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3D co-culture model of chronic lymphocytic leukemia bone marrow microenvironment predicts patient-specific response to mobilizing agents

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Key Points

• 3D co-culture model of CLL bone marrow microenvironment in a bioreactor allows to elucidate the mechanism involved in CLL tissue retention and mobilization
• The model identifies a patient specific role for HS1 protein in ibrutinib driven CLL cells mobilization from tissues
Abstract

Chronic Lymphocytic Leukemia (CLL) cells disseminate into supportive tissue microenvironments. To investigate the mechanisms involved in leukemic cell tissue retention we developed a 3D bone marrow (BM) microenvironment that recreates CLL-BM-stromal cells interactions inside a scaffold within a bioreactor. Our system allows the parallel analysis of CLL cells retained inside the scaffold and those released in the presence/absence of pharmacological agents, mimicking tissue and circulating cell compartments, respectively. CLL cells can be retained within the scaffold only in the presence of microenvironmental elements, which through direct contact down-regulate the expression of HS1 cytoskeletal protein in CLL cells. Consistent with this, the expression of HS1 was lower in CLL cells obtained from patients’ BM versus CLL cells circulating in the PB. Moreover, we demonstrate that CLL cells with inactive-HS1, impaired cytoskeletal activity and a more aggressive phenotype are more likely retained within the scaffold despite the presence of Ibrutinib, whose mobilizing effect is mainly exerted on those with active-HS1, ensuing dynamic cytoskeletal activity. This differential effect would not otherwise be assessable in a traditional 2D system and may underlie a distinctive resistance of single CLL clones. Notably, CLL cells mobilized in the peripheral blood of patients during Ibrutinib therapy exhibited activated HS1, underscoring that our model reliably mirrors the in vivo situation. The 3D model described herein is suitable to reproduce and identify critical CLL-BM interactions, opening the way to pathophysiological studies and the evaluation of novel targeted therapies in an individualized manner.
Introduction

Chronic Lymphocytic Leukemia (CLL) is characterized by a progressive expansion of clonal CD5+ B lymphocytes that accumulate and traffic between the Peripheral Blood (PB), Bone Marrow (BM) and secondary lymphoid organs 1,2. In those sites, CLL cells are extremely dependent on and reactive to the microenvironment (i.e. stromal, endothelial cells and immune cells) and proliferate in the so called “proliferation centers”, mainly found in the lymph nodes (LN), and/or sheltered in vaguely characterized niches in the BM 3,4,5. CLL cells accumulating within the tissues tend to spill over into the circulating blood where they acquire a more resting phenotype, indicating that the most clinically relevant events occur in tissues. This feature also underlines the importance of the hosting tissues in CLL which conceivably contribute to disease progression and ultimately to treatment resistance 6,3,5.

Cytoskeleton regulation is clearly implicated in the dynamic behavior of CLL cells, contributing to the homing and trafficking in and out of the tissues, also during treatment. In particular, we previously reported that the activated status of the cytoskeletal protein hematopoietic lineage cell-specific protein 1 (HS1) defines a distinct signalling pathway and cytoskeletal activity in CLL, while also having prognostic implications, with active/inactive forms of HS1 correlating with favorable or adverse prognosis, respectively 7,8,9. In parallel, we demonstrated that down-regulation of HS1 expression interferes with secondary lymphoid organ (LN and spleen) infiltration by CLL cells and leads to increased BM homing associated with impaired cytoskeletal activity 9,10. More recently, HS1 has been found to associate with ROR1 in enhancing CLL cell migration 11, further underlining its potential clinical significance.

New targeted therapies, namely kinase inhibitors, have multiple modes of action, including the mobilization of leukemic cells from tissues into the bloodstream, where CLL cells lose the protective effect exerted by the microenvironment, eventually becoming more susceptible to cell apoptosis 12,13,14. Effectively, the use of the BTK inhibitor Ibrutinib for CLL treatment has been a game-changer in the management of patients with this disease 14, although it is not curative and patients may relapse after several years of response 15. Inhibition of VLA-4-dependent adhesion of CLL cells to stroma and stromal components has been proposed as an explanation for the lymphocytosis induced by Ibrutinib treatment 16, while other studies suggest a role of Ibrutinib in modulating migration of CLL cells to chemokine gradients, in particular through CXCR4 17.
However, a major limitation of investigating tissue retention and egress (or mobilization) in CLL originates from the lack of suitable *in vitro* models for recreating the close interactions between leukemic cells and the microenvironment. Calissano et al. first showed a relationship between *in vivo* CLL cell kinetics and the expression of CD38, a protein involved in CLL cell retention and trafficking \(^4\). More recently, Pasikowska et al. reported differences between LN-derived CLL cells versus PB-derived cells by taking advantage of an *in vitro* system that models trans-endothelial migration \(^18\), while Chen et al. demonstrated the dynamic expression of CXCR4 following BTK inhibition *in vivo* in a CLL mouse model \(^17\). Despite these advances, none of the existing models is suitable for deeply characterizing what is occurring to human CLL cells in the tissues.

