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Lymphocyte migration and retention properties are affected by ibrutinib in chronic lymphocytic leukemia

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Abstract

The Bruton tyrosine kinase (BTK) inhibitor ibrutinib is widely used for treatment of patients with relapsed/refractory or treatment-naïve chronic lymphocytic leukemia (CLL). A prominent effect of ibrutinib is to disrupt the retention of CLL cells from supportive lymphoid tissues, by altering BTK-dependent adhesion and migration. To further explore the mechanism of action of ibrutinib and its potential impact on non-leukemic cells, we quantified multiple motility and adhesion parameters of human primary CLL cells and non-leukemic lymphoid cells. *In vitro*, ibrutinib affected CCL19-, CXCL12- and CXCL13-evoked migration behavior of CLL cells and non-neoplastic lymphocytes, by reducing both motility speed and directionality. De-phosphorylation of BTK induced by ibrutinib in CLL cells was associated with defective polarization over fibronectin and inability to assemble the immunological synapse upon B-cell receptor engagement. In patients' samples collected during a 6-month monitoring of therapy, chemokine-evoked migration was repressed in CLL cells and marginally reduced in T cells. This was accompanied by profound modulation of the expression of chemokine receptors and adhesion molecules. Remarkably, the relative expression of the receptors governing lymph node entry (CCR7) *versus* exit (S1PR1) stood out as a reliable predictive marker of the clinically relevant treatment-induced lymphocytosis. Together, our data reveal a multifaceted modulation of motility and adhesive properties of ibrutinib on both CLL leukemic cell and T-cell populations and point to intrinsic differences in CLL recirculation properties as an underlying cause for variability in treatment response.

Introduction

A paradigmatic shift in the clinical management of B-cell malignancies including chronic lymphocytic leukemia (CLL) has been the introduction of small molecule inhibitors of kinases composing the B-cell receptor (BCR) signalosome such as LYN, SYK, PI3K and Bruton tyrosine kinase (BTK).¹ Ibrutinib, a first-in-class BTK inhibitor has rapidly become a leading treatment of patients with relapsed/refractory or treatment-naïve CLL.²⁻⁶ A remarkable effect of ibrutinib is to cause a rapid and prolonged lymphocytosis due to the redistribution of CLL cells from lymphoid tissues to peripheral blood and a progressive resolution of adenopathy.⁷⁻

¹⁰ The displacement of leukemic cells away from the supportive lymphoid tissue microenvironment affects cell proliferation, survival and overall burden.^{11,12}

Current knowledge on the mechanistic basis of ibrutinib-evoked CLL redistribution points to the alteration of adhesion and motility steps governing tissue residency and homing. In particular, preclinical and clinical studies have established that BTK inhibition via ibrutinib directly alters CLL cell motility in response to chemokines^{13,14} and adhesion via very late antigen-4 (VLA-4).^{13,15,16} Ibrutinib treatment has also been shown to decrease expression of C-C motif chemokine receptor 7 (CCR7)¹⁷ and VLA-4,¹⁶ while increasing that of sphingosine-1-phosphate receptor 1

(S1PR1).¹⁷ Such alterations of receptor expression are expected to indirectly favor lymphoid organ egress over re-entry. Although redistribution lymphocytosis is a hallmark of ibrutinib treatment, important inter-patient variability in the degree and kinetics of lymphocytosis has been reported.^{5,7,11,16} In particular elevated VLA-4 expression by CLL cells in a subset of patients has been associated with reduced lymphocytosis, inferior nodal response and shorter progression-free survival.¹⁶ Whether other parameters related to tissue homing and retention might condition response to treatment remains to be investigated. A further open question relates to the leakiness of ibrutinib, which not only targets BTK but also the related interleukin-2-inducible T-cell kinase (ITK). Ibrutinib treatment induces phenotypic modifications of the T-cell compartment, such as a reduction in the proportions of Th2 and Treg cell subsets, which support CLL cell survival.^{18,19} Furthermore, both CLL and non-leukemic cells progressively acquire a quiescent-like status upon ibrutinib treatment with downregulation of genes involved in various processes, including cell adhesion and cell:cell interaction.²⁰ Whether ibrutinib might also alter the motility properties of T cells and other non-neoplastic lymphocytes remains to be investigated in order to gain broader insight into its immunomodulatory effects, because severe infections often jeopardize its efficacy.²¹ Using a combination of quantitative cell imaging approaches, we here explored the effects of ibrutinib on the ability of CLL cells to migrate directionally along chemokine gradients and to assemble BCR-evoked synapses. We also assessed the *in vivo* activity of ibrutinib by monitoring over a 6-month treatment period the expression of multiple adhesion and motility receptors at the surface of CLL cells and non-leukemic T cells.

Methods

Healthy donors and patients

The study on healthy peripheral blood mononuclear cells (PBMC) was approved by the French South-West and Overseas ethical committee and was registered at the French Ministry of Higher Education and Research (DC-2015-2488). CLL cells considered for the *in vitro* ibrutinib exposure experiments were from a registered PBMC cell bank described previously.²² The corresponding patients (n=32) had not been treated with ibrutinib at the time of blood sampling. Their mean age was 70.4 years (range, 45-85 years) and the male-to-female sex ratio was 0.58. CLL patients considered for the longitudinal follow-up of ibrutinib treatment are reported in Table 1. All patients were referred for CLL according to International Workshop on CLL criteria. The studies on PBMC isolated from healthy donors and CLL patients were performed in agreement with the guidelines of the Declaration of Helsinki.

Chemotaxis assays

Standard Transwell assays were used to monitor chemokine-evoked migration of CLL cells and non-neoplastic lymphocytes both upon *in vitro* exposure to ibrutinib and in cells recovered from treated patients (see *Online Supplementary Methods*).

Live recording of cell motility behaviors

The study of basal and chemokine-evoked motility of CLL cells and T cells upon ibrutinib exposure was further evaluated by recording cell tracks in dedicated imaging chambers (see *Online Supplementary Methods*).

Immunological synapse of chronic lymphocytic leukemia cells

The ability of ibrutinib-exposed CLL cells to assemble the immunological synapse was studied with a high-content cell imaging approach and by scanning electron microscopy (see *Online Supplementary Methods*).

Flow cytometry bar coding

The longitudinal analysis of the phenotype of CLL cells and T cells in patients treated with ibrutinib was conducted with a flow cytometry bar coding approach as described in the *Online Supplementary Methods* section and in *Online Supplementary Table S1*.

Statistical analysis

A D'Agostino & Pearson omnibus normality test was applied to all datasets. On that basis, a parametric test was applied to the datasets with a Gaussian distribution, whereas a nonparametric test was applied to the datasets that did not match a Gaussian distribution. The specific tests selected for each dataset are indicated in the corresponding legends. The statistical significance of the results was defined as: not statistically significant (ns) $P \geq 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Results

Ibrutinib impairs basal motility and chemokine-driven directional migration of chronic lymphocytic leukemia cells

The precise effects of ibrutinib on key aspects of cell motility, such as cell velocity and orientation in chemokine gradients has remained unexplored. PBMC from untreated CLL patients were exposed *in vitro* to 500 nM ibrutinib, a concentration comparable to that in the plasma of patients treated with a daily dose of 420 mg.²³ We confirmed that ibrutinib reduced the ability of CLL cells to migrate towards CCL19, CXCL12 and CXCL13 in Transwell assays (Figure 1A). A reduction in the proportion of migrating cells was observed for all three chemokines in most of the patients

Table 1. Characteristics of the ibrutinib-treated patients.

Patient	Gender	Age, years	Previous treatment lines, N	Response to IBRU (PFS), months	ALC at IBRU treatment start, x10 ⁹ /L	IBRU-induced lymphocytosis	LFA1	VLA4	IGHV98 status	Trisomy Chr12	Del Chr11q	Del Chr13q	Del Chr17p or mutated P53	Complex karyotype
CPT1	M	60	>2	24	74.16	Transient	Y	N	UM	N	N	N	N	Y
CPT2	F	65	>2	65	149	Sustained	Y	N	M	N	N	Y	Y	Y
CPT4	F	56	1	47	159.5	Transient	Y	N	UM	N	N	Y	Y	Y
CPT7	M	77	>2	52	390	Transient	Y	N	M	N	Y	Y	Y	Y
CPT8	F	70	1	63	159.4	None	N	N	UM	N	N	N	Y	N
CPT9	M	55	>2	11	57	Transient	Y	Y	UM	N	N	Y	N	N
CPT10	M	76	>2	59	8	None	Y	Y	M	Y	Y	Y	N	N
CPT11	F	70	>2	40	177.7	None	Y	N	UM	N	N	N	Y	Y
CPT12	F	74	>2	62	137.8	Sustained	Y	Y	UM	N	N	N	N	Y
CPT15	M	73	>2	50	47.3	Sustained	Y	Y	UM	N	Y	Y	N	N
CPT16	M	68	>2	34	189.8	Sustained	N	N	UM	N	Y	N	N	Y
CPT17	M	55	>2	59	180	Transient	N	N	UM	N	Y	N	Y	N
CPT18	M	76	>2	24	6.6	Sustained	N	Y	UM	N	Y	N	N	Y
CPT19	M	79	1	58	14.7	Sustained	N	Y	UM	N	N	N	Y	N
CPT20	M	85	1	58	9.7	Sustained	Y	Y	UM	Y	N	N	Y	Y
CPT21	M	53	>2	21	34.7	Transient	Y	N	UM	N	Y	N	N	N
CPT22	M	65	>2	57	199.6	None	Y	N	UM	Y	N	Y	N	N
CPT24	M	49	>2	45	88.6	None	Y	Y	M	N	N	N	N	N
CPT32	M	80	>2	51	140	Sustained	Y	N	UM	N	N	N	Y	N
CPT36	M	66	>2	31	70.2	Transient	Y	Y	UM	N	N	N	N	N

IBRU: ibrutinib; PFS: progression-free survival; ALC: absolute lymphocyte count; M: male; F: female; Y: yes; N: no; UM: unmutated; M: mutated; LFA1 and VLA4 expression cut-off: 2X fluorescence-minus-one value.

