Select Antitumor Cytotoxic CD8$^+$ T Clonotypes Expand in Patients with Chronic Lymphocytic Leukemia Treated with Ibrutinib

Maria Joao Baptista[1,2], Sivasubramanian Baskar[1], Erika M. Gaglione[1], Keyvan Keyvanfar[1], Inhye E. Ahn[1], Adrian Wiestner[1], and Clare Sun[1]

ABSTRACT

Purpose: In chronic lymphocytic leukemia (CLL), the T-cell receptor (TCR) repertoire is skewed and tumor-derived antigens are hypothesized as drivers of oligoclonal expansion. Ibrutinib, a standard treatment for CLL, inhibits not only Bruton tyrosine kinase of the B-cell receptor signaling pathway, but also IL2-inducible kinase of the TCR signaling pathway. T-cell polarization and activation are affected by ibrutinib, but it is unknown whether T cells contribute to clinical response.

Experimental Design: High-throughput TCRβ sequencing was performed in 77 longitudinal samples from 26 patients with CLL treated with ibrutinib. TCRβ usage in CD4$^+$ and CD8$^+$ T cells and granzyme B expression were assessed by flow cytometric analysis. Antitumor cytotoxicity of T cells expanded with autologous CLL cells or with antigen-independent anti-CD3/CD28/CD137 beads was tested.

Results: The clonality of the TCR repertoire increased at the time of response. With extended treatment, TCR clonality remained stable in patients with sustained remission and decreased in patients with disease progression. Expanded clonotypes were rarely shared between patients, indicating specificity for private antigens. Flow cytometry demonstrated a predominance of CD8$^+$ cells among expanded clonotypes. Importantly, bulk T cells from responding patients were cytotoxic against autologous CLL cells *in vitro* and selective depletion of major expanded clonotypes reduced CLL cell killing.

Conclusions: In patients with CLL, established T-cell responses directed against tumor are suppressed by disease and reactivated by ibrutinib.

See related commentary by Zent, p. 4465

Introduction

Antigen specificity of a T cell is mediated by binding of the T-cell receptor (TCR), and in particular, its variable region, to an antigen-derived peptide cognately associated with a MHC molecule. Up to 10^{15} unique TCR sequences are estimated to result from somatic recombination of component α and β chains (1). High-throughput TCR sequencing has accelerated understanding of this richly diverse repertoire of T cells in health and disease (2).

The TCR repertoire is more oligoclonal in chronic lymphocytic leukemia (CLL) than the healthy population (3). Some T-cell clonotypes are shared between patients with CLL, indicating the presence of common antigens. Although identification of these antigens is quite limited, viral infections and tumor-derived antigens have been implicated (4). In support of antitumor T cells, we previously described the expansion of select clonotypes in patients responding to treatment with the immunomodulatory drug lenalidomide (5).

The first-in-class irreversible Bruton tyrosine kinase (BTK) inhibitor ibrutinib is highly effective in CLL (6, 7). In addition to tumor intrinsic effects, treatment with ibrutinib reshapes the tumor microenvironment, which may, in part, be due to inhibition of kinases other than BTK. For example, ibrutinib inhibits IL2-inducible kinase (ITK) in T cells, which may influence T-cell polarization and activation in patients with CLL during treatment (8, 9). Ibrutinib enhances the activity of immune checkpoint inhibitors in different murine models of cancer (10). Some studies have shown that concomitant or antecedent administration of ibrutinib improves expansion of autologous anti-CD19 chimeric antigen receptor (CAR) T cells in patients with CLL (11) and antitumor cytotoxicity of CD19/CD3 bispecific antibodies in patient-derived xenografts (12). However, it is unknown whether the immunomodulatory properties of ibrutinib contribute to treatment response in CLL.

To address this question, we profiled the T-cell compartment in patients with CLL before treatment with ibrutinib, during remission, and at disease relapse. Our study integrated high-throughput sequencing of TCRβ, flow cytometric immunophenotyping, and *in vitro* cytotoxicity assays, revealing novel insights into T-cell dynamics during ibrutinib therapy.

Materials and Methods

Patients and samples

Patients with treatment naïve and relapsed/refractory (R/R) CLL were enrolled in a phase II open-label, single center study of ibrutinib monotherapy (ClinicalTrials.gov ID NCT01500733). Trial details were published previously (13). The trial was conducted in accordance with the U.S. Common Rule and approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (Bethesda, MD). All patients provided written informed consent. Longitudinal samples were available in 26 of 86 trial participants. Studies were

[1]Hematology Branch, National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland. [2]Lymphoid Neoplasms, Josep Carreras Leukaemia Research Institute, Badalona, Spain.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Authors: Clare Sun, Hematology Branch, National Heart Lung and Blood Institute, Bethesda, MD 20892. Phone: 301-402-1806; Fax: 301-496-8396; E-mail: clare.sun@nih.gov; and Adrian Wiestner, Hematology Branch, National Heart Lung and Blood Institute, Bethesda, MD 20892. Phone: 301-594-6855; Fax: 301-496-8396; E-mail: wiestnea@nhlbi.nih.gov

Clin Cancer Res 2021;27:4624–33

doi: 10.1158/1078-0432.CCR-20-4894

Translational Relevance

T-cell dysfunction in patients with chronic lymphocytic leukemia (CLL) manifests as impaired immune synapse formation, expression of inhibitory receptors, and reduced proliferative capacity. Although treatment with ibrutinib improves aspects of T-cell immunity, it is unknown whether these immunomodulatory properties contribute to disease control. To address this question, T-cell receptor (TCR) sequencing was used to track T-cell clonotypes in patients treated with ibrutinib. We observed oligoclonal expansion of antitumor cytotoxic $CD8^+$ T cells at response that was maintained in patients with sustained remission, but lost in patients who developed progressive disease. These data indicate preexisting immune surveillance mechanisms are reinvigorated during treatment with ibrutinib. In addition, the reversal in TCR clonality at progression suggests that Bruton tyrosine kinase–mediated signals contribute to immune evasion. Our findings provide a missing link between T-cell function and antitumor response in ibrutinib-treated CLL and rationale to combine ibrutinib with T cell–directed immunotherapy.

performed on cryopreserved peripheral blood mononuclear cells (PBMC) obtained before treatment, at the time of response, and either at disease progression or during sustained remission.

HLA typing

Patients were typed for class I (A, B, C) and class II (DR, DQ) alleles by means of PCR-SSP/SSO and/or Sanger SBT/NGS HLA typing at the HLA Laboratory of the NIH (Bethesda, MD).

High-throughput TCRβ sequencing

Approximately 2 μg of DNA was extracted from cryopreserved PBMCs to generate libraries with the immunoSEQ hsTCRB Kit (Adaptive Biotechnologies). Deep resolution sequencing was done with a target depth of 1 in 200,000 to 1 in 1 million T cells on the MiSeq instrument (Illumina Inc.). The average coverage was 10× for each input TCRβ clonotype.

Data were analyzed with ImmunoSEQ Data Assistant and ImmunoSEQ Analyzer (Adaptive Biotechnologies). One sample at the time of response failed quality control and was excluded. CDR3 sequences were studied at nucleotide and amino acid level and their relative frequencies quantified. T cell receptor beta variable gene (TRBV), cell receptor beta diversity gene, and cell receptor beta joining gene usage was identified according to the International ImMunoGeneTics information system definitions. T-cell clonality of each sample was calculated from the frequency distribution of TCR clonotypes as the inverse of the normalized Shannon entropy (14):

$$1 - \frac{\Sigma_{i=1}^{R} P_i \log_2 [P_i]}{\log_2 R}$$

R = the total number of rearrangements; i = each rearrangement; P_i = productive frequency of rearrangement i. Clonality values range between 0 (maximum diversity) and 1 (population with a single clone).

