29). RT-PCR products were gel-purified with the QIAGEN DNA purification columns (QIAGEN), ligated into the pCR2.1 vector (Invitrogen Life technologies), and transformed into *E. coli*/TOP10F' competent bacteria (Invitrogen

Life technologies). Colonies were chosen randomly and sequenced using the −20 universal primer or M13 primers.

Sequence analysis, definitions, and interpretation

Sequence data were analyzed using the IMGT/V-QUEST tool from the international ImMunoGeneTics information system (http://www.imgt.org). Only productive TRBV–TRBD–TRBJ gene rearrangements were included in the analysis.

Clonotypes were defined as TRBV–TRBD–TRBJ gene rearrangements using a particular TRBV gene and carrying a distinct TRB complementarity determining region 3 (CDR3) amino acid sequence. Clonotypes were considered expanded (forming clusters) when they contained ≥2 sequences, with the most expanded clonotype within a sample referred to as the immunodominant clonotype. The relative frequency of each clonotype/sample was calculated as the number of rearrangements corresponding to the clonotype divided by the total number of subcloned productive rearrangements for that particular sample. Clonality analysis was performed for samples with >10 sequenced productive rearrangements. For repertoire analysis, clonotypes rather than single rearrangements were considered to avoid potential biases due to expansion following antigenic stimulation.

All CLL TRB sequences were aligned and compared with the TRB sequences of the healthy control samples, as well as a panel of 5,717 nonredundant, well-annotated, and productive TRBV–TRBD–TRBJ rearrangement sequences from various entities (normal, autoreactive, and malignant T-cell clones) available to our group ($n = 1,715$) or retrieved from the IMGT/LIGM-DB sequence database (http://www.imgt.org/IMGTindex/antibodies.html; $n = 4,002$).

HLA typing

Typing of the HLA-A, -B, -C loci for low resolution and HLA-DRB1 locus for allelic level high-resolution determination was performed on the 2 healthy controls and 8 CLL samples (more specifically, the 4 pairs of CLL cases that were found to share common clonotypes, as described in the Results) by reverse PCR-SSOP (sequence-specific oligonucleotide probe) using a commercially available kit (LABtype RSSO, One Lambda).

Statistical analysis and visualization tools

Descriptive statistics for discrete parameters included counts and frequency distributions. For quantitative variables, statistical measures included means, medians, and min–max values. The significance of bivariate/multivariate relationships between variables was assessed using the unpaired t test, and for all comparisons, a significance level of $P = 0.05$ was set. All statistical analyses were performed using the statistical Package GraphPad Prism version 5.03 (GraphPad Software, Inc.).

Circos graphs were produced using freeware, licensed under GPL, and available at http://circos.ca (30).

Results

The T-cell gene repertoire of CLL is remarkably skewed

Overall, 1,378 productive CLL TRBV–TRBD–TRBJ gene rearrangements were included in the analysis, corresponding to 985 distinct clonotypes. Thirty-nine functional TRBV genes were identified. The most frequent gene was TRBV19, contributing to 108 clonotypes (11.0%), followed by TRBV27 (8.1%), TRBV12-3 (7.6%), TRBV7-9 (7.0%), TRBV6-5 (6.4%), and TRBV20-1 (5.1%). Thus, collectively, 6 TRBV genes accounted for almost

