

ORIGINAL ARTICLE

BTK inhibition results in impaired CXCR4 chemokine receptor surface expression, signaling and function in chronic lymphocytic leukemia

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Bruton's tyrosine kinase (BTK) is involved in the regulation of B-cell growth, migration and adhesion. The importance of BTK in cell trafficking is emphasized by the clonal contraction proceeded by lymphocytosis typical for the enzyme inhibitor, ibrutinib, in B-cell malignancies, including chronic lymphocytic leukemia (CLL). Here, we investigated BTK regulation of leukemic B-cell trafficking in a mouse model of aggressive TCL1 CLL-like disease. Inhibiting BTK by ibrutinib reduced surface membrane (sm) levels of CXCR4 but not CXCR5, CD49d and other adhesion/homing receptors. Decreased smCXCR4 levels resulted from blocking receptor signal transduction, which in turn aborted cycling from and to the membrane. This resulted in rapid re-distribution of CLL cells from spleens and lymph nodes into the circulation. CLL cells with impaired smCXCR4 from BTK inhibition failed to home to spleens. These functional changes mainly resulted from inhibition of CXCR4 phosphorylation at Ser339, mediated directly by blocking BTK enzymatic activity and indirectly by affecting the function of downstream targets PLC γ 2 and PKC ζ , and eventually synthesis of PIM-1 and BTK itself. Our data identify CXCR4 as a key regulator in BTK-mediated CLL-cell retention and have elucidated a complex set of not previously described mechanisms responsible for these effects.

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INTRODUCTION

Bruton's tyrosine kinase (BTK) is a key player in B-cell antigen receptor (BCR) signaling that regulates B-cell growth. In addition to BCR signaling, BTK participates in signal transduction through growth-factor receptors, Toll-like receptors, integrins and G-protein-coupled receptors such as CXCR4 and CXCR5.^{1,2} Among these, chemokine receptors and integrins modulate migration and adhesion of B cells to a microenvironment that promotes cell survival and proliferation.^{3,4}

Growing evidence supports a potential role for BTK in the trafficking of leukemic B cells as well. In chronic lymphocytic leukemia (CLL), CXCR4 (ref. 5) and the α 4 β 1 integrin VLA-4 (CD49d/CD29)⁶ are markers of disease course and outcome. Importantly, when the action of BTK is blocked, chemotaxis and adhesion of CLL cells, induced by CXCL12, CXCL13 and VCAM-1, are markedly reduced,⁷ as in normal B⁸ and pre-B⁹ cells. Furthermore, BTK inhibition by ibrutinib induces rapid lymph node (LN) and spleen shrinkage, believed to be due to impaired adhesion; this is associated initially with lymphocytosis and ultimately with lowered levels of leukemia cells in the blood of patients with CLL and other B-cell malignancies.^{10,11} These actions emphasize a key role for BTK in CLL-cell trafficking and survival.

In normal B cells, BCR stimulation promotes CXCR4 internalization through Syk, BTK, PLC γ 2 and PKC.¹² PIM-1, ERK/MAPK cascade activation^{3,13} and G-protein-coupled receptor kinases¹⁴ also regulate CXCR4 receptor trafficking and signaling. In addition, BTK might also associate directly with CXCR4 by interacting with the heterotrimeric G protein subunits G α ¹⁵ and G β γ .¹⁶ All these

suggest direct or indirect regulation of CXCR4 expression and function by BTK. Despite this information for normal B lymphocytes, evidence documenting the regulation of CLL B-cell trafficking and adhesion by BTK, and the breadth of mechanisms responsible for these actions, has not been codified *in vivo*. This might be especially important in CLL because the levels of chemokine receptors and integrins on CLL cells are heightened.^{7,17} Indeed, CLL cells have three- to fourfold higher levels of smCXCR4 compared with normal B lymphocytes.⁴

This *in vivo* study strove to document the effects of BTK inhibition and elucidate the mechanisms whereby BTK regulates B-cell migration and homing/retention in lymphoid tissues. To do this, we used ibrutinib, a BTK inhibitor with clinical benefit in CLL patients and a mouse model in which SCID mice are populated with murine leukemia cells (TCL1-192) from a TCL-1-bearing mouse.¹⁸ TCL1-192 leukemic cells express an unmutated, clonal VH11/V κ 14 BCR and are responsive to BCR crosslinking by endogenous phosphatidylcholine, hence requiring BTK's enzymatic activity. Adoptive transfer of these leukemic cells into SCID animals leads to aggressive disease similar to that observed in IGHV-unmutated CLL patients.¹⁹ Importantly, the cellular responses to ibrutinib treatment in this mouse model are very similar to CLL patients, including reduced tumor burden in spleens and LNs and transient lymphocytosis that has not been demonstrated in other animal models.¹⁷

We now show that ibrutinib treatment rapidly induces continuous egress of CLL cells into the circulation and prevents the return of cells to solid tissue sites, and that this is due not only to impaired BCR signaling but also deregulated smCXCR4

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signaling and expression. Ibrutinib caused these changes in CXCR4 recycling (internalization and re-expression) by preventing phosphorylation at Ser339 and indirectly by altering the function of downstream kinases PLC γ 2, PKC μ and PIM-1 and eventually the synthesis of BTK and PIM-1. Our results demonstrate for the first time a complex set of mechanisms responsible for ibrutinib's actions that contribute beneficially for patients.

MATERIALS AND METHODS

Study design

Animal studies were performed in accordance with experimental protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Feinstein Institute for Medical Research. TCL1-192 cells that had been transferred five times into 8-week-old female C.B-17 SCID (C.B-*Igh-1⁰/IcrTac-Prkd^{cscid}*) mice (Taconic Labs, Hudson, NY, USA) were used for all experiments in this study. For short-term experiments, mice received 5×10^6 TCL1-192 cells by retro-orbital injection followed by ibrutinib (25 mg/kg) or vehicle (1% HP beta-cyclodextrin) by oral gavage starting 2 weeks later, when CLL cells were detectable in the circulation. This experiment was carried out with 5 mice per treatment group per time point, and was repeated for three different time points, 1, 4 and 24 h, to ensure adequately powered sample sizes. For long-term treatment and survival assays, mice received treatment daily in drinking water starting at 2 or 4 weeks post tumor cell engraftment. Long-term treatment experiments were repeated three times with 10 animals per treatment group per time point; all the long-term treated animals were killed 6 weeks post tumor cell engraftment, the time when control mice appeared moribund. For survival experiments, there was a total of 20 mice per treatment group. Four treatment groups were followed for survival until mice appeared moribund or died. Sample sizes were estimated based on our previous report that showed a significant delay in disease progression with ibrutinib treatment using this same transfer model.¹⁷ Mice used for control and treatment groups were randomly selected from a pool of recipient animals with no pre-established inclusion/exclusion criteria. Investigators were not blinded.

Flow cytometric analyses and 5-bromodeoxyuridine (BrdU) incorporation assay

TCL1-192 cells were enumerated by incubation with fluorescein isothiocyanate anti-CD45R/B220 (553087), Phycoerythrin anti-CD5 (553023), APC-CXCR4 (558644), PerCPy5.5-CXCR5 (560528), Phycoerythrin-CD49d (553157; BD Biosciences, San Jose, CA, USA), PECy7 anti-CD39 (25-0391), eFluor 450 anti-CD69 (48-0691), A700 anti-CD62L (56-0621), PECy7 anti-CD5 (25-0051; eBioscience, San Diego, CA, USA), APC anti-S1PR1 (R&D, Minneapolis, MN, USA, FAB7089A) and PECy7 anti-CD29 (BioLegend, San Diego, CA, USA, 102222). BrdU incorporation assays were done following the manufacturer's protocol (BD Biosciences). Flow cytometry data were obtained with a BD LSRII machine (BD Biosciences), and analyzed with FlowJo software (FlowJo, LLC, Ashland, OR, USA).

