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Research paper

Ibrutinib restores immune cell numbers and function in first-line and relapsed/refractory chronic lymphocytic leukemia

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ABSTRACT

Ibrutinib positively modulates many T-cell subsets in chronic lymphocytic leukemia (CLL). To understand ibrutinib's effects on the broader landscape of immune cell populations, we comprehensively characterized changes in circulating counts of 21 immune blood cell subsets throughout the first year of treatment in patients with relapsed/refractory (R/R) CLL (n = 55, RESONATE) and previously untreated CLL (n = 50, RESONATE-2) compared with untreated age-matched healthy donors (n = 20). Ibrutinib normalized abnormal immune cell counts to levels similar to those of age-matched healthy donors. Ibrutinib significantly decreased pathologically high circulating B cells, regulatory T cells, effector/memory $CD4^+$ and $CD8^+$ T cells (including exhausted and chronically activated T cells), natural killer (NK) T cells, and myeloid-derived suppressor cells; preserved naive T cells and NK cells; and increased circulating classical monocytes. T-cell function was assessed in response to T-cell receptor stimulation in patients with R/R CLL (n = 21) compared with age-matched healthy donors (n = 18). Ibrutinib significantly restored T-cell proliferative ability, degranulation, and cytokine secretion. Over the same period, ofatumumab or chlorambucil did not confer the same spectrum of normalization as ibrutinib in multiple immune subsets. These results establish that ibrutinib has a significant and likely positive impact on circulating malignant and nonmalignant immune cells and restores healthy T-cell function.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common lymphoid malignancy in the United States and Europe [1,2], and is characterized by clonal expansion of malignant B cells. CLL is also characterized by profound immune dysregulation, including changes in the balance of T-cell subsets, chronic T-cell activation and exhaustion, changes in expression of inhibitory molecules and cytokine secretion, and induction of myeloid-derived suppressor cells (MDSCs) [3–6]. Impairment of innate and adaptive immunity is associated with pathophysiological changes of the underlying disease, and potentially leads to higher infection risk, as observed in clinical practice [4]. Immune dysfunction in CLL becomes more severe with increasing time from diagnosis and is exacerbated with chemotherapy [3,4]. Thus, patients with relapsed/refractory (R/R) CLL tend to present with profound immune suppression and are more prone to infections relative to earlier disease onset

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or previously untreated patients [7,8].

Ibrutinib is the only once-daily Bruton's tyrosine kinase (BTK) inhibitor to demonstrate significant progression-free survival (PFS) and overall survival benefit compared with established chemotherapy/chemoimmunotherapy regimens in CLL/small lymphocytic lymphoma (SLL) [9–14]. Infections have been observed in patients receiving ibrutinib, occurring most commonly within the first 6 months and gradually declining over time [8–10]. The subsequent decline of infection rates suggests that ibrutinib treatment may improve immune competence and/or that the gradual reduction in CLL disease burden by ibrutinib allows normalization of non-neoplastic immune cell function [15,16]. Besides BTK, ibrutinib covalently binds to interleukin-2–inducible T-cell kinase (ITK), a component of the T-cell receptor (TCR) signaling pathway [17]. Through BTK and ITK binding, ibrutinib may exert modulatory effects on B cells, MDSCs, mast cells, and T cells. Previous studies have shown that ibrutinib positively impacts specific T-cell subsets in CLL patients, increasing cytotoxic T-cell activation, suppressing regulatory T cells (Tregs), increasing TCR diversity, reducing programmed cell death 1 (PD-1)/programmed death ligand 1 expression, and normalizing inflammatory cytokines [15,18–21]. Mechanistic data from a phase 2 study showed that ibrutinib alleviates the inflammatory microenvironment in bone marrow, thereby decreasing immunosuppressive effects of CLL-B cells on macrophages and T cells [18,22]. However, ibrutinib's effects on the broader landscape of immune blood cell populations have not been fully described. To address this, we comprehensively characterized ibrutinib-related changes in peripheral immune cells and T-cell functions in patients with R/R or previously untreated CLL enrolled in RESONATE or RESONATE-2, respectively.

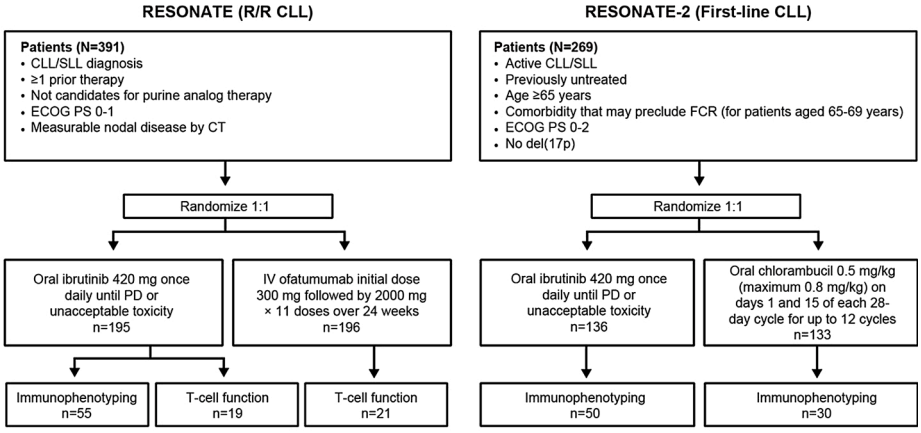
2. Materials and methods

2.1. Clinical studies and patient samples

Samples were collected from patients with R/R CLL treated with ibrutinib or ofatumumab in the phase 3 RESONATE study (NCT01578707) [23] and from patients with CLL treated with first-line ibrutinib or chlorambucil in the RESONATE-2 study (NCT01722487) [24] (Fig. 1A). Detailed clinical methods have been previously described [23,24]; both studies were approved by institutional review boards of participating institutions and were conducted according to the principles of the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice guidelines. All patients provided written informed consent.

We analyzed peripheral blood mononuclear cells (PBMCs) isolated by Ficoll density gradient from blood samples collected at baseline (pretreatment) and 1, 2–3, 5–6, 9, and 12 months post-treatment. Healthy donor ranges (HDRs) were obtained from peripheral blood samples from untreated, age-matched (> 50 years), healthy donors (n = 20, for first-line immunophenotyping; n = 18, for R/R T-cell function assessments), or from published reference values for healthy donors aged > 50 years (R/R immunophenotyping) [25].