In order to partially overcome this limitation, we have exploited and adapted to CLL a 3D co-culture model, already deeply validated for multiple myeloma, that is able to reproduce malignant cell-microenvironment interactions \(^19\). This 3D-model is based on the integrated use of cell-repopulated scaffolds and a rotating bioreactor. Such a combination allows establishing reciprocal interactions between tumoral and non-tumoral compartments inside the scaffolds and to promote CLL cell survival. Moreover, CLL cells can be recovered from both inside and outside the scaffolds, counted and characterized for the expression of lineage markers and of molecules putatively involved in their mobilization, providing the possibility to elucidate this mechanism, also in response to mobilizing agents, particularly Ibrutinib. As a proof-of-principle, we here provide evidence of HS1 modulation in the presence of the drug, ultimately regulating CLL cell tissue homing and egress. Moreover, we report that this innovative 3D-model is able to reliably reproduce the events occurring *in vivo* during homing and migration, thus potentially contributing to better understanding the pathogenic mechanisms leading to the dissemination and homing of CLL cells, particularly in response to treatment.
Methods

Human Ethics Statement
Patients with CLL were diagnosed according to the updated National Cancer Institute Working Group (NCIWG) guidelines. Peripheral blood (PB) samples were obtained after informed consent from patients who were (i) either untreated or off treatment for at least 6 months; or (ii) under Ibrutinib treatment. The study was approved by the Ospedale San Raffaele (OSR) ethics committee under the protocol VIVI-CLL entitled: “In vivo and in vitro characterization on CLL”; and the CERTH ethics committee on the application entitled “Molecular and functional studies of B cell malignancies”. Clinical and biological characteristics of patients with CLL who provided samples for the experiments are reported in supplementary Table 1.

Scaffold preparation
Scaffolds were populated as described by Belloni et al and adapted to CLL cells. Briefly: scaffold discs were cut from Spongostan™ sheets (Ethicon, Inc. USA) using a sterile 4 mm² biopsy punch and then pre-seeded with BM derived stromal cells HS5 (200,000/scaffold) in 96-well suspension culture plate (Greiner bio-one, Germany). Scaffolds were then transferred to 10ml High Aspect Ratio Vessels (HARV) in 1ml TCM (DMEM culture medium supplemented with 10% v/v FBS) and cultured overnight in the RCCSTM bioreactor at the lower rpm. Twenty-four hours later, CLL cells were added to the vessels, following the optimal CLL cells:stromal cells ratio, set in preliminary experiments (MEC1 cells=2x10⁶, primary CLL =3x10⁶). After 5 hours, vessels were filled with growth medium (RPMI1640 culture medium supplemented with respectively 10% v/v or 20% FBS for MEC-1 or primary CLL cells). At the end of the culture period, cells outside and inside the scaffold were recovered from the scaffolds by means of liberase (Roche) (25μg/ml) treatment for further analysis (see Supplementary Methods). The cells outside and inside the scaffold were counted using the trypan blue exclusion test for viability; showing that > 90% of the cells were viable. Alternatively, scaffolds were formalin-fixed for IF or lysed with 100 μl RIPA Buffer for WB analysis (see Supplementary Methods).

Bioreactor RCCSTM
3-D dynamic culture was performed using the RCCSTM bioreactor RCCS-4DQ equipped with four rotating 10 ml-HARV culture vessels, that work as culture chambers (Synthecon Inc., USA).
Vessels are provided with a gas exchange membrane made of silicon rubber, that allows optimal diffusion of O2. The bioreactor was kept inside an incubator, with humidified atmosphere at 37°C and 95% air 5% CO2. During the experimental procedures, RCCS™ operational conditions were settled and constantly monitored in order to keep the samples in a “free fall” condition, which minimizes the sedimentation of the scaffold while maximizing mass transfer and cell viability for extended culture period.

**Ibrutinib treatment in bioreactor**

After 72h of 3D dynamic culture in bioreactor supernatants were withdrawn from the vessels and centrifuged at 1500 RPM for 5’. Recovered cells were counted. Clarified supernatants were put again in the vessels with or without 10µM Ibrutinib. We compared 2 different concentrations of ibrutinib (1 and 10 µM) to exclude a possible role of cell apoptosis in the mobilization from the scaffold, due to the possible increased toxicity of ibrutinib at higher concentration, and we didn’t observe any significant differences (Supplementary Figure 2e). Cultures were stopped after 5 hours of treatment and cells in the supernatants and in the scaffolds were recovered and submitted to the above mentioned analysis (see Supplementary Methods).

**Results**

*Microenvironmental elements are required to establish a 3D-culture BM model for CLL*

We customized a new 3D co-culture model, previously validated by our group for myeloma cell survival 19 to recreate CLL and BM-stromal cell interactions inside a scaffold kept in culture in a rotating bioreactor (Figure 1a). We selected scaffolds made of spongostan that has an ultrastructure similar to the trabecular structure of the BM, also due to their superior performance in supporting CLL cell retention when compared to either gelatin or collagen coated beads (data not shown). To set the optimal experimental conditions, we first defined the best ratio of cellular components and the most appropriate co-culture medium in supporting cell viability (see Methods). The scaffolds were sequentially populated with the human BM-derived stromal cell line HS5 and the CLL cell line MEC1. Scaffolds retrieved from the vessels after 3 days of co-culture and submitted to confocal analysis showed that GFP-tagged MEC1 cells populated the entire scaffold efficiently and
homogeneously (Figure 1b). The model allows analysing in parallel CLL cells inside and outside the scaffold, revealing that HS5 cells are needed in order to efficiently retain MEC1 cells into the scaffold (Figure 1c, p=0.01).