Healthy donor PBMC TCRβ data originated from the immuneACCESS database (https://clients.adaptivebiotech.com/pub/TCRBv4-control). The ImmunoSEQ Analyzer was used to calculate the clonality of 13 age-matched (≥ 60 years) healthy controls. The median age was 65 years (range, 60–78).

Flow cytometry analysis of TRBV usage

PBMCs were enriched for T cells by negative selection by MACS using CD19 MicroBeads and LD columns (Miltenyi Biotec GmbH). Cell suspensions were preincubated with LIFE/DEATH fixable Aqua Blue dead cell stain (Invitrogen, Life Technologies Corporation) followed by surface staining with a custom panel of fluorescent-labeled antibodies against cell lineage and activation markers (Supplementary Table S1) and the IOTest Beta Mark TCR Vβ Repertoire Kit (Beckman Coulter Inc). Cells were then permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization Kit for intracellular staining. Antibodies were titrated prior to use and fluorescence minus one controls were created to set gates for positive events. Cells were acquired on a custom 19-color, 21-parameter special order LSRFortessa cell analyzer with high-throughput sampler (BD Biosciences). Data were analyzed using FlowJo v10.0.7 software (TreeStar Inc).

In vitro T-cell expansion systems

T cells were isolated from PBMCs obtained at the time of response by negative selection with the Pan T Cell Isolation Kit human and LS column MACS (Miltenyi Biotec GmbH). T cell–enriched products were cultured in 1:1 AIM-V:RPMI (Gibco, Life Technologies), 5% volume for volume FBS (SAFC), L-Glutamine, Penicillin, Streptomycin 100× (Gibco), 20 ng/mL of recombinant human IL2 (NCI Biological Research Branch PreClinical Repository), and 10 ng/mL of recombinant human IL7(Peprotech).

In the first setup, T cells were expanded for 7 days in a nonspecific manner with Dynabeads Human T-Activator CD3/CD28/CD137 (Gibco). In the second setup, T cells were cocultured for 7 days with autologous CLL cells at a ratio of 1:4 and 1 μg/mL of MEGACD40L Protein (Enzo Life Sciences; ref. 15). Dead cells were removed from the expanded T-cell products with the Dead Cell Removal kit and LS columns MACS (Miltenyi Biotec GmbH) before downstream analysis.

Antitumor cytotoxicity assay

Expanded T cells from each condition described above were mixed with autologous CLL cells labeled with Violet Proliferation Dye 450 (BD Horizon) at effector to target ratios of 3:1, 1:1, and 1:3. As a control for spontaneous cell death, CLL cells were also placed alone in culture. After 6 hours, cells were stained with TO-PRO-3 iodide (Molecular Probes Inc) and acquired on a BD LSRFortessa cell analyzer (BD Biosciences). Dead CLL cells were identified by double positivity for VPD450 and TO-PRO-3 iodide.

Cell sorting for depletion of select clonotypes

T cells were expanded with autologous CLL cells for 7 days then stained with anti-CD19 FITC (BD Pharmingen), anti-CD56 FITC (BD Biosciences), and with or without anti-TCR Vβ13.1-PE (Beckman Coulter). The anti-TCR Vß13.1 antibody recognizes TRBV6-5, TRBV6-6, and TRBV6-9 and was used to deplete TRBV6-5 T cells. FITC and PE double-negative cells were sorted with a BD FACSAria cell sorter (BD Biosciences) and evaluated for antitumor cytotoxicity against autologous CLL cells after 21 hours in coculture.

Statistical analysis

Descriptive statistics were used to summarize findings. χ^2 test or Fisher exact test were used to compare categorical variables and Mann–Whitney for continuous variables. Paired analyses using Wilcoxon test or paired t test were used to compare metrics over time. The level of significance for all statistical tests was two-tailed $P < 0.05$. Analysis were performed with SPSS v24.0 (IBM).

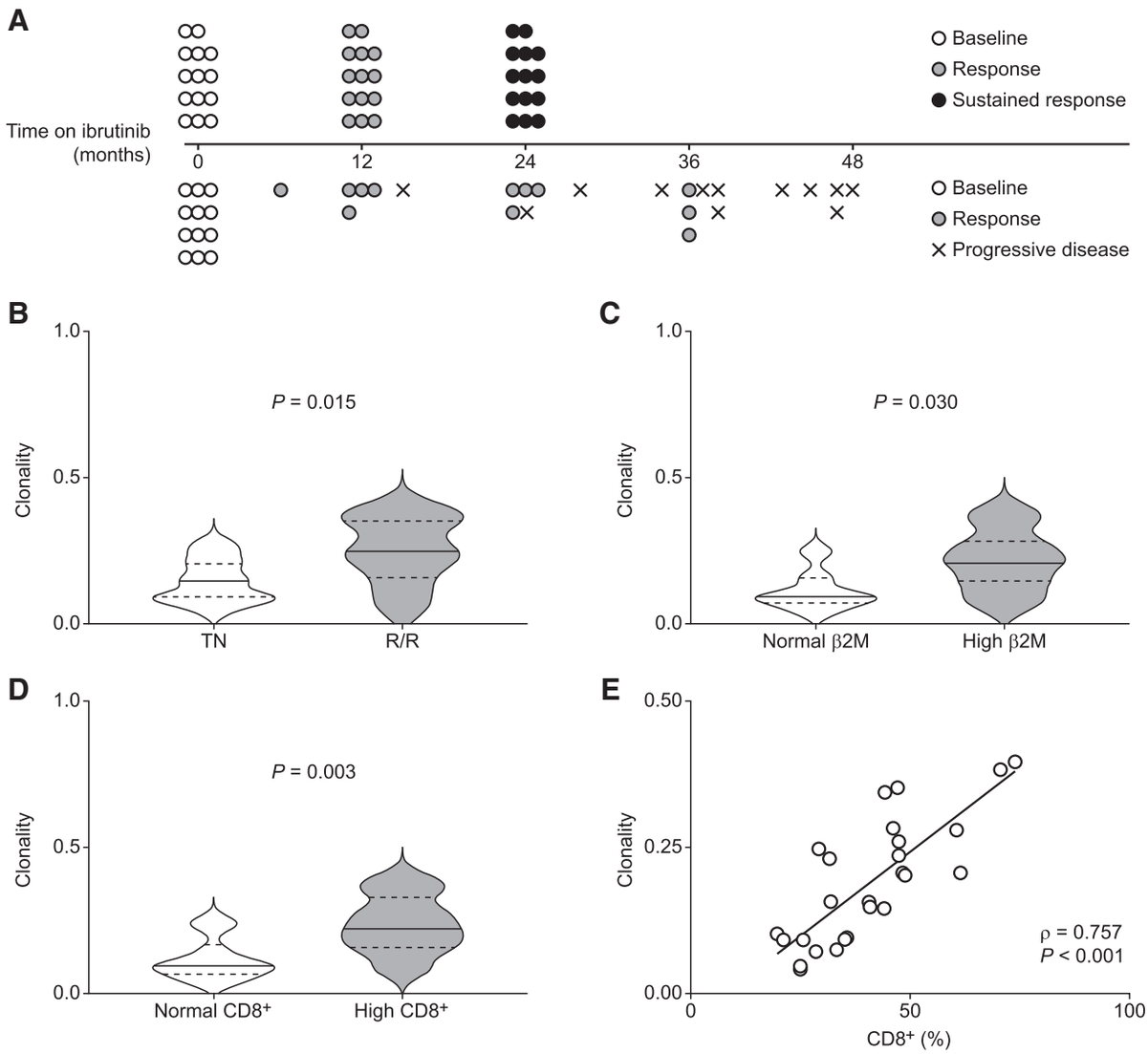

Figure 1.