BTK occupancy and western blot analysis

BTK occupancy in splenocytes was measured as previously described.²⁰ Protein expression was determined using standardized protocols with monoclonal antibodies reactive with the following proteins: BTK (clone 3533; Cell Signaling, Danvers, MA, USA; 1:1000), CXCR4 (clone 2074; Abcam, Cambridge, UK; 1:500), phospho-CXCR4 (clone 74012, Abcam; 1:500), PIM-1 (clone EP2645Y; Abcam; 1:10 000), GRK6 (clone D1A4, Cell Signaling; 1:1000), TCL1 (clone 4042; Cell Signaling; 1:1000) and β -actin (clone 8227; Abcam; 1:5000). Phosphorylation states of multiple PKC isoforms were tested by the phospho-PKC Antibody Sample Kit (Cell Signaling, catalog number 9921). Fluorescently stained protein bands were quantified using Molecular Dynamics ImageQuant 5.2 software (GE Healthcare, Waukesha, WI, USA).

Ca²⁺ flux analysis

TCL1-192 cells (5×10^6 cells/ml) were incubated in dye-free RPMI1640 medium supplemented with 5% FBS and 1 μ M fluorescent Ca²⁺ indicator Indo-1, acetoxymethyl ester (Invitrogen). 1×10^6 cells per tube were run for 30 s on LSRII to obtain a baseline reading, and then stimulated for 5 min by 5 μ g/ml F(ab')₂ fragments of anti-IgM polyclonal antibodies or 200 ng/ml CXCL12. Kinetic data were analyzed with FlowJo (BD Biosciences). The intracellular Ca²⁺ levels were calculated from the violet (440 nm) and green (530 nm) emissions and plotted against the time parameter.

CXCR4 recycling assay

For *ex vivo* assays, TCL1-192 cells (1×10^6 cells/ml), collected from mice previously treated with vehicle or ibrutinib, were seeded with or without 200 ng/ml CXCL12 for 2 h at 37 °C. After three washing steps with cold PBS to remove CXCL12, cells were resuspended in medium and incubated at 37 °C for 40 min. For *in vitro* assays, TCL1-192 cells were resuspended in medium containing 0.05% dimethylsulphoxide or ibrutinib at indicated concentrations with CXCL12 stimulation as described.²¹ After CXCL12 removal, dimethylsulphoxide or ibrutinib at the same concentration was added back in suspension medium to study their effects on CXCR4 re-expression.

In vivo cell trafficking assays

5×10^6 splenocytes collected from mice treated for 4 weeks were adoptively transferred into another set of SCID mice pre-treated with either vehicle or ibrutinib for 5 days. After transfer, mice continued to receive the same treatments for 24 or 72 h. To analyze trafficking of CXCR4^{dim}CD5^{br} leukemic cells, 5×10^6 sorted CXCR4^{dim}CD5^{br} TCL1-192 cells collected from animals treated for 4 weeks were injected into new recipients. Mice were killed 18 h later.

Statistics

Data represent means \pm s.d. or s.e.m., as stated in figure legends. Statistical significance was calculated using Prism software version 6.0 (GraphPad, La Jolla, CA, USA) from adequately powered sample sizes for two-tailed tests using two-way ANOVA, unpaired Student's *t*-test with Bonferroni correction for multiple comparisons, nonparametric Mann-Whitney test, or multiple *t*-test using the Holm-Sidak method, when applicable. Survival curves were analyzed by log-rank test. Correlations were assessed with the nonparametric Spearman correlation. Statistical significance was defined as $P < 0.05$. All exact P-values are provided in the figure legends.

RESULTS

Functional BTK is needed to retain CLL cells in lymphoid tissues

We studied BTK's involvement in CLL-cell retention in tissue niches by treating mice with ibrutinib 2 weeks after TCL1-192 leukemia cell injection, the time when CD19⁺CD5⁺ leukemic cells were detectable in the spleen and blood.¹⁹ Because oral administration of ibrutinib induces lymphocytosis within hours in CLL patients, in the first set of experiments we treated recipient mice once via gavage and killed them 1, 4 and 24 h later. Although control mice had negligible numbers of leukemic cells in the circulation, ibrutinib-treated animals exhibited a significant increase in circulating lymphocytes. This lymphocytosis occurred as early as 1 h and peaked at 4 h after the mice received ibrutinib (Figure 1a), indicating rapid uptake and action of the drug. In support of this, within 1 h of oral treatment, all BTK molecules in spleen-residing leukemia cells were occupied by ibrutinib (Supplementary Figure 1A).

We then analyzed the composition of blood and spleen cell populations in untreated and treated mice, analyzing the release of resting and recently divided leukemic cells identified by injecting BrdU at the time of treatment. After 1 h of BTK enzymatic inhibition, significantly higher numbers of BrdU⁺ TCL1-192 cells were observed in circulation (Supplementary Figure 1B). Twenty-four hours after, animals receiving ibrutinib still had significantly more BrdU⁺ leukemic cells in the blood (vehicle vs ibrutinib: 3.4–3.7% vs 18.5–22.4%; Figure 1b). Of note, the percentage of BrdU⁺ B cells in spleens very closely approximated the numbers in the blood, suggesting ibrutinib promotes emigration of leukemic cells into circulation proportional to that in tissues.

We next studied BTK-regulated CLL-cell retention in animals treated for 4 weeks. At this time point (6 weeks post tumor cell engraftment), most vehicle-treated mice were moribund; however, mice receiving ibrutinib appeared healthy with significantly lower absolute blood CLL-cell counts (Supplementary Figure 2A), much less organomegaly, and reduced leukemic cell infiltration in the spleens, liver and bone marrow (Supplementary Figure 2B). We again measured proliferating CLL cells by injecting BrdU 24 h before the end point of the study.