Next, we used MEC1 cells genetically modified to down-regulate HS1 expression (MEC1-HS1KD), already known to display increased BM homing capacity in vivo in a CLL xenograft model, and tested whether this could be reproduced in our 3D ex-vivo model. We co-cultured in 3D either MEC1-CNTR or MEC1-HS1KD cells with HS5 stromal cells as described above (Figure 1a) and observed that MEC1-HS1KD cells were significantly less than MEC1-CNTR outside the scaffolds (p=0.0013, Figure 1d). We then quantified by RT-qPCR the expression of HS1 in MEC1-CNTR cells cultured in 3D and found that HS1 is down-regulated in MEC1 cells retained inside the scaffold compared to the cells outside the scaffold (n=3 replicates; p=0.028, Figure 1e).

Collectively, these findings indicate that our 3D system can reliably reproduce the BM-CLL interactions occurring in vivo and further underscore the relevance of HS1 down-regulation as a putative mechanism associated with CLL cell retention in the BM microenvironment, as previously suggested in mouse models.

The BM microenvironment regulates HS1 expression in primary CLL cells in 3D co-culture

We then co-cultured primary leukemic CLL cells isolated from the PB of 6 patients with CLL in the spongostan scaffolds in the presence and absence of HS5 cells as represented in Figure 1a. We also confirmed for primary cells that stromal HS5 cells are needed to efficiently retain primary CLL cells in the scaffolds (p=0.0047, Figure 2a).

To further validate our model for CLL, we confirmed, by flow cytometry, the ability of CLL cells to retain the surface expression of their lineage markers CD19/CD5 throughout the whole culture period both inside and outside the scaffolds (supplementary Figure 1a). We then quantified the expression of CXCR4 by RT-qPCR and flow cytometry in CLL cells recovered inside and outside the scaffolds after 3 days of co-culture with HS5 cells and found that CXCR4 is down-regulated in the cells retained inside the scaffold (Figure 2b, n=8 p=0.015, supplementary Figure 1c, n=3 p=0.03), mimicking the in vivo finding of CXCR4 down-regulation in response to the binding of its cognate ligand CXCL12 (SDF-1α) (21). Next, we focused again on the HS1 gene and, similar to the findings in MEC1 cells (Figure 1e), we observed a significant down-regulation of HS1 in the CLL fraction inside the scaffolds as compared to the outside fraction (Figure 2c, n=8, p=0.005). These
findings raised the question of whether the expression of HS1 reflected a direct influence of the BM microenvironment or, conversely, a fraction of CLL cells constitutively expressing low levels of HS1 might preferentially home to the BM. In order to answer this question, we co-cultured primary CLL cells isolated from the PB of 10 patients with the stromal cell line HS5 in 2D monolayers, and evaluated the expression of HS1 by RT-qPCR. HS1 expression was significantly down-regulated after 24 hours of co-culture (Figure 2d, n=15, p<0.0001). We then co-cultured CLL primary cells from the PB of 8 patients with HS5 cells in the presence and absence of a trans-well (1μm pore) to avoid the direct tumor-stroma contact. HS1 expression was not down-regulated in the presence of the trans-well, (Figure 2e), suggesting that its regulation requires the direct contact between leukemic cells and the BM stromal microenvironment.

**HS1 is heterogeneously expressed in CLL tissues**

The results described above suggest that HS1 in CLL might be differentially expressed depending on the tissue where the leukemic cells are located. To assess whether our 3D model is recapitulating what occurs in vivo, we compared HS1 expression in primary human CLL cells from PB and BM. By RT-qPCR, we observed that HS1 expression was significantly down-regulated in CLL cells isolated from the BM as compared with paired samples isolated from the PB (Figure 3a, n=9, p=0.0498). In the same cohort of patients, we also confirmed CXCR4 down-regulation in the BM as compared to the PB (supplementary Figure 1b, n=10, p=0.003). When we quantified HS1 expression at single cell level in CLL cells isolated from paired PB and BM samples using the Image Stream instrument 22, we observed that the majority of CLL cells in the PB strongly expressed HS1, while in the BM a fraction of CLL cells were HS1 negative for the protein (Figure 3b, n=4, p=0.0187). Accordingly, immunohistochemistry (IHC) performed on BM (n=4, patients analysed) revealed a heterogeneous pattern of HS1 expression among patients (Figure 3c). This finding confirms that our 3D model can reliably reproduce the native BM microenvironment and provide significant insights on CLL cells in the tissues.

**CLL cells are mobilized from the scaffolds following Ibrutinib exposure**

On the evidence presented herein, HS1 appears to be involved in CLL cell compartmentalization, prompting the question whether it could also be involved in the process of CLL cell mobilization from the tissues. In order to address this point, we
exploited our 3D model as described in Figure 4a and evaluated whether the cytoskeletal activity of HS1 is also playing a role in CLL cell mobilization promoted by the BTK inhibitor Ibrutinib \(^1^6\). MEC1 co-cultured with HS5 within scaffolds were efficiently mobilized upon 5h of treatment with Ibrutinib (p=0.02), as shown by both the number of MEC1 cells recovered in the medium (Figure 4b) and by the confocal images of the scaffolds, which exhibited significant fewer GFP-tagged MEC1 cells in the untreated condition (p=0.002) (Figure 4c and supplementary movies 1-2). Of note, HS5 cells were not mobilized by the drug (supplementary Figure 2a).

We next studied the response to Ibrutinib of primary CLL samples (n=21) and found that the number of cells outside the scaffolds was significantly higher upon drug treatment as compared with the untreated samples (Figure 4d, n=21 p=0.0005). Consistent with this, confocal microscopy analysis performed on the scaffolds showed that they were depopulated of CLL cells after incubation with Ibrutinib (Figure 4e).

