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

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ABSTRACT

Chronic lymphocytic leukemia (CLL) cells disseminate into supportive tissue microenvironments. To investigate the mechanisms involved in leukemic cell tissue retention we developed a three-dimensional bone marrow (BM) microenvironment that recreates the interactions between CLL and BM stromal cells 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 downregulate 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 than in CLL cells circulating in the peripheral blood. Moreover, we demonstrate that CLL cells with inactive HS1, impaired cytoskeletal activity and a more aggressive phenotype are more likely to be 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 two-dimensional 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 three-dimensional model described herein is suitable for reproducing and identifying critical CLL-BM interactions, opening the way to pathophysiological studies and the evaluation of novel targeted therapies in an individualized manner.

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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 so-called “proliferation centers”,

mainly found in the lymph nodes, and/or sheltered in vaguely characterized niches in the BM.³⁻⁵ CLL cells accumulating within 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 host tissues in CLL which conceivably contribute to disease progression and ultimately to treatment resistance.^{3,5,6}

Cytoskeleton regulation is clearly implicated in the dynamic behavior of CLL cells, contributing to the homing and trafficking in and out of 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 signaling pathway and cytoskeletal activity in CLL, while also having prognostic implications, with the active and inactive forms of HS1 correlating with a favorable or adverse prognosis, respectively.⁷⁻⁹ In parallel, we demonstrated that downregulation of HS1 expression interferes with secondary lymphoid organ (lymph nodes 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.¹²⁻¹⁴ 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 lymph node-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 happening to human CLL cells in the tissues.

In order to partially overcome this limitation, we have exploited, and adapted to CLL, a three-dimensional (3D) co-culture model, already thoroughly validated for multiple myeloma, which 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. This combination enables reciprocal interactions to be established 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 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

Study subjects and ethics statement

Patients with CLL were diagnosed according to the updated National Cancer Institute Working Group guidelines.²⁰ 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 “San Raffaele” Hospital ethics committee under the protocol VIVI-CLL entitled: “*In vivo* and *in vitro* characterization on CLL”; and the CERTH ethics committee in response to the application entitled “Molecular and functional studies of B cell malignancies”.

The clinical and biological characteristics of the patients with CLL who provided samples for the experiments are reported in *Online Supplementary Table S1*.

Scaffold preparation

Scaffolds were populated as described by Belloni *et al.*¹⁹ 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 plates (Greiner bio-one, Germany). Scaffolds were then transferred to 10 mL High Aspect Ratio Vessels (HARV) in 1 mL TCM (DMEM culture medium supplemented with 10% v/v fetal bovine serum) and cultured overnight in the RCCS™ bioreactor at the lower speed (rpm). Twenty-four hours later, CLL cells were added to the vessels, using the optimal ratio of CLL cells to stromal cells established in preliminary experiments (MEC1 cells=2x10⁶, primary CLL cells=3x10⁶). After 5 h, vessels were filled with growth medium (RPMI1640 culture medium supplemented with 10% v/v or 20% fetal bovine serum for MEC1 or primary CLL cells, respectively). At the end of the culture period, cells outside and inside the scaffold were recovered from the scaffolds by means of liberase (Roche) (25 µg/mL) treatment for further analysis (see *Online Supplementary Methods*). The cells outside and inside the scaffold were counted using the trypan blue exclusion test for viability, which showed that more than 90% of the cells were viable. Alternatively, scaffolds were formalin-fixed for IF or lysed with 100 µL RIPA buffer for western blotting analysis (see *Online Supplementary Methods*).