Figure 1.
TRBV gene repertoire. A, TRBV gene repertoire analysis at the CLL cohort level and comparison of TRBV gene frequencies within the expanded clonotypes versus the polyclonal background (clonotypes that were identified only once, "single clonotypes"). The fourth column corresponds to the two age-matched healthy samples. The first 10 TRBV genes on the x-axis are the most frequent within the CLL cohort. The final TRBV gene (TRBV24-1) was added to facilitate comparison of the CLL TRBV gene repertoire to that of the healthy controls. The P values relate to the TRBV gene frequency within the CLL cohort versus the healthy controls. B, comparison of the TRBV gene repertoire among the four subgroups within the CLL cohort, subset #1, subset #2, subset #4, and non-subset cases.
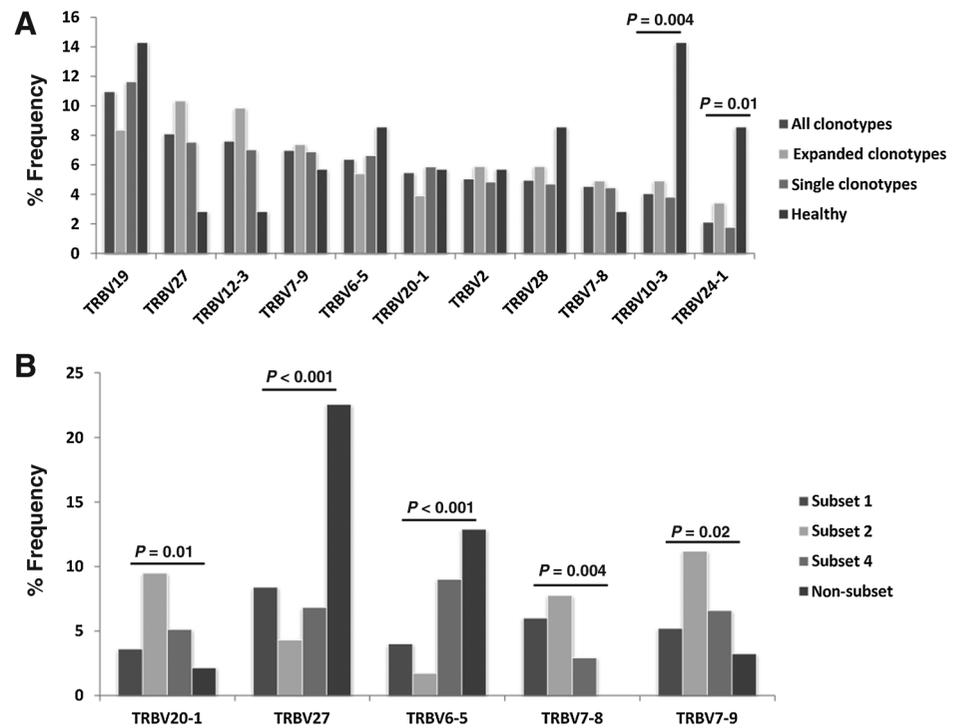
half of all clonotypic rearrangements, highlighting a remarkable bias within the CLL TRBV gene repertoire. Importantly, analysis of the healthy control samples revealed a different pattern of TRBV gene usage, with clear overrepresentation of the TRBV10-3 and TRBV24-1 genes ($P = 0.004$ and $P = 0.01$, respectively), and underrepresentation of the TRBV27 and TRBV12-3 genes (P values did not reach statistical significance, likely due to the small size of the respective sequence dataset; Fig. 1A). Detailed immunogenetic information regarding gene frequencies and TRB CDR3 length and composition are provided in the Supplementary Data.

When comparing the TRBV gene usage among the four patient groups within our cohort (i.e., subsets #1, #2 and #4, as well as non-subset cases), several differences were noted. More specifically, (i) clonotypic rearrangements belonging to cases with heterogeneous BcR IGs were significantly enriched for TRBV27 and TRBV6-5 genes, whereas TRBV7-8, TRBV20-1, and TRBV7-9 were underrepresented; and (ii) clonotypic rearrangements belonging to subset #2 cases were significantly enriched for TRBV20-1, TRBV7-9, and TRBV7-8 gene usage (Fig. 1B).

In total, 73 samples from 58 patients with CLL were analyzed. The great majority of patients exhibited oligoclonality. More specifically, when samples with ≤10 sequenced productive rearrangements were excluded, 52 of the remaining 57 samples (91%), corresponding to 40 of 45 cases (89%), were found to carry clusters of identical rearrangements (≥2; expanded clonotypes). For the remaining 5 cases, the failure to detect expanded clonotypes may be attributed to their significantly ($P = 0.02$) lower median number of subcloned productive TRBV–TRBD–TRBJ rearrangement sequences.

For the 52 CLL samples with expanded clonotypes, the number of expanded clonotypes/sample ranged from 1 to 11 (median, 3), whereas the relative frequency of each clonotype/sample, ranged from 2.7% to 80% (median, 10%). The relative frequency of the

immunodominant clonotype ranged from 4.9% to 70.4% (median, 16.2%) and the cumulative frequency of all expanded clonotypes/sample ranged from 10.5% to 88.9% (median, 44.8%). The respective values concerning all analyzed CLL samples ($n = 57$) are shown in Table 1. Of relevance, in the CLL case where peripheral blood and lymph node from the same time point were cross-examined, only the lymph node sample exhibited oligoclonality (cumulative frequency of expanded clonotypes 42.9%). This finding may also be relevant for the 5 CLL cases lacking expanded clonotypes in the peripheral blood: put differently, it cannot be a priori excluded that such cases carry expanded T-cell clones albeit with a distinct tissue localization.

We then assessed clonality separately for each subgroup of patients, that is, subset #1, subset #2, subset #4, and cases with non-subset BcR IG rearrangements. Unexpectedly, subset #2 was substantially less oligoclonal in comparison to subset #4, as well as patients with heterogeneous BcR IG rearrangements (median cumulative frequency of expanded clonotypes 23.8% vs. 48.3%, $P = 0.03$ and 51.1%, $P = 0.01$, respectively; Table 1; Figs. 2 and 3).