Clonality of the TCRβ repertoire at baseline. **A,** Outline of patient samples. Serial TCRβ sequencing was performed at three timepoints per patient: baseline, time of response, and sustained response or PD. Dots above the horizontal line represent samples collected from patients in sustained response. Dots below the horizontal line represent samples from patients who developed PD during treatment with ibrutinib. **B–D,** Baseline clonality in patients with treatment naïve (TN, $n = 15$) versus R/R ($n = 11$) CLL ($P = 0.015$), normal ($n = 7$) versus high ($n = 19$) serum β2-microglobulin levels ≥3.5 mg/L ($P = 0.030$), and normal ($n = 10$) versus high ($n = 16$) $CD8^+$ T-cell counts ≥850/μL ($P = 0.003$). Horizontal lines indicate quartiles. **E,** Scatter plot of percentage of $CD8^+$ T cells versus clonality ($\rho = 0.757$, $P < 0.001$).

Data availability

The TCRβ sequencing data have been deposited in the the immuneACCESS database (https://clients.adaptivebiotech.com/pub/baptista-2021-ccr).

Results

TCRβ sequencing of patients on ibrutinib monotherapy

We studied the TCRβ repertoire of 26 patients with CLL treated with ibrutinib monotherapy. Eleven patients (42%) had relapsed or refractory (R/R) disease, 17 (65%) were immunoglobulin heavy-chain variable gene (IGHV) unmutated, and 19 (73%) had deletion of chromosome 17p (Supplementary Table S2). Responses were partial in 24 patients and complete in 2 patients. After a median follow-up of 68 months, 14 patients remained in remission and 12 developed progressive disease (PD).

Next-generation sequencing (NGS) of the TCRβ repertoire was performed on samples collected at baseline and during remission, after a median time on treatment of 12 months (range, 6–36; **Fig. 1A**). For each patient, TCRβ sequencing was performed on a third sample that was obtained during sustained remission or at the time of PD (**Fig. 1A**). All remission and PD samples were collected on ibrutinib therapy. The mean (range) number of productive templates and rearrangements were 255,043 (9,496–1,127,768) and 94,082 (3,719–443,861), respectively, across all samples analyzed.

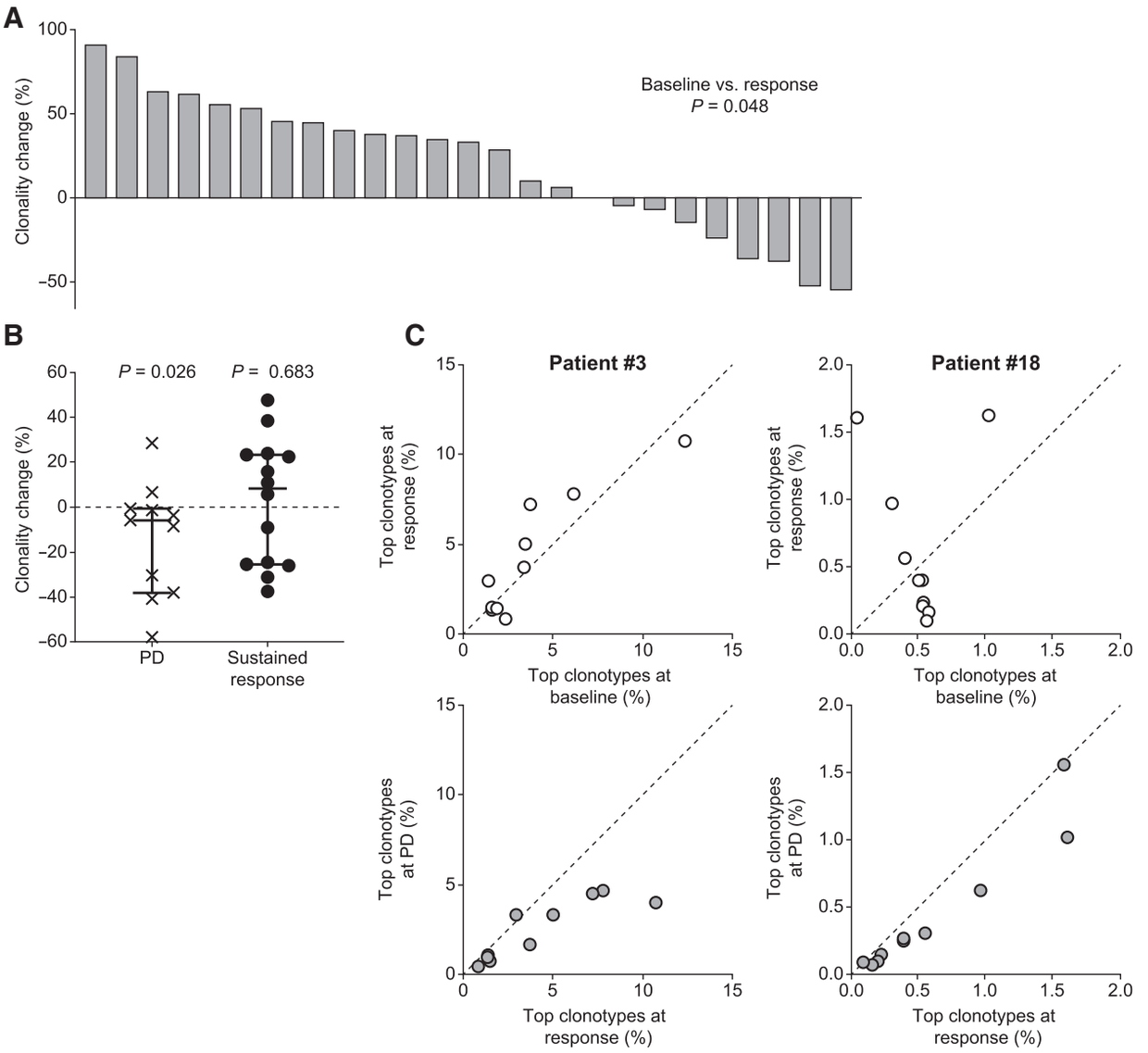

Figure 2.

Change in clonality of the TCRβ repertoire during treatment with ibrutinib. **A,** Change in clonality of each patient from baseline to response. Clonality significantly increased at response ($P = 0.048$). **B,** Change in clonality from response to PD or from response to sustained response. Clonality significantly decreased at PD ($n = 11$, $P = 0.026$), but did not change during sustained remission ($n = 14$, $P = 0.683$). **C,** Frequency of the 10 most abundant clonotypes at baseline versus response and at response versus PD in 2 representative patients.

