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

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Conflict of interest

The authors have declared that no conflict of interest exists.

Author contribution

J.R.-B. performed experiments, analyzed data, and wrote the paper. A.Mu., N.R. and A.Mo. designed, performed and analyzed experiments. M.C. and S.G. designed and applied analytical solutions. O.D., R.P. and S.C. performed experiments and analyzed data. L.Y. and A.Q.-M. provided CLL samples, participated in research design and scientific discussions. L.D. designed the research, supervised data analysis and wrote the paper.

Data availability

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

The Bruton’s 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 BCR engagement. In patient 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 underlying cause for variability in treatment response.
Introduction

A paradigm shift in the clinical management of B-cell malignancies including CLL has been the introduction of small molecule inhibitors of kinases composing the BCR signalosome such as LYN, SYK, PI3K and 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 for 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 motility in response to chemokines\(^13\),\(^14\) and adhesion via VLA-4\(^13\),\(^15\),\(^16\). Ibrutinib treatment has also been shown to decrease expression of CCR7\(^17\) and VLA-4\(^16\), while increasing that of S1PR1\(^17\). Such receptor expression alteration is 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 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\textsuperscript{18, 19}. Furthermore both CLL and non leukemic cells progressively acquire a quiescent-like status upon ibrutinib treatment with down-regulation of genes involved in various processes, including cell adhesion and cell:cell interaction\textsuperscript{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 immuno-modulatory effects, because severe infections often jeopardize its efficacy\textsuperscript{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 patient cohorts

The study on healthy 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 PBMCs cell bank described previously. The corresponding patients (n=32) had not been treated with ibrutinib at the time of blood sampling. Mean age was 70.4 years (range 45-85 years) and M/F 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 IWCLL criteria. The studies on PBMC isolated from healthy donor and CLL patients were performed in agreement with the guidelines of the Declaration of Helsinki.

Chemotaxis assays

Standart Transwell assays were used to monitor chemokine-evoked migration in CLL cells and non-neoplastic lymphocytes both upon in vitro exposure to ibrutinib and in cells recovered from treated patients (see Suppl 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 Suppl Methods).
Immunological synapse of CLL 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 Suppl 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 Suppl Methods section and in Suppl 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. ns : p ≥ 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 CLL cells

The precise effects of ibrutinib on key aspects of cell motility, such as cell velocity and orientation in chemokine gradients has remained unexplored. PBMCs 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. We confirmed that ibrutinib reduced the ability of CLL cells to migrate towards CCL19, CXCL12 and CXCL13 in Transwell assays (Fig 1A). Reduction in the proportion of migrating cells was observed for all 3 chemokines in most of the patients (respectively 32, 30 and 29 out of 32). The proportion of migrating cells was reduced in 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 (Fig 1B-C). Addition of CCL19 evoked an increase in velocity for both control and ibrutinib-treated CLL cells (Fig 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 (Fig 1E). Accordingly, the Forward Migration Index of treated cells was reduced (Fig 1F-G). Treatment of CLL cells with acalabrutinib, a more selective inhibitor of BTK, resulted in comparable inhibition of migration along CCL19 gradients (Suppl Fig S1A-C), indicating that the observed ibrutinib effects 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\textsuperscript{25-28}, flow cytometry analysis showed that, as compared to healthy B cells, CLL cells expressed low to barely detectable levels of the integrins LFA-1 and VLA-4 at their surface depending on the 8 tested patients (Fig 2A). In this context, we tested 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\textsuperscript{29}. During a 20-min interaction of CLL cells with coated ICAM-1, we could not detect cell spreading, activation of pBTK, nor actin remodeling (Fig 2B-C). BCR stimulation mostly stimulated p-BTK, but not spreading. Interestingly, combined BCR and ICAM-1 stimulated cell spreading for the 2 patients expressing intermediate 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 (Suppl Fig S2). These data suggests 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 (Fig 2C) and flow cytometry (Suppl Fig 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 2 patients expressing LFA-1 (Fig 2C). Complementary analysis of the assembly of the synapse upon BCR and LFA-1 triggering in 13 patients confirmed the impact of ibrutinib at reducing BTK phosphorylation, cell spreading but also actin cytoskeleton organization (Suppl Fig S4A-B). To gain more insight in the alteration of CLL synapse assembly
upon ibrutinib exposure, we assessed cell morphology by Scanning Electron Microscopy (SEM). 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 Ab emitted filopodia and lamellipodia protrusions, associated with spreading and a partial loss of microvilli (Fig 2D). Remarkably, in vitro exposure to ibrutinib prevented the ability of CLL cells to spread over the ICAM-1/anti-BCR Ab 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 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 ITK⁹, 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 T cells migrating towards CCL19, CXCL12 and CXCL13 in most of the 32 tested CLL samples (Fig 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 recording (Suppl Fig 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 5
donors were submitted to Transwell assays, in which the emigrated cells where stained with CD19, CD3 and CD56 antibodies to distinguish and count the proportion of migrating cells within the subsets of B cells, T cells and NK cells. All 3 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 (Fig 3B). T cells from the 5 donors displayed a robust migration in response to CCL19 and CXCL12, while migration to CXCL13 was restricted to one donor, probably because of 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 (Fig 3B). Finally, our data show that also the migration of the NK-cell subset was affected by ibrutinib, both in terms of basal migration and CXCL12-evoked migration (Fig 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 lymphocytes subsets of healthy donors.