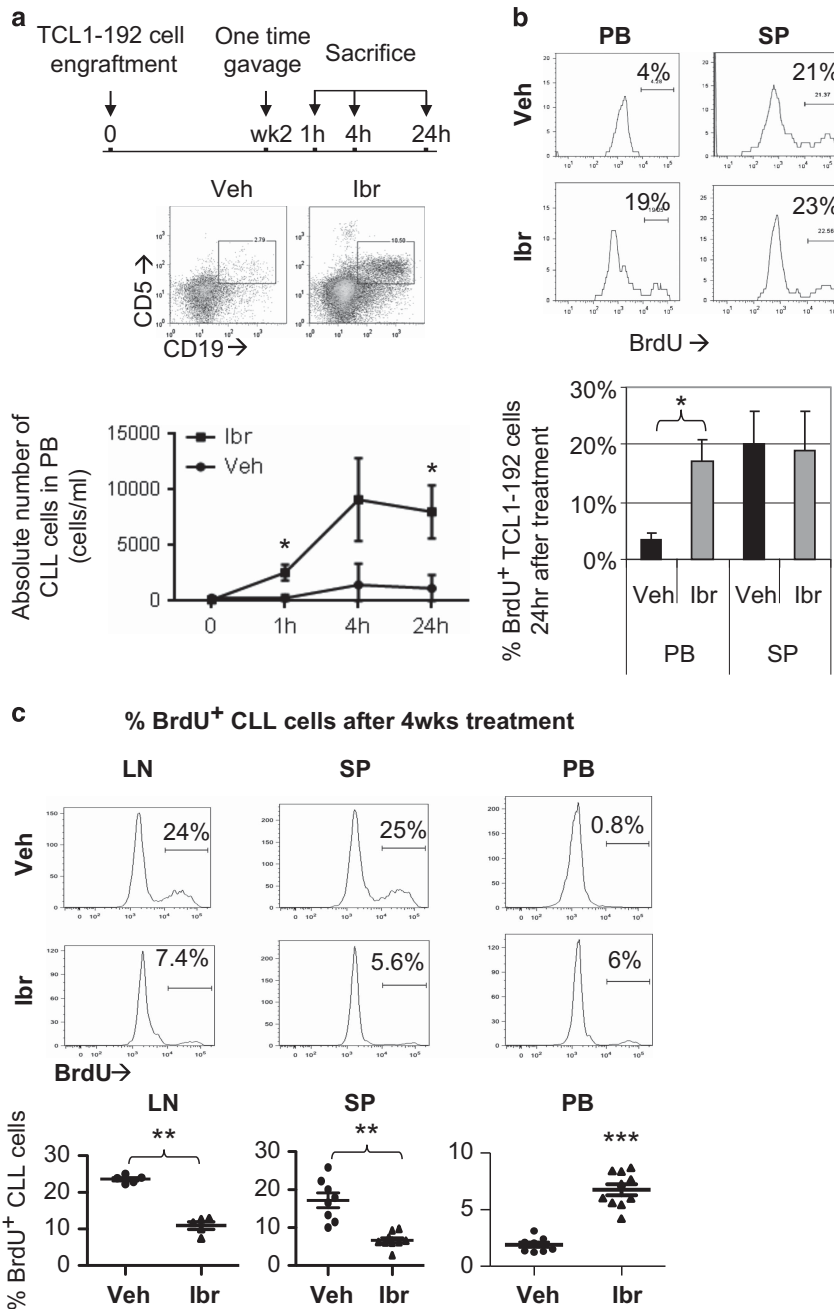


Figure 1. Mobilization of lymphocytes into the peripheral circulation after BTK inhibition. **(a)** Significant increases in TCL1-192 cell counts in PB were observed in ibrutinib (Ibr)-treated animals 1 and 24 h after treatment via oral gavage ($*P < 0.05$ by multiple *t*-test using the Holm–Sidak method, $n = 5$ for each time point). The representative data shown were obtained from peripheral blood samples collected 4 h after the treatment. **(b)** Increased numbers of BrdU⁺ leukemia cells were seen in PB 24 h ($*P = 0.016$, $n = 5$) after Ibr treatment. **(c)** TCL1-192 cell proliferation as detected by BrdU incorporation in LN ($n = 5$) and SP ($n = 8$) was significantly reduced after Ibr treatment for 4 weeks ($**P < 0.01$); however, percentage of BrdU⁺ B cells in PB was increased ($***P < 0.001$, $n = 10$). *P*-values shown in **b** and **c** were calculated using the Mann–Whitney nonparametric test; ‘NS’ indicates non-significant *P*-values. All data plots represent the mean \pm s.d. Representative examples are shown on the top of each panel. PB, peripheral blood; SP, spleen; Veh, vehicle; wk, week.

Interestingly, although BTK inhibition abrogated the proliferation of CLL cells in spleens and LNs, ibrutinib-treated animals still displayed BrdU-labeled cells in the blood, despite their much lower levels of circulating leukemia cells (Figure 1c). Notably, the percentage of BrdU-labeled cells in the blood ($6.7\% \pm 0.05$) was again higher than in control mice ($\sim 2\%$), while similar to those in spleens ($5.9\% \pm 0.08$).

Thus, inhibition of BTK’s enzymatic activity rapidly and continuously led to re-distribution of cells from tissues into the

circulation, highlighting the involvement of BTK in tissue retention. This suppressed CLL-cell retention may minimize the chance of malignant B cell to receive growth signaling in spleens and LNs.

BTK influences smCXCR4 expression and signaling

We further dissected BTK-dependent mechanisms regulating retention of CLL cells by studying the effect of ibrutinib treatment

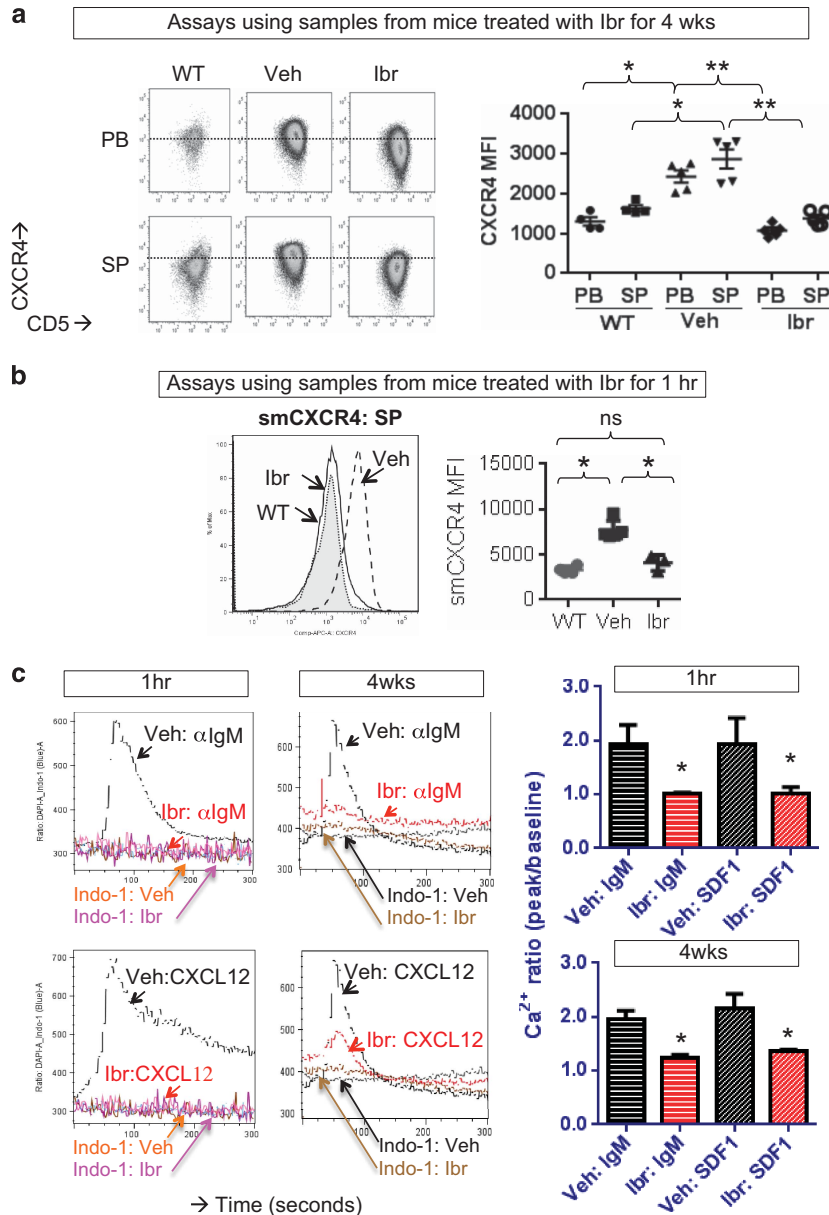


Figure 2. Rapidly reduced smCXCR4 levels after BTK inhibition. **(a)** Reduced smCXCR4 mean fluorescence intensity (MFI) on leukemic cells 4 weeks (wks) after ibrutinib (Ibr) treatment reached levels similar to wild-type (WT) normal B cells ($*P < 0.05$, $**P < 0.01$, $n = 5$). Right panels shown are representative flow cytometry data. **(b)** SP cells collected 1h after Ibr treatment had significantly reduced smCXCR4 MFI compared with those from vehicle (Veh) group ($*P = 0.03$, $n = 5$); no difference was seen between Ibr-treated cells versus WT normal B cells ($P = 0.23$, $n = 5$). **(c)** Ca^{++} flux results shown on the left are representative data from three experiments performed in triplicate. Normalized ratios of peak to baseline Ca^{++} flux in response to CXCL12 and anti-IgM pAbs are displayed on the right. These indicate significantly reduced Ca^{++} mobilization in SP-residing TCL1-192 cells after 1 h or 4 wks of Ibr treatment ($*P = 0.028$). All data plots represent the mean \pm s.d. P -values shown were calculated by Mann-Whitney test.

on specific molecular components known to control this process. Among all the trafficking/migration and adhesion molecules assessed (Figure 2a and Supplementary Figure 3), including CXCR4, CXCR5 and CD49d that have BTK in their signaling cascade, as well as CD29, CD62L, CD39, CD69 and S1PR1, only smCXCR4 was significantly reduced levels on leukemic cells after 4 weeks of ibrutinib treatment; this was seen in both blood and spleen samples (Figure 2a). Overexpressed smCXCR4 in CLL⁴ was significantly reduced in spleens within 1 h (Figure 2b). However, notably, the total amount of CXCR4 protein remained unchanged after ibrutinib treatment (Supplementary Figure 3), suggesting the effect of BTK might be on receptor trafficking within the cell.