Oligoclonality of the TCRβ repertoire is associated with CD8$^+$ T cells

To compare the TCRβ repertoire between patients and in each patient over time, we calculated the T-cell clonality of each sample, which measures the number of unique TCR clonotypes and the extent to which clonotypes dominate the repertoire. At baseline, the median clonality was 0.1795 [interquartile range (IQR), 0.0923–0.2643] in 26 patients with CLL compared with 0.0837 (IQR, 0.0445–0.1609) in age-matched healthy donors. Higher clonality was associated with adverse risk factors such as R/R CLL ($P = 0.015$; **Fig. 1B**) and increased plasma β2-microglobulin ≥3.5 mg/L ($P = 0.030$; **Fig. 1C**). Furthermore, we observed a strong correlation between clonality and the absolute number, as well as percentage, of circulating CD8$^+$ T cells ($\rho = 0.757$, $P < 0.001$; **Fig. 1D** and **E**).

Ibrutinib alters T-cell composition and the TCRβ repertoire

Circulating CD3$^+$, CD4$^+$, and CD8$^+$ T-cell counts were increased at baseline and significantly decreased after the initiation of ibrutinib therapy (Supplementary Fig. S1). With extended treatment, T-cell counts remained stable in patients with sustained remission and increased in patients who developed PD (Supplementary Fig. S1).

In contrast to the reduction in T-cell count, clonality significantly increased during treatment with ibrutinib ($P = 0.048$; **Fig. 2A**).

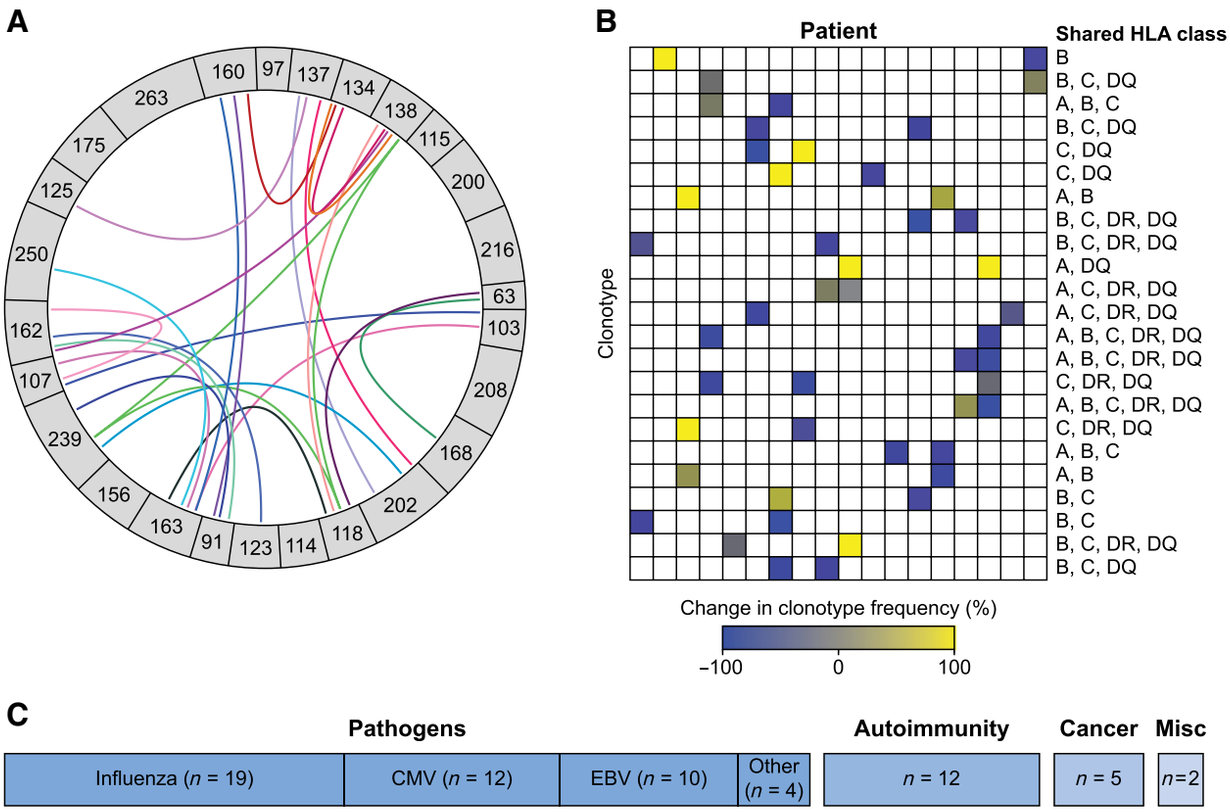

Figure 3.

Public clonotypes. **A,** Circus plot showing shared clonotypes between patients as connecting lines. Each outer slice represents a patient with the total number of overrepresented clonotypes as indicated. **B,** Heatmap of the change in frequency of shared clonotypes during treatment. **C,** Number and relative proportion of previously annotated clonotypes categorized into pathogens, autoimmunity, cancer, and miscellaneous.

Clonality decreased in 8 patients between baseline and response. Repeat TCRβ sequencing in patients with sustained remission showed stable clonality ($P = 0.683$ comparing on-treatment samples collected 12 months apart). Conversely, in patients who developed PD, clonality decreased at relapse ($P = 0.026$), returning to baseline levels (**Fig. 2B**). Consistent with these findings, we observed the relative expansion of predominant clonotypes at the time of response to ibrutinib followed by contraction at relapse (representative patients in **Fig. 2C**).

Public TCRβ clonotypes do not expand on treatment

To address the possible contribution of common antigens in shaping the TCRβ repertoire, we searched for public clonotypes that were shared between patients or were previously annotated in public databases. The analysis was narrowed to overrepresented clonotypes comprising ≥ 0.05% of the repertoire in each patient because overrepresented clonotypes are more likely to be clinically relevant. Between 91 and 263 overrepresented clonotypes were identified in each patient for a total of 4,004 clonotypes across 26 patients (**Fig. 3A**).

Eighteen of 26 patients shared ≥ 1 clonotype (**Fig. 3A**). In total, 23 shared clonotypes were identified; only one clonotype was shared by > 2 patients (**Fig. 3A**). Patients who shared clonotypes also had ≥ 1 HLA serotypes in common. Seven patients had common HLA serotypes from MHC class I only. The mean (±SEM) frequency of shared clonotypes at baseline was 0.234 (± 0.056) % and significantly decreased to 0.157 (± 0.033) % during treatment with ibrutinib ($P = 0.007$; **Fig. 3B**).

Out of 4,004 overrepresented clonotypes, 52 were previously annotated in the McPAS-TCR database (Supplementary Table S3) (16), of which 8 were present in ≥ 2 patients. Twenty (77%) patients had ≥ 1 previously annotated clonotype, most commonly associated with viral infections ($n = 41$, 78.8%), followed by autoimmune conditions ($n = 12$, 23.1%), and cancer ($n = 5$, 9.6%; **Fig. 3C**). In some instances, the same clonotype was reported in different diseases, while other viral epitopes were recognized by ≥ 2 clonotypes, reflecting convergent recombination of TCRs across different patients (17). The frequency of previously annotated clonotypes in these 20 patients did not change between baseline [0.339 (± 0.091) %] and at the time of response [0.391 (± 0.131) %, $P = 0.347$].

Taken together, public clonotypes were uncommon and contracted or remained stable on treatment. The observed increase in TCRβ clonality, therefore, reflected the expansion of private clonotypes at the time of ibrutinib response.