**CLL patient treatment with ibrutinib reduces migratory capacities of leukemic CLL cells**

Following the characterization of the in vitro effects of ibrutinib on CLL cells and non-neoplastic lymphocyte subsets, we evaluated its in vivo impact on cell motility along the course of treatment. For that purpose, we monitored longitudinally 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 (Fig 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 (Fig 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 harbor lower sensitivity than CLL cells in vivo.

**Alteration of CLL 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 5 samples (pre-treatment, months 1, 2, 3 and 6 post treatment) from a given patient in a single tube (Suppl Fig S6A-C). Although the CLL cells from the 20 tested patients expressed initially heterogeneous levels of motility receptors, ibrutinib reproducibly reduced the expression of CCR7, CXCR5, CCR5, S1PR1 and CXCR3 over time (Fig 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 (Fig 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 (Fig 5B). Noticeably, 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 (Fig 5C). We further explored whether the levels of the integrins LFA-1 and VLA-4 previously shown to be associated to CLL recirculation steps might relate to the 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 5 consecutive time points (Suppl Fig 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 (Suppl Fig S7A-C and S8A-C). Together this longitudinal analysis reveals that ibrutinib treatment progressively reprograms the repertoire of key motility/adhesion receptors at the surface of CLL cells, but that distinct features among patient subgroups (e.g. patients with low integrin expression) are maintained along treatment. We then asked if 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 (Suppl Fig 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 lowest levels at the 6-month time point, while the expression of the other tested receptors did not vary significantly. In CD8+ T cells, the expression of CCR7, CXCR5, CXCR3 and BTLA diminished progressively to reach lowest levels at the 6-month time point, 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 modulating selectively a set of motility and activation markers in T cells.

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

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, the herein studied ibrutinib-treated CLL patient cohort displayed very heterogeneous redistribution lymphocytosis, ranging from sustained lymphocytosis to early decline of leukemic cells in the blood (Fig 6A). According to the criteria defined by Herman and colleagues, patients were clustered into 3 sub-groups that harbored either sustained lymphocytosis, transient lymphocytosis or no lymphocytosis (Fig 6B and 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 receptors known to instruct CLL homing and motility behaviors, CCR7 and S1PR1 emerged as the ones most relating to the magnitude of the redistribution lymphocytosis, when considering their expression level along the 6-month follow-up period (Fig 6C). CCR7 expression appeared to relate inversely to lymphocytosis, in agreement with the function of CCR7 to instruct homing to and retention in LN. In opposite, S1PR1 expression appeared to relate positively to lymphocytosis, in agreement with the function of S1PR1 to instruct exit of CLL cells from the LN 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 (Fig 6D). This is reminiscent of previous data in murine T cells showing coordinated up-regulation of S1PR1 and down-regulation of CCR7, as a switch mechanism to favor egress over
retention\textsuperscript{30}. The S1PR1/CCR7 ratio was then calculated as a molecular marker of the egress / retention equilibrium in individual patients (Fig 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 at 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 to 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 individual 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 explores how ibrutinib affects CLL adhesion and 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. It finally 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\textsuperscript{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 to in vivo measurements\textsuperscript{23}, did not completely blunt migration nor adhesion in CLL cells, suggesting that alternative BTK-independent pathways can sustain these cellular activities. Directional migration was affected to a comparable level upon ibrutinib and acalabrutinib treatment, reinforcing the notion that BTK activity is particularly central to govern chemotaxis in CLL. The inhibition of chemokine-induced migration at different time points 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 the 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 $\text{CXCR4}^{\text{bright}}\text{CD5}^{\text{dim}}$ 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 however argues against the possibility that CXCR3 participates to 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 TME interactions$^{15,16}$, we failed to detect an effect of ibrutinib on the expression of this integrin. Instead, we measured a progressive down-regulation of the adhesion receptors LFA-1, LFA-3, CD11c and CD44, pointing to a wide 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 both depend on a methylation process related to the trisomy12 status$^{28,34}$. Further analysis indicated that samples with low integrin expression had high CD44. 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 TME, has been shown more recently to be particularly relevant for CLL progression$^{36}$. Our study further 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 that retained LFA-1 expression. This is in 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 about 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 scRNA-seq 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 identify that the S1PR1/CCR7 ratio reflecting the exit/entry rate of CLL cells is differently set in individual patients prior to treatment and that it is associated to 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 the 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 that is not impacted by treatment. This is probably related to mechanisms in place to co-regulate the expression of these 2 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
overcoming 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\textsuperscript{40, 42}.