As expected, the expression levels of HS1 of cells moving out of the scaffolds decreased while they remained lower in CLL cells inside the scaffold in both untreated and treated (ibrutinib) settings (supplementary Figure 2b). Conversely, CXCR4 expression on CLL cells inside the scaffold decreased following Ibrutinib exposure confirming previous \textit{in vivo} results by Chen et al. \(^1^7\) (supplementary Figure 2c, p=0.03). In parallel, we observed that CXCL12 levels in the medium did not significantly change during the drug treatment (supplementary Figure 2d).

\textbf{Inactive-HS1 cells are less efficiently mobilized following Ibrutinib treatment}

To elucidate the mechanism underlying CLL mobilization, we evaluated whether HS1-mediated cytoskeletal rearrangements might be involved. We have previously shown that HS1 activation differs among patients with CLL and is associated to the clinical course (Active-HS1 and Inactive-HS1 associated with favorable and adverse prognosis, respectively) \(^1^0\). CLL cells with Active-HS1 (carring HS1 phosphorylated in Y378 as result of WB analyis, data not shown) show efficient cytoskeletal functionality, while Inactive-HS1 CLL cells (not phosphorylated in HS1-Y378) display reduced cytoskeletal activity associated with a higher propensity to accumulate within the BM microenvironment \(^1^0\). We detected that both Active-HS1 and Inactive-HS1 CLL cells are capable to home the scaffolds without significant differences (data not shown). Interestingly, we observed that the CLL cells released from the scaffolds upon exposure to Ibrutinib were enriched for those with Active-HS1 (10 cases) as compared to those with Inactive-HS1 (10 cases),
Similarly, when we studied, in the same co-culture model, HS1 activation i.e. the levels of HS1-Y378 by WB, in primary CLL cells (n=7) with or without Ibrutinib, we observed that the levels of HS1-Y378 were higher in the cells released in the supernatant as compared to those retained inside the scaffolds (Figure 5b, n=7, p=0.0035). These results demonstrate that more aggressive CLL cells (i.e. Inactive-HS1) are less efficiently mobilized from the BM surrogate scaffold and confirm that the segregation of CLL cells between the two compartments is not random but rather affected by the drug that, in turn, mediated changes in HS1 activation. Arguably, therefore, our 3D model may discriminate between cases that will more robustly respond to the CLL cell mobilizing effect of Ibrutinib.

**CLL cells mobilized into the PB by Ibrutinib show active-HS1 during the first weeks of treatment**

We finally analysed HS1 activation in CLL cells of patients (n=8) under Ibrutinib treatment for different time periods (from week 1 to week 12) and correlated it with the lymphocyte count into the PB at the different time points. In accordance with the results obtained in our 3D-model (Figure 5b), we observed that PB CLL cells from 6/8 patients displayed HS1 activation during the first weeks (week 1, 2, 3) of treatment as compared to the basal level (Figure 6a), in parallel with an increase in lymphocyte count into the PB. In contrast, peripheral lymphocytosis was not seen in the 2 cases where HS1 did not undergo activation. In parallel, we analysed HS1 expression in CLL cells by both WB and RT-qPCR and found that HS1 expression increased during the first weeks of treatment in all patients analysed either at the protein or at the gene level, however we could not find a correlation with circulating lymphocyte count (*supplementary Figure 3a*). Altogether, these results indicate that our 3D model can mirror the events occurring *in vivo* during Ibrutinib therapy.
Discussion

The clinical scenario of CLL is rapidly changing especially thanks to new targeted therapies, nevertheless the disease is still incurable. CLL is strongly influenced by the tissue microenvironment, as also evidenced by the fact that circulating CLL cells are more sensitive to drug-induced apoptosis, suggesting that a supportive microenvironment is necessary for leukemic cells survival. This encourages the development of mobilizing agents and points to the key role of the cytoskeleton in recirculation and accumulation of CLL cells in different tissues.

A key prerequisite for investigating the mechanisms underlying human CLL cell homing and mobilization is the capability to reliably and accurately reproduce in vitro a native CLL tissue microenvironment, so far unavailable. Our previous studies pointed to the importance of the BM microenvironment showing a specific homing of aggressive CLL (with Inactive-HS1) to this site in vivo in mouse models. For this reason, we customized for CLL a 3D-BM model based on scaffolds within a bioreactor, following our previously published experience from the analysis of multiple myeloma cell survival and response to Bortezomib. The model allows us to analyze in parallel CLL cells retained inside the reconstructed BM microenvironment and those recovered outside. We were able to define BM stromal cells as the minimal requirement to support CLL cell retention and homing inside the scaffolds, paving the way for future improvements aimed at testing the individual contribution of additional cellular players in this process. The observation that BM stromal cells support the retention of a consistent albeit variable fraction of primary CLL cells among different patients indicates that CLL compartmentalization in a bioreactor is not a random phenomenon. The demonstration of differential HS1 expression and activation status of CLL cells inside and outside the scaffolds, along with a similar modulation of CXCR4 expression, indicates a potential molecular basis for this process that indeed mirrors the events taking place in vivo in the BM and PB.