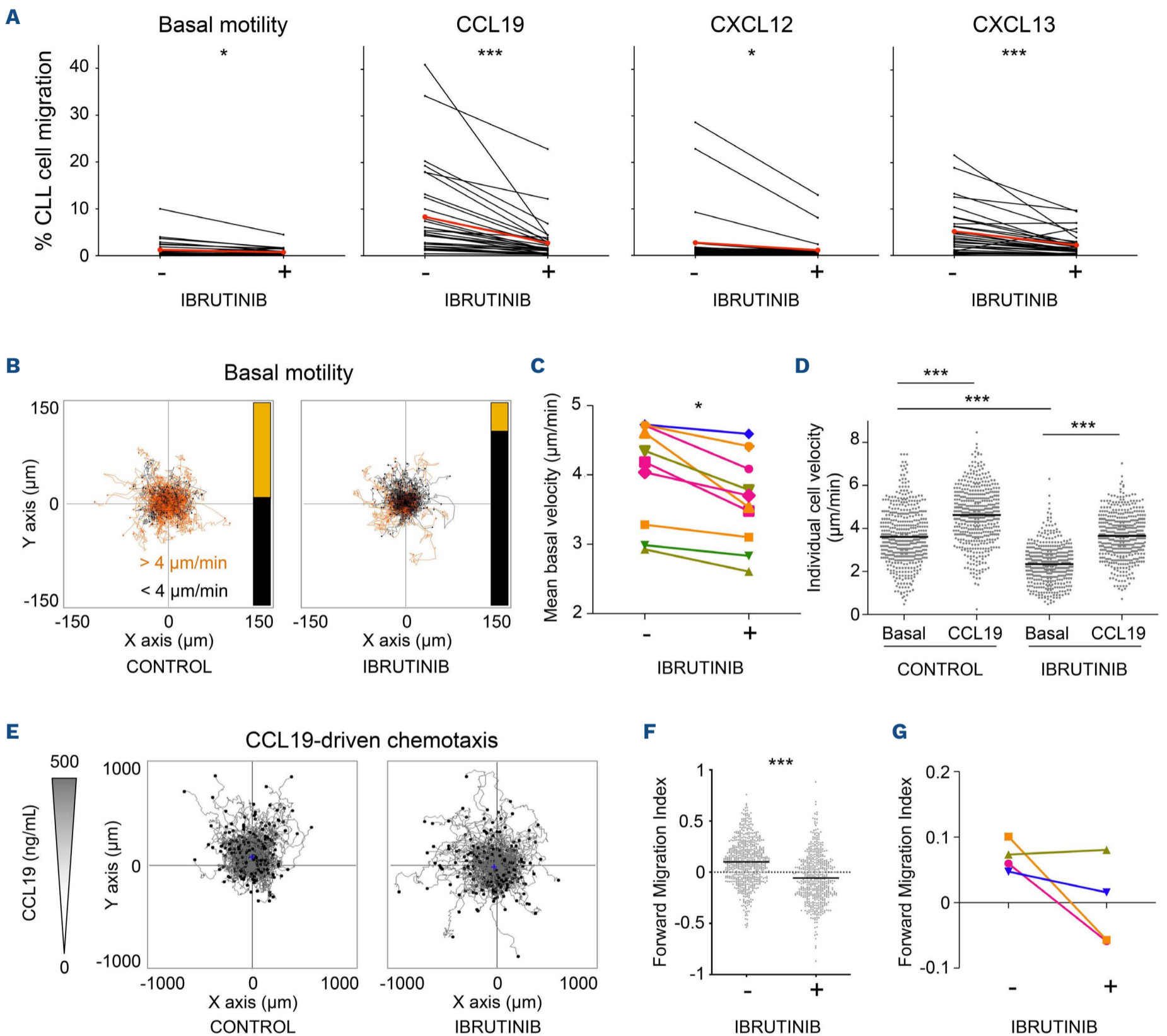


Figure 1. *In vitro* exposure to ibrutinib reduces random motility and chemokine-evoked directional motility in chronic lymphocytic leukemia cells. (A) Control or ibrutinib-treated peripheral blood mononuclear cells (PBMC) were seeded into Transwell inserts and exposed to CXCL12, CXCL13 or CCL19. Migrating chronic lymphocytic leukemia (CLL) cells were counted after 6 h by flow cytometry. Data represent the mean value from duplicate wells. N=32 patients. The mean of patients is indicated in red. A paired *t* test was applied to estimate the statistical significance of ibrutinib inhibition. Wilcoxon matched-pairs signed rank tests were applied. (B) Control or ibrutinib-treated PBMC were seeded on fibronectin and leukemic cells were tracked by live imaging. Panels show 2 h tracks, color-coded using a $4 \mu\text{m}/\text{min}$ threshold for mean speed; the bar on the right summarizes the proportion of cells over (orange) and under (black) the threshold (control 104 vs. 114, ibrutinib 32 vs. 186). (C) Comparison of mean basal velocity between control and ibrutinib-treated CLL cells. N=10 patients. A Wilcoxon matched-pairs signed rank test was applied. (D) Control or ibrutinib-treated PBMC were seeded on fibronectin in the presence or absence of CCL19 and tracked to calculate individual CLL cell velocity. One-way analysis of variance (ANOVA) with Bonferroni multiple comparison tests were applied. Results from a representative experiment out of four are shown. (E) Control or ibrutinib-treated PBMC were seeded on fibronectin, exposed to a CCL19 chemokine gradient and imaged. Panels show tracks of 441 control and 337 ibrutinib-treated CLL cells. (F) Forward Migration Index (FMI) along the CCL19 gradient (Y axis) of cells tracked in (E). Values for individual cells and mean are presented. The Mann-Whitney test was applied to compare conditions. (G) FMI index along the CCL19 gradient (Y axis) of cells from four CLL patients (mean values). Two-way ANOVA (mixed model) and Bonferroni multiple comparison tests were applied and revealed statistically significant reduction of the FMI upon ibrutinib treatment for three of the four patients. * $P < 0.05$; *** $P < 0.001$.

(respectively 32, 30 and 29 out of 32). The proportion of migrating cells was reduced on average by 40%. A reduction of random transmigration was also measured in the absence of chemokine (21 out of 32 patients). To gain insight into how ibrutinib affects CLL motility, we tracked CLL cell displacement using dedicated chemotaxis microslides. Following *in vitro* exposure to ibrutinib, the basal motility of CLL cells was affected as indicated by shorter migration tracks and reduced velocity (Figure 1B, C). Addition of CCL19 evoked an increase in velocity for both control and ibrutinib-treated CLL cells (Figure 1D), indicating that ibrutinib did not prevent chemokine sensing. In contrast, ibrutinib strongly affected the ability of CLL cells to migrate directionally along a CCL19 gradient (Figure 1E). Accordingly, the Forward Migration Index of treated cells was reduced (Figure 1F, G). Treatment of CLL cells with acalabrutinib, a more selective inhibitor of BTK,²⁴ resulted in comparable inhibition of migration along CCL19 gradients (*Online Supplementary Figure S1A-C*), indicating that the observed effects of ibrutinib were attributable to BTK inhibition. Collectively, these data reveal that ibrutinib affects the basal motility of CLL cells. In addition, although treated cells are responsive to CCL19 in terms of velocity, they fail to migrate directionally toward this chemokine.