Importantly, the healthy control samples did not exhibit oligoclonality. Collectively, of the 35 clonotypes that were

Table 1. Clonality analysis for samples with >10 subcloned productive TRBV–TRBD–TRBJ sequences

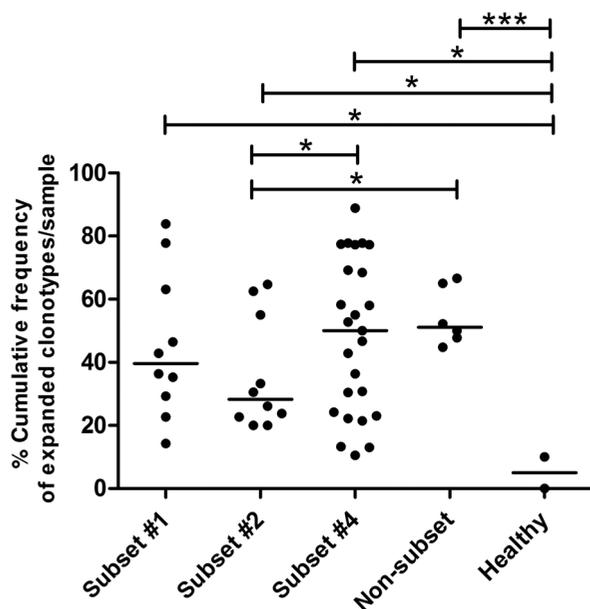|	Cases	Samples	Sequences/ sample [median (range)]	Expanded clonotypes [median (range)]	Relative frequency of the dominant clonotype (%)
Subset #1	12	12	23 (14–41)	4 (0–11)	13.3 (0–21.4)
Subset #2	13	13	20 (12–36)	2 (0–3)	13.6 (0–50)
Subset #4	17	26	21 (11–36)	3 (0–8)	18.2 (0–70.4)
Non-subset	6	6	23 (14–30)	5 (3–7)	16.7 (13–30)
Total	48	57	22 (11–41)	3 (0–11)	14.8 (0–70.4)

Vardi et al.

Figure 2.
The percentage cumulative frequency of all expanded clonotypes per sample analyzed separately for each subgroup of patients with CLL, that is, subset #1, subset #2, subset #4, non-subset cases, and the age-matched healthy controls. Each dot corresponds to a sample with >10 subcloned productive sequences, and horizontal lines indicate the median value for each subgroup of patients. The T-cell gene repertoire of subset #2 patients was significantly less oligoclonal in comparison to subset #4 patients, as well as patients with heterogeneous BcR IG rearrangements. The healthy controls were essentially polyclonal. Differences of median values at level of statistical significance $P < 0.05$ and $P < 0.01$ are marked with * and ***, respectively.

identified, 34 concerned "singletons" that is appeared only once whereas only a single clonotype was detected twice (median cumulative frequency, 5.0%; Fig. 2).

The T-cell repertoire fluctuates; however, the same subclone may persist over time

To assess clonal dynamics over time, we tested longitudinal samples from 5 subset #4 patients (2–6 samples per patient, median 3); the sampling period ranged from 18 to 42 months (median, 28 months). With the exception of one patient who displayed a relatively stable TRBV gene repertoire, all other patients exhibited variable TRBV gene frequencies at the different time points analyzed, suggestive of clonal drift (Fig. 4A); however overall, in 4 of 5 cases (80%), at least one clonotype was found to persist over at least two different time points (Fig. 4B). Interestingly, the immunodominant clone differed at each time point.

Of relevance, the TRBV gene repertoire was also found to differ substantially when both the peripheral blood sample and the lymph node sample from the same case and time point were examined, with the two samples presenting distinct TRBV gene frequencies.

Shared clonotypes between different patients

Cluster analysis of the TRB CDR3 amino acid sequences of all cases identified 4 distinct TRB CDR3 amino acid sequences used by pairs of patients. In 2 of 4 such pairs of patients, the TRBV gene was the same, thus translating to a shared ("stereotyped" or "public") clonotype; interestingly, these 2 pairs involved patients

assigned to subset #4. The remaining 2 pairs carrying identical TRB CDR3 amino acid sequences exhibited discordant TRBV gene usage; one of these pairs involved a patient assigned to subset #1 and a subset #4 patient, whereas the other involved a subset #2 patient and a non-subset patient (Table 2).

Given that antigen recognition by T-cell receptors occurs in the context of the major histocompatibility complex proteins, we then investigated the respective gene loci (HLA typing) in these 8 cases (the 4 pairs of patients carrying identical TRB CDR3s) to search for possible matches and found that within each pair of patients at least one gene match was found among the 8 loci tested (Supplementary Data). For 2 of 4 CLL pairs, this "implicated," shared HLA gene was also found to be expressed by the healthy controls (Supplementary Data); however, none of the aforementioned "public" clonotypes were detected in the latter.