Expanding clonotypes are composed of $CD8^+$ T cells

Given the strong association between clonality and $CD8^+$ T cells, we hypothesized that $CD8^+$ clonotypes expanded on ibrutinib therapy. To address this question, we sought to determine the subset composition and select function of predominant clonotypes. Baseline and on-treatment samples were available in 8 of 26 patients for flow cytometric analysis of TCRVβ (TRBV) usage and expression of CD4, CD8, and

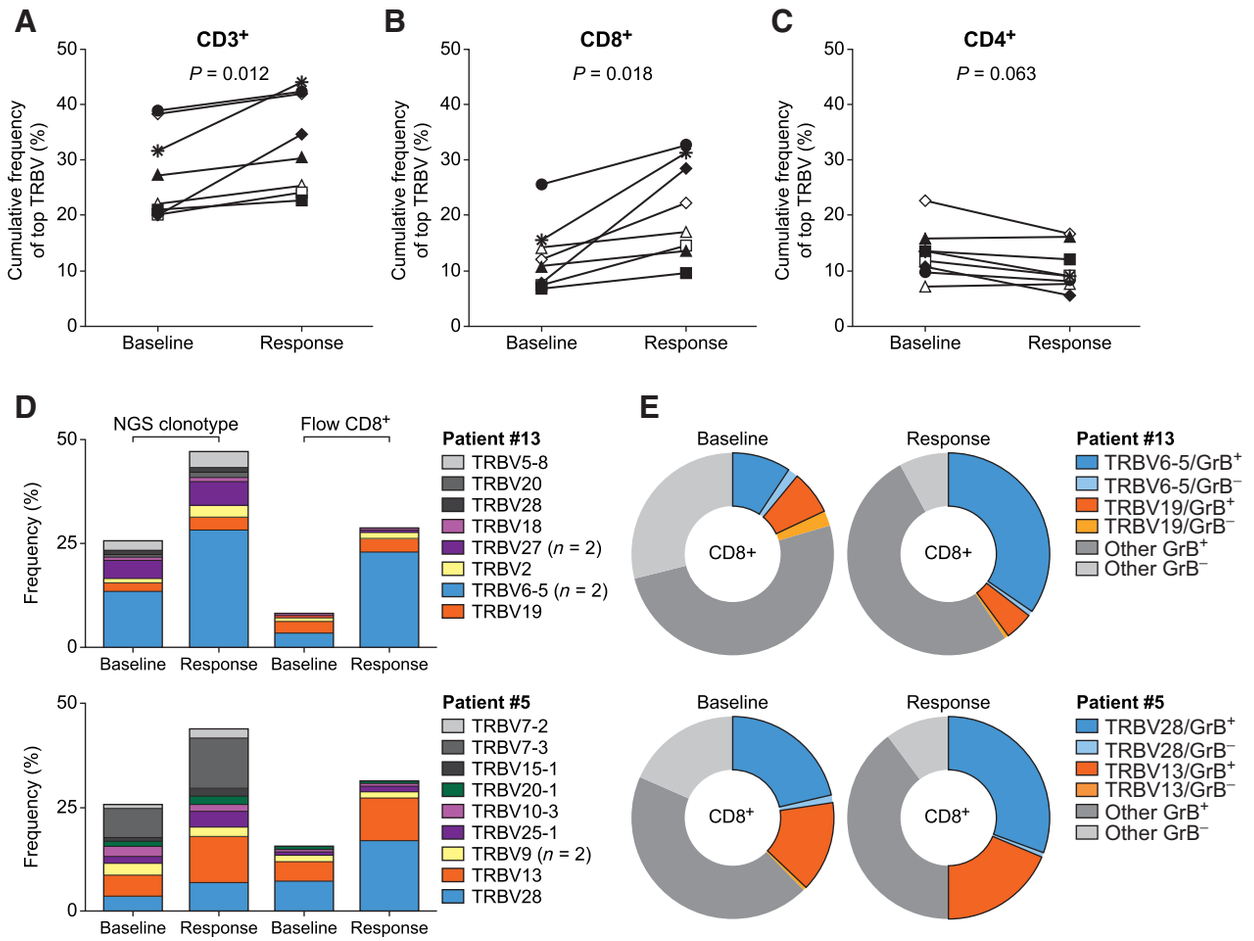

Figure 4.

Expansion of predominant CD8$^+$ clonotypes. **A–C,** Cumulative frequency of the 10 most abundant TRBV families among all live CD3$^+$ cells in each patient at baseline and at the time of ibrutinib response ($n = 8$ patients). The cumulative frequency of CD3$^+$ and CD8$^+$ clonotypes significantly increased at response ($P = 0.012$ and $P = 0.018$, respectively). **D,** Frequency of predominant clonotypes as detected by NGS among all productive templates and of the corresponding CD8$^+$ TRBV families among all live CD3$^+$ cells as measured by flow cytometry. Gray bars indicate TRBV families that did not have commercially available fluorescent-labeled antibodies. **E,** Pie chart of the most expanded clonotypes and expression of granzyme B (GrB) among all live CD8$^+$ cells. CD8$^+$ GrB$^+$ clonotypes comprising >2% of the repertoire at the time of response are shown in color.

granzyme B. On-treatment samples were collected after 12 months of ibrutinib, except for one sample from Patient #15, which was collected after 36 months. These samples matched the timepoints used for TCRβ sequencing. Of note, some clonotypes using the same TRBV gene could not be differentiated by flow cytometry and a median of 3 (range, 2–4) TRBV families per patient were not recognized by commercially available antibodies.

Overall, flow cytometric analysis of TRBV frequencies was strongly correlated with NGS data (Supplementary Fig. S2). The cumulative frequency of predominant TRBV families significantly increased after treatment with ibrutinib ($P = 0.012$; **Fig. 4A**). This increase was driven by the expansion of CD8$^+$ T cells within the top TRBV families while CD4$^+$ T cells contracted on therapy (**Fig. 4B** and **C**).

In each patient, we tracked and profiled individual TRBV families during ibrutinib therapy. Expansion of predominant TRBV families was not uniform within the CD8$^+$ T-cell subset (**Fig. 4D**; Supplementary Fig. S3A). Furthermore, expanding TRBV CD8$^+$ subsets expressed granzyme B (**Fig. 4E**; Supplementary Fig. S3B). Thus, specific cytotoxic clonotypes underlay the increase in TCR oligoclonality at the time of ibrutinib response.

Expanding CD8$^+$ clonotypes recognize and kill CLL cells

We hypothesized that expanding clonotypes recognize tumor antigens and partake in the overall response to ibrutinib. To drive disease-biased clonotypic expansion, T cells were cultured in the presence of autologous CLL cells. As comparison, T cells were expanded with anti-CD3/CD28/CD137 beads to activate antigen-independent TCR signaling (**Fig. 5A**). There was more T-cell expansion after 7 days with anti-CD3/CD28/CD137 beads compared with autologous CLL cells (Supplementary Fig. S4). Cytotoxicity assays were performed by mixing each of the expanded T-cell products with autologous CLL cells at a range of effector to target ratios.