Ibrutinib impairs BTK phosphorylation associated with immunological synapse assembly

The finding that ibrutinib affects CLL cell basal motility independently from chemokines suggests that this drug might also affect the ability of CLL cells to spread following adhesive stimuli such as during immunological synapse assembly. In agreement with previous studies,²⁵⁻²⁸ flow cytometry analysis showed that, as compared to healthy B cells, CLL cells expressed low to barely detectable levels of the integrins lymphocyte function-associated antigen 1 (LFA-1) and VLA-4 at their surface, based on the eight patients who were tested (Figure 2A). In this context, we studied the impact of ibrutinib on the assembly of the immunological synapse upon LFA-1 and BCR engagement by extracting metrics pertaining to cell spreading, BTK phosphorylation and actin cytoskeleton organization using a high-content confocal imaging pipeline.²⁹ During a 20-min interaction of CLL cells with coated intercellular adhesion molecule 1 (ICAM-1), we could not detect cell spreading, activation of pBTK, nor actin remodeling (Figure 2B, C). BCR stimulation mostly stimulated pBTK, but not spreading. Interestingly, combined BCR and ICAM-1 stimulated cell spreading for the two patients expressing intermediate-level LFA-1, but not for those with extremely low LFA-1 levels. This effect appeared to be dependent on a direct ICAM-1 to LFA-1 interaction since it was lost upon addition of a LFA-1 blocking antibody (*Online Supplementary Figure S2*). These data suggest that CLL cells that retain some level of LFA-1 expression are more prone to spread upon

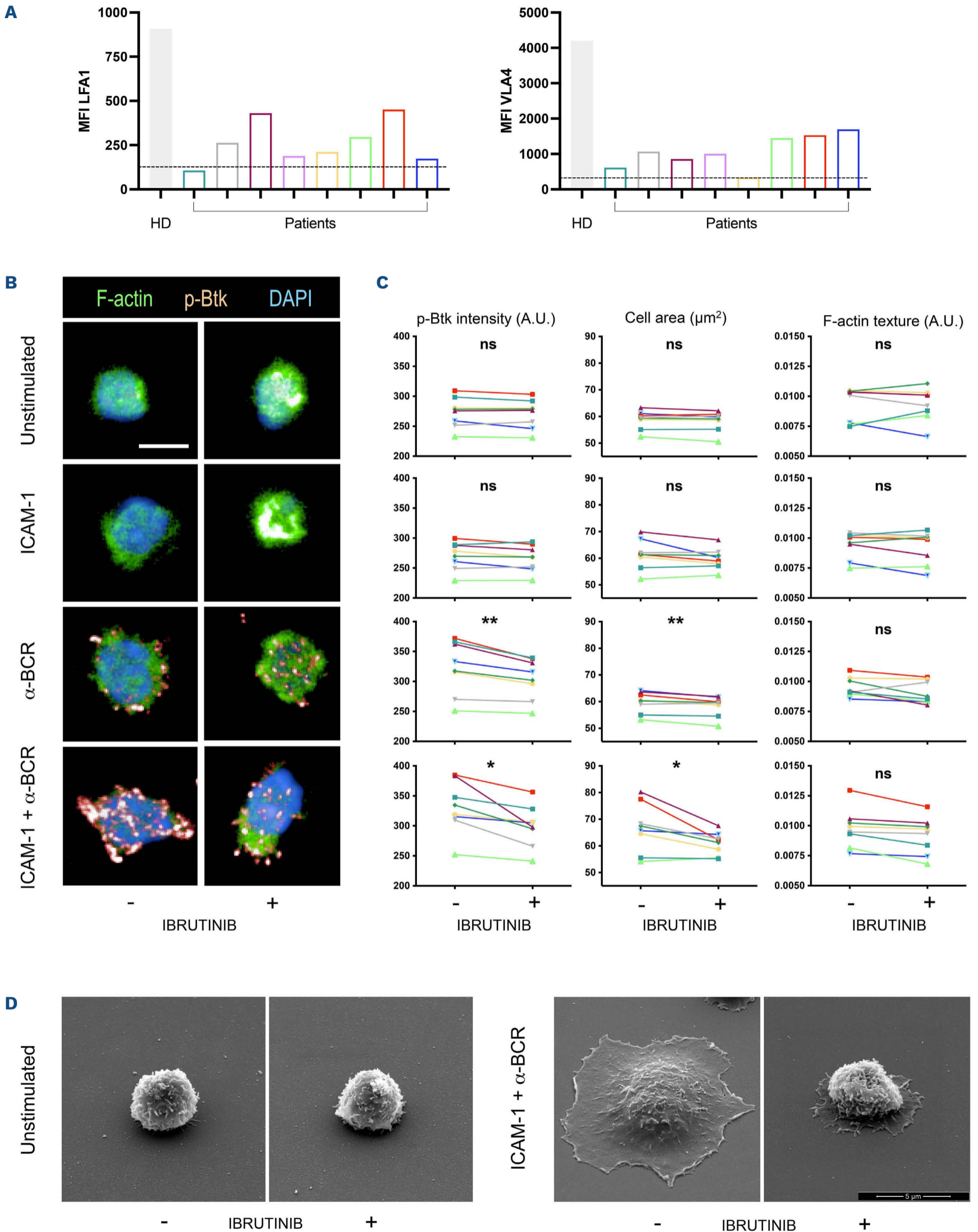
BCR engagement. *in vitro* exposure to ibrutinib markedly reduced the intensity of the BCR-evoked phosphorylated BTK signal, as assessed by automated cell imaging (Figure 2C) and flow cytometry (*Online Supplementary Figure S3A-C*). *In vitro* ibrutinib exposure also reduced the emission of protrusions emanated from the cell body, which directly impacted the spreading area, in particular for the two patients expressing LFA-1 (Figure 2C). Complementary analysis of the assembly of the synapse upon BCR and LFA-1 triggering in 13 patients confirmed the impact of ibrutinib on reducing BTK phosphorylation, cell spreading but also actin cytoskeleton organization (*Online Supplementary Figure S4A, B*).

To gain more insight into the alteration of CLL synapse assembly upon ibrutinib exposure, we assessed cell morphology by scanning electron microscopy. While CLL cells on PLL displayed a rather spherical shape and a surface covered by a dense network of microvilli, CLL cells on ICAM-1/anti-BCR antibody emitted filopodia and lamellipodia protrusions, associated with spreading and a partial loss of microvilli (Figure 2D). Remarkably, *in vitro* exposure to ibrutinib prevented the ability of CLL cells to spread over the ICAM-1/anti-BCR antibody-coated surface. Collectively, our data indicate that BTK is essential for CLL synapse assembly. These data complete our understanding of the effects of ibrutinib on CLL cell biology by suggesting that the blockade of BTK phosphorylation has a prominent impact on F-actin remodeling, sustaining emission of pro-adhesive structures in response to integrin and BCR stimulation.

Ibrutinib impairs chemokine-evoked motility in non-neoplastic lymphocytes

Although evidence has been collected that ibrutinib targets the function not only of CLL cells but also that of non-neoplastic lymphocytes via BTK or ITK,¹⁹ its potential impact on the motility of non-neoplastic B, T and NK cells has not been assessed yet. We first evaluated whether the ability of T cells from untreated CLL patients to respond to chemokines would be affected. Transwell assays on total CLL PBMC, in which T-cell migration could be assessed following CD3 staining, revealed that *in vitro* exposure to ibrutinib reduced the proportion of T cells migrating towards CCL19, CXCL12 and CXCL13 in most of the 32 tested CLL samples (Figure 3A). The level of inhibition was not complete, implying that upon ibrutinib treatment non-neoplastic T cells might retain some of their sensitivity to chemokines. In agreement, the motility of T cells over fibronectin was reduced, as assessed by live microscopy recordings (*Online Supplementary Figure S5A, B*).

To further explore the activity of ibrutinib in affecting the migration of non-neoplastic lymphocytes, we next sought to test its effects on B, T and NK cells from healthy individuals. PBMC from five donors were submitted to Trans-



Continued on following page.

Figure 2. *In vitro* exposure to ibrutinib impairs immunological synapse assembly in chronic lymphocytic leukemia cells. (A) Flow cytometry analysis of the expression of LFA-1 and VLA-4 at the surface of chronic lymphocytic leukemia (CLL) cells. Histograms represent mean fluorescence intensity in B cells from one healthy donor and in CLL cells from eight patients. The dashed line represents the background signal (fluorescence minus one). (B) Representative images of CLL cells stained for F-actin, phosphorylated BTK (p-BTK) and DAPI, and imaged on an automated confocal microscope. (C) Following automated recognition of individual cells based on the DAPI and F-actin staining, p-BTK intensity, cell area and F-actin texture were quantified for each cell. $N > 100$ cells/condition. Mean parameter values for the eight patients analyzed in (A) are shown. Wilcoxon matched-pairs signed rank tests were applied to estimate the statistical significance of ibrutinib inhibition. (D) Scanning electron microscopy micrographs of representative unstimulated CLL cells or CLL cells spreading over ICAM-1/anti-BCR antibody. CLL cells were exposed *in vitro* to ibrutinib, as indicated. ns: not statistically significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$. MFI: mean fluorescence intensity; HD: healthy donor; A.U.: arbitrary units.

well assays, in which the emigrated cells were stained with CD19, CD3 and CD56 antibodies to distinguish and count the proportion of migrating cells within the subsets of B, T and NK cells. All three tested chemokines, CCL19, CXCL12 and CXCL13, increased the proportion of migrating B cells over the basal values. Very clearly, *in vitro* exposure to ibrutinib systematically reduced the proportion of migrating B cells for all tested donors and chemokines (Figure 3B). T cells from the five donors displayed a robust migration in response to CCL19 and CXCL12, while migration to CXCL13 was restricted to one donor, probably because of a limited proportion of CXCR5-expressing T cells. Ibrutinib exposure reduced the proportion of migrating T cells upon CCL19 and CXCL12 stimulation, but also in the context of basal migration (Figure 3B). Finally, our data show that the migration of the NK-cell subset was also affected by ibrutinib, both in terms of basal migration and CXCL12-evoked migration (Figure 3B). These data therefore provide the evidence that ibrutinib reduces both basal and chemokine-evoked migration in non-neoplastic T cells from CLL patients, as well as in the main lymphocyte subsets of healthy donors.