Hits with public database sequences

We next compared all productive CLL TRBV–TRBD–TRBJ gene rearrangement sequences from our study ($n = 1,378$) against the TRBV–TRBD–TRBJ gene rearrangement sequences obtained from the two healthy control samples ($n = 36$) and also a panel of 5,717 nonredundant, productive TRBV–TRBD–TRBJ gene rearrangement sequences from various entities. Only a single TRB CDR3 sequence from our CLL dataset was found to have an identical match within the public database; notably, this particular TRB CDR3 sequence participated in one of the aforementioned "pairs" with identical TRB CDR3. More specifically, the match concerned a shared TRB CDR3 sequence between two patients with CLL
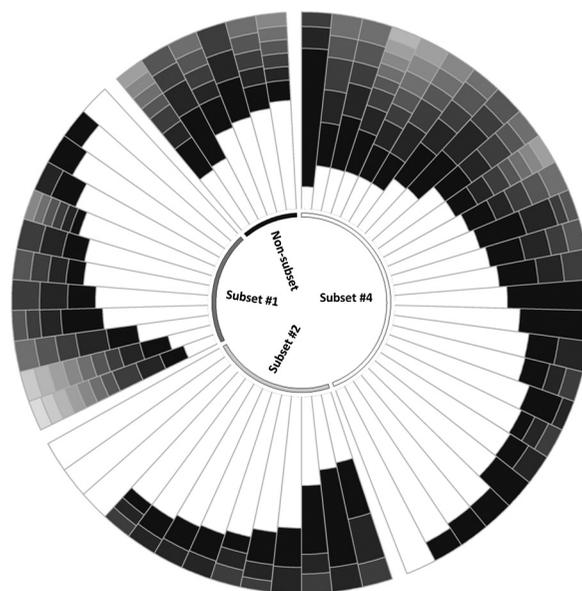

Figure 3.
T-cell clonality analysis for each subgroup of patients (subset #1, subset #2, subset #4 and cases with heterogeneous BcR IG rearrangements). Each lane corresponds to a sample with >10 subcloned productive sequences and each tile of a lane corresponds to the relative size of different expanded clonotypes within a sample. The inner tile (shown in white in all instances) depicts the polyclonal background, that is, the cumulative frequency of all single clonotypes, within a sample. Samples belonging to subset #2 cases appear to be less oligoclonal both in terms of the relative size of the polyclonal background and in terms of the number of expanded clonotypes per sample.
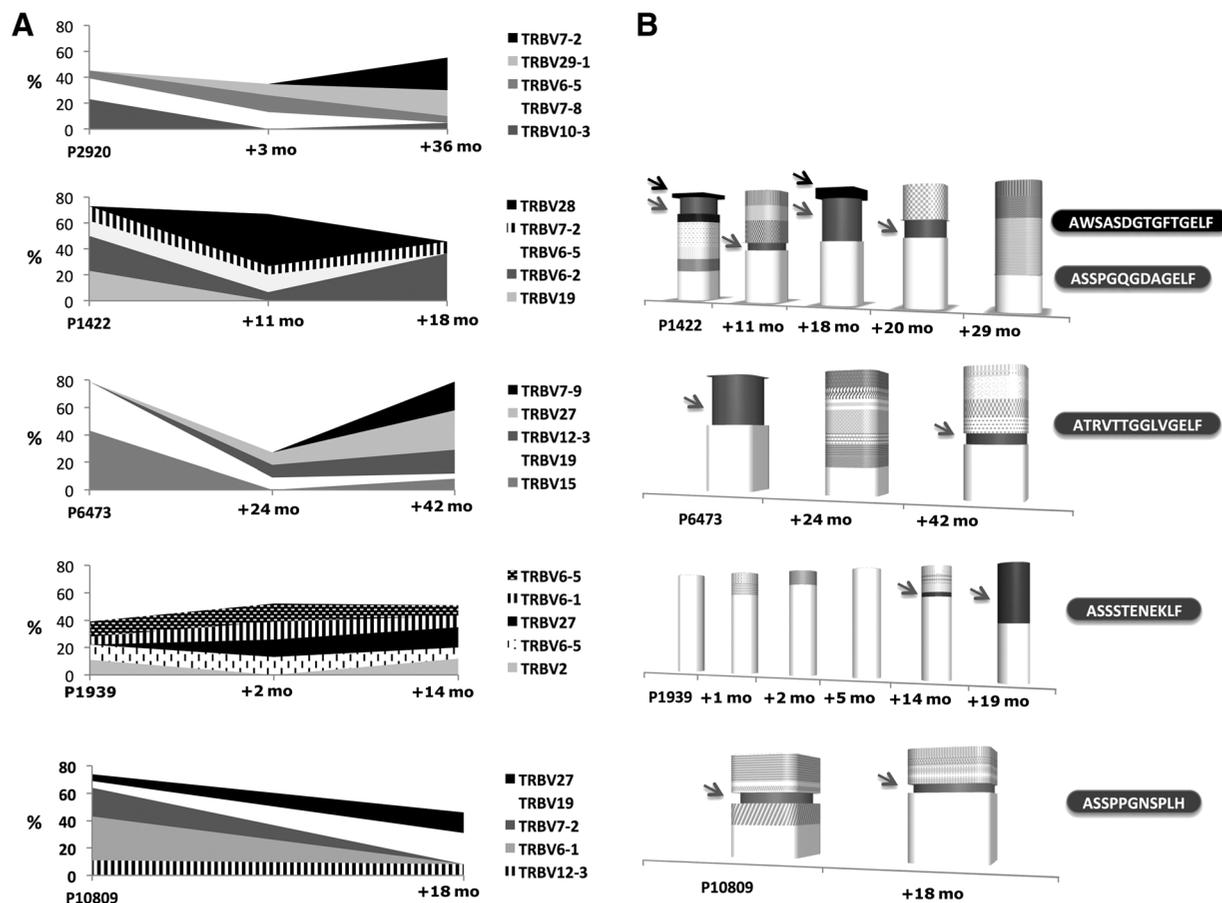
Figure 4.
TRBV gene repertoire and clonal dynamics over time. A, representation of how the TRBV gene frequencies fluctuate over time in the 5 subset #4 patients with CLL for whom successive samples were analyzed. Only the 5 most frequent TRBV genes and time points with ≥10 sequenced rearrangements are depicted. Solid shades are used for TRBV genes with frequencies that fluctuate ≥10% over time, whereas pattern fill is used for TRBV genes with relatively stable frequencies over time (i.e., fluctuate < 10%). B, clonal drift. Each column corresponds to a distinct time point and each tile to a specific clonotype. The basal tiles (depicted in white in all instances) represent the polyclonal background at each time point. Despite the fluctuating nature of the TCR β repertoire, certain clonotypes, indicated by arrows, persist over time.