Autologous T cells cocultured with CLL cells killed CLL cells in a dose-dependent manner and were more efficient than bead-expanded T cells (**Fig. 5B**). To understand this difference in cytotoxicity, we compared T-cell composition and function between the expanded

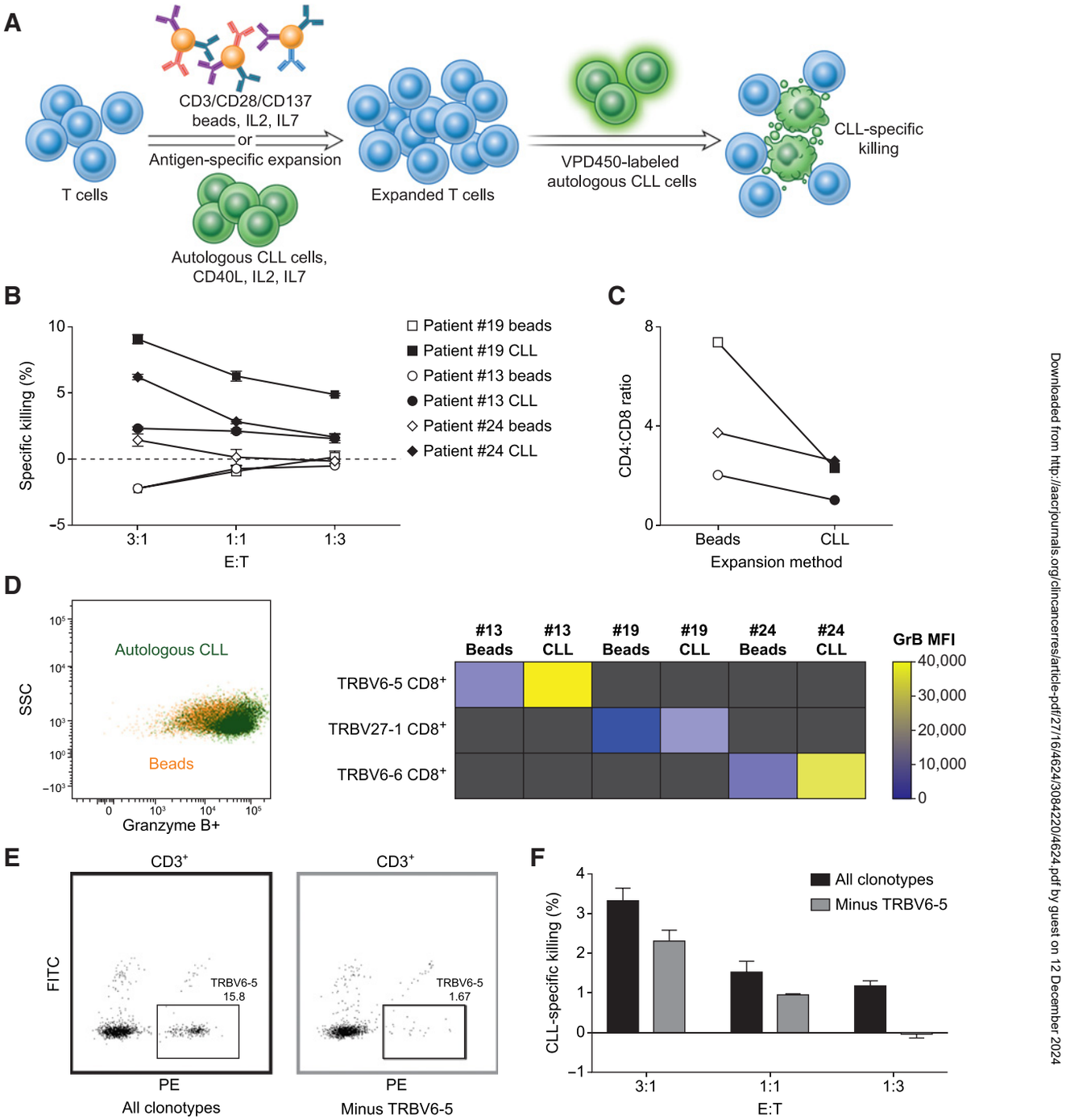

Figure 5.

Autologous CLL cells drive the *in vitro* proliferation of antitumor cytotoxic T cells. **A,** Schematic representation of the two *in vitro* T-cell expansion conditions. **B,** Percentage of all VPD450$^+$ CLL cells killed by T cells expanded with beads (white symbols) or cocultured with autologous CLL cells (black symbols) subtracted by percentage of spontaneous cell death. Dead CLL cells were TO-PRO-3$^+$ VPD450$^+$. Mean and SD of 2–3 experimental replicates are shown at the indicated effector: target (E:T) ratios. **C,** CD4:CD8 ratio of the two expanded T-cell products. **D,** Dot plot of granzyme B (GrB) expression of the most abundant CD8$^+$ clonotype in the two expanded T-cell products from a representative patient. Heatmap of GrB mean fluorescent intensity (MFI) of the most abundant CD8$^+$ clonotype in each patient. **E,** Frequency of TRBV6–5 in bulk T cells expanded with autologous CLL cells (left) or T cells after FACS of cells negative for TRBV 6-5 (right) in Patient #13. FITC$^-$ PE$^+$: TRBV6-5/6-6/6-9; FITC$^+$ PE$^+$: TRBV 6-6; FITC$^+$ PE$^-$: TRBV12-3/12-4. **F,** Percentage of all VPD450$^+$ CLL cells killed by each T-cell product shown in **E**.

T-cell products. T cells cocultured with CLL cells had a lower CD4: CD8 ratio and higher granzyme B expression than bead-expanded T cells (**Fig. 5C** and **D**). PD-1 expression in $CD3^+$, $CD8^+$, and the most abundant $CD8^+$ clonotype in each patient was not significantly different between the two conditions (Supplementary Fig. S5).

In Patient #13, TRBV6-5 was the most overrepresented TRBV family at baseline and showed the greatest expansion during treatment with ibrutinib by both NGS and flow cytometry. Moreover, 97.8% of TRBV6-5 $CD8^+$ cells expressed granzyme B at the time of response. We, therefore, sought to test the antitumor cytotoxicity of TRBV6-5 clonotypes by comparing CLL-specific killing between bulk T cells and T cells depleted of the TRBV6-5 family. Removal of TRBV6-5 T cells reduced CLL-specific killing compared with bulk T cells (**Fig. 5E**). These data support the expansion of tumor-specific cytotoxic T cells in patients treated with ibrutinib.

Discussion

A hallmark of the adaptive immune system is the ability to specifically target antigens. Mechanisms employed by cancer cells to evade immune surveillance and attack include suppression of antigen presentation, inhibition, and exclusion of T cells, and recruitment of immunosuppressive cells (18). CLL cells benefit from the tumor supportive properties of $CD4^+$ T cells and inactivation of $CD8^+$ T cells (19). In addition to facilitating disease progression, immune dysregulation in CLL manifests as blunted responses to vaccination (20), opportunistic infections (21), and an increased risk of second primary malignancies (22). Restoring immunocompetence has been an elusive goal in CLL despite the introduction of less immunosuppressive targeted therapies as patients continue to experience complications of immunodeficiency (23–25).

Expansion of preexisting clonotypes during treatment with ibrutinib or idelalisib was previously observed by Vardi and colleagues (26). In contrast, Yin and colleagues reported increasing TCR diversity in patients with CLL treated with ibrutinib (27). Notably, Yin and colleagues studied patients with relapsed or refractory disease, including some who were treated with concomitant rituximab. Chemoimmunotherapy fundamentally alters the TCR repertoire by eliminating pretreatment clonotypes (26) and rituximab has been associated with skewing of the TCR repertoire (28, 29).