A



B

Baseline Characteristics	R/R CLL RESONATE		First-line CLL RESONATE-2		
	T-cell Function Assessment		Immunophenotyping		
Treatment	Ibrutinib	Ofatumumab	Ibrutinib	Ibrutinib	Chlorambucil
Number of patients	19	21	55	50	30
Males, %	68	81	58	64	57
Age, years, median (range)	63 (39-78)	67 (41-86)	69 (41-86)	73 (65-83)	73 (65-88)
Number of prior therapies, median (range)	3 (1-8)	1 (1-5)	3 (1-12)	0	0
Rai stage, %					
I	32	29	27	16	33
II	0	29	16	36	27
III	16	24	18	18	20
IV	53	19	38	30	20
Cytogenetics, %					
Del(13q)	73	72	54	43	36
Del(11q)	39	30	32	19	14
Del(17p)	32	10	46	0	0
Trisomy 12	14	5	20	13	29
Complex karyotype	33	16	35	3	0
IGHV unmutated, %	75	56	72	62	46
ALC, $\times 10^9$/L, median (range)	44.4 (4-131)	48.9 (6-183)	57.9 (0.7-340)	64.8 (8-350)	80.5 (2-212)

Fig. 1. Patient populations. (A) Complete study design of RESONATE (R/R CLL) and RESONATE-2 (first-line CLL) clinical trials. (B) Baseline characteristics of patients studied. ALC: absolute lymphocyte count; CT: computed tomography; ECOG PS: Eastern Cooperative Oncology Group performance status; FCR: fludarabine + cyclophosphamide + rituximab; CLL: chronic lymphocytic leukemia; IV: intravenous; PD: progressive disease; R/R: relapsed/refractory; SLL: small lymphocytic lymphoma.

2.2. Flow cytometry immunophenotyping

PBMCs were thawed, washed, treated with Benzonase Nuclease (Sigma-Aldrich, St. Louis, MO, USA), and prewarmed (1 h) before staining with antibodies according to manufacturers' recommendations (Supplementary Tables S1 and S2). Circulating B cells, T cells, natural killer (NK) cells, NKT cells, classical/nonclassical monocytes, and monocytic MDSCs were identified using multiparametric flow cytometry (Supplementary Table S3). Granulocytic MDSCs were not assessed because they were likely removed during Ficoll separation. Cells were acquired on FACSAria II and LSRFortessa X-20 flow cytometers (BD Biosciences); analyses were performed with FlowJo software (BD Biosciences). Absolute counts were calculated by multiplying the frequency of each population among total lymphocytes by absolute lymphocyte counts (ALCs) obtained from whole-blood assessment on the day of collection.

2.3. T-cell function assessment

Effects of treatment on T-cell proliferative ability, degranulation, and cytokine secretion upon ex vivo TCR stimulation in the R/R CLL setting were compared with healthy donors. PBMCs were stained with antibodies against CD3, CD4, CD8, CD19, annexin V, and LIVE/DEAD fixable aqua dead cell stain (Supplemental Table S4) before and after 4 days of in vitro stimulation with 2.5 μg/mL anti-CD3 and 10 μg/mL anti-CD28. T-cell proliferation was assessed at day 4 with carboxyfluorescein succinimidyl ester (CFSE) staining. Lytic proteins and cytokines were quantified in culture supernatant at day 3 using the LEGENDplex Human CD8/NK panel (BioLegend, San Diego, CA, USA). To differentiate direct treatment effects from indirect changes resulting from disease resolution, we selected from each treatment arm similar numbers of responders and non-responders at the 6-month timepoint (end of ofatumumab treatment), based on investigator-assessed response per International Workshop on Chronic Lymphocytic Leukemia criteria [26].

2.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism8 (GraphPad Software, San Diego, CA, USA). Mann-Whitney 2-tailed test was performed to compare cohorts (baseline CLL vs. healthy donors; ibrutinib vs. comparators; R/R vs. first-line setting). Wilcoxon matched-pairs signed-rank test was used to determine statistically significant changes versus baseline (pretreatment); $P \leq 0.05$ was considered significant.

3. Results

3.1. Ibrutinib treatment positively impacts critical circulating immune cells

A competent immune system requires adequate presence and coordinated activities of innate and adaptive immune cells. Positive effects on these immune cells may enhance CLL treatment effectiveness [3]. At 6 months, PFS rates were superior for ibrutinib versus ofatumumab in RESONATE and versus chlorambucil in RESONATE-2 (88% vs. 65% and 98% vs. 78%, respectively). However, at this timepoint, reductions in circulating total B-cell counts were smaller with ibrutinib (63% vs. 94% and 58% vs. 97%, respectively) [23,24], showing that PFS improvement does not solely correlate to the decrease in circulating tumor cells. We performed immunophenotyping of PBMCs from patients in those trials (Fig. 1B) to better characterize the impact of treatment on circulating lymphocytes and myeloid cells, and to delineate differences between first-line and R/R cohorts. As previously described [27–29], ibrutinib-treated patients experienced transient lymphocytosis with an increase in B cells that largely subsided to baseline levels within 3 months followed by a steady decrease over the subsequent 9 months (Fig. 2A). T cells, NKT cells, and NK cells were also increased during lymphocytosis (Fig. 2B-D-E, first-line CLL). With longer treatment, ibrutinib restored abnormally elevated T-cell counts to within HDR (Fig. 2B). NKT cells, important mediators of immune responses and tumor immunosurveillance [30], were abnormally elevated in CLL patients at baseline but were brought near HDR with ibrutinib treatment (Fig. 2D). Despite initial lymphocytosis, median NK cell counts were within HDR throughout ibrutinib treatment (Fig. 2E). Overall, patterns of changes over time were similar between first-line and R/R cohorts. In addition to lymphocytes, myeloid cells contribute to the tumor–immune balance. Macrophages can adopt 1 of 2 divergent phenotypes in the tumor microenvironment: M1 tumor-associated macrophages (TAMs) have immune-boosting and cancer-killing potential and M2 TAMs are immunosuppressive and promote tumor survival and spreading [31]. Because assessment for TAMs requires invasive biopsies, we investigated their circulating precursors, classical and nonclassical monocytes, as surrogates for M1 and M2 subsets, respectively [32]. Compared with healthy donors, classical monocytes were significantly lower in CLL patients ($P < 0.0001$; Fig. 2C, baseline). First-line ibrutinib progressively restored classical monocyte counts to near HDR (Fig. 2C) and maintained nonclassical monocytes (Supplementary Fig. S1C), whereas chlorambucil significantly and persistently reduced classical monocyte counts as well as NK- and T-cell counts (all $P < 0.0001$; Fig. 2B-C-E) to below HDR. In effect, chlorambucil reduced all major circulating immune cells, while ibrutinib provided some normalization by decreasing B, T, and NKT cells; increasing classical monocytes; and maintaining NK cells throughout treatment.