We next determined if ibrutinib treatment impaired CXCR4 signal transduction potential of leukemia cells, rendering them functionally inert. Splenocytes from mice were exposed for 5 min to activate CXCR4 by native ligands (CXCL12), or the BCR by surrogate ligands (anti-IgM pAbs). For short- (1 h) or long- (4 weeks) term ibrutinib treatment *in vivo* blocked both BCR and CXCR4 signaling pathways, as measured by Ca^{++} flux (Figure 2c).

Collectively, these results indicate that smCXCR4 expression and signaling are impaired in BTK-treated cells, indicating the dual role of BTK in CXCR4 and BCR signaling, which together that contribute to the support of leukemic cell growth in LNs and spleens (Figure 1c).

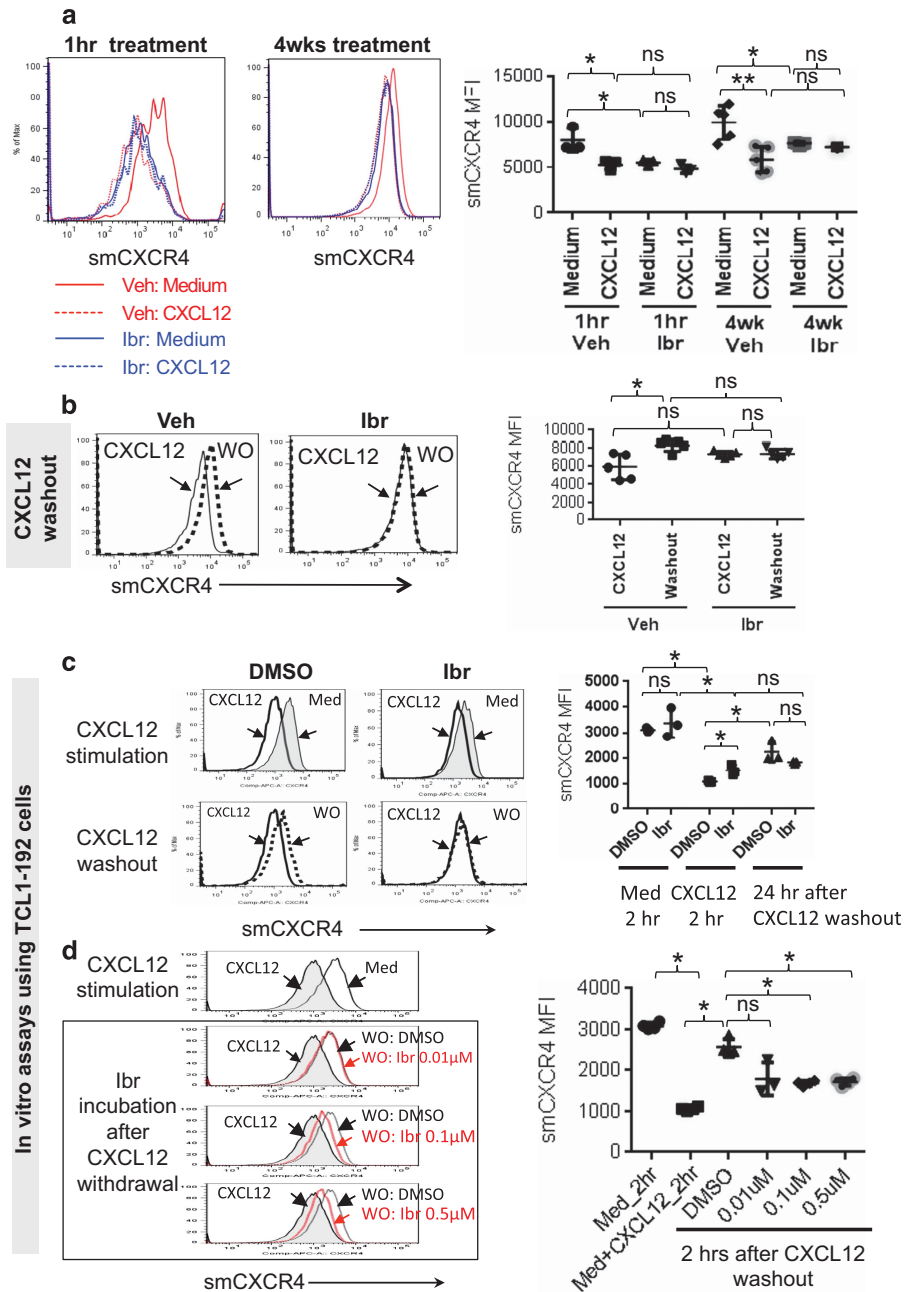


Figure 3. BTK-inhibited CLL cells do not internalize and re-express smCXCR4 after CXCL12 exposure. All data plots represent the mean \pm s.d. (a) TCL1-192 cells from SP of mice treated for 1 h ($n=3$) or 4 weeks (wks; $n=5$) were seeded in culture medium (Med: RPMI1640+10% FBS) with or without CXCL12. After 2 h incubation at 37 °C, cells from vehicle (Veh) but not ibrutinib (lbr) groups had significantly reduced smCXCR4 (** $P < 0.01$). (b) Re-expression of smCXCR4 after CXCL12 removal was only seen in splenocytes collected from Veh mice ($*P=0.03$), but not from mice treated with lbr for 4 wks ($n=5$). (c) Significantly higher smCXCR4 levels remained on 0.1 μ M lbr-treated cells than those on dimethylsulphoxide (DMSO)-treated cells ($*P < 0.05$, $n=4$). lbr-treated cells also failed to re-express smCXCR4 after CXCL12 removal. (d) TCL1-192 cells were exposed *in vitro* to DMSO or lbr after CXCL12 washout ($n=4$). lbr treatment at $\geq 0.1 \mu$ M significantly reduced smCXCR4 re-expression ($*P < 0.05$). Left panels in c, d are representative histograms of smCXCR4 of two independent experiments performed in triplicate. P -values shown were calculated by unpaired t -test using nonparametric Mann–Whitney test. 'NS' indicates non-significant P -values. WO, wash out.

BTK regulates CXCR4 internalization and re-expression

Reduction of smCXCR4 levels without obvious changes in total CXCR4 in BTK-inhibited cells suggested that CXCR4 recycling (internalization and re-expression) had been altered and not that enhanced CXCR4 degradation had occurred. Indeed, *in vivo* ibrutinib treatment for 1 h and for 4 weeks completely prevented smCXCR4 internalization stimulated by CXCL12 (Figure 3a). In addition, reappearance of smCXCR4 after CXCL12 removal,

which occurred normally in the absence of ibrutinib, did not take place in ibrutinib-treated cells (Figure 3b).

We next tested effects of ibrutinib on CXCR4 re-cycling *in vitro*. TCL1-192 cells were first exposed to 0.1 μ M ibrutinib for 2 h at 37 °C in the absence or presence of CXCL12. Again, after ligand exposure, internalized smCXCR4 levels were significantly reduced by ibrutinib (Figure 3c). In addition, whereas CXCL12 removal resulted in significant re-expression of smCXCR4 on control cells,

the level of CXCR4 re-expression on ibrutinib-treated cells did not change significantly after ligand withdrawal (Figure 3c). Finally, exposing TCL1-192 cells to ibrutinib ($\geq 0.1 \mu\text{M}$) after CXCL12 withdrawal, significantly reduced the re-expression levels of smCXCR4 (Figure 3d).