In our study, we showed that ibrutinib not only increases TCR clonality, but also improves the cytotoxicity of select antitumor clonotypes. For T cells, antigen recognition is encoded by the TCR. Using TCRβ sequencing, we followed the dynamics of T-cell antigen specificities during the course of treatment. Clonality of the TCR repertoire was higher at baseline than reported in healthy age-matched individuals and further increased on treatment, driven by the expansion of preexisting private $CD8^+$ clonotypes. Bulk T cells were cytotoxic against autologous CLL cells *in vitro*. Antitumor cytoxicity was reduced upon depletion of the most expanded clonotypes from bulk T cells. The private nature of expanding clonotypes implicates tumor neoantigens as the targets of antitumor T cells. Because $CD8^+$ T cells were present at low frequency relative to clonal B cells, limited sample availability precluded selective depletion of putative antitumor clonotypes in additional patients. This single patient experiment should not be interpreted in isolation, but rather alongside the increase in T-cell clonality at response detected by TCRβ sequencing, orthogonal flow cytometric validation of expanding $CD8^+$ clonotypes, and specific killing of autologous CLL cells by expanded T cells.

Allogeneic stem cell transplantation was an early demonstration of T cell–mediated eradication of CLL cells (30). More recently, anti-CD19 CAR T-cell therapy was shown to induce a high rate of minimal residual disease negative remissions (31). While $CD8^+$ T cells delayed CLL progression in the TCL1 mouse model (32), antitumor reactivity of autologous T cells expressing endogenous TCRs had not been demonstrated in patients with CLL prior to our report. The expansion and functional recovery of preexisting clonotypes during treatment with ibrutinib indicate that antitumor T cells were previously established but inactivated. The on-target and off-target effects of ibrutinib influence many immune cell populations. Ibrutinib inhibits ITK, a proximal member of the TCR signaling pathway (8). Myeloid-derived suppressor cells, which suppress T-cell activation and proliferation, express BTK and are inhibited by ibrutinib (33). It is also well established that CLL cells impair the function of T cells within their microenvironment (34). These factors could contribute to the improved health of T cells during treatment with ibrutinib. Importantly, our study revealed the differential effects of ibrutinib on specific T-cell clonotypes by preferentially expanding antitumor T cells and enhancing their cytotoxicity. It is unknown whether second-generation BTK inhibitors with increased kinase selectivity would have the same effect on T cells.

We observed a reversal of TCR clonality in patients who subsequently progressed on therapy in contrast to the persistence of predominant clonotypes in patients with sustained response. This finding together with an increase in absolute T-cell counts suggest a return of the T-cell compartment to a pre-ibrutinib state driven by resurgent disease. Indeed, there are more circulating T cells in patients with untreated CLL than age-matched healthy donors and during treatment with ibrutinib T-cell numbers contract into the normal range (9, 35). Progressive CLL during treatment with ibrutinib is most often caused by acquired mutations in *BTK* that restore BTK-dependent signaling in tumor cells (36). Therefore, we hypothesize that BTK-mediated signals, such as IL10 production (37) and expression of inhibitory ligands (38) by CLL cells, directly contribute to the ensuing reversal in TCR clonality and possibly, immune evasion. Conversely, spontaneous tumor regression, a rare phenomenom in CLL, has been associated with reduced expression of exhaustion markers and more robust proliferation of T cells (39).

This study has limitations. The timing of sample collection from patients in sustained remission and those who subsequently developed PD were not matched. However, in patients with sustained remission, we showed that clonality of the TCR repertoire was stable over a 12-month period. We could not investigate the TCR repertoire in lymph node samples as most patients on ibrutinib do not have lymph nodes that are amenable to biopsy even after just a few months of treatment. The patient population was enriched for biologically high-risk disease and included treatment naïve and relapsed CLL. While these factors could influence the generalizability of our findings, shifts in TCR clonotypes were present in most patients and focusing on high-risk disease is arguably of clinical interest. Functional assays could only be conducted in a small number of patients due to the challenges of identifying candidate TCR clonotypes by Vβ antibody staining and sample availability.

In summary, our study showed that ibrutinib expands antitumor $CD8^+$ clonotypes and enhances their cytotoxicity, providing a missing link between antigen specificity and function of T cells in patients with CLL treated with ibrutinib. Ibrutinib appears to reinvigorate preexisting immune surveillance mechanisms and skew the TCR repertoire

further toward tumor recognition and cytotoxicity. Harnessing autologous antitumor T cells could provide avenues for disease eradication.

Authors' Disclosures

M.J. Baptista reports personal fees from AstraZeneca outside the submitted work. A. Wiestner reports grants from Pharmacyclics during the conduct of the study; grants from Acerta Pharma, Merck, Nurix, Genmab, and Verastem outside the submitted work. C. Sun reports grants from Genmab outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

M.J. Baptista: Data curation, formal analysis, investigation, visualization, writing–original draft, writing–review and editing. **S. Baskar:** Investigation, writing–review and editing. **E.M. Gaglione:** Project administration, writing–review and editing. **K. Keyvanfar:** Investigation, writing–review and editing. **I.E. Ahn:** Resources, writing–review and editing. **A. Wiestner:** Conceptualization, supervision, funding acquisition, writing–original draft, writing–review and editing. **C. Sun:** Conceptualization, data curation, formal analysis, supervision, investigation, visualization, writing–original draft, writing–review and editing.

Acknowledgments

The authors thank the patients and their families. M.J. Baptista acknowledges the support and encouragement of her direct supervisor in Spain, José Tomás Navarro, during her stay at the National Heart, Lung, and Blood Institute (NHLBI).

This study was supported by the Intramural Research Program of the NHLBI and Pharmacyclics, an Abbvie company. M.J. Baptista was supported by fellowships from Fundación Española de Hematología y Hemoterapia. I.E. Ahn is supported by the American Society of Hematology Scholar Award.

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 December 18, 2020; revised March 15, 2021; accepted April 15, 2021; published first April 19, 2021.