We here also focused 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. Noticeably, 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\textsuperscript{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 scRNA-seq analysis\textsuperscript{20}.

In conclusion, our study provides novel insight in the mechanism of action of ibrutinib in the context of CLL treatment. Our study identifies a set of mechanisms that are reproducibly altered by ibrutinib both in 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.
References


## Table 1: characteristics of the ibrutinib-treated patients

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Y = YES; N= NO; UM= unmaturated; M= mutated; LFA1 and VLA4 expression cut-off: 2X FMO value.
**Figure legends**

**Figure 1. In vitro exposure to ibrutinib reduces random motility and chemokine-evoked directional motility in CLL cells.** (A) Control or ibrutinib treated PBMCs were seeded into Transwell inserts and exposed to CXCL12, CXCL13 or CCL19. Migrating CLL cells were counted after 6 h by flow cytometry. Data represent mean value from duplicate wells. N=32 patients, 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 PBMCs were seeded on fibronectin and leukemic cells were tracked by live imaging. Panels show 2 h tracks, color-coded using a 4 µm min⁻¹ threshold for mean speed; 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. Wilcoxon matched-pairs signed rank test was applied. (D) Control or ibrutinib treated PBMCs were seeded on fibronectin in the presence or absence of CCL19 and tracked to calculate individual CLL cell velocity. One way ANOVA with Bonferroni multiple comparison tests were applied. Results from a representative experiment out of 4 are shown. (E) Control or ibrutinib treated PBMCs 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 along the CCL19 gradient (Y axis) of cells tracked in E. Values for individual cells and mean are represented. Mann-Whitney test was applied to compare conditions. (G) Forward Migration Index along the CCL19 gradient (Y axis) of cells from 4 CLL patients (mean values). Two-ways ANOVA (mixed
model) and Bonferroni multiple comparison tests were applied and revealed statistically significant reduction of FMI upon ibrutinib for 3 out of 4 patients.

Figure 2. In vitro exposure to ibrutinib impairs immunological synapse assembly in CLL cells. (A) Flow cytometry analysis of the expression of LFA-1 and VLA-4 at the surface of CLL cells. Histograms represent Mean Fluorescence Intensity in B cells from one healthy donor and in CLL cells from 8 patients. Dashed line represents background signal (Fluorescence minus one). (B) Representative images of CLL cells stained for F-actin, phosphorylated 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 8 patients analysed in A are shown. Wilcoxon matched-pairs signed rank tests were applied to estimate the statistical significance of ibrutinib inhibition. (D) SEM 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.

Figure 3. In vitro exposure to ibrutinib reduces chemokine-evoked migration of non-neoplastic lymphocytes from CLL patients and healthy donors. (A) Control or ibrutinib treated PBMCs from 32 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 mean value from duplicate wells. Mean of patients is indicated in red. Wilcoxon matched-pairs signed rank test were applied to estimate the statistical significance of ibrutinib inhibition. (B) Control or ibrutinib treated PBMCs from 5 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 mean value from duplicate wells. Paired $t$-tests were applied.