3.2. Ibrutinib maintains T_{naive} counts and normalizes the expanded effector pool typically seen in CLL

T cells constitute a crucial component of adaptive immunity; a competent immune system must gradually evolve T cells from the pool of naive T (T_{naive}) cells to fully differentiated effector memory cells. When T_{naive} cells encounter a cognate antigen, they undergo maturation through distinct stages, including stem cell memory (T_{SCM}), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), and terminal effector memory (T_{TEM}) [33,34]. Alterations to those stages could compromise immune response [35]. The distribution of T-cell developmental subsets is dysregulated in CLL patients, with elevated terminally differentiated T cells and reduced T_{naive} cells [5]. Similar to data from Long et al. [15], we confirmed that the CLL-associated increase in T-cell counts was not equally distributed across T-cell developmental stages (Fig. 3A-B, baseline). Early developmental stages (T_{naive} and T_{SCM}) were low at baseline, particularly $CD4^+$ T_{naive} in R/R patients, whereas later stages (T_{TM}, T_{EM}, and $CD8^+$ T_{TEM}), were highly elevated relative to HDR. Ibrutinib significantly reduced effector and memory T-cell counts toward HDR and preserved T_{naive} (Fig. 3A-B). Normal T_{SCM} counts were also preserved, and high $CD8^+$ T_{SCM} counts in R/R CLL were reduced to HDR with ibrutinib. These data show that ibrutinib normalized pathogenically elevated T-cell counts from any developmental stage in both R/R and first-line cohorts. In contrast, chlorambucil reduced T cells across all developmental stages, including the already too low $CD4^+$ and $CD8^+$ T_{naive} counts (Fig. 3B).

3.3. Ibrutinib decreases pseudo-exhausted T cells and immunosuppressive cell populations

T-cell exhaustion is characterized by increased anergy and diminished immune responsiveness. Effector and memory T cells in CLL patients are frequently pseudo-exhausted due to chronic activation by CLL-B cells and interactions with other immunosuppressive cells, such as Tregs and MDSCs [5,36,37]. Ibrutinib therapy is associated with substantial reversal of T cell exhaustion [21]. In our study, elevated numbers of dysfunctional or pseudo-exhausted T cells, encompassing

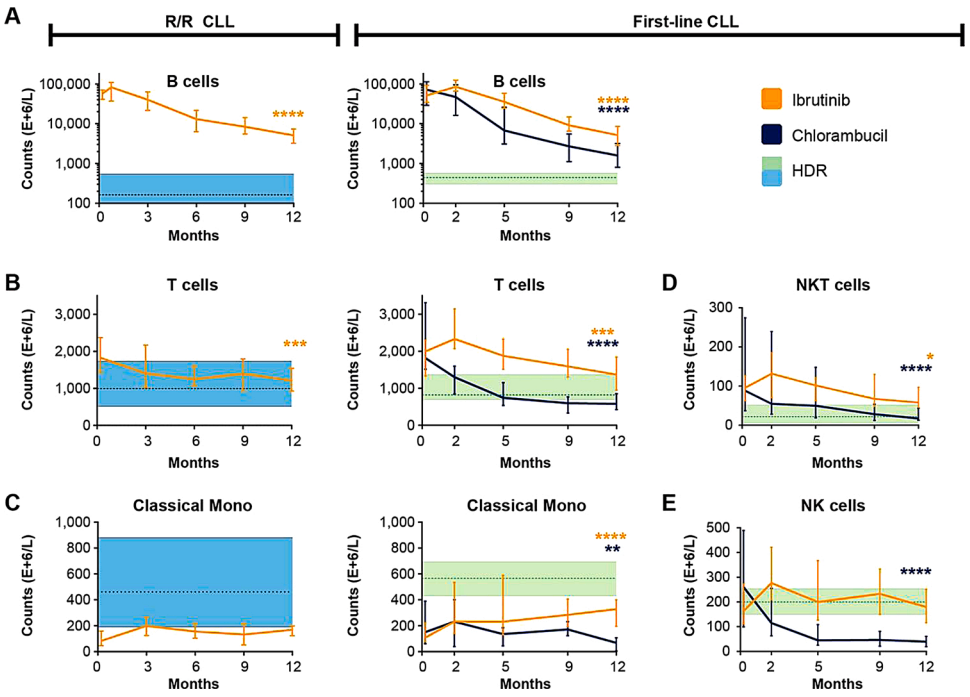


Fig. 2. Ibrutinib restores major immune cell populations to near healthy range. (A-E) Liquid nitrogen cryopreserved peripheral blood mononuclear cells from healthy donors, as well as patients enrolled in RESONATE (ibrutinib treatment) and RESONATE-2 (ibrutinib or chlorambucil treatment) were analyzed by flow cytometry to assess changes in immune cell numbers during treatment. Changes in (A) B cells, (B) T cells, (C) classical monocytes, (D) NKT cells, and (E) NK cells counts in circulation are reported. Data represent median and 95% confidence intervals. HDRs are represented by the interquartile range in blue or green, with the median in dash line. The blue HDR represents reference values for healthy donors aged > 50 years as published by Bisset et al [25]. The green HDR represents data from 20 healthy donors aged > 50 years (median 63.5 years; range, 51-76), assessed along with samples from RESONATE-2 in first-line CLL. Statistically significant changes reported in this figure were determined using Wilcoxon matched-pairs signed rank test at the 12-month timepoint in comparison to baseline (pretreatment) (*P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.001). CLL: chronic lymphocytic leukemia; HDR: healthy donor range; Mono: monocytes; NK: natural killer; NKT: natural killer T cell; R/R: relapsed/refractory. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

exhausted T cells (Texh: PD-1$^+$) and long-term activated T cells (T_{LTA}: CD38$^+$HLA-DR$^+$), are found before treatment (Fig. 4A-B). Notably, CD4$^+$ and CD8$^+$ T_{LTA} counts were 6-fold higher in R/R versus first-line CLL ($P < 0.0001$; Supplementary Fig. S1B), likely resulting from prolonged exposure to an immunosuppressive environment, extended chronic stimulation, and possibly secondary effect of previous therapies. Ibrutinib reduced both Texh and T_{LTA} counts toward HDR in first-line and R/R CLL (Fig. 4A-B), while chlorambucil lowered Texh cells only.

Elevated circulating Tregs have been observed in CLL and are associated with poor prognosis [38]. As previously reported for ibrutinib treatment in R/R CLL [39], Tregs returned to healthy levels in the first-line and R/R cohorts (Fig. 4C). MDSCs also exert immunosuppressive effects on T cells and expand with cancer progression, inflammation, and infection [6]. Monocytic MDSCs were significantly decreased by first-line ibrutinib treatment (Fig. 4D; not assessed in R/R CLL).