Bioreactor RCCS™

The 3-D dynamic culture was performed using the RCCS™ bioreactor RCCS-4DQ equipped with four rotating 10 mL-HARV culture vessels, which work as culture chambers (Synthecon Inc., USA).¹⁹

Vessels are provided with a gas exchange membrane made of silicon rubber, which allows optimal diffusion of O₂. The bioreactor was kept inside an incubator, in a humidified atmosphere, at 37°C with 95% air and 5% CO₂. During the experimental proce-

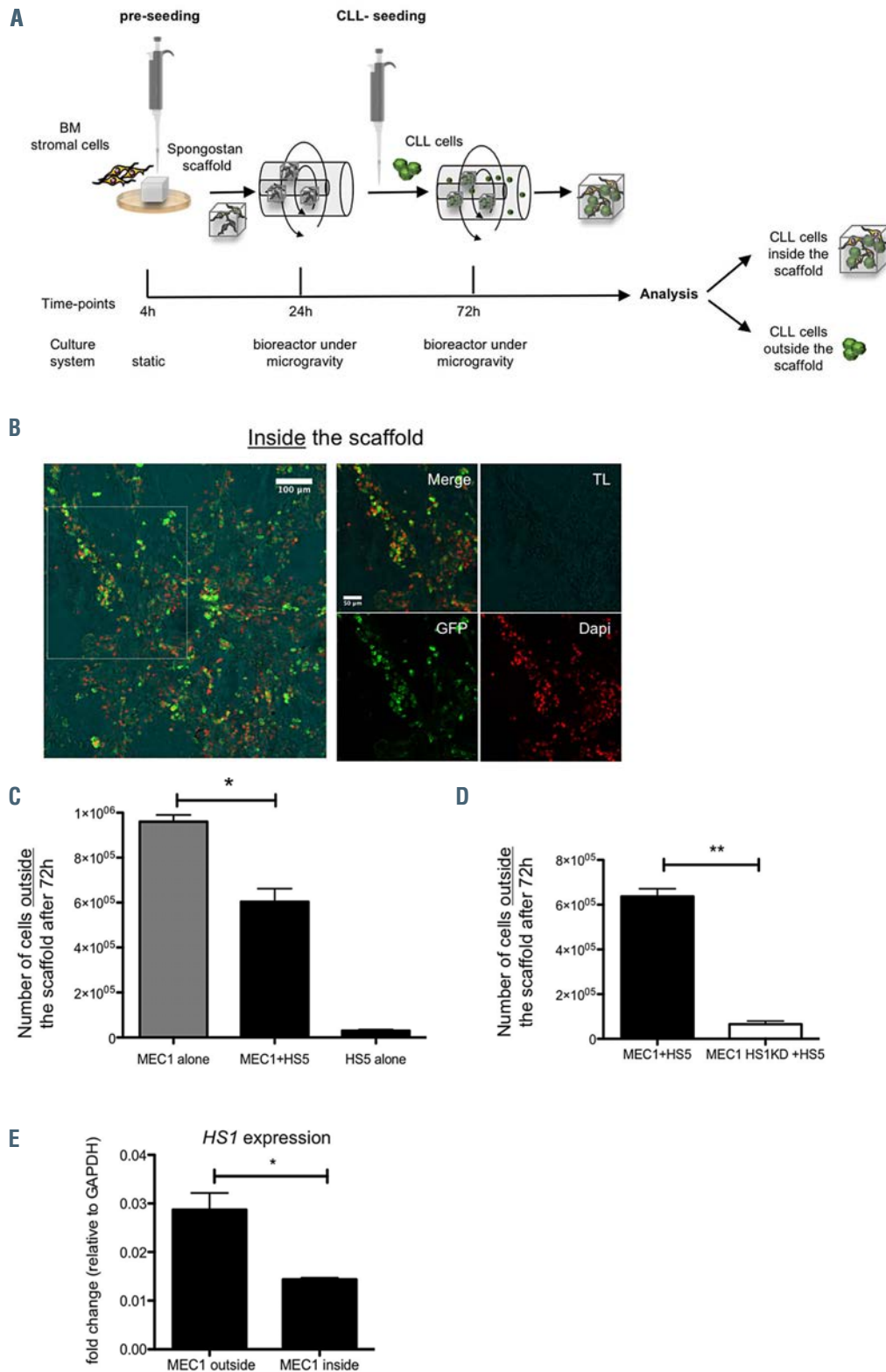


Figure 1. Optimization of the 3D model. (A) Schematic representation of the experimental setup: HS5 cells were seeded into 3D Spongostan scaffolds and cultured under microgravity in a RCCSTM bioreactor for 24 h. MEC1 cells or primary CLL cells were added to the same scaffold and co-cultured for 72 h under microgravity. At the end of the incubation period, cells and scaffolds were collected and analyzed. (B) Representative confocal section taken from an X,Y,Z-stack of a representative scaffold, after 72 h of MEC1-GFP culture in the bioreactor. MEC1 cells were distinguished from HS5 cells by their smaller size. Each experiment was run in triplicate. MEC1 cells were significantly retained into the scaffold only in the presence of HS5 cells (* $P=0.01$). (D) The graph shows the total number of cells recovered from the medium outside the scaffold after 72 h of dynamic culture in the bioreactor of MEC1 control cells and MEC1-HS1KD cells: the latter were retained in the scaffold more than MEC1 control cells (** $P=0.0013$). (E) Evaluation of *HS1* expression by quantitative real-time polymerase chain reaction analysis in MEC1 control cells inside and outside the scaffolds (experiment run in triplicate) showing that *HS1* is down-regulated inside the scaffolds (* $P=0.028$).

dures, the operational conditions of the RCCS™ were set and constantly monitored in order to keep the samples in a “free fall” condition, which minimizes sedimentation of the scaffold while maximizing mass transfer and cell viability for the extended culture period.

Ibrutinib treatment in the bioreactor

After 72 h of 3D dynamic culture in the bioreactor, supernatants were withdrawn from the vessels and centrifuged at 1,500 rpm for 5 min. Recovered cells were counted. Clarified supernatants were put into the vessels again, with or without 10 μ M ibrutinib. We compared two different concentrations of ibrutinib (1 and 10 μ M) to exclude a possible role of cell apoptosis in the mobilization from the scaffold, due to the possible increased toxicity of ibrutinib at the higher concentration, and did not observe any significant differences (Online Supplementary Figure S2E). Cultures were stopped after 5 h of treatment and cells in the supernatants and in the scaffolds were recovered and submitted to the above mentioned analysis (see Online Supplementary Methods).

Results

Microenvironmental elements are required to establish a 3D culture bone marrow model for chronic lymphocytic leukemia

We customized a new 3D co-culture model, previously validated by our group for myeloma cell survival¹⁹ 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, which has an ultra-structure similar to the trabecular structure of BM, also because of their superior performance in supporting CLL cell retention 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 for 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