Kinase inhibitors, such as the BTK inhibitor Ibrutinib, influence the kinetics of leukemic cell recirculation and, interestingly, mobilize CLL cells more efficiently from the LN as compared to the BM, suggesting a specific tissue-dependent effect. Taking advantage of our 3D-BM model, we here provide evidence that CLL cells mobilized from the scaffolds upon Ibrutinib exposure are mainly those with Active-HS1 and suggest that Ibrutinib may
exert its mobilizing effect through HS1 activation. It remains to be elucidated whether Ibrutinib directly or indirectly affects HS1 activation on CLL cells into the scaffold. We have evidence that Ibrutinib does not affect HS1 activation in the absence of BM-derived stromal cells (data not shown), suggesting a specific role exerted by the microenvironment possibly toward HS1 downregulation following direct contact with BM-derived stromal cells. Accordingly, we observed that HS1 undergoes activation during the first weeks of Ibrutinib treatment in patients. We may then infer that CLL cells with low/inactive HS1 preferentially home in the BM niche where they encounter a protective microenvironment against the mobilization effect promoted by Ibrutinib. This further indicates that our 3D model may reproduce the events occurring in vivo under Ibrutinib treatment and may help understand the slower clearance of the BM in patients.

In conclusion, we here present and validate a reproducible 3D BM model for the comprehension of the mechanisms underlying CLL tissue retention and mobilization, but also capable of predicting patient-specific efficacy of CLL mobilizing agents (Figure 7 schematic summary). This may serve in the future as a precision medicine tool to test these and other drugs acting through similar mechanisms in a more suitable system as compared to traditional 2D models where the dynamic effects of treatments cannot be inferred. Moreover, it represents the first step towards the development of new and more complex 3D in-vitro models mimicking different microenvironments such as LN.
Disclosure of Potential Conflicts of Interest
The authors declare no conflict of interest. PG and KS received honoraria and research funding from AbbVie and Janssen outside this project.

Authors’ Contributions
CS, wrote the manuscript. CS, FB, DB, FVS, LP, performed the experiments and analysed the data. LS, PG, KS provided patients’ and clinical information. MP, LB performed IHC. DZ performed image stream analysis. VRC helped in confocal microscopy. EF, VRC, PG, MP and KS revised the manuscript.

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References


Figure Legends

Figure 1. 3D model optimization.
1a) Schematic representation of the experimental setup: HS5 cells are seeded into 3D spongostan scaffolds and cultured under microgravity in a RCCSTM bioreactor for 24h. MEC1 cells or primary CLL cells are added to the same scaffold and co-cultured for 72h under microgravity. At the end of incubation time, cells and scaffolds are collected and analysed. 1b) Representative confocal section taken from a X,Y, Z-stack of a representative scaffold, after 72h culturing MEC1-GFP in bioreactor. Nuclei were stained with Hoechst and the scaffold was imaged by transmitted light (TL). The square-marked region of interest is also shown at higher magnification for each acquired channel. 1c) The graph shows the total number of cells recovered in the medium outside the scaffold after 72h of dynamic culturing in the bioreactor. MEC1 cells were distinguished from HS5 cells according to their smaller size. Each experiment was run in triplicate. MEC1 cells were significantly retained into the scaffold only in the presence of HS5 cells (*p=0.01). 1d) The graph shows the total number of cells recovered in the medium outside the scaffold after 72h of dynamic culture in the bioreactor of MEC1 CNTR cells and MEC1-HS1KD cells: the latter were retained in the scaffold more than MEC1 CNTR (**p=0.0013). 1e) Evaluation of HS1 expression by RT-qPCR in MEC1-CNTR cells inside and outside the scaffolds (run in triplicate) showing that HS1 is down-regulated inside the scaffolds (*p=0.028).

Figure 2. HS1 expression is regulated by the stromal BM microenvironment
2a) The graph shows the total number of cells recovered in the medium outside the scaffold after 72h of dynamic culture in the bioreactor. For the experiments with primary CLL cells, control samples were run using CLL cells alone and HS5 cells alone under the same culture conditions. CLL cells are retained inside the scaffolds only in the presence of HS5 (***p=0.0047). 2b and 2c) The line plots show CXCR4 and HS1 expression respectively as determined by RT-qPCR in primary CLL cells collected inside the scaffolds or in the outside medium. CLL cells retained inside the scaffold expressed significantly lower CXCR4 (*p=0.015) and HS1 (***p=0.005) levels. 2d) Line plot illustrating the down-regulation of HS1 expression in CLL cells isolated from PB when they are in direct co-culture with HS5 cells as determined by RT-qPCR. (****p<0.0001). 2e) Line plot illustrating the HS1 expression in CLL cells isolated from PB when they are cultured in a 1μm pore transwell without direct contact with HS5 cells.
Figure 3. Analysis of HS1 expression in CLL cells isolated from PB and BM.

3a) HS1 mRNA expression levels (determined by RT-qPCR) in primary CLL cells isolated from the PB and the BM of patients (n=9). HS1 is significantly downregulated in the BM if compared to PB of the same patient (*p=0.0498).

3b) Image Stream analysis of intra-clonal expression of HS1 in PB vs BM from CLL patients (n=4). By gating on the CLL pool (CD5+CD19+), we found mainly within BM a population that was negative for HS1 expression (red rectangle and arrow indicate a representative image of a single CLL cell negative for HS1). We also found a population positive for HS1 (black rectangle and arrow indicate a representative image a single CLL cell positive for HS1). The percentage of the PB and BM populations negative for HS1 are also shown (right panel) (*p=0.0187).

3c) IHC analysis of HS1 expression in BM sections

Figure 4. Ibrutinib treatment in 3D co-culture.