(P1812 and Swe-1025) of our cohort (P1812 used the TRBV10-3 gene, whereas Swe-1025 used the TRBV19 gene) and a single entry in the IMGT/LIGM-DB sequence database (Accession number AM041155, using the TRBV19 gene). The latter corresponded to a CD8^{+} T-cell clone specific for a highly immunogenic Epstein-Barr virus (EBV) epitope (EPLPQGQLTAY). This clone is known to

be HLA-B*35:01–restricted (31), and indeed, our two patients with CLL carrying this clonotype were both found to express HLA-B*35.

When following a less stringent threshold (>85%) for amino acid identity, several additional "hits" were identified concerning similar TRB CDR3s, as listed in the Supplementary Data.

Table 2. Pairs of patients that harbor clonotypes with identical amino acid TRB CDR3 sequences

Patient ID	Subset	TRBV gene	TRBD gene	TRBJ gene	Shared CDR3 AA sequence
P1626	#4	TRBV15	TRBD1	TRBJ2-2	ATRVTTGGLVGELF
P1939	#4				
P1422	#4	TRBV 30	TRBD1	TRBJ2-2	AWSASDGTGFTGELFF
P2920	#4				
Swe-1065	#1	TRBV7-2	TRBD2	TRBJ1-5	ASSLGTNQPQH
P3870	#4	TRBV7-9	TRBD1		
P1812	Non-subset	TRBV10-3	TRBD1	TRBJ1-5	ASSTGDSNQPQH
Swe-1025	#2	TRBV19			

Abbreviations: AA, amino acid; CDR3, complementarity determining region 3; TRBD, T-cell receptor β diversity gene; TRBJ, T-cell receptor β joining gene; TRBV, T-cell receptor β variable gene.

Discussion

We investigated the T-cell receptor gene repertoire in the largest CLL cohort studied so far for this purpose. We focused on patients with CLL expressing stereotyped BcR IG and assigned to the most populated CLL-specific subsets, namely #1 (the largest within U-CLL, clinically aggressive), #2 (the largest overall, mix of U-CLL and M-CLL, clinically aggressive), and #4 (the largest within M-CLL, particularly indolent; refs. 9, 10, 32). We also investigated patients with CLL carrying non-subset BcR IG rearrangements, so as not to disregard the possibility of identifying T-cell immunogenetic features that are ubiquitous in CLL, irrespective of the particular IG receptor that is expressed. Our cohort was intentionally biased towards subset #4, where ample evidence suggests ongoing interactions with the selecting antigen (33–36). For this reason, we also performed longitudinal immunoprofiling of five subset #4 patients, aiming to explore the possibility of clonal drift. Clonotypes were considered expanded when they contained ≥2 sequences, and while this definition may seem arbitrary, the identification of a specific rearrangement more than once using a low-throughput method, such as subcloning, justifies considering this clone as expanded.

The findings reported herein provide solid evidence for TRB gene repertoire skewing and oligoclonality in the great majority of patients with CLL analyzed. This is in line with previous reports of oligoclonality within the T-cell repertoire in CLL; however, ours is the first molecular documentation of this phenomenon, as previous studies were based solely on flow cytometric analysis and spectratyping (25–28). Importantly, within our study, T-cell oligoclonality was found to be universal in CLL and not confined to cases carrying stereotyped BcR IGs. The differences in TRBV gene usage between CLL and age-matched healthy samples argue that the reported TRB gene frequency skewing is CLL-biased and not an age-related repertoire restriction phenomenon. Although the small number of analyzed healthy samples precludes definitive conclusions, the relative lack of clonality within these healthy controls (at least with this low-throughput approach) efficiently highlights the greater extent of oligoclonality that characterizes CLL samples as compared with age-matched healthy controls, thus providing reassuring evidence that the reported TRB repertoire skewing is indeed CLL-biased.

That notwithstanding, TRB gene repertoire analysis performed separately for each subgroup of patients, that is, subset #1, subset #2, subset #4 and non-subset cases revealed that each subgroup exhibited varying TRBV gene restrictions, alluding to different antigenic elements driving T-cell selection. This is in line with previous studies detailing distinct antigenic stimulation that drives the selection and expansion of the malignant B-cell clone in CLL subsets carrying different stereotyped BcR IGs (6–10, 33, 34, 37–39). Counter-arguing, if certain antigenic elements were responsible for the TRBV gene repertoire skewing within each subgroup, one would expect the skewing to remain stable over time. However, with a single exception, all cases profiled over successive time points exhibited fluctuating frequencies of TRBV genes, although certain clonotypes did persist.