Overall, the above data suggest BTK regulates smCXCR4 expression by modulating recycling of the receptor. In addition, some receptors remain on the surface membrane (sm) because of impeded internalization because of blocked ligand-mediated signaling.

Mechanisms responsible for the regulation of CXCR4 recycling by BTK

In CLL patient B cells, overexpression of smCXCR4 correlates with hyper-phosphorylated Ser339 residues.^{21,22} Similar results were also observed in TCL1-192 cells (Figure 4a). BTK inhibition blocked CXCR4 phosphorylation, but not total CXCR4, in spleen-residing

leukemia cells from animals treated for 4 weeks; levels of Ser339-phosphorylated CXCR4 were considerably diminished, approximating levels observed in cells from wild-type mice. Notably, this reduction correlated tightly with smCXCR4 expression (Figure 4a).

We next tested if BTK enzymatic activity/expression correlates with smCXCR4 levels. To do this, we used the same dose of ibrutinib to treat a group of mice for only 2 weeks starting at 4 weeks post TCL1-192 cell transfer. This treatment approach delayed CLL progression but survival was shorter than the group of mice treated for a total 4 weeks starting at week 2 (Supplementary Figure 4). Interestingly, analyzing leukemia cells from mice treated with ibrutinib for either 2 or 4 weeks showed a progressive fall in not only BTK enzymatic activity but also BTK protein levels, which correlated directly with reduced smCXCR4 expression (Figure 4b and Supplementary Figure 5A).

Finally, we examined the levels and phosphorylation states of kinases known to be responsible for CXCR4 phosphorylation,

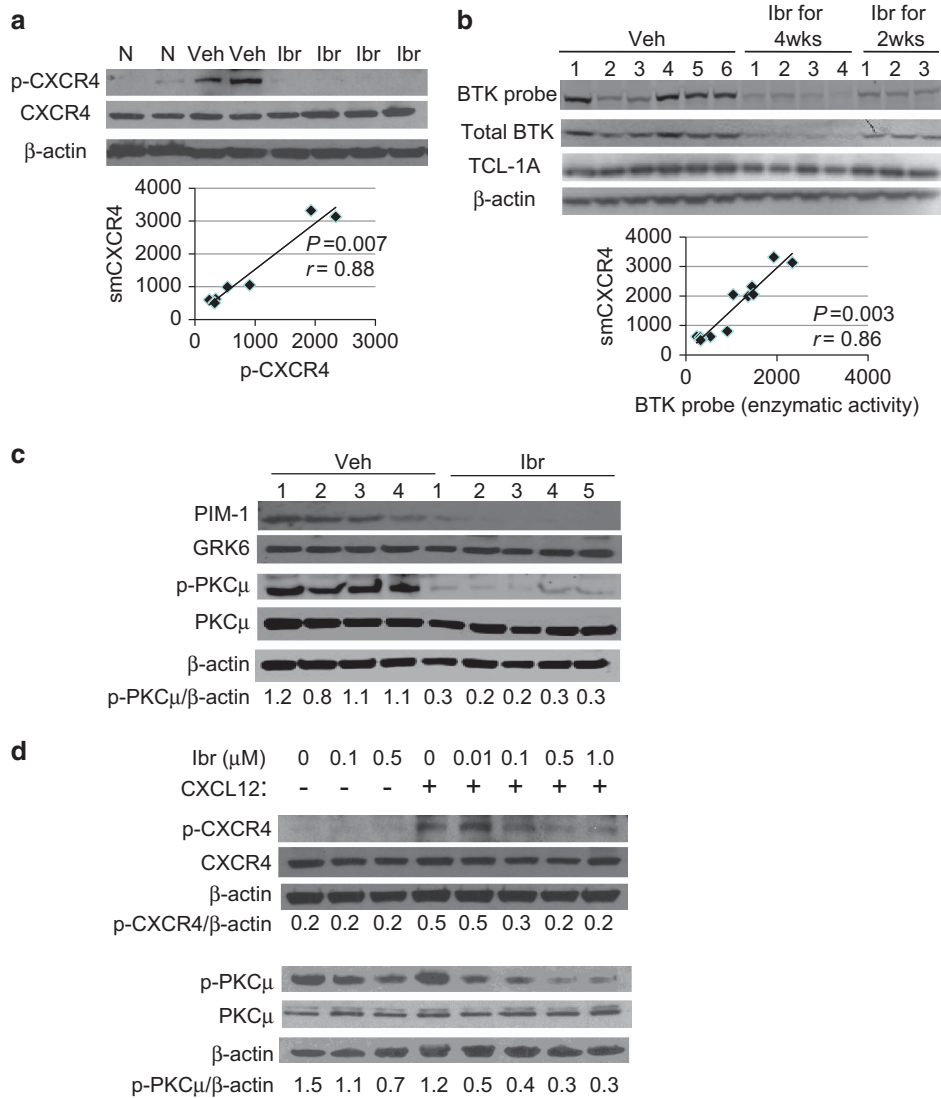


Figure 4. BTK inhibition blocks CXCL12-induced CXCR4 phosphorylation at Ser339. **(a)** *In vivo* ibrutinib (lbr) treatment for 4 weeks (wks) reduced levels of pCXCR4 but not total CXCR4 in TCL1-192 SP cells. N, normal SP B cells. **(b)** lbr treatment for 4 or 2 wks reduced total BTK levels in TCL1-192 SP cells. **(a, b)** Spearman correlation coefficient of smCXCR4 expression (measured as mean fluorescence intensity) versus relative pCXCR4 protein levels ($r=0.88$, $P=0.007$) and BTK enzymatic activity assessed by BTK probe assay ($r=0.86$, $P=0.003$). **(c)** BTK inhibition reduced levels of PIM-1 and p-PKC μ but not GRK6. **(d)** *In vitro* lbr treatment at dosages $\geq 0.1 \mu\text{M}$ for 2 h inhibited CXCL12-induced CXCR4 phosphorylation (pCXCR4), but not total CXCR4. Phosphorylation of PKC μ (p-PKC μ) but not total PKC μ was inhibited in TCL1-192 cells after 2 h of *in vitro* lbr treatment at doses $> 0.1 \mu\text{M}$. The quantified ratios of pCXCR4 and p-PKC μ are shown below the bands in **c** and **d**. Veh, vehicle.

including GRK6, PIM-1 and PKC; the latter is an upstream regulator of PIM-1 (ref. 23) and a downstream target of BCR signaling.²⁴ BTK inhibition significantly reduced PIM-1, but not GRK6 protein levels (Figure 4c). When testing the phosphorylation state of PKC isoforms in splenocytes collected after 4 weeks of treatment, we found BTK inhibition diminished levels of phosphorylated PKC μ (p-PKC μ) that is constitutively associated with BTK²⁵ and is regulated by BCR/PLC γ 2 signaling.²⁶ Total levels of neither PKC μ nor any other PKC isoforms tested were diminished by ibrutinib treatment (Figure 4c and Supplementary Figure 5C).

Finally, we repeated these analyses on TCL1-192 cells treated with ibrutinib *in vitro*. Ibrutinib treatment ($\geq 0.1 \mu\text{M}$) for only 2 h reduced CXCL12-stimulated CXCR4 phosphorylation at Ser339 (Figure 4d). A reduction in p-PKC μ , but not other isoforms, was also observed with ibrutinib exposure *in vitro*. Total protein levels of CXCR4, PIM-1 and BTK remained unchanged in these short-term ibrutinib-treated samples (Figure 4d and Supplementary Figure 5C).

Collectively, these data indicate that BTK regulates smCXCR4 signaling and recycling by allowing ligand-receptor interactions to result in phosphorylation of CXCR4 at Ser339 of its intracellular domain. This in turn induces phosphorylation and activation of PLC γ 2 (as reported in our previous study¹⁷), PIM-1 and PKC μ , but

not GRK6. The data also indicate that BTK is involved in a feed-forward action that supports its own synthesis and that of PIM-1; the latter is likely occurring because of the need for BTK to allow BCR-mediated signaling, which is needed to foster production of PIM-1 and BTK itself.