Ibrutinib treatment of chronic lymphocyte leukemia patients reduces migratory capacities of the leukemic cells

Following the characterization of the *in vitro* effects of ibrutinib on CLL cells and non-neoplastic lymphocyte subsets, we evaluated ibrutinib's *in vivo* impact on cell motility along the course of treatment. For that purpose, we longitudinally monitored a cohort of 20 CLL patients who received ibrutinib monotherapy (Table 1). PBMC were collected immediately prior to treatment and after 1, 2, 3 and 6 months of treatment implementation. PBMC were frozen until completion of the sample collection and then thawed in parallel to test chemokine-evoked migration of CLL cells and T cells in Transwell assays. In line with the inhibitory effect of ibrutinib *in vitro*, the migration of CLL cells was reduced upon ibrutinib therapy. This was most evident upon CCL19 stimulation at 1 and 2 months of treatment with a drop in migration for nearly all of the 20 patients (Figure 4A). The effect of ibrutinib treatment on the ability of CLL cells to migrate to CXCL13 and CXCL12 (not

shown) could not be interpreted because of a generally low migratory response. Although our *in vitro* study pointed to a potent effect of ibrutinib on the basal and chemokine-evoked migration of T cells, the effect of ibrutinib treatment *in vivo* had a very modest effect on T-cell migration (Figure 4B). This suggests that although T cells appear to be as sensitive as CLL cells to ibrutinib in the context of an acute *in vitro* exposure, they might have lower sensitivity than CLL cells *in vivo*.

Alteration of chronic lymphocytic leukemia and T-cell homing receptor expression during ibrutinib treatment

Beyond affecting intrinsic adhesive and motility properties, ibrutinib might affect CLL recirculation *in vivo* also by altering the expression of receptors relevant for migration and adhesion. We here examined the expression of a large panel of receptors for chemotactic factors, adhesion molecules and co-receptors. To ensure the highest accuracy in the comparison of serially collected samples, we implemented a flow cytometry bar-coding approach to stain in parallel five samples (pre-treatment, months 1, 2, 3 and 6 after treatment) from a given patient in a single tube (*Online Supplementary Figure S6A-C*). Although the CLL cells from the 20 tested patients initially expressed heterogeneous levels of motility receptors, ibrutinib reproducibly reduced the expression of CCR7, CXCR5, CCR5, S1PR1 and CXCR3 over time (Figure 5A). Interestingly, the effect of ibrutinib treatment on CXCR3 expression followed a distinct pattern with an almost complete loss of expression after 2 months. In sharp contrast, CXCR4 expression did not vary along the 6-month follow-up period. These data point to a selective effect of ibrutinib in reprogramming the repertoire of motility receptors in CLL cells. Ibrutinib treatment also resulted in selective effects towards the expression of adhesion molecules (Figure 5B). While the expression of LFA-1, LFA-3, CD11c and CD44 progressively diminished upon treatment, the expression of VLA-4 remained unchanged and that of ICAM-1 increased (Figure 5B). Notably, ibrutinib treatment led to a progressive and marked reduction of the expression of the co-receptors BTLA and CD276, while the expression of LAG-3 remained stable (Figure 5C).

We further explored whether the levels of the integrins

LFA-1 and VLA-4, previously shown to be associated with CLL recirculation steps,²⁶ might relate to the levels of other examined markers or delineate a particular response to treatment. We observed that the samples that were negative for LFA-1 remained so over the five consecutive time points (*Online Supplementary Figure S7A-C*), indicating that this was a stable trait not impacted by treatment. Interestingly, both LFA-1-negative samples and VLA-4-

negative samples, which partially overlapped, tended to express high CD44 (*Online Supplementary Figures S7A-C* and *S8A-C*). Together this longitudinal analysis revealed that ibrutinib treatment progressively reprograms the repertoire of key motility/adhesion receptors at the surface of CLL cells, but that distinct features among subgroups of patients (e.g., patients with low integrin expression) are maintained along treatment.

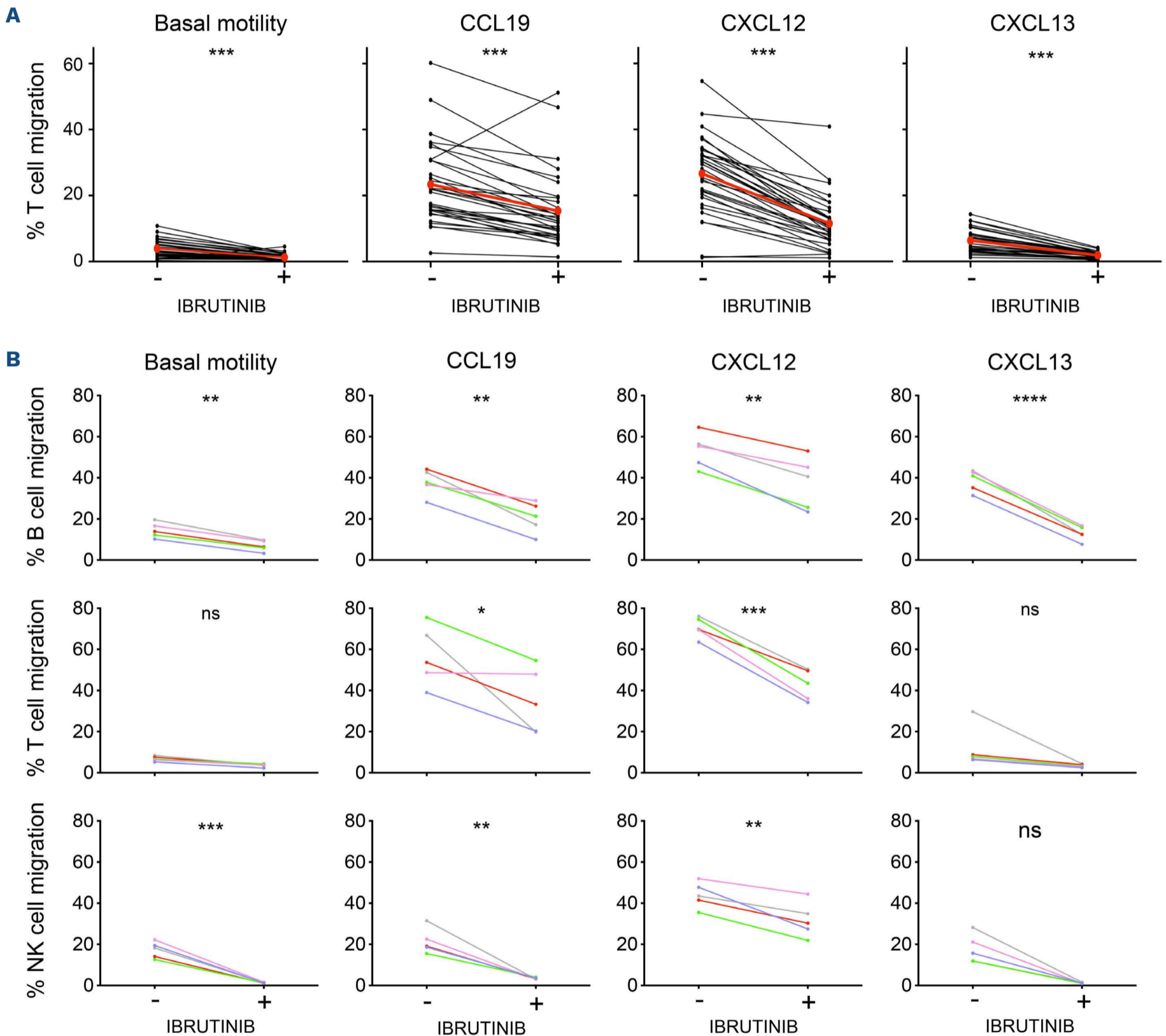


Figure 3. *In vitro* exposure to ibrutinib reduces chemokine-evoked migration of non-neoplastic lymphocytes from chronic lymphocytic leukemia patients and healthy donors. (A) Control or ibrutinib-treated peripheral blood mononuclear cells (PBMC) from 32 chronic lymphocytic leukemia (CLL) patients were seeded into Transwell inserts and exposed to CXCL12, CXCL13 or CCL19 for 6 h at 37°C. Migration of non-neoplastic T cells was quantified by flow cytometry. Data represent the mean value from duplicate wells. The mean of patients is indicated in red. Wilcoxon matched-pairs signed rank tests were applied to estimate the statistical significance of ibrutinib inhibition. (B) Control or ibrutinib-treated PBMC from five healthy donors were seeded into Transwell inserts and exposed to CXCL12, CXCL13 or CCL19 for 6 h at 37°C. Migration of B cells, T cells and NK cells was quantified by flow cytometry following specific staining of the migrated cells. Data represent the mean value from duplicate wells. Paired *t* tests were applied. ns: not statistically significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