3.4. Ibrutinib improves T-cell survival

Tumor-specific T cells expand following TCR stimulation, but contract subsequently via mechanisms, such as activation-induced cell death and programmed cell death [40]. We examined cell death induced by ex vivo TCR stimulation in samples from R/R patients. The R/R cohort was selected due to its higher T_{LTA} count compared to first-line, likely leading to more pronounced T-cell dysfunction. At baseline, median ALC was comparable between ibrutinib and ofatumumab arms (Fig. 1B), as were percentages of B and T cells (approximately 80% and 3%, respectively). Following TCR stimulation, T cells from CLL patients had higher rates of apoptosis (annexin V$^+$) at baseline compared with healthy donors ($P < 0.0001$; Fig. 5C), consistent with a chronically activated phenotype. Although both treatments reduced T-cell apoptosis over time and independently of patient response, the effect with ofatumumab was more rapid (Fig. 5D). To better understand whether ibrutinib's effect on apoptotic cells could be masked by annexin V$^+$–activated T cells [41,42], we assessed T-cell apoptosis immediately after thawing. Compared with healthy donors, CLL patients had 50% more apoptotic CD4$^+$ and CD8$^+$ T cells at baseline ($P < 0.0001$; Fig. 5A). After 6 months of treatment, ofatumumab did not significantly alter T-cell apoptosis. In contrast, ibrutinib decreased T-cell apoptosis, especially CD8$^+$ T-cell apoptosis ($P = 0.0103$; Fig. 5B). No correlation was observed with response, suggesting that the effect was not a by-product of reduced disease burden. These data suggest that ibrutinib's true effect on apoptotic cells upon TCR stimulation is masked by Annexin V$^+$ on activated T cells.

Nonapoptotic cell death (NACD; annexin V– aqua^{hi}) fractions at baseline in stimulated T-cell cultures were similar between CLL patients and healthy donors (Fig. 5E). After 6 months of treatment, ibrutinib decreased NACD post-TCR stimulation by 53% (Fig. 5F), while ofatumumab had no significant effect.

3.5. Ibrutinib improves T-cell proliferation potential

Upon antigen stimulation, clonal expansion of T cells is necessary for a successful immune response. T-cell proliferation is impaired in CLL patients [5] and this impairment may lead to immunodeficiency. To assess T-cell proliferative ability, we stimulated PBMCs with αCD3/CD28 for 4 days in vitro and measured T-cell division index. Proliferative ability of T cells from most ibrutinib-treated patients improved with treatment, whereas ofatumumab impaired CD4$^+$ and CD8$^+$ T-cell proliferation (Fig. 6A). At 6 months (end of ofatumumab treatment), T-cell proliferative ability in ofatumumab-treated patients decreased by 49% from baseline ($P = 0.0032$) but improved by 28% in ibrutinib-treated patients ($P = 0.0012$; Fig. 6B). During both treatments, T-cell proliferation was greater for cytotoxic T cells than for helper T cells; this finding is of significance given the crucial role of CD8$^+$ T cells in antitumor immunity. To delineate therapy-specific effects from overall disease status, we assessed changes in T-cell proliferation within response subgroups (Fig. 6C). At 6 months, T-cell proliferation was greater in responders versus non-responders for ibrutinib (+39%

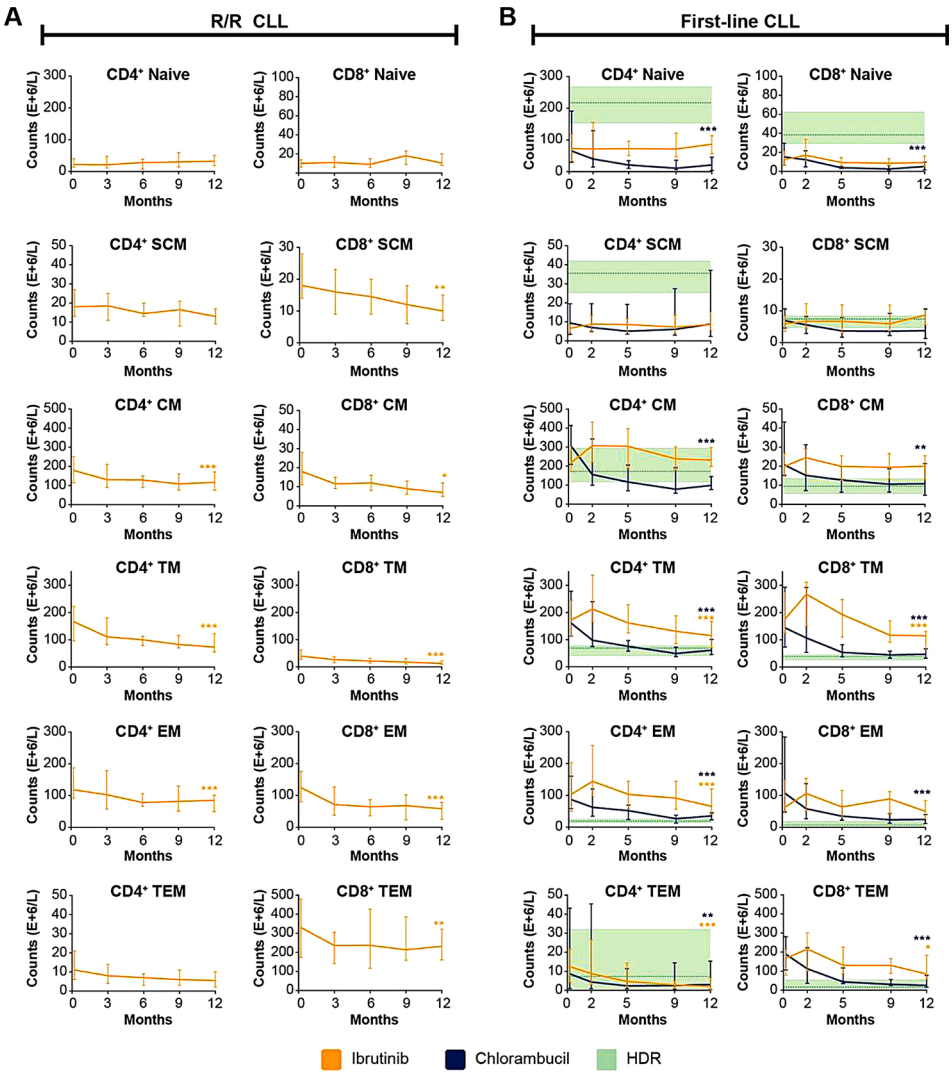


Fig. 3. Ibrutinib preserves early T-cell development stages, while reducing abnormally elevated effector/memory populations. T-cell populations were analyzed by flow cytometry in (A) R/R CLL and (B) first-line CLL. Data represent median and 95% confidence intervals. Healthy donor range (HDR) is represented by the interquartile range in green and median in dash line. The HDR represents data from 20 heathy donors aged > 50 years (median 63.5 years; range, 51-76), assessed along with samples from RESONATE-2 in first-line CLL. Statistically significant changes reported in this figure were determined using Wilcoxon matched-pairs signed rank test at the 12-month timepoint in comparison to baseline (pretreatment) (*$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.005). CLL: chronic lymphocytic leukemia; CM: central memory; EM: effector memory; HDR: healthy donor range; R/R: relapsed/refractory; SCM: stem cell memory; TM: transitional memory; TEM: terminal effector memory. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