BTK regulates smCXCR4 (re)-expression and function in CLL B lymphocytes

We next tested the effects of BTK on smCXCR4 re-expression and the homing capacity of CLL B cells. First, we sorted the CXCR4^{dim}CD5^{br} subpopulation of leukemic B cells that is enriched in recently divided cells (Supplementary Figure 6), emigrating from lymphoid tissues to the periphery.²⁷ Even though the CXCR4^{dim}CD5^{br} populations sorted from the blood of vehicle- and ibrutinib-treated animals had similarly low smCXCR4 expression (771 \pm 46 vs 313 \pm 28, Figure 5a), most vehicle-treated leukemic cells were able to re-express smCXCR4 and migrate to the spleen within 18 h of cell transfer. However, ibrutinib-treated leukemic cells remained in the blood, with only a small number of cells able to re-express smCXCR4 and migrate to spleens (Figures 5b and c).

We then studied if continuous ibrutinib treatment was required to block BTK-mediated CLL-cell homing to tissue niches.

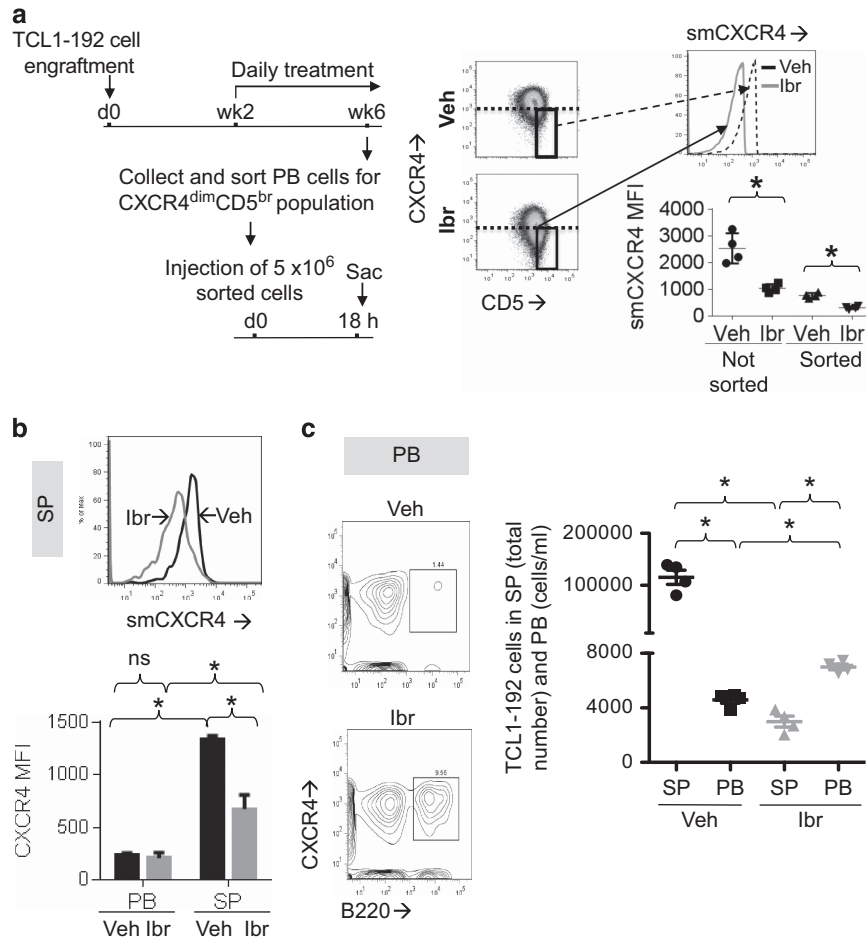


Figure 5. BTK inhibition blocks smCXCR4 re-expression and CLL-cell homing. **(a)** 5x10⁶ CXCR4^{dim}CD5^{br} cells sorted from PB of animals treated for 4 weeks (wks; n = 4 per group) were injected into SCID animals; 18 h later mice were killed. The representative histogram plot and dot plots of smCXCR4 indicate relatively similar smCXCR4 mean fluorescence intensity in donor cells obtained and sorted from vehicle (Veh) and ibrutinib (lbr)-treated mice (*P < 0.05). **(b)** lbr-mobilized TCL1-192 cells migrating to SP exhibit persistently lower smCXCR4 than control cells (P = 0.028, n = 4). **(c)** Significantly higher numbers of lbr-mobilized TCL1-192 cells remain in PB than SP (P = 0.03, n = 4). In contrast, the opposite pattern is seen for Veh-treated cells with significantly higher numbers of CLL cells in SP than PB (P = 0.03). Plots shown represent mean \pm s.d. Representative examples of B220⁺ cells obtained from PB 18 h post cell engraftment are shown on the left. P-values shown were calculated by Mann-Whitney multiple t-test. 'NS' indicates non-significant.