4a) Schematic representation of the experimental setup: HS5 cells are seeded into 3D spongostan scaffolds and cultured under microgravity in a RCCS™ bioreactor for 24h. MEC1 cells or primary CLL cells are added to the same scaffold and co-cultured for 72 hours under microgravity. At the end of the incubation time, the supernatant was collected and depleted from the cells. The same supernatant was added again to the culture with or without Ibrutinib for 5h. At the end of the incubation time the scaffolds were collected and analysed. 4b) The histogram plot shows the total number of cells (MEC1-GFP+ HS5) that migrated outside the scaffold after 72h of dynamic culture in bioreactor in the presence of 10μM Ibrutinib (5h) or RPMI medium only (untreated), MEC1 cells were significantly mobilized (*p=0.02) from the scaffolds. 4c) On the left, representative confocal images of of the scaffolds analysed in panel 4b, and on the right the histogram showing the mean number of MEC1 GFP+ cells quantified in the scaffold by counting the GFP+ cells in 4 different stacks for both treated and untreated conditions (p=0.002). 4d) Line plot illustrating the effect of 10μM Ibrutinib treatment on the mobility of primary PB-derived CLL cells that were recovered outside the scaffold (***p=0.0005). 4e) Representative confocal images of representative examples of the scaffolds analysed in panel 4d.

Figure 5. HS1 activation following Ibrutinib treatment.

5a) Dot plot showing the number of CLL cells mobilized outside the scaffold after Ibrutinib treatment and fractionated according to HS1 activation. HS1-Active CLL cells were
mobilized more efficiently from the scaffolds compared to the HS1-Inactive (*p=0.04).

5b) The graph shows the densitometric analysis of the active HS1-Y378 phosphorylated form in CLL cells retained inside or recovered outside the scaffolds with or without Ibrutinib treatment. The CLL cells mobilized and recovered outside the scaffold show significantly higher HS1 activation than that the cells retained inside the scaffold (**p=0.0035).

**Figure 6. HS1 activation and expression in patients during Ibrutinib treatment**

6) Top left panel: schematic representation of the experimental protocol. PB was collected at 1/2/3/4 and 8/12 weeks after drug treatment, PB lymphocyte (LY) count was determined at each time point with an hemocytometer and CLL cells isolated and stored frozen at -80°C. For each patient sample, we plotted in the graph the WB quantified by densitometry for HS1-Y378 and the number of lymphocytes in the PB. The values are normalized to the basal level (n=8 patients).

**Figure 7. Schematic summary of the presented model.**
a

**HS1**

fold change (relative to GAPDH)

---

PB  | BM
---|---

* 

b

**CD19+ CD5+**

Intensity_MC_CD5

CD19+ CD5+

96.6%

CD19+ CD5+

2.85%

% HS1 negative cells gated on CD19+ CD5+ cells

---

PB  | BM
---|---

* 

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**IHC Bone marrow**

---

HS1
a

Number of cells outside the scaffold after ibrutinib

HS1-Active  HS1-Inactive

*  

b

3D culture

Densitometric analysis HS1 Y378/β-actin

Untreated  +ibrutinib 5h

inside  outside  inside  outside

**
CLL cells + BM stromal cells → 3D scaffold

Bone marrow-niche

Bioreactor

Peripheral blood

+ ibrutinib

STOP HS1 inactive

GO HS1 active
Supplementary Methods

Cell cultures and human primary samples purification. The MEC1 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% volume/volume (v/v) Foetal Bovine Serum (FBS) and 15 mg/ml Gentamicin (complete RPMI) at 37°C and 5% CO2. The HS5 human bone marrow stromal cell line was obtained from the American Type Culture Collection (ATTC) and cultured in DMEM medium (GIBCO) supplemented with 10% v/v FBS, 15 mg/ml Gentamicin at 37°C and 10% CO2. Leukemic CD19 cells were negatively selected from fresh peripheral blood using the RosetteSep B-lymphocyte enrichment kit (StemCell Technologies). The purity of all preparations was always higher than 99%, and the cells co-expressed CD19 and CD5 on their surface as assayed by flow cytometry (Navios; Beckman Coulter); preparations were virtually devoid of natural killer (NK) cells, T lymphocytes, and monocytes. MEC1-CNTR-GFP and MEC1-HS1KD cells have been generated and cultured as previously described \(^9\).

2D co-culture. 150,000 HS5 stromal cells/well were seeded the day before the co-culture onto 24-well plates and the next day 1x10^6 primary cells where added. After 24h of co-culture CLL cells were recovered from the plates.

Cell treatment. Primary CLL cells were either left untreated or treated with 10µM Ibrutinib (Selleck) for 30’, 5h in 2D and 3D cultures at the concentration of 3x10^6 cells/ml in complete RPMI medium.