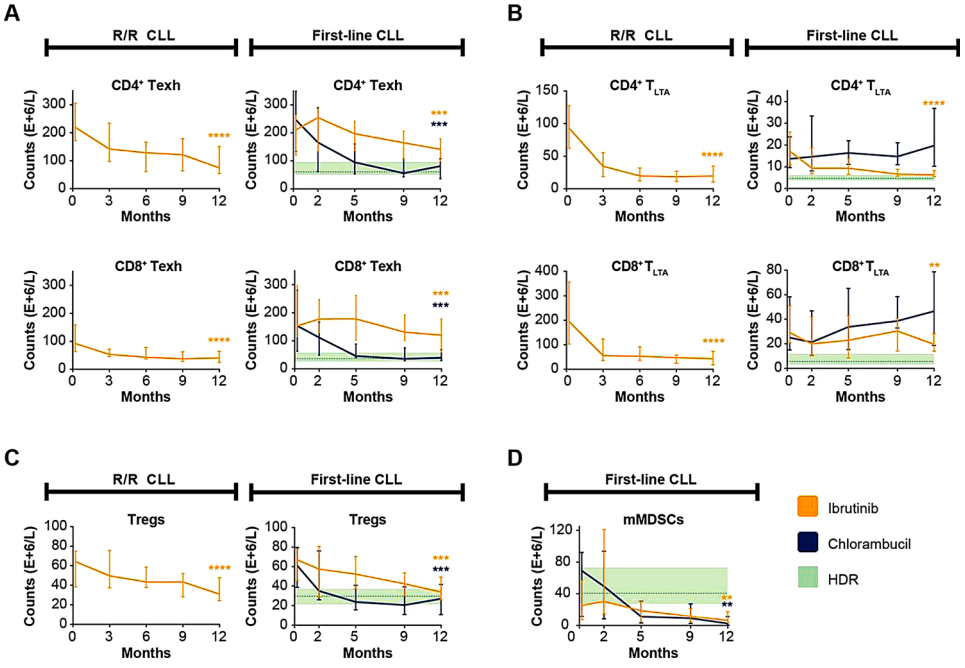


Fig. 4. Ibrutinib reduces abnormally elevated pseudo-exhausted T cells and immunosuppressive cells. Dysfunctional T-cell populations, such as (A) exhausted PD-1^{+} T cells and (B) chronically/long-term activated T cells (CD38^{+} HLADR^{+}), were analyzed by flow cytometry, along with immunosuppressive (C) Tregs and (D) monocytic MDSCs. Data represent median and 95% confidence intervals. HDR is represented by the interquartile range in green and median in dash line. The HDR represents data from 20 heathy donors aged > 50 years, assessed along with samples from RESONATE-2 in first-line CLL. Statistically significant changes reported in this figure were determined using Wilcoxon matched-pairs signed rank test at the 12-month timepoint in comparison to baseline (pretreatment) (*$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.005; ****$P <$ 0.001). CLL: chronic lymphocytic leukemia; HDR: healthy donor range; LTA: long-term activation; mMDSCs: monocytic myeloid-derived suppressor cells; PD-1: programmed cell death 1; R/R: relapsed/refractory; Texh: exhausted T cells; Tregs: regulatory T cells. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

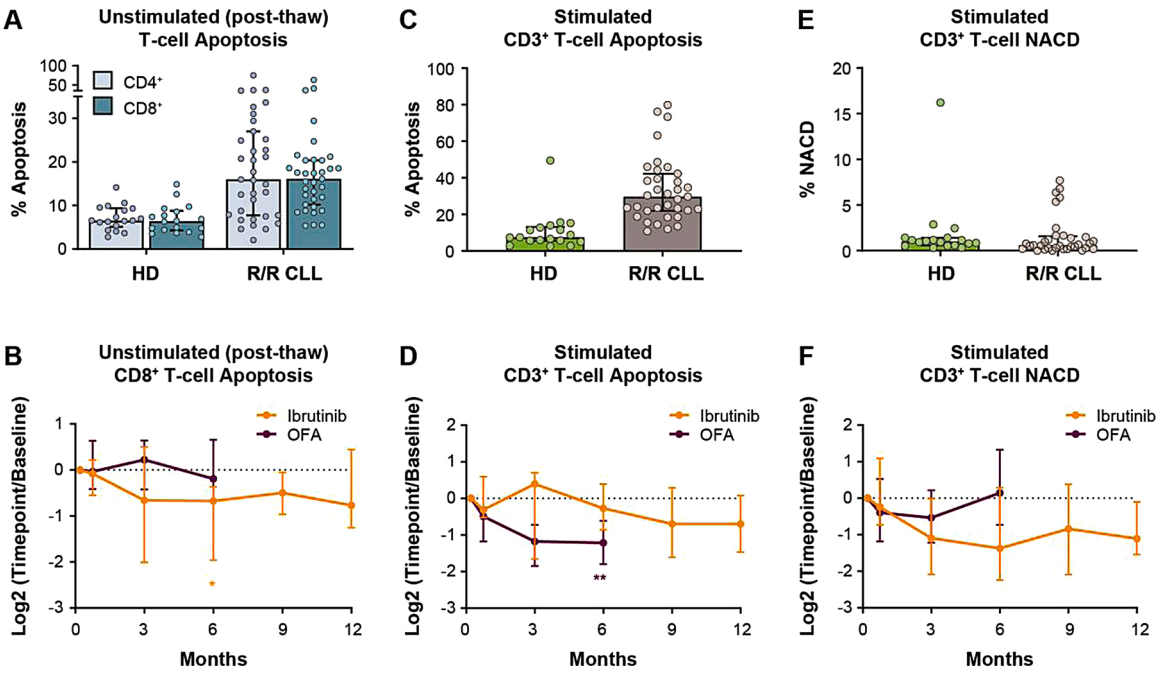


Fig. 5. Ibrutinib increases T-cell survival in R/R CLL. T-cell apoptosis (A-D) was assessed with annexin V by flow cytometry immediately after thawing, which indicates the unstimulated status (A, B), and post 4 days of anti-CD3/CD28 stimulation (C, D). T-cell nonapoptotic cell death was also assessed by flow cytometry after 4 days of anti-CD3/CD28 stimulation (E-F). Baseline statuses were compared to healthy donors (A, C, E), and treatment effects normalized to each patient's individual baseline are reported over time (B, D, F). Data represent median and interquartile range. Treatment-specific statistically significant changes were assessed at the 6-month timepoint in comparison to baseline using Wilcoxon matched-pairs signed rank test, and only statistically significant changes are reported (*$P < 0.05$; **$P < 0.01$). CLL: chronic lymphocytic leukemia; HD: healthy donors; NACD: nonapoptotic cell death; OFA: ofatumumab; R/R: relapsed/refractory; TCR: T-cell receptor.

vs. + 6%; $P = 0.0078$) and ofatumumab (–20% vs. –96%; $P = 0.0371$), suggesting that disease resolution correlates with T-cell proliferation. However, T-cell proliferation was significantly higher for ibrutinib than for ofatumumab in both responders and non-responders, indicating that ibrutinib improved T-cell proliferative ability beyond improvements related to disease burden. Increased proliferation did not correlate with T-cell or B-cell counts, nor with lymphocytosis status.