Subgroup analysis also revealed that clonality is less evident in certain subsets, especially subset #2. The underlying reason for this remains elusive; however, it should be noted that subset #2 cases follow an aggressive clinical course, even when harboring mutated BcR IGs and were recently found to display a particularly

high frequency of SF3B1 mutations that may somehow render the cells less dependent on T-cell interactions through, as yet, unidentified mechanisms (37, 40–42). Indeed, whether T-cell oligoclonality is associated with disease course and outcome will require the study of larger cohorts of patients.

A notable finding in the present study was the identification of shared ("public") clonotypes among different patients. According to estimates of normal T-cell repertoire diversity, the chance that this may happen by serendipity is negligible (43, 44). The possibility that this resulted from cross-contamination was also ruled out by the fact that shared clonotypes were identified in subcloning experiments performed at different times. Therefore, we have to assume that "public" clonotypes present in different patients with CLL stem from selection by the same or closely similar antigenic elements. Could these be the same antigens that are selecting the malignant B-cell clone?

If this were the case, one would expect that common clonotypes would only be identified among patients belonging to the same subset (and sharing the respective HLA alleles). Indeed, identical clonotypes were found only among subset #4 patients with at least one common HLA allele. However, if a more relaxed definition of "clonotype" was adopted, for example, involving the same amino acid TRB CDR3 sequence yet with different TRBV genes, then common clonotypes were also found among patients in different subgroups. Thus, the possibility exists that the common antigenic drive concerns tumor-associated antigens, that is, proteins specifically expressed by the CLL cells. Alternatively, it could be related to shared, persistent subclinical infections.

To further interrogate the nature of antigenic elements that interfere with T-cell selection in CLL, we performed extensive cross-entity comparisons. Notably, within a panel of 5,717 productive TRBV–TRBD–TRBJ rearrangement sequences, only one identical match was found, which corresponded to the TRB CDR3 sequence shared by two patients with CLL from our cohort and a CD8^{+} T-cell clone from the literature, highly specific for an immunogenic epitope from the BZLF1 antigen of EBV (31). Interestingly, this clone is part of a "public" T-cell receptor repertoire, that is, a highly constrained, HLA-restricted T-cell receptor repertoire that is usually elicited in response to bulged viral peptides and is common among HLA-B*35:01 individuals (31, 45). Given that both patients with CLL also expressed HLA-B*35, it is reasonable to say that, at least on this occasion, the identified shared clonotype stemmed from EBV antigenic stimulation. This is not altogether unexpected, given that the immunocompromise of CLL is a predisposing factor for reactivation of chronic herpes virus infections (46); furthermore, it is relevant to mention our previous finding that demonstrated that certain patients with CLL, especially those using the IGHV4-34 gene, have persistent subclinical infection by EBV, indicating that viral antigens/superantigens might facilitate clonal expansion (47).

In conclusion, the present study provides clear molecular evidence for TRBV gene repertoire skewing among T cells in patients with CLL belonging to major stereotyped subsets as well as non-subset patients, thereby strongly supporting antigen selection in disease pathogenesis. The finding of identical or highly similar clonotypes among different cases raises the possibility that shared antigenic epitopes may be relevant for clonal selection of T cells. Whether the antigens that drive T-cell repertoire restriction are identical/related to those implicated in the selection of CLL

progenitors or the malignant cells themselves remains to be clarified.

Disclosure of Potential Conflicts of Interest

K. Stamatopoulos reports receiving commercial research grants from Glaxo-SmithKline, Novartis Hellas, Roche SA. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A. Agathangelidis, R. Rosenquist, K. Stamatopoulos
Development of methodology: E. Stalika, M. Karypidou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Siorenta, A. Anagnostopoulos, L.-A. Sutton
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Vardi, A. Agathangelidis, R. Rosenquist, L.-A. Sutton, K. Stamatopoulos
Writing, review, and/or revision of the manuscript: A. Vardi, A. Agathangelidis, R. Rosenquist, P. Ghia, L.-A. Sutton, K. Stamatopoulos
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Karypidou, A. Anagnostopoulos, L.-A. Sutton
Study supervision: A. Agathangelidis, A. Hadzidimitriou, K. Stamatopoulos