RT-qPCR. RNA was isolated from cell lines and primary samples with ReliaPrep RNA Cell mini Prep System (Promega) according to the manufacturer’s instructions. cDNA was synthesized according to the manufacturer’s protocol using maxim RevertAid H minus First Strand cDNA Synthesis Kit reagents (Thermo Fisher scientific). RTq-PCR analysis was performed using Titan HotTaq Probe qPCR mix (BioAtlas) and in an ABI7900 Thermal Cycler instrument (Applied Biosystem). Quantification of HS1 and CXCR4 transcripts (Applied Biosystem probes) was performed according to the Ct method, using GAPDH as the housekeeping gene.
Western Blot analysis. Western Blots (WB) were performed as described previously. Briefly, cells were lysed on ice for 15 minutes in RIPA Buffer (Santa Cruz Biothecnology), containing fresh phosphatase inhibitor cocktail (phosphoSTOP; Roche, Mannheim, Germany) and complete protease inhibitor cocktail (Roche). Cells were then centrifuged at 13,200 rpm for 15 minutes at 4°C, and supernatants were collected and stored at −80°C until further use. Protein content was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s instructions. 30 μg of total protein were supplemented with NuPage Sample Buffer (4x) and NuPage Sample Reducing Agent (10x) and loaded onto 4–12% sodium dodecyl sulfate-polyacrylamide gradient gels (Invitrogen), then transferred to nitrocellulose membranes (Thermo Scientific). Membranes were blocked for 15 minutes in PBS-Tween containing 5% BSA and incubated overnight with primary antibodies, followed by species-specific horseradish peroxidase-conjugated secondary antibodies (diluted 1:5000) for 1h. Membranes were probed with the following primary antibodies: mouse anti-HS1 from Cell Signaling; anti-Y378-HS1 from Cell Signaling primary antibody was used at 1:1000 dilution. Anti-mouse IgG HRP-linked (1:5000 dilution) was purchased from GE Healthcare. All WB were normalized to Rabbit anti-Actin HRP linked (clone AC-15, Sigma Aldrich) diluted 1:50000. Amersham ECL Western Blotting Analysis System from GE Healthcare was used to visualize immuno-reactive bands. Quantification of relative protein expression levels was performed by Uvitec software analysis (https://www.uvitec.co.uk). We used beta-actin to compare HS1 activation at different time points in the same patient due to the variable level of expression of the total HS1 overtime.

Image stream sample preparation. HS1 expression was analysed by imaging flow cytometry using ImagestreamX MarkII (Amnis, Merck). We used 2x10^6 CLL cells isolated from PB or BM. Briefly, single cell suspensions were fixed with PAF2% for 10 minutes at 37°C. Cells were then washed with PBS-1%BSA and permeabilized by exposure to methanol (stored at -20°C) for 8 minutes in ice. Cells were washed and incubated with anti-human CD19-CD5 (Miltenyi) and HS1 (BD, conjugated with alexa-fluor 488 APEX antibody labelling Thermofisher), for 30 minutes at room temperature and then imaged by ImageStream X MarkII imaging flow cytometer using the 60X_0.9NA objective. The system is equipped with a 6-channel camera and 405nm, 488nm and 642nm lasers. Excitation laser settings were as follows: 405nm (8mW), 488nm (150mW), 642nm (110mW). At least 3x10^4 events were collected for each sample and the images were analysed using the IDEAS 6.2
software. Single-stained samples were acquired with identical laser settings of the samples but without Bright Field illumination and were used for compensation. First, cells were gated for cells in focus using the gradient root mean square feature and then single cells were identified using area and aspect ratio features on the bright field image. CLL cells were identified by their positivity for CD5 and CD19 using the Intensity Feature for both staining; among those cells, we identified 2 populations with different HS1 expression levels, once again relying on the Intensity feature.

**Immunohistochemistry (IHC).** Bone marrow biopsies were retrieved from pathology files of Ospedale San Raffaele in Milan. Four μm thick sections were obtained from Bouin-fixed, paraffin-embedded specimens and de-paraffinized in xylene, rehydrated in ethanol, put in 0.1 M citric acid pH 6.0 solution, heated in microwave and cooled at room temperature. Endogenous peroxidases were quenched with 3% H2O2. Slides were incubated with 3% BSA and monoclonal antibody HS1 (Cell Signalling) at a 1:50 dilution for 30 minutes, followed by incubation with biotinylated goat anti-rat IgG diluted 1:200 and with streptavidin 1:500 for 30 min. Slides were then incubated with 3,3’-diaminobenizidine tetrahydrochloride (DAB) for 5 min and then counterstained with Mayer Haematoxylin. Histological analysis was performed with a Zeiss Axioskop 40 optical microscope equipped with a Zeiss AxioCam MRC.

**Immunofluorescence staining and confocal microscopy.** 3D-spongostan scaffolds, after 72h culture in RCCSTM bioreactor, were washed with PBS and fixed with 4% v/v PAF (Sigma-Aldrich) for 1h at room temperature. Cells were permeabilized in Blocking Buffer (Blocking Buffer: 0.1% w/v BSA, 10% v/v FBS in PBS), containing 0.3% v/v Triton-X100 (Sigma Aldrich). To limit unspecific antibody binding, 3D samples were then incubated with Blocking Buffer (Blocking Buffer: 0.1% w/v BSA, 10% v/v FBS in PBS) for 30 minutes at room temperature. Cells were stained for 2 hours with Phalloidin Atto565 (diluted1:50 in Blocking Buffer; Sigma Aldrich), followed by Hoechst (diluted1:2000 in PBS; Invitrogen) for 5 minutes. 3D scaffolds were placed on Cell Imaging Dish 170 m and 35x10 mm (from Eppendorf), whereas 2D sample slides were mounted using ProLong Gold antifade reagent (Invitrogen) as mounting media. Images were acquired on an inverted TCS SP8 SMD Laser Scanning Confocal microscope (Leica Microsystems) equipped with a white laser and an 40x (NA 1.3) oil objective. The supplementary movies are the 3D rendering of confocal z-stacks. Representative optical sections of confocal z-stacks in figures 1 and 4 were
processed using FIJI (ImageJ) software. All images were acquired at the same framerate format 1024x1024.