References

1. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395–402.
2. Calis JJ, Rosenberg BR. Characterizing immune repertoires by high throughput sequencing: strategies and applications. Trends Immunol 2014;35:581–90.
3. Vardi A, Vlachonikola E, Karypidou M, Stalika E, Bikos V, Gemenetzi K, et al. Restrictions in the T-cell repertoire of chronic lymphocytic leukemia: high-throughput immunoprofiling supports selection by shared antigenic elements. Leukemia 2017;31:1555–61.
4. Blanco G, Vardi A, Puiggros A, Gomez-Llonin A, Muro M, Rodriguez-Rivera M, et al. Restricted T cell receptor repertoire in CLL-like monoclonal B cell lymphocytosis and early stage CLL. Oncoimmunology 2018;7:e1432328.
5. Aue G, Sun C, Liu D, Park JH, Pittaluga S, Tian X, et al. Activation of Th1 immunity within the tumor microenvironment is associated with clinical response to lenalidomide in chronic lymphocytic leukemia. J Immunol 2018; 201:1967–74.
6. Woyach JA, Ruppert AS, Heerema NA, Zhao W, Booth AM, Ding W, et al. Ibrutinib regimens versus chemoimmunotherapy in older patients with untreated CLL. N Engl J Med 2018;379:2517–28.
7. Shanafelt TD, Wang XV, Kay NE, Hanson CA, O'Brien S, Barrientos J, et al. Ibrutinib-rituximab or chemoimmunotherapy for chronic lymphocytic leukemia. N Engl J Med 2019;381:432–43.
8. Dubovsky JA, Beckwith KA, Natarajan G, Woyach JA, Jaglowski S, Zhong Y, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood 2013;122:2539–49.
9. Niemann CU, Herman SE, Maric I, Gomez-Rodriguez J, Biancotto A, Chang BY, et al. Disruption of *in vivo* chronic lymphocytic leukemia tumor-microenvironment interactions by ibrutinib–findings from an investigator-initiated phase II study. Clin Cancer Res 2016;22:1572–82.
10. Sagiv-Barfi I, Kohrt HE, Czerwinski DK, Ng PP, Chang BY, Levy R. Therapeutic antitumor immunity by checkpoint blockade is enhanced by ibrutinib, an inhibitor of both BTK and ITK. Proc Natl Acad Sci U S A 2015; 112:E966–72.
11. Fraietta JA, Beckwith KA, Patel PR, Ruella M, Zheng Z, Barrett DM, et al. Ibrutinib enhances chimeric antigen receptor T-cell engraftment and efficacy in leukemia. Blood 2016;127:1117–27.
12. Robinson HR, Qi J, Cook EM, Nichols C, Dadashian EL, Underbayev C, et al. A CD19/CD3 bispecific antibody for effective immunotherapy of chronic lymphocytic leukemia in the ibrutinib era. Blood 2018;132:521–32.
13. Ahn IE, Farooqui MZH, Tian X, Valdez J, Sun C, Soto S, et al. Depth and durability of response to ibrutinib in CLL: 5-year follow-up of a phase 2 study. Blood 2018;131:2357–66.
14. Emerson RO, Sherwood AM, Rieder MJ, Guenthoer J, Williamson DW, Carlson CS, et al. High-throughput sequencing of T-cell receptors reveals a homogeneous repertoire of tumour-infiltrating lymphocytes in ovarian cancer. J Pathol 2013; 231:433–40.
15. Neelapu SS, Baskar S, Gause BL, Kobrin CB, Watson TM, Frye AR, et al. Human autologous tumor-specific T-cell responses induced by liposomal delivery of a lymphoma antigen. Clin Cancer Res 2004;10:8309–17.
16. Tickotsky N, Sagiv T, Prilusky J, Shifrut E, Friedman N. McPAS-TCR: a manually curated catalogue of pathology-associated T cell receptor sequences. Bioinformatics 2017;33:2924–9.
17. Li H, Ye C, Ji G, Han J. Determinants of public T cell responses. Cell Res 2012;22: 33–42.
18. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, adaptive, and acquired resistance to cancer immunotherapy. Cell 2017;168:707–23.
19. Roessner PM, Seiffert M. T-cells in chronic lymphocytic leukemia: guardians or drivers of disease? Leukemia 2020;34:2012–24.
20. Whitaker JA, Shanafelt TD, Poland GA, Kay NE. Room for improvement: immunizations for patients with monoclonal B-cell lymphocytosis or chronic lymphocytic leukemia. Clin Adv Hematol Oncol 2014; 12:440–50.
21. Wadhwa PD, Morrison VA. Infectious complications of chronic lymphocytic leukemia. Semin Oncol 2006;33:240–9.
22. Kumar V, Ailawadhi S, Bojanini L, Mehta A, Biswas S, Sher T, et al. Trends in the risk of second primary malignancies among survivors of chronic lymphocytic leukemia. Blood Cancer J 2019;9:75.
23. Al-Sawaf O, Zhang C, Tandon M, Sinha A, Fink AM, Robrecht S, et al. Venetoclax plus obinutuzumab versus chlorambucil plus obinutuzumab for previously untreated chronic lymphocytic leukaemia (CLL14): follow-up results from a multicentre, open-label, randomised, phase 3 trial. Lancet Oncol 2020;21: 1188–200.
24. Pleyer C, Ali MA, Cohen JI, Tian X, Soto S, Ahn IE, et al. Effect of Bruton tyrosine kinase inhibitor on efficacy of adjuvanted recombinant hepatitis B and zoster vaccines. Blood 2021;137:185–9.
25. Ahn IE, Jerussi T, Farooqui M, Tian X, Wiestner A, Gea-Banacloche J. Atypical Pneumocystis jirovecii pneumonia in previously untreated patients with CLL on single-agent ibrutinib. Blood 2016;128:1940–3.
26. Vardi A, Vlachonikola E, Papazoglou D, Psomopoulos F, Kotta K, Ioannou N, et al. T cell dynamics in chronic lymphocytic leukemia under different treatment modalities. Clin Cancer Res 2020;26:4958–69.
27. Yin Q, Sivina M, Robins H, Yusko E, Vignali M, O'Brien S, et al. Ibrutinib therapy increases T cell repertoire diversity in patients with chronic lymphocytic leukemia. J Immunol 2017;198:1740–7.
28. Papalexandri A, Karypidou M, Stalika E, Kotta K, Touloumenidou T, Zerva P, et al. Skewing of the T-cell receptor repertoire in patients receiving rituximab after allogeneic hematopoietic cell transplantation: what lies beneath? Leuk Lymphoma 2019;60:1685–92.
29. Stasi R, Del Poeta G, Stipa E, Evangelista ML, Trawinska MM, Cooper N, et al. Response to B-cell depleting therapy with rituximab reverts the abnormalities of T-cell subsets in patients with idiopathic thrombocytopenic purpura. Blood 2007;110:2924–30.
30. Dreger P, Dohner H, Ritgen M, Bottcher S, Busch R, Dietrich S, et al. Allogeneic stem cell transplantation provides durable disease control in poor-risk chronic lymphocytic leukemia: long-term clinical and MRD results of the German CLL Study Group CLL3X trial. Blood 2010;116: 2438–47.

31. Turtle CJ, Hay KA, Hanafi LA, Li D, Cherian S, Chen X, et al. Durable molecular remissions in chronic lymphocytic leukemia treated with CD19-specific chimeric antigen receptor-modified T cells after failure of ibrutinib. J Clin Oncol 2017; 35:3010–20.
32. Hanna BS, Roessner PM, Yazdanparast H, Colomer D, Campo E, Kugler S, et al. Control of chronic lymphocytic leukemia development by clonally-expanded CD8(+) T-cells that undergo functional exhaustion in secondary lymphoid tissues. Leukemia 2019;33:625–37.
33. Stiff A, Trikha P, Wesolowski R, Kendra K, Hsu V, Uppati S, et al. Myeloid-derived suppressor cells express Bruton's tyrosine kinase and can be depleted in tumor-bearing hosts by ibrutinib treatment. Cancer Res 2016;76:2125–36.
34. Riches JC, Ramsay AG, Gribben JG. Immune dysfunction in chronic lymphocytic leukemia: the role for immunotherapy. Curr Pharm Des 2012;18: 3389–98.
35. Catovsky D, Miliani E, Okos A, Galton DA. Clinical significance of T-cells in chronic lymphocytic leukaemia. Lancet 1974;2:751–2.
36. Ahn IE, Underbayev C, Albitar A, Herman SE, Tian X, Maric I, et al. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. Blood 2017;129:1469–79.
37. Alhakeem SS, McKenna MK, Oben KZ, Noothi SK, Rivas JR, Hildebrandt GC, et al. Chronic lymphocytic leukemia-derived IL-10 suppresses antitumor immunity. J Immunol 2018;200:4180–9.
38. Long M, Beckwith K, Do P, Mundy BL, Gordon A, Lehman AM, et al. Ibrutinib treatment improves T cell number and function in CLL patients. J Clin Invest 2017;127:3052–64.
39. Kwok M, Oldreive C, Rawstron AC, Goel A, Papatzikas G, Jones RE, et al. Integrative analysis of spontaneous CLL regression highlights genetic and microenvironmental interdependency in CLL. Blood 2020;135:411–28.