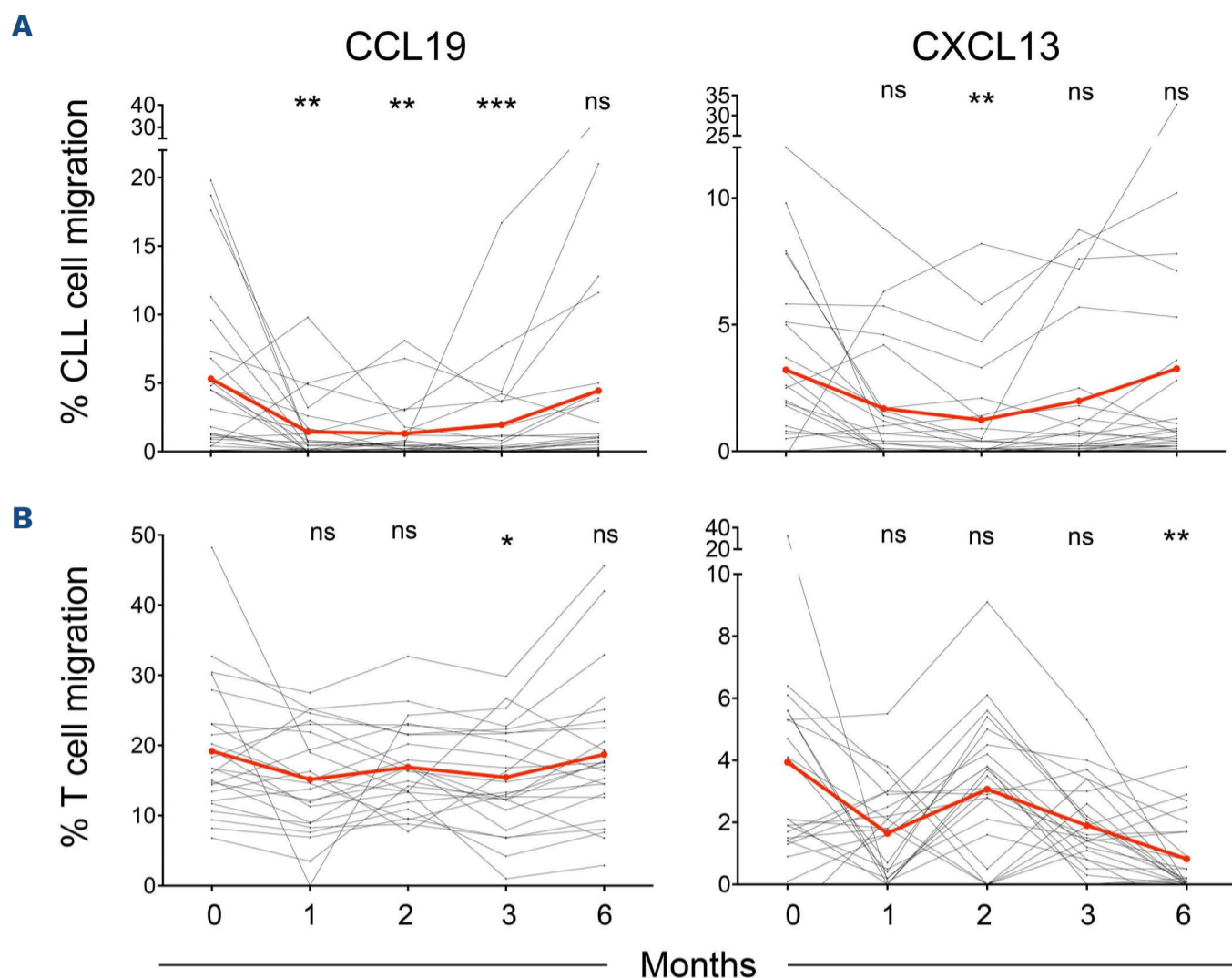


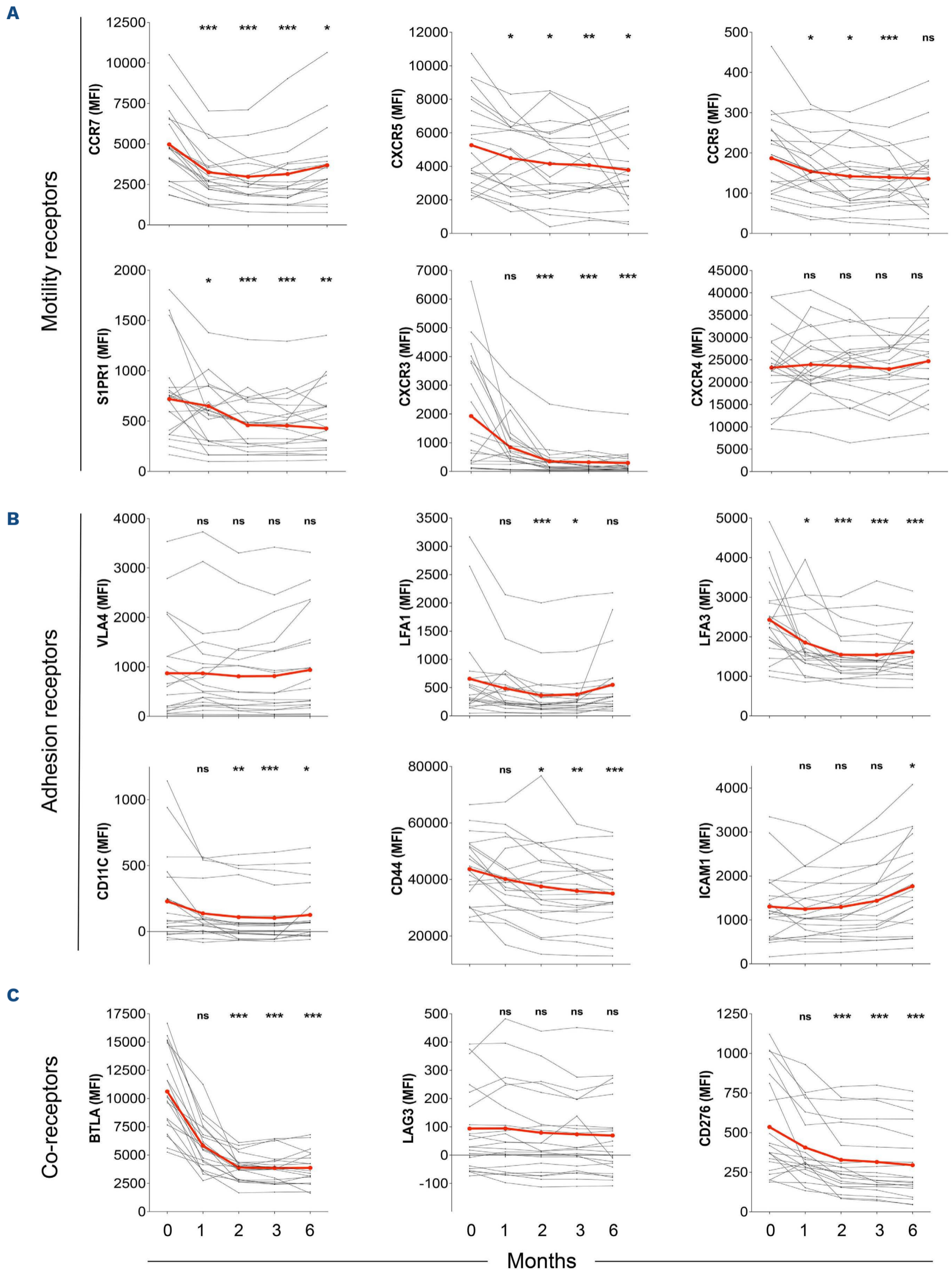
Figure 4. Ibrutinib treatment leads to a progressive reduction of chemokine-evoked migration in chronic lymphocytic leukemia cells. (A) Peripheral blood mononuclear cells (PBMC) from 20 chronic lymphocytic leukemia (CLL) patients were collected before and 1, 2, 3 and 6 months after the initiation of ibrutinib treatment. The five longitudinally collected and frozen samples were thawed together, seeded into Transwell inserts and exposed to CCL19 or CXCL13 for 6 h at 37°C. Migration of CLL cells was quantified by flow cytometry. Data represent the mean value from duplicate wells. The mean of patients is indicated in red. A Friedman test with Dunn multiple comparison tests were applied to estimate the statistical significance of ibrutinib inhibition as compared to the pre-treatment values. (B) Migration of T cells was quantified by flow cytometry from the same experiments as those presented in (A). Data represent the mean value from duplicate wells. The mean of patients is indicated in red. A Friedman test with Dunn multiple comparison tests with respect to pre-treatment values were applied. ns: not statistically significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

We then investigated whether ibrutinib would also alter the expression of receptors for chemotactic factors, adhesion molecules and co-receptors at the surface of non-neoplastic CD4⁺ and CD8⁺ T cells (*Online Supplementary Figures S9A-C and S10A-C*). As compared to the CLL cell compartment, less pronounced modulation of receptor expression was detected at the surface of T cells. In CD4⁺ T cells, the expression of CCR7, CXCR5, CCR5, CXCR3, VLA-4, LFA-1, LFA-3, and BTLA diminished progressively to reach their lowest levels at the 6-month timepoint, while the expression of the other tested receptors did not vary significantly. Similarly, in CD8⁺ T cells, the expression of CCR7, CXCR5, CXCR3 and BTLA diminished progressively to reach lowest levels at the 6-month timepoint, while the expression of the other tested receptors did not vary significantly. We additionally measured the expression of perforin, granzyme B and LAMP-1 as a proxy for the cytotoxic potential of CD8⁺ T cells. Ibrutinib was found to have no impact on the expression of those molecules over the course of treatment. Together, these data indicate a moderate and progressive effect of

ibrutinib treatment in selectively modulating a set of motility and activation markers in T cells.