Ibrutinib treatment in bioreactor. After 72h of 3D dynamic culture in RCCS™ bioreactor, supernatants were withdrawn from the vessels and centrifuged at 1500 RPM for 5 min. Recovered cells were counted. Clarified supernatants were put again in the vessels with or without 1 and 10μM ibrutinib. Cultures were stopped after 5h of treatment and cells in the supernatants and in the scaffolds were recovered and submitted to the above-mentioned analyses. Ibrutinib-driven mobilization of CLL cells from the scaffolds was calculated with the following formula:

\[
(Mobilized \text{ cells} = \text{cells outside the scaffold with } ibrutinib - \text{cells outside the scaffold UNTREATED}) \text{ each sample is normalized for the total amount of seeded cells}
\]

ELISA. We collected the culture medium of the cells co-cultured in the bioreactor and the plasma from the PB of the CLL patient and we used them to measure the quantity of soluble SDF-1. We used Human CXCL12/SDF1-α Quantikine Elisa Kit (R&D Systems) and followed the manufacturer’s instructions. Read wavelength was 450nm, whereas correction wavelength was set at 540nm. Plates were read with iMark Microplate Reader (Biorad).

Flow Cytometry. Flow Cytometry analysis was performed on the cells outside the scaffold and inside the scaffold, in this case after digestion with 500μL liberase (Roche) (25μg/ml) for 15min at 37°C, we next stopped the reaction with 500 μL of FBS (euroclone). We washed the cells with 3mL PBS (euroclone) and centrifuged at 1500rpm for 5min. We next added the antibodies: CD19 PEVio770 (2 μL), CD5 PE (1μL), CXCR4 VioBright FITC (2μL) (Miltenyi Biotech). We incubated for 20min at room temperature in the dark. We next washed with 3 mL PBS and resuspended the sample in 500 μL PBS. We read the sample at the flow cytometer (Navios; Beckman Coulter). Data were analysed by FCS express software.

Cell line genotyping. 10 ng of DNA of MEC1 CNTR, MEC1 HS1-KD and HS5 were purified with QiAmp DNA Mini Kit (Qiagen) and amplified through PCR with GenePrint ® 10 System (Qiagen) and sold Eurofins Genomics Standard FLA Service to perform genotyping. Data was analysed with DSMZ Online STR Analysis (https://www.dsmz.de/services/services-human-and-animal-cell-lines/online-str-analysis.html). We confirmed the identity of all the cell lines analysed. MEC1 CNTR cells are reported in supplementary figure 4.
**Statistical analysis.** Student’s t-test was performed for statistical analysis. Mann-Whitney unpaired t test and Wilcoxon signed paired t test were used for non-parametric comparisons of data sets (*p ≤0.05; **p ≤0.01; ***p ≤0.001; ****p ≤0.0001).
Supplementary figures

Supplementary Figure 1. **a)** Flow cytometry analysis shows the presence of the leukemic clone based on the expression of CD19 and CD5 expression, both outside (upper panel) and inside (lower panel) the scaffold after 72h in bioreactor. The red circle shows the presence of HS5 cells distinguishable by size from CLL cells inside the scaffold and the blue circle shows the lymphocytes present in both fractions. **b)** CXCR4 mean fluorescent intensity (MFI) measured by flow cytometry analysis on CLL cells (gate on CD19 and CD5 expressing cells) outside and inside the scaffold (patients n=3, p=0.03). **c)** CXCR4 mRNA expression levels in primary CLL cells isolated from the PB and the BM of patients (n=9) and determined by RT-qPCR (**p=0.003).
Supplementary Figure 2. a) The histogram plot shows the total number of HS5 cells recovered in the medium outside the scaffold after 72h of dynamic culture in bioreactor with or without Ibrutinib treatment. b) HS1 expression levels determined by RT-qPCR in CLL cells retained inside the scaffold; CLL cells were obtained from 7 patients before and after Ibrutinib treatment. c) RT-qPCR (left panel) and flow cytometry analysis (right panel) of CXCR4 expression levels in CLL cells derived from patients' PB and retained inside the scaffolds (n=4) before and after Ibrutinib treatment (p=0.03). d) CXCL12 quantification by Elisa. e) CLL cells mobilized from the scaffold after 5h exposition to ibrutinib (1µM and 10 µM), (n=3).
Supplementary Figure 3. PB was collected at 1/2/3/4 and 8/12 weeks after drug treatment, PB lymphocyte (LY) count (LY) was determined at each time point with an hemocytometer and CLL cells isolated and stored frozen at -80°C. For each patient sample, we plotted in the graph the densitometry quantification of HS1 by WB, HS1 expression determined by RT-qPCR and the number of lymphocytes in the PB. The values are normalized to the basal level (n=8 patients).
Supplementary Figure 4. Genotyping analysis of DNA Short Tandem Repeats (STR) by DSMZ Online STR Analysis software. Comparison to validate DNA MEC1 CNTR is shown.

Supplementary movie 1-2. Representative 3D-rendering of a confocal z-stacks showing MEC1-CNTR cells retained into scaffolds after 72h co-culturing with HS5 cells in the presence (movie1) or in the absence of Ibrutinib (movie2) treatment.
**Supplementary Table 1.** Clinical and biological characteristics of the patients used for the experiments reported in the manuscript (n= 63). Samples were collected from patients with stable or progressive disease course. For those who received treatment with Ibrutinib response to treatment is reported in the last column (PR: partial response; PD: progressive disease; CR: complete response). NA=not available.

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*Note: CR: complete response.*