**Figure 4. Ibrutinib treatment leads to progressive reduction of chemokine-evoked migration in CLL cells.** (A) PBMCs from 20 CLL patients were collected before and at 1, 2, 3 and 6 months after ibrutinib treatment initiation. The 5 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 mean value from duplicate wells. Mean of patients is indicated in red. Friedman test with Dunn's 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 mean value from duplicate wells. Mean of patients is indicated in red. Friedman test with Dunn's Multiple Comparison tests respect to pre-treatment values were applied.

**Figure 5. Ibrutinib treatment affects differentially the expression of chemokine receptors, adhesion molecules and coreceptors in CLL cells.** (A) PBMCs from 20 CLL patients were collected before and at 1, 2, 3 and 6 months after ibrutinib treatment initiation. The 5 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 of the indicated motility receptors. Data represent the longitudinal evolution of the expression of the indicated receptors as MFI on CLL leukemic cells for each patient (black dots and lines) and as a mean (red dots and lines). Friedman test with Dunn’s Multiple Comparison tests 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).

**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 CLL patients (black dots and lines). Mean values are shown in red. (B) Fold change ALC for the CLL patient cohort divided according to the indicated subgroup. (C) Level of expression (MFI) of CCR7 and S1P1 at the indicated time points 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 the indicated time points along ibrutinib treatment. Colors represent lymphocytosis subgroups, as indicated in (B). Two-ways 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.
Rey-Barroso et al. - Figure 3

A

Basal motility

% T cell migration

CCL19

***

CXCL12

***

CXCL13

***

IBRUTINIB

IBRUTINIB

IBRUTINIB

IBRUTINIB

B

Basal motility

% B cell migration

CCL19

**

CXCL12

**

cxcl13

***

ns

IBRUTINIB

IBRUTINIB

IBRUTINIB

IBRUTINIB

% T cell migration

NK cell migration

IBRUTINIB

IBRUTINIB

IBRUTINIB

IBRUTINIB

ns
Rey-Barroso et al. - Figure 4

A

CCL19

% CLL cell migration

% T cell migration

% T cell migration

0 1 2 3 6

0 1 2 3 6

Months

B

CXCL13

** ** *** ns

ns ** ns ns ns

ns ns ns ns ns

**

0 1 2 3 6

0 1 2 3 6

Months
Lymphocyte migration and retention properties affected by ibrutinib in Chronic Lymphocytic Leukemia

by Javier Rey-Barroso et al.

Supplementary material content

- Supplementary Methods
- Supplementary Reference
- Supplementary Table S1
- Supplementary Figures S1 to S10
Supplementary Methods

Chemokine-evoked migration upon in vitro exposure to ibrutinib
Frozen PBMCs samples from CLL patients or healthy donors were thawed in RPMI 1640 GlutaMax, supplemented with 10% Fetal Calf Serum medium (RPMI-10% FCS) and let rest for 1 h at 37°C. Then, 50,000 cells were seeded in the upper compartments of Transwell-96 well plates (Corning) with a pore size of 5 µm in the presence or absence of 500 nM ibrutinib. The lower chamber of the wells was filled with RPMI-10% FCS medium containing 1000 ng ml⁻¹ CCL19, 500 ng ml⁻¹ CXCL12 or 1000 ng ml⁻¹ CXCL13. Medium without chemokine was used as a control for basal migration. After 6 h of incubation, migrated cells present on the lower chamber of the wells were stained 30 min at 4°C with anti-CD3 and anti-CD19 antibodies, as well as with anti-CD56 antibodies in the case of healthy donors. Then, 100 µl was retrieved from the lower chambers and analyzed on a MACSQuant Q10 cytometer (Miltenyi) to extract total cell counts and proportions of CD3⁺, CD19⁺ and CD56⁺ subsets using the MACSQuantify Software (Miltenyi). Migration for each subset was calculated as percentage to positive control wells in which cells were seeded directly in the lower chamber.