Ibrutinib-driven redistribution lymphocytosis relates to relative expression of S1PR1/CCR7

Redistribution lymphocytosis has been related to the efficiency of ibrutinib in individual patients, highlighting the importance of CLL trafficking on treatment outcome. In agreement with previous reports,^{7,10,16} the herein studied ibrutinib-treated CLL cohort of patients displayed very heterogeneous redistribution lymphocytosis, ranging from sustained lymphocytosis to early decline of leukemic cells in the blood (Figure 6A). According to the criteria defined by Herman and colleagues,⁷ patients were clustered into three subgroups that had either sustained lymphocytosis, transient lymphocytosis or no lymphocytosis (Figure 6B, Table 1). Given the unique collection of motility parameters collected longitudinally in our cohort, we then sought to search for markers that may account for the inter-individual heterogeneity in lymphocytosis. Among the tested re-



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Figure 5. Ibrutinib treatment differentially affects the expression of chemokine receptors, adhesion molecules and co-receptors in chronic lymphocytic leukemia cells. (A) Peripheral blood mononuclear cells (PBMC) from 20 chronic lymphocytic leukemia (CLL) patients were collected before and 1, 2, 3 and 6 months after the initiation of ibrutinib treatment. The five longitudinally collected and frozen samples were thawed together, pre-stained with distinct concentrations of CellTrace™ Violet, mixed in a single tube and stained with antibodies specific for the indicated motility receptors. Data represent the longitudinal evolution of the expression of the indicated receptors as mean fluorescence intensity on CLL leukemic cells for each patient (black dots and lines) and as a mean (red dots and lines). A Friedman test with Dunn multiple comparison tests with respect to pre-treatment values were applied. (B) The indicated adhesion receptors were studied with the same approach as in (A). (C) The indicated co-receptors were studied with the same approach as in (A). ns: not statistically significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. MFI: mean fluorescence intensity.

ceptors known to instruct CLL homing and motility behaviors, CCR7 and S1PR1 emerged as the ones most related to the magnitude of the redistribution lymphocytosis, when considering their expression level along the 6-month follow-up period (Figure 6C). CCR7 expression appeared to relate inversely to lymphocytosis, in agreement with the function of CCR7 to instruct homing to and retention in lymph nodes. In contrast, S1PR1 expression appeared to relate positively to lymphocytosis, in agreement with the function of S1PR1 to instruct exit of CLL cells from lymph nodes to the blood circulation. Interestingly, our analysis further pointed to a negative correlation between the expression of CCR7 and S1PR1 on CLL cells among individual patients (Figure 6D). This is reminiscent of previous data in murine T cells showing coordinated upregulation of S1PR1 and downregulation of CCR7, as a switch mechanism to favor egress over retention.³⁰

The S1PR1/CCR7 ratio was then calculated as a molecular marker of the egress/retention equilibrium in individual patients (Figure 6E). Remarkably, this ratio was >1 (in favor of egress) in patients with sustained lymphocytosis, while it was <1 (in favor of retention) in patients with reduced lymphocytosis. This ratio was at a value close to 1 (egress and retention in equilibrium) for patients with intermediate lymphocytosis. Interestingly, the S1PR1/CCR7 ratio remained very stable over time for each patient, although the expression levels of S1PR1 and CCR7 decreased upon treatment. This was explained by a very parallel decrease of S1PR1 and CCR7 expression during the course of treatment. Collectively, these data suggest that a pre-existing heterogeneity in the turnover of CLL in lymphoid organs associated with the levels of S1PR1 and CCR7 accounts for the magnitude of ibrutinib-induced redistribution lymphocytosis in individual CLL patients.

Discussion

Understanding the mode of action of ibrutinib on CLL biology and identifying molecular markers accounting for treatment efficacy are key to the optimization of individualized clinical management. This study provides novel insight into the effects of ibrutinib on the motility properties of CLL cells and non-neoplastic lymphocytes. It also ex-

plores how ibrutinib affects CLL adhesion and the immunological synapse. It quantifies the *in vivo* impact of ibrutinib treatment on the motility capacity of these cell subsets along a 6-month follow-up. Finally, it identifies CCR7 and S1PR1 as key motility receptors that determine the degree of treatment-induced lymphocytosis.

Beyond the well-described inhibitory effect of ibrutinib on CLL adhesion and migration,¹³⁻¹⁵ our study identifies the precise parameters accounting for the activity of ibrutinib on CLL cell adhesion and motility. *In vitro* exposure of leukemic cells from CLL patients to ibrutinib induced a very reproducible reduction in chemokine-evoked migration. However, the concentration of 500 nM ibrutinib tested *in vitro*, in accordance with *in vivo* measurements,²³ did not completely blunt migration or adhesion of CLL cells, suggesting that alternative BTK-independent pathways can sustain these cellular activities. Directional migration was affected to a comparable degree upon ibrutinib and acalabrutinib treatment, reinforcing the notion that BTK activity is particularly central to governing chemotaxis in CLL. The inhibition of chemokine-induced migration at different timepoints after ibrutinib treatment was comparable to that measured upon *in vitro* exposure, suggesting that the daily delivery of ibrutinib might exert an acute effect on chemokine responsiveness via the maintenance of BTK inhibition.

An additional effect of treatment might relate to profound remodeling of the expression of multiple key adhesion and motility receptors, in particular CCR7 and CXCR5, which might account, at least in part, for the measured drop in responsiveness to CCL19 and CXCL13. Similarly to CCR7 and CXCR5, CCR5 and S1PR1 were progressively reduced. In our study, global CXCR4 expression remained stable over the course of treatment. It should be noted, however, that ibrutinib treatment has been reported to increase the proportion of long-term circulating CXCR4^{bright}CD5^{dim} cells and to result in an adaptation mechanism via the FOXO1-GAB1-pAKT axis to promote survival in cells unable to home back to lymphoid niches.^{31,32} The most drastic alteration of expression was observed for CXCR3, whose expression dropped massively. CXCR3 expression has been shown to inversely mirror the activation status of CLL and to be related to a good prognosis.³³ The drop in CXCR3 does, however, argue against the possibility that CXCR3 participates

in treatment efficacy. How this might affect CLL migration towards inflammatory sites rich in CXCR3 ligands remains to be investigated.

In contrast to previous reports that focused on VLA-4 as an important integrin for CLL trafficking and tumor micro-environment interactions,^{15,16} we failed to detect an effect of ibrutinib on the expression of this integrin. Instead, we measured a progressive downregulation of the adhesion receptors LFA-1, LFA-3, CD11c and CD44, pointing to a broad alteration of the CLL adhesive potential. Low expression of LFA-1 and VLA-4 partially overlapped across the examined samples, indicating that the regulation of LFA-1 and VLA-4 expression might be coupled, in line with the fact that both depend on a methylation process re-

lated to trisomy 12 status.^{28,34} Further analysis indicated that samples with low integrin expression had high CD44 levels. This is interesting in light of the fact that CD44 has been shown to support CLL survival in the context of the lymphoid organs.³⁵ It is therefore unexpected that CLL with low/negative integrin expression are associated with high CD44. However, the CD44v6 isoform, which is induced by the tumor microenvironment, has been shown recently to be particularly relevant for CLL progression.³⁶ Our study also provides insight into the assembly of the immunological synapse of CLL cells and its impairment by ibrutinib. While the BCR was the main trigger of BTK phosphorylation, LFA-1 potentiated synaptic spreading at least in patients who retained LFA-1 expression. This is in