3.6. Ibrutinib improves T-cell effector function

Impairment in T-cell effector function is a key characteristic of CLL [5,37]. Normally, upon antigen recognition and costimulatory signals, effector T cells release an array of cytokines and lytic proteins to potentiate immune responses. Cytokines further support T-cell maturation and proliferation, and attract fresh macrophages, neutrophils, and other lymphocytes to attack infected or cancerous cells. Lytic proteins, such as granzyme-A, granzyme-B, granulysin, and perforin, are responsible for degranulation and lysis of targeted cells [33,34]. To further characterize ibrutinib's impact on T-cell effector function, we quantified cytokines and lytic proteins released into the supernatant following 3 days of in vitro TCR stimulation with PBMCs from R/R patients treated with ibrutinib or ofatumumab. At baseline, less cytokine release was observed in the ibrutinib arm (Fig. 6D-E), suggesting greater immune dysfunction, which correlates with the higher prevalence of poor prognostic features in this group (Fig. 1B). Compared with healthy donors, R/R CLL patients at baseline had impaired secretion of IL-4, IL-6, IL-10, and IL-17A but enhanced secretion of IL-2 and tumor necrosis factor α (TNF-α) (Fig. 6D-E). Six months post-treatment, ofatumumab only significantly improved secretory ability of IL-6 and IL-17A (Fig. 6E). In contrast, almost all cytokines measured (IL-4, IL-6, IL-10, IL-17A, and TNF-α) were significantly increased with ibrutinib at 6 months and stabilized thereafter (Fig. 6D). Degranulation of granzyme-A, granzyme-B, granulysin, and perforin, a key effector function of $CD8^+$ T cells, was impaired in R/R CLL patients compared with healthy donors (Fig. 6F). At 6 months, TCR-induced degranulation of lytic proteins exhibited similar improvement in both treatment arms, except for granzyme-B, which improved to a greater extent with ibrutinib (Fig. 6G). At month 12, improvements in cytokine release and lytic protein degranulation observed with ibrutinib were maintained and degranulation was largely restored to HDR. These improvements did not correlate with patient response or T-cell counts, suggesting that they were therapy-specific.

4. Discussion

Single-agent ibrutinib has shown superiority over established therapies in patients with CLL/SLL in both the first-line and R/R settings [9–14]. Patients enrolled in RESONATE and RESONATE-2 differ substantially with respect to number of prior therapies (median 3 vs. 0, respectively), cytogenetics, and other prognostic factors. Accordingly, we considered it important to characterize these cohorts separately to fully understand treatment effect and time to immune recovery in each cohort. We comprehensively assessed the effects of ibrutinib on circulating immune cell counts and select functions over a 12-month period. All ibrutinib-treated patients in the current analysis were progression-free throughout this time frame. When appropriate, observations were compared with randomized comparator treatments (chlorambucil or ofatumumab) and healthy donor samples to understand and differentiate CLL disease-related effects. Functional analyses were performed in R/R CLL patients only, as these patients are expected to have more pronounced T-cell dysfunction than previously untreated patients. Additionally, R/R patients were further evaluated based on clinical response per iwCLL criteria to explore treatment-related effects compared with indirect effects of disease resolution, although it should be noted that clinical response may not fully correlate with disease burden. Together with other reports [15,18–21], the current results