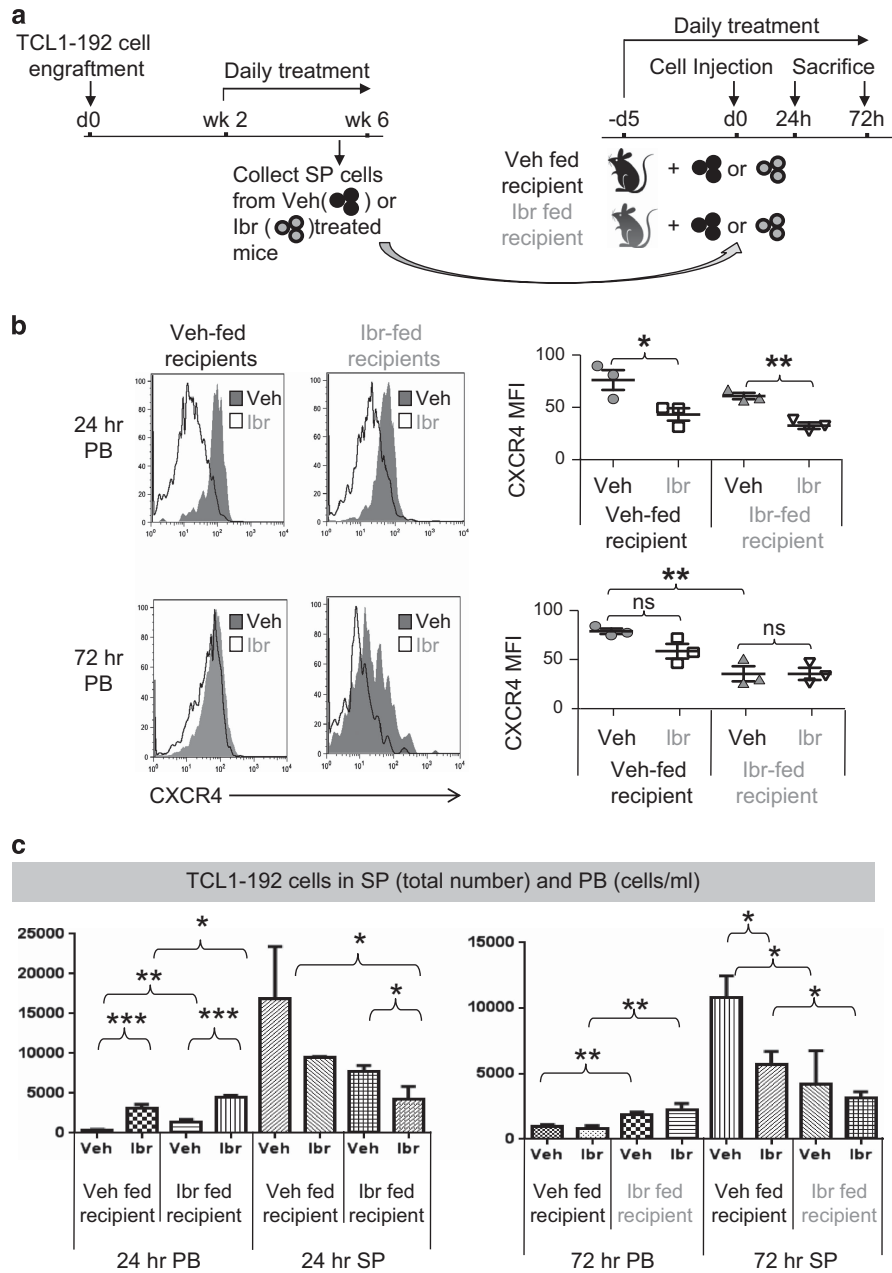


Figure 6. Continuous treatment with ibrutinib (Ibr) is required to prevent smCXCR4 re-expression. **(a)** 5×10^6 SP TCL1-192 cells collected from mice receiving vehicle (Veh) or Ibr for 4 weeks (wks) were injected into SCID mice previously treated daily for 5 days. All 24 SCID recipients were treated continuously for 24 or 72 h. **(b)** At 24 h, tumor cells previously exposed to Ibr (white) had significantly lower smCXCR4 compared with those exposed to Veh (gray), regardless of whether they were subsequently transferred into Veh- or Ibr-treated mice ($*P < 0.05$, $**P < 0.01$; $n = 3$ per group). At 72 h, only Ibr-treated recipients had leukemic cells with reduced smCXCR4, regardless of the donor from which the cells were taken. **(c)** At 24 h, mice that received leukemic cells previously exposed to Ibr had a higher number of CLL cells in PB, but lesser number in SP, compared with those exposed to Veh, regardless of the treatment recipients received. At 72 h, only Ibr- but not Veh-treated recipients had higher numbers of circulating cells in PB ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 3$ for each group). Plots are mean \pm s.d. *P*-values were calculated by unpaired parametric *t*-test. 'NS' indicates non-significant *P*-values.

Splenocytes from mice treated for 4 weeks were injected into SCID recipients receiving either vehicle or ibrutinib, and then the recipient mice were killed 24 or 72 h later (Figure 6a). Splenocytes from ibrutinib-treated mice that were transferred into vehicle-fed animals maintained, for at least 24 h, lower smCXCR4 than those transferred into ibrutinib-fed animals (Figure 6b, Top). However, at 72 h, smCXCR4 levels of both ibrutinib-treated and vehicle-treated splenocytes were comparable in the ibrutinib-deficient environment. Correspondingly, when cells from vehicle-treated animals were placed in an ibrutinib-containing environment, smCXCR4

levels dropped progressively after 72 h (mean fluorescence intensity: 78.8 ± 2.9 vs 35.6 ± 7.7 , $P = 0.006$; Figure 6b, bottom). Consistent with reduced levels of smCXCR4, at 24 h, more ibrutinib-treated TCL1-192 cells remained in the blood of ibrutinib-fed animals, whereas animals receiving vehicle-treated cells had more leukemic B cells in their spleens, regardless of the type of treatment recipient mice received (Figure 6c). After 72 h, ibrutinib-exposed recipients had higher percentages of cells in their circulation.

Together, these data demonstrate that BTK is needed for homing of leukemic B lymphocytes to lymphoid tissues

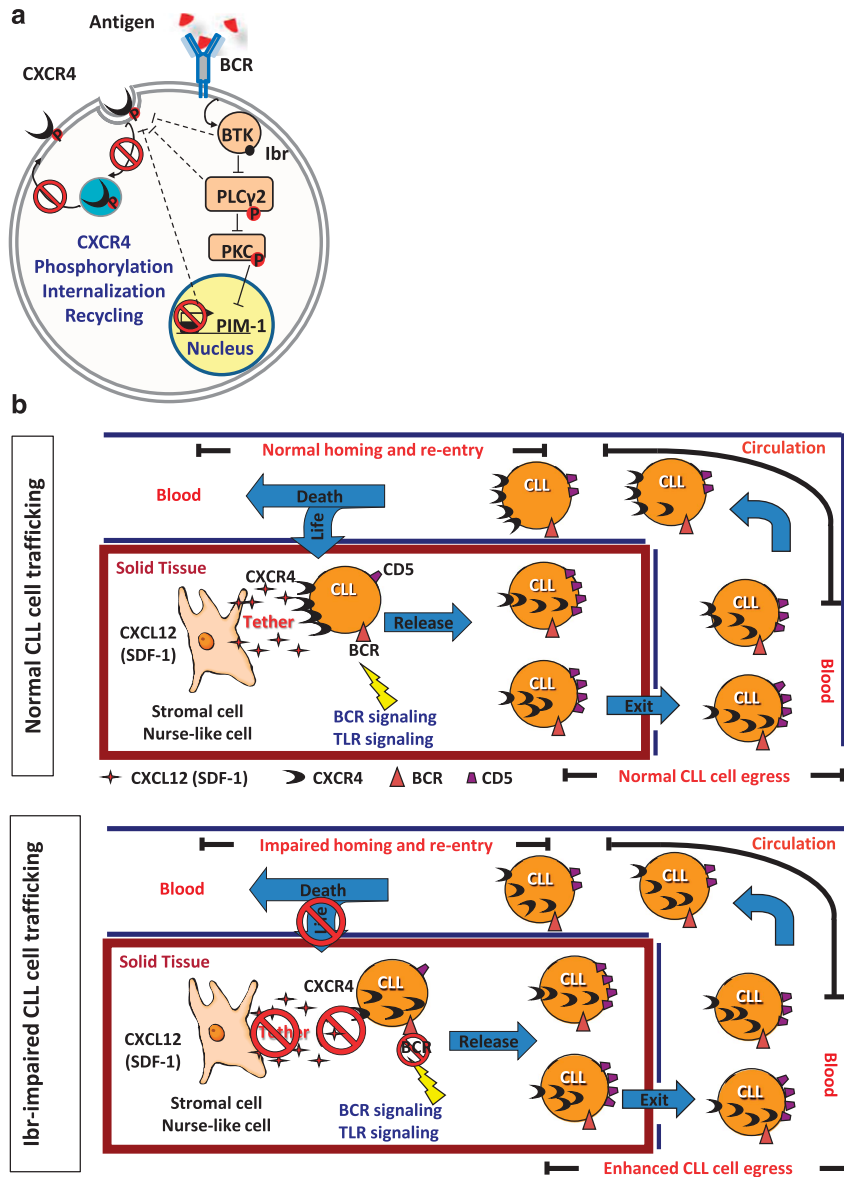


Figure 7. Mechanisms whereby BTK phosphorylates CXCR4 and regulates CXCR4 signaling, recycling and B-cell tissue homing and retention. **(a)** Inhibition of BTK (Figure 4b) and its downstream targets such as PLC γ 2,¹⁷ PKC μ and PIM-1 (Figure 4c), diminishes CXCR4 phosphorylation, recycling and consequently lowering smCXCR4 expression. **(b)** (Top) Hypothetical model of the lifecycle of CLL B cells. In solid tissues, CXCR4–CXCL12 interactions enhance the tethering of CLL cells to stroma. Cellular activation, possibly by (auto)antigen stimulation, promotes higher CD5 expression, CXCR4 internalization and detachment of CLL cells from stroma. In circulation, recently divided CLL cells (CXCR4^{dim}CD5^{bright}) may begin to re-express CXCR4. Eventually, CXCR4^{bright}CD5^{dim} CLL cells have the greatest chance of re-entering solid tissue and receiving pro-survival signals. Those that do not re-enter die by survival signal-deprivation/exhaustion. (Bottom) BTK inhibition results in impaired CXCR4- and BCR-signaling and further lowered smCXCR4 levels to more effectively terminate stromal tethering. The majority of BTK-inhibited CLL cells are unable to re-express sufficient levels of functional smCXCR4 and thereby fail to re-enter niches, leading to greater survival signal-deprivation/exhaustion and cell death. Through all these actions, BTK inhibition delays CLL disease progression. Ibr, ibrutinib; TLR, toll-like receptor.

and this requires ongoing synthesis of the enzyme. This is consistent with the beneficial effects of BTK inhibition in leukemia patients¹⁰ and leukemia-bearing mice,¹⁷ and the requirement that inhibition be maintained for therapeutic efficacy.²⁸ By blocking the retention and homing of CLL cells mediated at least by CXCR4, BTK inhibition delayed disease progression and improved overall survival (Supplementary Figure 4).