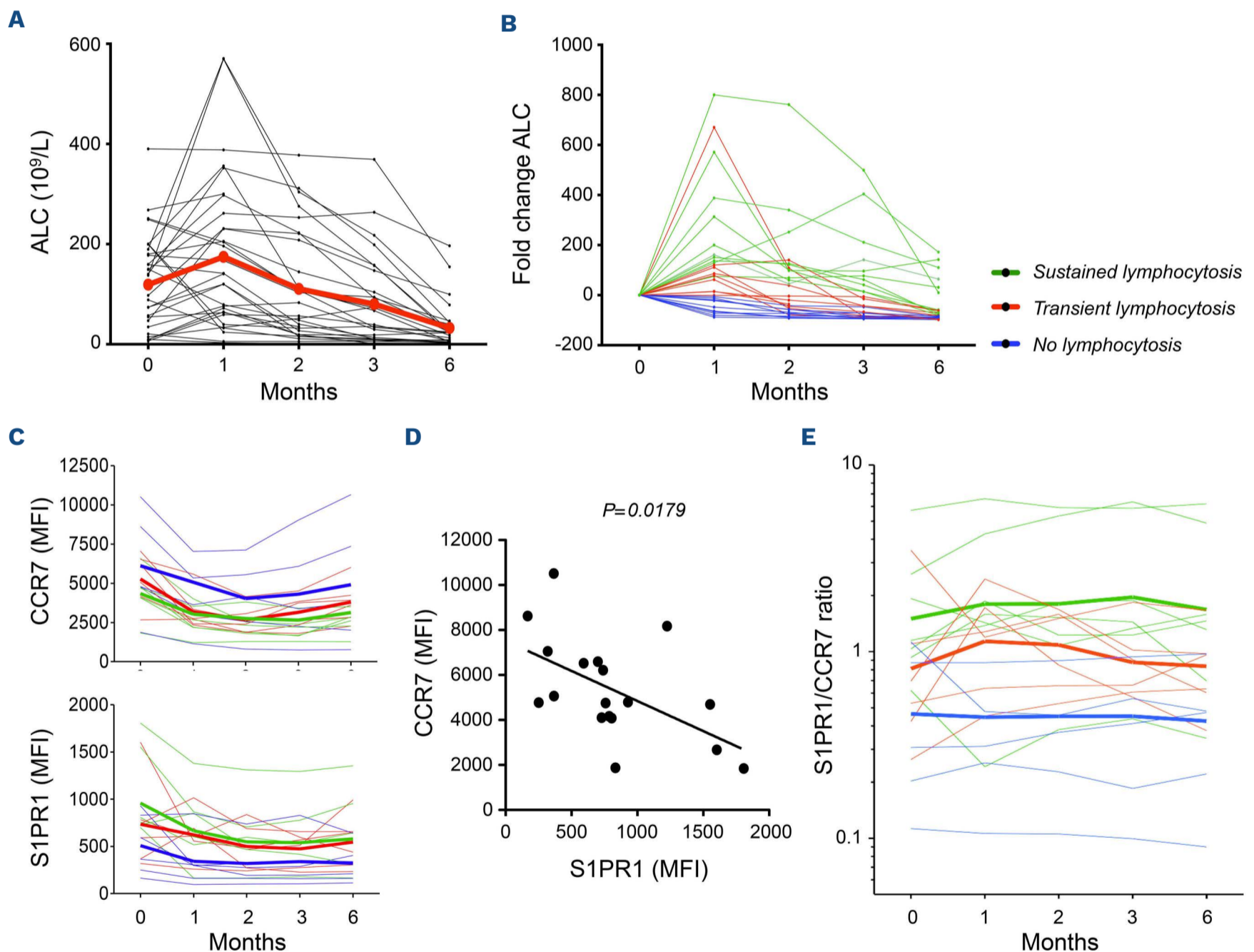


Figure 6. The S1PR1/CCR7 ratio is associated with the rate of ibrutinib-induced lymphocytosis. (A) Absolute leukocyte counts (ALC) are plotted during the course of ibrutinib treatment for individual chronic lymphocytic leukemia (CLL) patients (black dots and lines). Mean values are shown in red. (B) Fold change of ALC for the CLL patient cohort divided according to the indicated subgroup. (C) Level of expression (as determined by mean fluorescence intensity) of CCR7 and S1P1 at various timepoints after treatment in individual patients with colors indicating lymphocytosis subgroups, as indicated in (B). (D) Correlation analysis of the expression of CCR7 and S1P1 expression at the surface of CLL cells from individual patients prior to ibrutinib treatment initiation. (E) Normalized ratio of S1P1 and CCR7 expression at the surface of CLL cells from individual patients at various timepoints along ibrutinib treatment. Colors represent lymphocytosis subgroups, as indicated in (B). Two-way analysis of variance (ANOVA) (mixed model) was applied: lymphocytosis subgrouping accounted for 27.42% of the total variance (after adjusting for matching) with $P=0.0702$. One-way ANOVA test at $M=0$ of subgroup medians showed differences with $P=0.0602$. MFI: mean fluorescence intensity.

line with the notion that BCR signaling via BTK activates integrin-dependent adhesion.^{37,38} In this context, ibrutinib exposure severely impaired immunological synapse assembly by blocking BTK phosphorylation and associated actin remodeling. Complementary investigation of the role of additional co-receptors in CLL synapse assembly and their sensitivity to ibrutinib would be of interest, also because of the expression modulation observed by cytometry. The profound reprogramming of the motility/adhesion/interaction potential of CLL cells upon treatment is in line with an erosion of CLL identity, as characterized by single-cell RNA-sequencing analysis.²⁰ Our data further support a model whereby intrinsic differences in the recirculation properties of CLL cells, governed by S1PR1 and CCR7 expression, play a pivotal role in the individual response to ibrutinib. In particular, we identified that the S1PR1/CCR7 ratio, reflecting the exit/entry rate of CLL cells, is set differently in individual patients prior to treatment and that it is associated with the redistribution behavior of the CLL cells upon treatment. A state of imbalance in the expression of S1PR1 and CCR7 has been previously proposed to contribute to retaining CLL cells in the stromal microenvironment.^{17,39,40} *In vitro* data have pointed to the ability of ibrutinib to increase S1PR1 and decrease CCR7, thereby leading to a normalization of the imbalance between CCR7 and S1PR1 to favor CLL redistribution. However, our study shows that prolonged *in vivo* treatment leads to decreased expression of both S1PR1 and CCR7. Remarkably, the S1PR1/CCR7 ratio at the surface of CLL cells emerges as a set value in individual patients which is not affected by treatment. This is probably related to mechanisms in place to co-regulate the expression of these two receptors,⁴¹ agreeing with the inverse correlation between S1PR1 and CCR7 in individual patients. How could the S1PR1 axis be targeted to promote the egress of ibrutinib-resistant CLL? Larger cohorts might be used to validate the robustness of the S1PR1/CCR7 ratio to predict ibrutinib responsiveness. Then, combination therapy might help to overcome the S1PR1-related egress deficiency. In particular, SYK and PI3K inhibitors may promote S1P-mediated egress, in particular by relieving BCR-mediated repression of S1PR1 expression.^{40,42}

We also focused here on the off-target effects of ibrutinib on non-neoplastic cell subsets. Our *in vitro* exposure experiments showed a decrease in T-cell transmigration in response to CCL19 and CXCL12 in both CLL patients and healthy donors. Basal motility of T cells was also affected by ibrutinib. Notably, these *in vitro* effects did not translate into a substantial alteration of chemokine-evoked migration in T cells from treated CLL patients along our longitudinal follow-up study, in agreement with preserved CCR7-dependent migration of T cells in ibrutinib-treated

CLL patients.⁴³ Interestingly, expression of CXCR3 on T cells was strongly affected by ibrutinib treatment, suggesting that T-cell homing properties might be altered *in vivo*. Our study further points to cell adhesion and cell:cell interactions as pathways affected during the course of ibrutinib treatment, in parallel in CLL cells and non-neoplastic lymphocytes. This observation is in agreement with a recent single-cell RNA-sequencing analysis.²⁰

In conclusion, our study provides novel insight into the mechanism of action of ibrutinib in the context of CLL treatment. We identified a set of mechanisms that are reproducibly altered by ibrutinib in both CLL cells and non-neoplastic lymphocytes. Furthermore, our data reinforce the notion that the intrinsically low turnover of CLL cells from secondary lymphoid organs is a major mechanism of resistance to treatment efficacy. In particular the balance between CCR7 and S1PR1, which appears to be set in individual patients and to not vary during the course of treatment appears as a potential marker to predict treatment efficacy.

Disclosures

No conflicts of interest to disclose.

Contributions

JR-B performed experiments, analyzed data, and wrote the paper. AMu, NR, and AMo designed, performed and analyzed experiments. MC and SG designed and applied analytical solutions. OD, RP and SC performed experiments and analyzed data. LY and AQ-M provided CLL samples, participated in research design and scientific discussions. LD designed the research, supervised data analysis and wrote the paper.

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Data-sharing statement

The image-based and flow cytometry-based data reported in the study have not been deposited in a public repository but are available from the corresponding author upon request.