References

1. Hashimoto S, Dono M, Wakai M, Allen SL, Lichtman SM, Schulman P, et al. Somatic diversification and selection of immunoglobulin heavy and light chain variable region genes in IgG+ CD5+ chronic lymphocytic leukemia B cells. J Exp Med 1995;181:1507–17.
2. Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin Invest 1998;102:1515–25.
3. Dighiero G, Hamblin TJ. Chronic lymphocytic leukaemia. Lancet 2008;371:1017–29.
4. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V (H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94:1848–54.
5. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94:1840–7.
6. Tobin G, Thunberg U, Johnson A, Eriksson I, Soderberg O, Karlsson K, et al. Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vlambda2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope. Blood 2003;101:4952–7.
7. Tobin G, Thunberg U, Karlsson K, Murray F, Laurell A, Willander K, et al. Subsets with restricted immunoglobulin gene rearrangement features indicate a role for antigen selection in the development of chronic lymphocytic leukemia. Blood 2004;104:2879–85.
8. Ghiotto F, Fais F, Valetto A, Albesiano E, Hashimoto S, Dono M, et al. Remarkably similar antigen receptors among a subset of patients with chronic lymphocytic leukemia. J Clin Invest 2004;113:1008–16.
9. Stamatopoulos K, Belessi C, Moreno C, Boudjograh M, Guida G, Smilevska T, et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood 2007;109:259–70.
10. Agathangelidis A, Darzentas N, Hadzidimitriou A, Brochet X, Murray F, Yan XJ, et al. Stereotyped B-cell receptors in one-third of chronic lymphocytic leukemia: a molecular classification with implications for targeted therapies. Blood 2012;119:4467–75.
11. Herman SE, Mustafa RZ, Gyamfi JA, Pittaluga S, Chang S, Chang B, et al. Ibrutinib inhibits BCR and NF-kappaB signaling and reduces tumor proliferation in tissue-resident cells of patients with CLL. Blood 2014;123:3286–95.
12. Brown JR, Byrd JC, Coutre SE, Benson DM, Flinn IW, Wagner-Johnston ND, et al. Idelalisib, an inhibitor of phosphatidylinositol 3-kinase p110delta, for relapsed/refractory chronic lymphocytic leukemia. Blood 2014;123:3390–7.
13. Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. N Engl J Med 2013;369:32–42.
14. Gorgun G, Holderried TA, Zahrieh D, Neuberg D, Gribben JG. Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. J Clin Invest 2005;115:1797–805.
15. Ramsay AG, Johnson AJ, Lee AM, Gorgun G, Le Dieu R, Blum W, et al. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. J Clin Invest 2008;118:2427–37.
16. Bagnara D, Kaufman MS, Calissano C, Marsilio S, Patten PE, Simone R, et al. A novel adoptive transfer model of chronic lymphocytic leukemia suggests a key role for T lymphocytes in the disease. Blood 2011;117:5463–72.
17. Ramsay AG, Clear AJ, Fatah R, Gribben JG. Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. Blood 2012; 120:1412–21.
18. Ramsay AG, Evans R, Kiaii S, Svensson L, Hogg N, Gribben JG. Chronic lymphocytic leukemia cells induce defective LFA-1-directed T-cell motility by altering Rho GTPase signaling that is reversible with lenalidomide. Blood 2013;121:2704–14.
19. Devereux S. Two-faced T cells in CLL. Blood 2011;117:5273–4.
20. Zenz T. Exhausting T cells in CLL. Blood 2013;121:1485–6.
21. Totterman TH, Carlsson M, Simonsson B, Bengtsson M, Nilsson K. T-cell activation and subset patterns are altered in B-CLL and correlate with the stage of the disease. Blood 1989;74:786–92.
22. Foa R, Giovarelli M, Jemma C, Fierro MT, Lusso P, Ferrando ML, et al. Interleukin 2 (IL 2) and interferon-gamma production by T lymphocytes from patients with B-chronic lymphocytic leukemia: evidence that normally released IL 2 is absorbed by the neoplastic B cell population. Blood 1985;66:614–9.
23. Cantwell M, Hua T, Pappas J, Kipps TJ. Acquired CD40-ligand deficiency in chronic lymphocytic leukemia. Nat Med 1997;3:984–9.
24. Rossi E, Matutes E, Morilla R, Owusu-Ankomah K, Heffernan AM, Catovsky D. Zeta chain and CD28 are poorly expressed on T lymphocytes from chronic lymphocytic leukemia. Leukemia 1996;10:494–7.
25. Farace F, Orlanducci F, Dietrich PY, Gaudin C, Angevin E, Courtier MH, et al. T cell repertoire in patients with B chronic lymphocytic leukemia. Evidence for multiple in vivo T cell clonal expansions. J Immunol 1994; 153:4281–90.
26. Rezvany MR, Jeddi-Tehrani M, Osterborg A, Kimby E, Wigzell H, Mellstedt H. Oligoclonal TCRBV gene usage in B-cell chronic lymphocytic leukemia: major perturbations are preferentially seen within the CD4 T-cell subset. Blood 1999;94:1063–9.
27. Goolsby CL, Kuchnio M, Finn WG, Peterson L. Expansions of clonal and oligoclonal T cells in B-cell chronic lymphocytic leukemia are primarily

Vardi et al.

restricted to the CD3(+)CD8(+) T-cell population. Cytometry 2000;42: 188–95.

28. Zaborsky N, Holler C, Geisberger R, Asslaber D, Gassner FJ, Egger V, et al. B cell receptor usage correlates with the sensitivity to CD40 stimulation and the occurrence of CD4+ T cell clonality in chronic lymphocytic leukemia. Haematologica 2015;100:e307–10.

29. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003;17: 2257–317.

30. Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, et al. Circos: an information aesthetic for comparative genomics. Genome Res 2009;19:1639–45.

31. Miles JJ, Elhassen D, Borg NA, Silins SL, Tynan FE, Burrows JM, et al. CTL recognition of a bulged viral peptide involves biased TCR selection. J Immunol 2005;175:3826–34.

32. Baliakas P, Hadzidimitriou A, Sutton LA, Minga E, Agathangelidis A, Nichelatti M, et al. Clinical effect of stereotyped B-cell receptor immuno-globulins in chronic lymphocytic leukemia: a retrospective multicentre study. Lancet Haematol 2014;1:e74–84.

33. Sutton LA, Kostareli E, Hadzidimitriou A, Darzentas N, Tsaftaris A, Ana-gnostopoulos A, et al. Extensive intraclonal diversification in a subgroup of chronic lymphocytic leukemia patients with stereotyped IGHV4-34 recep-tors: implications for ongoing interactions with antigen. Blood 2009;114: 4460–8.

34. Kostareli E, Sutton LA, Hadzidimitriou A, Darzentas N, Kouvatsi A, Tsaftaris A, et al. Intraclonal diversification of immunoglobulin light chains in a subset of chronic lymphocytic leukemia alludes to antigen-driven clonal evolution. Leukemia 2010;24:1317–24.

35. Sutton LA, Kostareli E, Stalika E, Tsaftaris A, Anagnostopoulos A, Darzentas N, et al. Temporal dynamics of clonal evolution in chronic lymphocytic leukemia with stereotyped IGHV4-34/IGKV2-30 antigen receptors: longi-tudinal immunogenetic evidence. Mol Med 2013;19:230–6.

36. Vardi A, Agathangelidis A, Sutton LA, Chatzouli M, Scarfo L, Mansouri L, et al. IgG-switched CLL has a distinct immunogenetic signature from the common MD variant: ontogenetic implications. Clin Cancer Res 2013;20: 323–30.

37. Tobin G, Thunberg U, Johnson A, Thorn I, Soderberg O, Hultdin M, et al. Somatically mutated Ig V(H)3-21 genes characterize a new subset of chronic lymphocytic leukemia. Blood 2002;99:2262–4.

38. Chu CC, Catera R, Zhang L, Didier S, Agagnina BM, Damle RN, et al. Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: implications for patient outcome and cell of origin. Blood 2010;115:3907–15.

39. Murray F, Darzentas N, Hadzidimitriou A, Tobin G, Boudjogra M, Scielzo C, et al. Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis. Blood 2008;111:1524–33.

40. Thorselius M, Krober A, Murray F, Thunberg U, Tobin G, Buhler A, et al. Strikingly homologous immunoglobulin gene rearrangements and poor outcome in VH3-21-using chronic lymphocytic leukemia patients inde-pendent of geographic origin and mutational status. Blood 2006;107: 2889–94.

41. Strefford JC, Sutton LA, Baliakas P, Agathangelidis A, Malcikova J, Plevova K, et al. Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of SF3B1 and subset #2. Leukemia 2013;27:2196–9.

42. Rossi D, Spina V, Bomben R, Rasi S, Dal-Bo M, Bruscaggin A, et al. Association between molecular lesions and specific B-cell receptor subsets in chronic lymphocytic leukemia. Blood 2013;121:4902–5.

43. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P. A direct estimate of the human alphabeta T cell receptor diversity. Science 1999;286:958–61.

44. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat Rev Immunol 2004;4:123–32.

45. Tynan FE, Reid HH, Kjer-Nielsen L, Miles JJ, Wilce MC, Kostenko L, et al. A T cell receptor flattens a bulged antigenic peptide presented by a major histocompatibility complex class I molecule. Nat Immunol 2007;8:268–76.

46. Hermouet S, Sutton CA, Rose TM, Greenblatt RJ, Corre I, Garand R, et al. Qualitative and quantitative analysis of human herpesviruses in chronic and acute B cell lymphocytic leukemia and in multiple myeloma. Leuke-mia 2003;17:185–95.

47. Kostareli E, Hadzidimitriou A, Stavroyianni N, Darzentas N, Athanasiadou A, Gounari M, et al. Molecular evidence for EBV and CMV persistence in a subset of patients with chronic lymphocytic leukemia expressing stereo-typed IGHV4-34 B-cell receptors. Leukemia 2009;23:919–24.

Clinical Cancer Research

Antigen Selection Shapes the T-cell Repertoire in Chronic Lymphocytic Leukemia

Anna Vardi, Andreas Agathangelidis, Evangelia Stalika, et al.

Updated version	Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-14-3017
Supplementary Material	Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/09/02/1078-0432.CCR-14-3017.DC1

Cited articles	This article cites 47 articles, 28 of which you can access for free at: http://clincancerres.aacrjournals.org/content/22/1/167.full#ref-list-1
Citing articles	This article has been cited by 6 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/22/1/167.full#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, use this link http://clincancerres.aacrjournals.org/content/22/1/167. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.