Chemokine-evoked migration during the course of ibrutinib treatment
Frozen PBMCs samples from CLL patients from the CompuTreatCLL cohort before and after 1, 3 and 6 months of ibrutinib treatment were thawed in RPMI-10% FCS medium and let rest for 1 h at 37°C. Then, 50,000 cells were seeded in the upper compartments of Transwell-96 well plates (Corning) with a pore size of 5 µm. The lower chamber of the wells was filled with RPMI-10% FCS medium containing 1000 ng ml⁻¹ CCL19, 500 ng ml⁻¹ CXCL12 or 1000 ng ml⁻¹ CXCL13.
Medium without chemokine was used as a control for basal migration. After 6 h of incubation, migrated cells present on the lower chamber of the wells were stained 30 min at 4°C with anti-CD3 and anti-CD19 antibodies and analyzed using the procedure described above.

**Live recording of basal and chemokine-evoked cell motility**

PBMCs from CLL patients were cultured at $5 \times 10^6$ cells ml$^{-1}$ in RPMI-10% FCS supplemented with 5 μM CpG ODN 2006 (InvivoGen) and 100 IU ml$^{-1}$ human recombinant IL-2 (Peprotech) for 48 h. Cells were pre-incubated 30 min at 37°C with anti-CD3-PE antibody in order to distinguish T cells from CLL cells. Then, 100,000 cells resuspended in RPMI-10% FCS in the presence or absence of 500 nM ibrutinib were seeded in each channel of a µ-Slide VI 0.4 slide (Ibidi) coated with 5 μg ml$^{-1}$ fibronectin. After 3 h, cells were imaged for a 12-h period at 37 °C and 5% CO$_2$ using on a Zeiss apotome microscope, collecting red and bright field channels with a 10x/0.45 NA objective. T cell position was identified using red channel, then subtracted via image treatment on the bright field channel in order to extract CLL cell positions. Tracks were quantified using the image J plugin Trackmate for both cell types. Tracks of at least 20 min and a minimum displacement of 10 μm were selected for speed measurements. For chemokine evoked motility experiments, CCL19 was added to the medium at 500 ng ml$^{-1}$.

**Migration along CCL19 gradients**

PBMCs from CLL patients were cultured at $5 \times 10^6$ cells ml$^{-1}$ in RPMI-10% FCS supplemented with 5 μM CpG ODN 2006 (InvivoGen) and 100 IU ml$^{-1}$ human recombinant IL-2 (Peprotech) for 48 h. Cells were pre-incubated 30 min at 37°C with anti-CD3-PE antibody in order to distinguish T cells from CLL cells. Then, 50,000 cells resuspended in RPMI-10% FCS in the
presence or absence of 500 nM ibrutinib or acalabrutinib were loaded into the central transversal channel of ibitreat 3D chemotaxis µ-slides (Ibidi) coated with 5 μg ml⁻¹ fibronectin and incubated at 37 °C for 30 min to allow cell attachment. Then, 0–500 ng ml⁻¹ CCL19 gradients were set following the manufacturer’s instructions. Gradient linearity was verified with a 10% dextran-FITC solution. Cell migration was assessed by recording 1 image of red and bright field channels per min for 14 h with an Apotome microscope (Zeiss) and a 5X / 0.15 NA objective in temperature and CO₂ controlled conditions. As previously described, CLL and T cell tracks were isolated by image treatment and quantified using the image J plugin Trackmate. Tracks of at least 20 min and a minimum displacement of 10 μm were selected for speed measurements. Chemotaxis plots and migration parameters (FMI-Y and total speed) were obtained using the Chemotaxis and Migration tool from Ibidi.

**Morphological analysis of ibrutinib-exposed CLL cells by high-content cell imaging**

PBMCs from CLL patients were cultured at 5 × 10⁶ cells ml⁻¹ in RPMI-10% FCS supplemented with 5 μM CpG ODN 2006 (InvivoGen) and 100 IU ml⁻¹ human recombinant IL-2 (Peprotech) for 48 h. BTK phosphorylation and morphological descriptors were analyzed in parallel in CLL cells from multiple patients upon in vitro ibrutinib exposure using a recently developed high-content cell imaging platform adapted to the study of lymphoid cell populations. Briefly, 384-well PhenoPlates (PerkinElmer) were coated with either 0.1 mg ml⁻¹ poly-L-lysine, 2 μg ml⁻¹ recombinant human ICAM-1-Fc chimera (R&D Systems), 10 μg ml⁻¹ anti-BCR antibodies or a combination of ICAM-1 and anti-BCR antibodies. CLL patient cells were seeded at 10.000 cells per well (3 replicates per condition) and incubated for 15 min at 37°C in the presence or absence of 500 nM ibrutinib. Cells were fixed by adding a small volume of 16% paraformaldehyde (3%
Rey-Barroso et al. Motility alterations in ibrutinib-treated CLL