DISCUSSION

The E μ -TCL1 mouse model resembles aggressive human CLL based on the development of *IGHV*-unmutated leukemic clones.²⁹

It also leads to the development of U-CLL-like subsets with stereotyped *IGHV-D-J* rearrangements²⁹ that are virtually identical to sequences previously determined from murine B-1 cells and murine autoantibodies to phosphatidylcholine, bromelain-treated erythrocytes and nuclear components.³⁰ The model used here employs a U-CLL-like clone from a TCL1 Tg animal (TCL1-192) that expresses a canonical anti-phosphatidylcholine BCR that binds and responds to this autoantigen.¹⁹ Thus, this clone can serve as a model of a stereotyped, autoantigen-reactive U-CLL subset analogous to those expressing stereotyped *IGHV-D-J* rearrangements that react with autoantigens, resembling but not limited to U-CLL subsets that use *IGHV1-69* such as subset 6 that binds myosin heavy chain IIA.^{31,32}

Using this model and a small-molecule inhibitor approach, we have defined the mechanisms responsible for BTK-mediated CXCR4 expression and signaling in CLL (Figure 7a), thereby providing *in vivo* evidence for BTK's regulation of CLL B-cell migration and homing (Figure 7b). Our novel findings include documentation that:¹ blocking BTK's enzymatic activity, which inhibits CXCR4-mediated signaling by preventing phosphorylation of the receptor at Ser339, leads to a rapid and continuous release of leukemia cells into the circulation, and this release associates tightly with decreased smCXCR4 expression;² BTK inhibition indirectly alters the function of PKC μ , which is downstream of CXCR4 signaling, contributing to a further impairment of CXCR4 phosphorylation and internalization/re-expression;³ inhibition of BTK eventually results in decreased synthesis of PIM-1 and BTK itself, enhancing the molecular and functional effects of enzyme blockade; and⁴ diminished levels and function of smCXCR4 *in vivo* prevent leukemic cells from re-entering protective niches, and those actions require uninterrupted enzymatic inhibition.

BTK inhibition promoted release of leukemia cells into the circulation, not only in ≤ 1 h after ibrutinib administration, but also after 4 weeks of treatment when circulating leukemic cell counts were significantly reduced (Figure 1). This continuous cell egress included the most recently divided cells, the wellspring of clonal evolution as new genomic abnormalities favoring disease progression are introduced during DNA replication.³³ Our results agree with a recent study in CLL patients demonstrating that ibrutinib releases Ki-67⁺ cells into the peripheral blood at percentages similar to those in LNs.³⁴ The rapid resolution of lymphocytosis could be due to ibrutinib-promoted cell death. However, this might be difficult to measure as dying tumor cells are quickly taken up by phagocytosis. Indeed, by analyzing Annexin-V and 7-amino-actinomycin D expression on fresh isolated cells, we were not able to detect ibrutinib-promoted cell death in circulating or tissue-resident cells (Supplementary Figure 2C).

The rapid and continuous release of leukemia cells from lymphoid tissues induced by ibrutinib's occupation of available BTK (Figure 1) resulted from two actions on CXCR4: selective lowering of membrane receptor levels without affecting total receptor amounts, and blocking CXCR4 signal transduction after ligand binding (Figure 2). The reduction in smCXCR4 in ibrutinib-treated cells was relatively specific, as it was not found for other BTK-regulated molecules that play a role in leukemic cell migration and adhesion, including CXCR5 and CD49d (Supplementary Figure 3). Our results are consistent with the reduction of CXCR4 reported in ibrutinib-treated patients with CLL⁷ and mantle cell lymphoma,^{35,36} and with the finding that resistance to ibrutinib occurs in patients with Waldenstrom's macroglobulinemia with CXCR4 mutations.³⁷

In the normal state, smCXCR4 is rapidly internalized and re-expressed after ligand binding by a process dependent on phosphorylation at Ser339.³⁸ Here we found that BTK inhibition rapidly and continuously impairs recycling of CXCR4 (Figure 3) by blocking CXCL12-induced phosphorylation of CXCR4 at Ser339 (Figure 4a). Inhibition of Ser339 phosphorylation also resulted indirectly by preventing activation of downstream targets such as PLC γ 2 (shown previously in this model, ref. 17) and PKC μ (Figures 4c and d), which is activated by chemokine stimulation.³⁹ In addition, ongoing BTK inhibition eventually led to a reduction in the protein levels of PIM-1, a target of BTK and PKC,²³ and of BTK itself, which requires BCR stimulation to regulate its synthesis.⁴⁰ These data are consistent with the protein levels of BTK and PIM-1 positively correlating with smCXCR4 density in CLL and myeloma cells.^{21,41}

The transfer of BTK-inhibited cells into secondary recipients, given either ibrutinib or vehicle control, helped elucidate the consequences of impairing smCXCR4 expression, especially those on leukemia cell trafficking. For secondary recipients that were continuously treated with ibrutinib, homing of leukemic

B lymphocytes to lymphoid tissues was aborted because of the inability to re-synthesize BTK (Figures 5 and 6). In the absence of newly produced BTK, CLL-cell homing remained impaired, making leukemic cells susceptible to death by depriving sustaining inputs through the BCR, CXCR4 and other trophic receptors. To our knowledge, this is the first *in vivo* demonstration of these phenomena due to BTK blockade.

In addition, TCL1-192 cells remaining in tissues after BTK inhibition displayed dysfunctional BCR and CXCR4 signaling (Figure 2c) that could contribute to reduced leukemic cell growth in spleens and LNs (Figure 1c). These actions of ibrutinib delayed disease progression despite the aggressiveness of this leukemia model (Supplementary Figure 4), although ultimately the animals succumbed to the disease. This may be akin to CLL patients who relapse when receiving ibrutinib mono-therapy.¹⁰ Because a small percentage of dividing cells was still present in 4-week-treated animals (Figure 1c), a finding that differs somewhat from the apparent complete inhibition of leukemia cell proliferation documented in previously untreated patients receiving ibrutinib,⁴² some cells in the TCL1-192 population may have developed compensatory mutations that subverted BTK blockade, as seen in a minority of patients treated with ibrutinib.⁴³ Indeed, resistance might occur more frequently in TCL1-192 recipients than in patients because of the higher number of proliferating cells in this transfer system.¹⁹ Finally, the actions of ibrutinib on CLL B-cell migration could also involve changes in the tumor microenvironment. For example, in mantle cell lymphoma, a reduction in serum levels of CCL4, CCL22 and CXCL13 occurs in patients receiving ibrutinib.³⁵

Altogether, using TCL1-192 cells that mimic human CLL and ibrutinib, the therapeutically valuable BTK inhibitor, we have uncovered a complex set of mechanisms responsible for the regulation of CXCR4 expression and function. Considering the need of human CLL cells for ancillary survival and growth signals, inhibition of BTK function would lead to a loss of tumor volume by preventing replenishment after spontaneous or drug-induced death, and by subverting leukemia cell retention in and homing back to sustaining tissue niches (Figure 7). The dual action of ibrutinib on CXCR4 as well as BCR function delays disease progression and prolongs survival.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Conception and design of the study were done by S-SC, NC; development of methodology was done by S-SC, BYC, NC; acquisition of data was done by S-SC, SC, TT, SH; analysis and data interpretation were done by S-SC, BYC, BAS, NC; manuscript writing and review were done by S-SC, BYC, BAS, JAB, KRR, NC.

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Supplementary Information accompanies this paper on the Leukemia website (<http://www.nature.com/leu>)