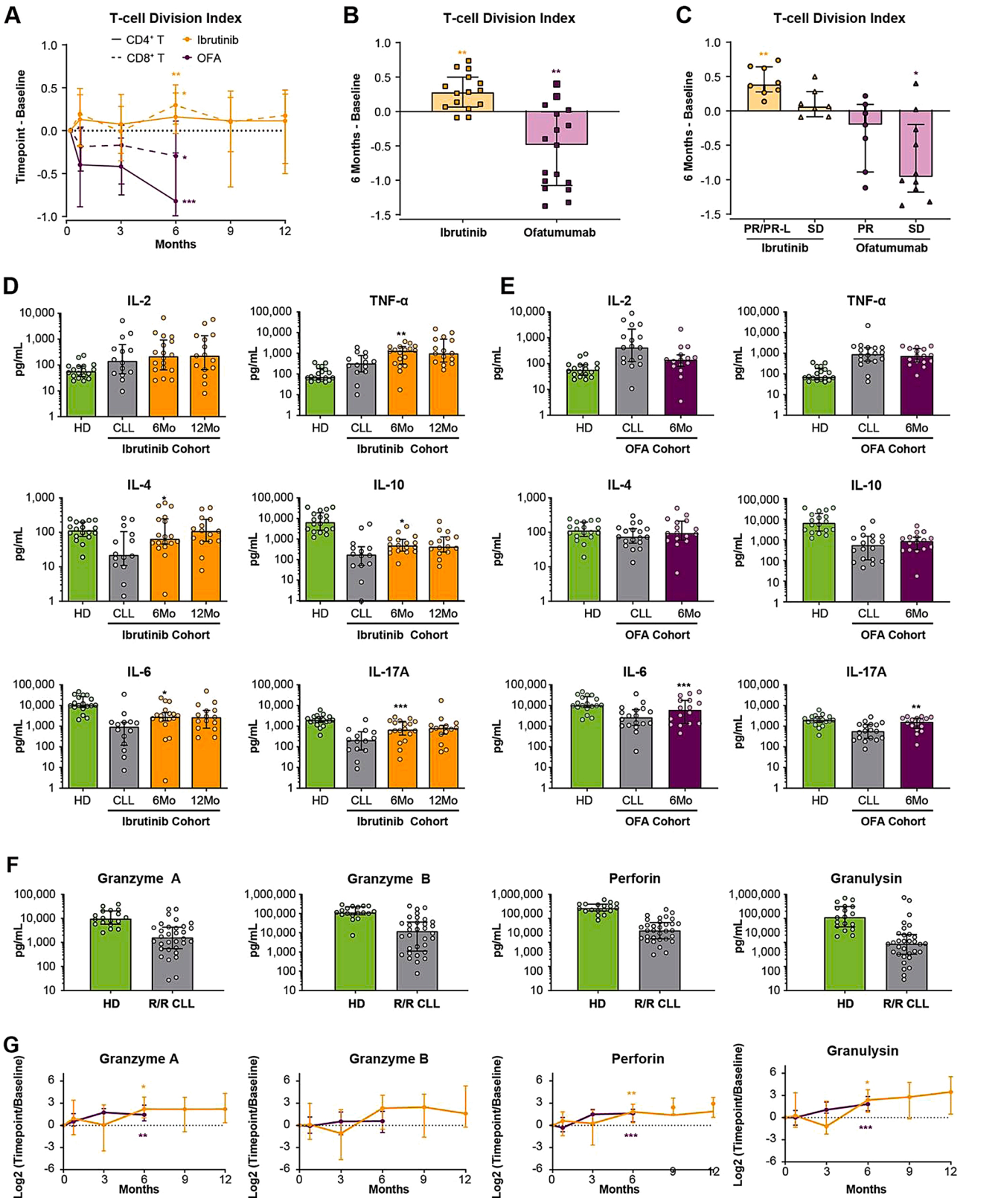


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Fig. 6. Ibrutinib increases T-cell proliferation and restores T-cell function in R/R CLL. Cells were stained with CFSE prior to TCR stimulation with anti-CD3/CD28 for 4 days. (A-C) Cell division index was calculated based on dilution of the CFSE signal measured by flow cytometry and represent the average number of cell divisions that a cell in the original population has undergone [division index = sum (i * N(i) / 2^i) / sum (N(i) / 2^i), where "i" is division number (undivided = 0), and "N(i)" is the number of events in division "i"]. Treatment effects over time are normalized to each patient's individual baseline. Division indexes for $CD4^+$ and $CD8^+$ T cells (A) are reported, along with overall T-cell division indexes for ibrutinib and ofatumumab treatment at 6 months, the end of ofatumumab treatment (B). T-cell division index at 6 months post-treatment was compared between responders (partial response with or without lymphocytosis, PR/PR-L) and non-responders (stable disease, SD) based on investigator-assessed response per iwCLL criteria (C). (D-E) Cytokine and lytic protein concentrations in the supernatants of ex vivo PBMC cultures were measured by LEGENDPlex assay after 3 days of anti-CD3/CD28 stimulation. Cytokines produced from (D) the ibrutinib cohort and (E) the ofatumumab cohort at baseline and 6-12 months post-treatment. Baseline from both cohorts were found to be different and therefore data from each cohort are individually compared to age-matched healthy donors. (F-G) Lytic proteins produced from both ibrutinib and ofatumumab cohorts were similar at baseline and are compared to healthy donors (F). Over time changes in degranulation are reported normalized to each patient's individual baseline (G). Data represent median and interquartile range. Statistically significant changes reported in this figure were determined using Wilcoxon matched-pairs signed rank test at the 6-month timepoint in comparison to baseline (pretreatment) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$). CFSE: carboxyfluorescein succinimidyl ester; CLL: chronic lymphocytic leukemia; IL: interleukin; iwCLL: International Workshop on Chronic Lymphocytic Leukemia; OFA: ofatumumab; PBMC: peripheral blood mononuclear cell; PR/PR-L: partial response without or with lymphocytosis; R/R: relapsed/refractory; SD: stable disease; TCR: T-cell receptor; TNF-α: tumor necrosis factor α.

show that ibrutinib offers a unique protective and restorative effect on T cells compared to other treatments.

An existing pool of T_{naive} cells is needed to initiate adaptive immunity when tumorigenic or infectious antigens are encountered. T_{naive} cell counts are near HDR in previously untreated patients but significantly lower in R/R patients ($P = 0.0091$; Supplementary Fig. S1A). Our data showed that T_{naive} cell counts were protected under ibrutinib, but decreased under chlorambucil in the first-line cohort. Another study reported reduced T_{naive} cells following treatment with alemtuzumab or fludarabine/cyclophosphamide [36]. Long et al. previously described preservation of T_{naive} cells during the first 5 months of ibrutinib treatment [15]; our analyses affirmed that this preservation continued over 12 months. Ibrutinib had no impact on T_{naive} counts and they remained overall below healthy donor levels. Long et al. also described increased effector and effector memory T cells during ibrutinib treatment in R/R patients with persistent lymphocytosis [15], a finding that seemingly contradicted an earlier report [18] of decreased T cells at 6–12 months in a mixed first-line and R/R cohort. By including timepoints encompassing periods studied by both groups, we confirmed that the impact of ibrutinib on T cells differs during the lymphocytosis period (typically the first few months on treatment) relative to subsequent months. The initial increase in effector, effector memory and exhausted T cells was only observed during lymphocytosis, followed by a decrease and ultimately normalization with continued ibrutinib treatment. Ibrutinib is thought to induce lymphocytosis by preventing CXCR4-mediated homing of CLL-B cells to lymph nodes [43]. Consequently, T cells previously under co-opted pressure by CLL-B cells are also released from lymph nodes [18]. In the circulation, without constant stimulation by CLL-B cells T cells stop proliferating and some are eliminated, resulting in reduction and normalization of overall T-cell count [22].

Unlike the first-line setting where $CD8^+$ T_{SCM} counts were normal at baseline, the abnormally high level of $CD8^+$ T_{SCM} in R/R patients was reduced. Normalization of T-cell subsets across T-cell development stages, coupled with improved T-cell proliferative ability, suggest that ibrutinib supports repopulation and functional recovery of the adaptive immune system. Compared with ofatumumab, ibrutinib-treated patients in our study carried more risk factors associated with poor prognosis, including del(17p), del(11q), and a greater number of prior treatments (Fig. 1B) [2]. Results of T-cell functional analyses are thus unlikely to be biased in favor of ibrutinib. Our data indicate that 6 months of ibrutinib treatment is required to significantly improve T-cell function, coinciding with the timing of the decrease in infection rates observed in the clinical setting [8,15]. Restoration of immunoglobulin A levels may also contribute to the decline in infection rates after 6 months of ibrutinib treatment [16]. Recent long-term analyses revealed that prevalence of upper respiratory tract infection of any grade in RESONATE-2 was 13% in the first year (n = 135), 7% in the second year (n = 123), and 8% in the fifth year (n = 89) of ibrutinib treatment [9]. In RESONATE, occurence of grade ≥ 3 infections gradually decreased from 29% in the first year (n = 195) to 17% in the second year (n = 160) and 9% during the sixth year of treatment (n = 57) [10]. Similar decreases in infection rates following ibrutinib treatment were also observed in mantle cell lymphoma [44]. The lower infection risk observed from these larger cohorts indicates ibrutinib's effect in multiple disease settings beyond the small group of patients assessed here.

Cancer progression is facilitated by multiple mechanisms that contribute to tumor evasion of the host immune system, including accumulation of Tregs and MDSCs in the tumor microenvironment [40,45]. Malignant CLL-B cells co-opt MDSCs, which impair T-cell function by promoting expansion of Tregs [6]. Tregs and MDSCs together weaken host immunity and support growth and survival of CLL-B cells and opportunistic pathogens. Beyond this indirect effect on T cells, ibrutinib may also influence them directly through ITK inhibition or other mechanisms. In an ex vivo experiment, ibrutinib treatment was sufficient to reactivate exhausted cytolytic T-cell function in a co-culture of T and CLL-B cells [46]. Attribution to ITK inhibition is supported by a treatment-induced shift of CD4+ cells toward a tumor-suppressive Th1 phenotype, originally observed in αβ T cells [17] and more recently in cytotoxic Vγ9Vδ2 T cells [47]. We hypothesize that, in the clinical setting, ITK inhibition may also underlie the significant reduction in the incidence and severity of infusion-related reactions (IRR) observed with ibrutinib/obinutuzumab versus chlorambucil/obinutuzumab in the iLLUMINATE trial (25% vs. 58% any grade IRR; 3% vs. 9% grade ≥ 3 or serious IRR) [11]. Although conclusive data are not yet available, the same mechanism also likely mitigates the incidence of severe cytokine-release syndrome (CRS) observed with ibrutinib combined with anti-CD19 chimeric antigen receptor T-cell infusion (JCAR014) versus JCAR014 monotherapy (0% vs. 25% grade ≥ 3 CRS) [48]. In this regard, the context-dependent effect of ibrutinib on T cells is particularly intriguing: ibrutinib reactivates anergic T cells and improves engraftment [49] to enhance antitumor effect, but downregulates undesirable inflammatory responses [48].