final concentration) 15 min at 37°C. They were then permeabilized in permeabilization buffer (eBioscience) and stained with AlexaFluor488-conjugated anti-CD19 antibodies, phalloidin-AlexaFluor555 (ThermoFisher Scientific), AlexaFluor647-conjugated anti-phospho-BTK antibodies (BD Biosciences) and DAPI. An anti-CD18 antibody (TS1/18) was used to assess whether the spreading upon ICAM-1/anti-BCR antibody stimulation was dependent on the engagement of LFA-1. The specificity of the phospho-BTK staining was verified by using an isotype antibody. Images were acquired on an automated spinning disk confocal device (Opera Phenix, PerkinElmer) equipped with a 40x 1.1 NA Plan Apochromat water immersion objective and sCMOS cameras (16 bits, 2160 × 2160 pixels, 6.5 μm pixel size). Image datasets were processed with Harmony software (PerkinElmer) to extract phospho-BTK intensity, cell area and F-actin texture from the CD19+ CLL cells at the confocal plane corresponding to the cell to substrate interface.

**Scanning electron microscopy imaging**

Following exposure to 500 nM ibrutinib for 30 min, were seeded for 30 min on coverslips coated with PLL or ICAM-1 + anti-BCR antibody, then fixed for 10 min in a 0.2 M sodium cacodylate buffer (pH 7.4) containing 2% paraformaldehyde (Electron Microscopy Sciences 15710) and 2.5% glutaraldehyde (Electron Microscopy Sciences 16220), then washed with distilled water. Samples were dehydrated through a graded series (25–100%) of ethanol, transferred in acetone and subjected to critical point drying with CO₂ in a Leica EM CPD300. Samples were then sputter-coated with 3 nm platinum with a Leica EM MED020 evaporator. They were examined and photographed with an FEI Quanta FEG250 electron microscope.
Flow cytometry bar coding for longitudinal analysis of surface receptors in leukemic cells and T cells

PBMCs from CLL patients, that had been collected and frozen along the course of ibrutinib treatment, were thawed and counted. For each of the 5 time points (0, 1, 2, 3 and 6 months after treatment initiation), samples were labeled with a specific concentration (0, 0.04, 0.2, 1 and 3 μM, respectively) of CellTrace™ Violet (Invitrogen) during 20 min at 37°C. Barcoded samples were washed, pooled and labeled with Fixable Viability Dye-eFluor780 (Affymetrix eBioscience) during 15 min at 4°C. The viability of the tested samples was between 90 and 97%. Pooled barcoded samples were stained 30 min at 4°C for extracellular markers with antibodies listed in Suppl Table S1. Samples were subsequently fixed with 2% paraformaldehyde and stained 30 min at 4°C for intracellular markers with antibodies listed in Suppl Table S1. Data were acquired on a BD FACSCalibur cytometer and analyzed using Flowjo software, by separating the different longitudinal samples on the basis of the CTV staining.