References

- Burger JA, O'Brien S. Evolution of CLL treatment - from chemoimmunotherapy to targeted and individualized therapy. *Nat Rev Clin Oncol*. 2018;15(8):510-527.
- Brown JR, Hillmen P, O'Brien S, et al. Extended follow-up and impact of high-risk prognostic factors from the phase 3 RESONATE study in patients with previously treated CLL/SLL. *Leukemia*. 2018;32(1):83-91.
- Byrd JC, Furman RR, Coutre SE, et al. Ibrutinib treatment for first-line and relapsed/refractory chronic lymphocytic leukemia: final analysis of the pivotal phase Ib/II PCYC-1102 study. *Clin Cancer Res*. 2020;26(15):3918-3927.
- Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2013;369(1):32-42.
- Farooqui MZ, Valdez J, Martyr S, et al. Ibrutinib for previously untreated and relapsed or refractory chronic lymphocytic leukaemia with TP53 aberrations: a phase 2, single-arm trial. *Lancet Oncol*. 2015;16(2):169-176.
- Gribben JG, Bosch F, Cymbalista F, et al. Optimising outcomes for patients with chronic lymphocytic leukaemia on ibrutinib therapy: European recommendations for clinical practice. *Br J Haematol*. 2018;180(5):666-679.
- Herman SE, Niemann CU, Farooqui M, et al. Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia*. 2014;28(11):2188-2196.
- O'Brien S, Furman RR, Coutre SE, et al. Ibrutinib as initial therapy for elderly patients with chronic lymphocytic leukaemia or small lymphocytic lymphoma: an open-label, multicentre, phase 1b/2 trial. *Lancet Oncol*. 2014;15(1):48-58.
- Wodarz D, Garg N, Komarova NL, et al. Kinetics of CLL cells in tissues and blood during therapy with the BTK inhibitor ibrutinib. *Blood*. 2014;123(26):4132-4135.
- Woyach JA, Smucker K, Smith LL, et al. Prolonged lymphocytosis during ibrutinib therapy is associated with distinct molecular characteristics and does not indicate a suboptimal response to therapy. *Blood*. 2014;123(12):1810-1817.
- Burger JA, Li KW, Keating MJ, et al. Leukemia cell proliferation and death in chronic lymphocytic leukemia patients on therapy with the BTK inhibitor ibrutinib. *JCI Insight*. 2017;2(2):e89904.
- Burger JA, Montserrat E. Coming full circle: 70 years of chronic lymphocytic leukemia cell redistribution, from glucocorticoids to inhibitors of B-cell receptor signaling. *Blood*. 2013;121(9):1501-1509.
- de Rooij MF, Kuil A, Geest CR, et al. The clinically active BTK inhibitor PCI-32765 targets B-cell receptor- and chemokine-controlled adhesion and migration in chronic lymphocytic leukemia. *Blood*. 2012;119(11):2590-2594.
- Ponader S, Chen SS, Buggy JJ, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing in vitro and in vivo. *Blood*. 2012;119(5):1182-1189.
- Herman SE, Mustafa RZ, Jones J, Wong DH, Farooqui M, Wiestner A. Treatment with ibrutinib inhibits BTK- and VLA-4-dependent adhesion of chronic lymphocytic leukemia cells in vivo. *Clin Cancer Res*. 2015;21(20):4642-4651.
- Tissino E, Benedetti D, Herman SEM, et al. Functional and clinical relevance of VLA-4 (CD49d/CD29) in ibrutinib-treated chronic lymphocytic leukemia. *J Exp Med*. 2018;215(2):681-697.
- Patrussi L, Capitani N, Martini V, et al. Enhanced chemokine receptor recycling and impaired S1P1 expression promote leukemic cell infiltration of lymph nodes in chronic lymphocytic leukemia. *Cancer Res*. 2015;75(19):4153-4163.
- Dubovsky JA, Beckwith KA, Natarajan G, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. *Blood*. 2013;122(15):2539-2549.
- Long M, Beckwith K, Do P, et al. Ibrutinib treatment improves T cell number and function in CLL patients. *J Clin Invest*. 2017;127(8):3052-3064.
- Rendeiro AF, Krausgruber T, Fortelny N, et al. Chromatin mapping and single-cell immune profiling define the temporal dynamics of ibrutinib response in CLL. *Nat Commun*. 2020;11(1):577.
- Varughese T, Taur Y, Cohen N, et al. Serious infections in patients receiving ibrutinib for treatment of lymphoid cancer. *Clin Infect Dis*. 2018;67(5):687-692.
- Gonnord P, Costa M, Abreu A, et al. Multiparametric analysis of CD8(+) T cell compartment phenotype in chronic lymphocytic leukemia reveals a signature associated with progression toward therapy. *Oncoimmunol*. 2019;8(4):e1570774.
- Gallais F, Ysebaert L, Despas F, et al. Population pharmacokinetics of ibrutinib and its dihydrodiol metabolite in patients with lymphoid malignancies. *Clin Pharmacokinet*. 2020;59(9):1171-1183.
- Byrd JC, Harrington B, O'Brien S, et al. Acalabrutinib (ACP-196) in relapsed chronic lymphocytic leukemia. *N Engl J Med*. 2016;374(4):323-332.
- Till KJ, Harris RJ, Linford A, Spiller DG, Zuzel M, Cawley JC. Cell motility in chronic lymphocytic leukemia: defective Rap1 and alphaLbeta2 activation by chemokine. *Cancer Res*. 2008;68(20):8429-8436.
- Hartmann TN, Grabovsky V, Wang W, et al. Circulating B-cell chronic lymphocytic leukemia cells display impaired migration to lymph nodes and bone marrow. *Cancer Res*. 2009;69(7):3121-3130.
- Riches JC, O'Donovan CJ, Kingdon SJ, et al. Trisomy 12 chronic lymphocytic leukemia cells exhibit upregulation of integrin signaling that is modulated by NOTCH1 mutations. *Blood*. 2014;123(26):4101-4110.
- Hutterer E, Asslaber D, Caldana C, et al. CD18 (ITGB2) expression in chronic lymphocytic leukaemia is regulated by DNA methylation-dependent and -independent mechanisms. *Br J Haematol*. 2015;169(2):286-289.
- German Y, Vulliard L, Kamnev A, et al. Morphological profiling of human T and NK lymphocytes by high-content cell imaging. *Cell Rep*. 2021;36(1):109318.
- Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity*. 2008;28(1):122-133.
- Pavlasova G, Borsky M, Seda V, et al. Ibrutinib inhibits CD20 upregulation on CLL B cells mediated by the CXCR4/SDF-1 axis. *Blood*. 2016;128(12):1609-1613.
- Seda V, Vojackova E, Ondrisova L, et al. FoxO1-GAB1 axis regulates homing capacity and tonic AKT activity in chronic lymphocytic leukemia. *Blood*. 2021;138(9):758-772.
- Ganghammer S, Gutjahr J, Hutterer E, et al. Combined CXCR3/CXCR4 measurements are of high prognostic value in chronic lymphocytic leukemia due to negative co-operativity of the receptors. *Haematologica*. 2016;101(3):e99-102.
- Zucchetto A, Caldana C, Benedetti D, et al. CD49d is overexpressed by trisomy 12 chronic lymphocytic leukemia

- cells: evidence for a methylation-dependent regulation mechanism. *Blood*. 2013;122(19):3317-3321.
35. Fedorchenko O, Stiefelhagen M, Peer-Zada AA, et al. CD44 regulates the apoptotic response and promotes disease development in chronic lymphocytic leukemia. *Blood*. 2013;121(20):4126-4136.
36. Gutjahr JC, Szenes E, Tschach L, et al. Microenvironment-induced CD44v6 promotes early disease progression in chronic lymphocytic leukemia. *Blood*. 2018;131(12):1337-1349.
37. Spaargaren M, Beuling EA, Rurup ML, et al. The B cell antigen receptor controls integrin activity through Btk and PLCgamma2. *J Exp Med*. 2003;198(10):1539-1550.
38. Montresor A, Toffali L, Rigo A, Ferrarini I, Vinante F, Laudanna C. CXCR4- and BCR-triggered integrin activation in B-cell chronic lymphocytic leukemia cells depends on JAK2-activated Bruton's tyrosine kinase. *Oncotarget*. 2018;9(80):35123-35140.
39. Sic H, Kraus H, Madl J, et al. Sphingosine-1-phosphate receptors control B-cell migration through signaling components associated with primary immunodeficiencies, chronic lymphocytic leukemia, and multiple sclerosis. *J Allergy Clin Immunol*. 2014;134(2):420-428.
40. Till KJ, Pettitt AR, Slupsky JR. Expression of functional sphingosine-1 phosphate receptor-1 is reduced by B cell receptor signaling and increased by inhibition of PI3 kinase delta but not SYK or BTK in chronic lymphocytic leukemia cells. *J Immunol*. 2015;194(5):2439-2446.
41. Capitani N, Patrussi L, Trentin L, et al. S1P1 expression is controlled by the pro-oxidant activity of p66Shc and is impaired in B-CLL patients with unfavorable prognosis. *Blood*. 2012;120(22):4391-4399.
42. Borge M, Remes Lenicov F, Nannini PR, et al. The expression of sphingosine-1 phosphate receptor-1 in chronic lymphocytic leukemia cells is impaired by tumor microenvironmental signals and enhanced by piceatannol and R406. *J Immunol*. 2014;193(6):3165-3174.
43. Mateu-Albero T, Marcos-Jimenez A, Wissmann S, et al. Ibrutinib does not impact CCR7-mediated homeostatic migration in T-cells from chronic lymphocytic leukemia patients. *Cancers*. 2022;14(11):2729.