The samples derived from RESONATE and RESONATE-2 allowed us to characterize the immune status of previously untreated and R/R CLL patients. The R/R population, which reportedly has a higher rate of infections than previously untreated patients [7,8], was found to have more immune cell subsets with aberrant counts. For example, R/R patients appear to have up to 3 times fewer $CD4^+$ T_{naive} cells and 5-fold higher chronically activated T_{LTA} cells compared with previously untreated patients at baseline (Supplementary Fig. S1A-B). Overall, ibrutinib treatment provided immune reconstitution for both cohorts. Normalization was more complete in the first-line cohort though, in regard to T_{naive}, T_{LTA}, and classical monocytes (Supplementary Fig. S1). While chemoimmunotherapy-based regimens have been considered standard care for first-line treatment of young, fit patients with mutated *IGHV*, recent data from ECOG-ACRIN showed that ibrutinib/rituximab improved PFS and overall survival, and reduced grade ≥ 3 infectious complications compared with chemoimmunotherapy (20.3% with fludarabine/cyclophosphamide/rituximab vs. 10.5% with ibrutinib/rituximab; $P < 0.0001$) [13]. Quantitative immunophenotype and functional

data presented here support the hypothesis that improved clinical outcomes associated with first-line ibrutinib treatment are attributable, at least in part, to this therapy's beneficial immune reconstitution effects when compared with chemotherapy-based regimens. In contrast, the process of rebuilding the immune system is incomplete and lengthier if patients have received prior therapies, such as chemotherapy-based regimens, before ibrutinib.

Here, we comprehensively characterized changes in circulating counts of 21 immune subsets as well as T-cell function throughout a full year of treatment and demonstrate that ibrutinib has a significant, progressively positive impact on both malignant and nonmalignant immune cells in CLL. These positive effects on circulating nonmalignant immune cells may contribute to long-term CLL disease control, overall health status, and decreased susceptibility to infection.

Data availability

Requests for access to individual participant data from clinical studies conducted by Pharmacyclics LLC, an AbbVie Company, can be submitted through Yale Open Data Access (YODA) Project site at http://yoda.yale.edu

Authors' contributions

IGS, LKB, and AM were responsible for conception and design. IGS developed the methodology. Acquisition of data (acquired and managed patients, provided facilities) was done by IGS, HYH, TJK, JAB, JCB, SO, SPM, NEK, PH, and JCB. Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis) was performed by IGS, HYH, LKB, and AM. Writing, review, and/or revision of the manuscript was completed by all authors. Administrative, technical, or material support (i.e. reporting or organizing data, constructing databases) was provided by IGS and LKB. JPD and AM supervised.

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Conflict of interest statement

IGS reports employment with Pharmacyclics LLC, an AbbVie Company, and stock or other ownership in AbbVie. LKB reports former employment with Pharmacyclics LLC, an AbbVie Company; employment at Bolt Biotherapeutics; and stock or other ownership in AbbVie. HYH reports employment with Pharmacyclics LLC, an AbbVie Company; and stock or other ownership in AbbVie. TJK reports a consulting/advisory role for AbbVie, Genentech-Roche, Gilead, Pharmacyclics LLC, an AbbVie Company, and Celgene; and research funding from AbbVie, Genentech-Roche, Pharmacyclics LLC, an AbbVie Company, and Oncternal. JAB reports honoraria from and consulting/advisory role for Janssen; research funding from Gilead, TG Therapeutics, Pharmacyclics LLC, an AbbVie Company, and BeiGene; speakers bureau for and travel, accommodations, or other expenses from Gilead, TG Therapeutics, Pharmacyclics LLC, an AbbVie Company, Novartis, and Janssen. JCBa reports a consulting/advisory role for Genentech, Gilead, AstraZeneca and Sandoz; research funding from Oncternal Therapeutics; and honoraria from Janssen. SO reports a consulting/advisory role for Amgen, Astellas, Celgene, GlaxoSmithKline, Janssen Oncology, Aptose Biosciences, Vaniam Group, AbbVie, Alexion, Verastem, Eisai, Juno Therapeutics, Gilead, Pharmacyclics LLC, an AbbVie Company, TG Therapeutics, Pfizer, and Sunesis; and research funding from Kite, Regeneron, Acerta, Gilead, Pharmacyclics LLC, an AbbVie Company, TG Therapeutics, Pfizer, and Sunesis. SPM reports honoraria from and consulting/advisory role for Roche, AbbVie, Janssen, Gilead, and GlaxoSmithKline; research funding from Roche, AbbVie, and Janssen; and speakers bureau for Roche, AbbVie, Janssen, and Gilead. NEK reports a consulting/advisory role for MorphoSys, Infinity Pharm, Celgene, Agios, CytomX Therapeutics, AstraZeneca, Pharmacyclics LLC, an AbbVie Company, Rigel, Dava Pharmaceuticals, and Oncotracker; and research funding from Acerta, Pharmacyclics LLC, an AbbVie Company, Tolero, MEI Pharma, Celgene, and Sunesis. PH reports honoraria from, consulting/advisory role for, and research funding from Janssen, Pharmacyclics LLC, an AbbVie Company, and AbbVie; and travel, accommodations, or other expenses from Janssen and AbbVie. JCBy reports a consulting/advisory role for Janssen; research funding from Pharmacyclics LLC, an AbbVie Company, Gilead, TG Therapeutics, and BeiGene; speakers bureau from Pharmacyclics LLC, an AbbVie company, Gilead, TG Therapeutics, Novartis and Janssen; and travel, accommodations, or other expenses from Pharmacyclics LLC, an AbbVie Company, Janssen, Novartis, Gilead, and TG Therapeutics. IDL reports employment with Pharmacyclics LLC, an AbbVie Company, and spouse employment with The Permanente Medical Group; and stock or other ownership in AbbVie, Gilead Sciences, Clovis, Infinity, The Permanente Medical Group, and Reviva Pharmaceuticals. JPD and AM report employment with and stock or other ownership in Pharmacyclics LLC, an AbbVie Company.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2020.106432.

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