Reference

Table S1. Antibodies used for flow cytometry bar coding analysis

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*Intracellular staining
Supplementary Figure S1. Acalabrutinib impairs the directional migration of CLL cells along CCL19 gradients. (A) Control or acalabrutinib treated PBMCs were seeded on fibronectin, exposed to a CCL19 chemokine gradient and imaged. Panels show tracks of control and ibrutinib treated CLL cells. (B) Forward Migration Index along the CCL19 gradient (Y axis) of cells tracked in A. Values for individual cells and mean are represented. Mann-Whitney test was applied. (C) Forward Migration Index along the CCL19 gradient (Y axis) of cells from 4 CLL patients (mean values). Two-ways ANOVA (mixed model) and Bonferroni multiple test were applied and shown statistically significant differences for 4 out of 4 patients.
Supplementary Figure S2. Contribution of the ICAM-1-LFA-1 axis to CLL immunological synapse assembly. (A) 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 8 patients as in Figure 2. Wilcoxon matched-pairs signed rank tests were applied to estimate the statistical significance of anti-LFA-1 (TS1/18) antibody inhibition.
Supplementary Figure S3. Ibrutinib abrogates BCR-evoked BTK phosphorylation in CLL patients. (A) Representative confocal images of unexposed and ibrutinib-exposed treated PBMCs, stimulated with coated anti-BCR Ab and stained for F-actin, phosphorylated BTK and DAPI. (B) Confocal images were exploited to extract phosphorylated BTK intensity values in cells treated and stimulated in the indicated conditions. One way ANOVA with Bonferroni Multiple Comparison tests were applied (C) Parallel analysis of phosphorylated BTK intensity by flow cytometry.
Supplementary Figure S4. In vitro exposure to ibrutinib impairs immunological synapse assembly.
(A) Representative images of CLL cells stained for F-actin, phosphorylated BTK and DAPI, and imaged on an automated confocal microscope. (B) 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 13 patients are shown. Wilcoxon matched-pairs signed rank tests were applied to estimate the statistical significance of ibrutinib inhibition.
Supplementary Figure S5. Ibrutinib reduces the velocity of T cells from CLL patients over fibronectin. (A) Control or ibrutinib treated PBMCs were seeded on fibronectin and T cells were tracked by live imaging. Panels show 2 h tracks, color-coded using a $8 \mu m/min$ threshold for mean speed; bar on the right summarizes the proportion of cells over (orange) and under (black) the threshold. (B) Comparison of mean basal velocity between control and ibrutinib treated samples. N=10 patients. Wilcoxon matched-pairs signed rank tests was applied.
Supplementary Figure S6. Flow cytometry bar-coding strategy for the longitudinal assessment of CLL and T cell receptor expression. (A) Gating strategy used to analyse pooled CLL PBMC samples after prestaining with different Pacific Blue dye dilutions. (B) Histogram representation of the pooled samples (M0: pre-treatment, M1-6: months post treatment initiation). (C) Expression along the course of ibrutinib treatment of the indicated motility receptors in gated CLL leukemic cells from one representative patient.
Supplementary Figure S7. Kinetics of surface receptor expression as a function of LFA-1 expression status in CLL patients treated with ibrutinib. (A) Longitudinal evolution of the expression of the indicated receptors as MFI on CLL leukemic cells of patients with residual LFA-1 expression (black lines) or negative LFA-1 expression (blue lines) and as a mean of all patients (red lines). (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). Two-ways ANOVA (mixed model) and Bonferroni multiple test were applied, not showing statistically significant differences between LFA-1 subgroups.
Supplementary Figure S8. Kinetics of surface receptor expression as a function of VLA-4 expression status in CLL patients treated with ibrutinib. (A) Longitudinal evolution of the expression of the indicated receptors as MFI on CLL leukemic cells of patients with residual VLA-4 expression (black lines) or negative VLA-4 expression (blue lines) and as a mean of all patients (red lines). (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). Two-ways ANOVA (mixed model) and Bonferroni multiple tests were applied, showing statistically significant differences in CD44 expression at months 2, 3 and 6 of treatment between VLA-4 subgroups.
Supplementary Figure S9. Ibrutinib treatment affects differentially the expression of chemokine receptors, adhesion molecules and activation markers in CD4+ T cells. (A) PBMCs from 20 CLL patients were collected before and at 1, 2, 3 and 6 months after ibrutinib treatment initiation. The 5 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 of the indicated motility receptors. Data represent the longitudinal evolution of the expression of the indicated receptors on CD4+ T cells as MFI for each patient (black dots and lines) and as a mean (red dots and lines). Friedman test with Dunn's Multiple Comparison tests were applied to estimate the statistical significance of the impact of ibrutinib treatment as compared to the pre-treatment values. (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).
Supplementary Figure S10. Ibrutinib treatment affects differentially the expression of chemokine receptors, adhesion molecules and activation markers in CD8+ T cells. (A) PBMCs from 20 CLL patients were collected before and at 1, 2, 3 and 6 months after ibrutinib treatment initiation. The 5 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 of the indicated motility receptors. Data represent the longitudinal evolution of the expression of the indicated receptors on CD8+ T cells as MFI for each patient (black dots and lines) and as a mean (red dots and lines). Friedman test with Dunn's Multiple Comparison tests were applied to estimate the statistical significance of the impact of ibrutinib treatment as compared to the pre-treatment values. (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). (D) The indicated cytotoxicity-related molecules were studied with the same approach as in (A).