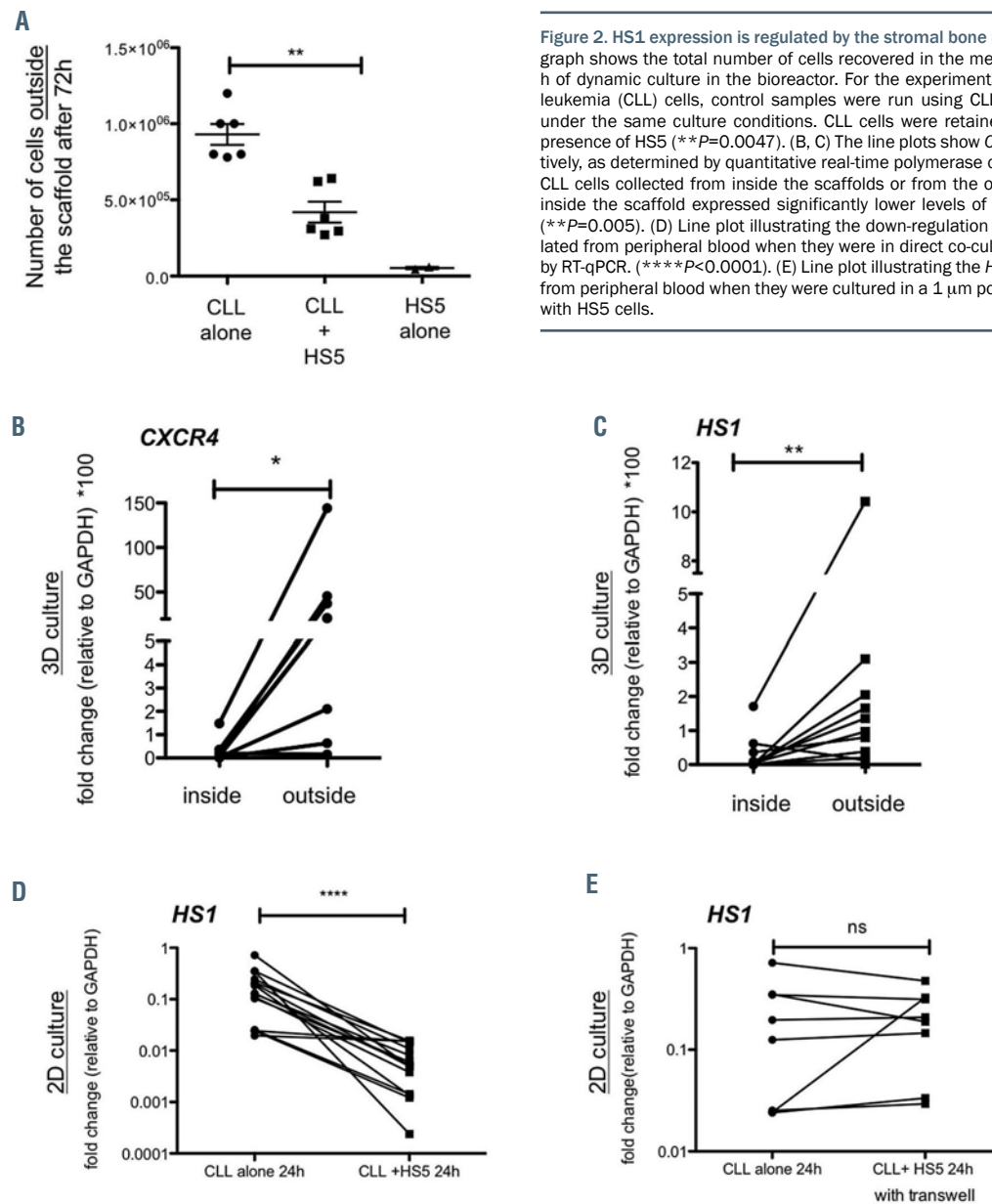


Figure 2. *HS1* expression is regulated by the stromal bone marrow microenvironment. (A) The graph shows the total number of cells recovered in the medium outside the scaffold after 72 h of dynamic culture in the bioreactor. For the experiments with primary chronic lymphocytic leukemia (CLL) cells, control samples were run using CLL cells alone and HS5 cells alone under the same culture conditions. CLL cells were retained inside the scaffolds only in the presence of HS5 (** $P=0.0047$). (B, C) The line plots show *CXCR4* and *HS1* expression, respectively, as determined by quantitative real-time polymerase chain reaction (RT-qPCR) in primary CLL cells collected from inside the scaffolds or from the outside medium. CLL cells retained inside the scaffold expressed significantly lower levels of *CXCR4* ($n=8$; * $P=0.015$) and *HS1* (** $P=0.005$). (D) Line plot illustrating the down-regulation of *HS1* expression in CLL cells isolated from peripheral blood when they were in direct co-culture with HS5 cells, as determined by RT-qPCR. (**** $P<0.0001$). (E) Line plot illustrating the *HS1* expression in CLL cells isolated from peripheral blood when they were cultured in a 1 μ m pore trans-well without direct contact with HS5 cells.

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 parallel analysis of CLL cells inside and outside the scaffold, revealing that HS5 cells were needed in order to retain MEC1 cells

efficiently within the scaffold (Figure 1C).

Next, we used MEC1 cells genetically modified to downregulate HS1 expression (MEC1-HS1KD), already known to display increased BM homing capacity *in vivo* in a CLL xenograft model,⁹ and tested whether this could be reproduced in our 3D *ex-vivo* model. We co-cultured in 3D either

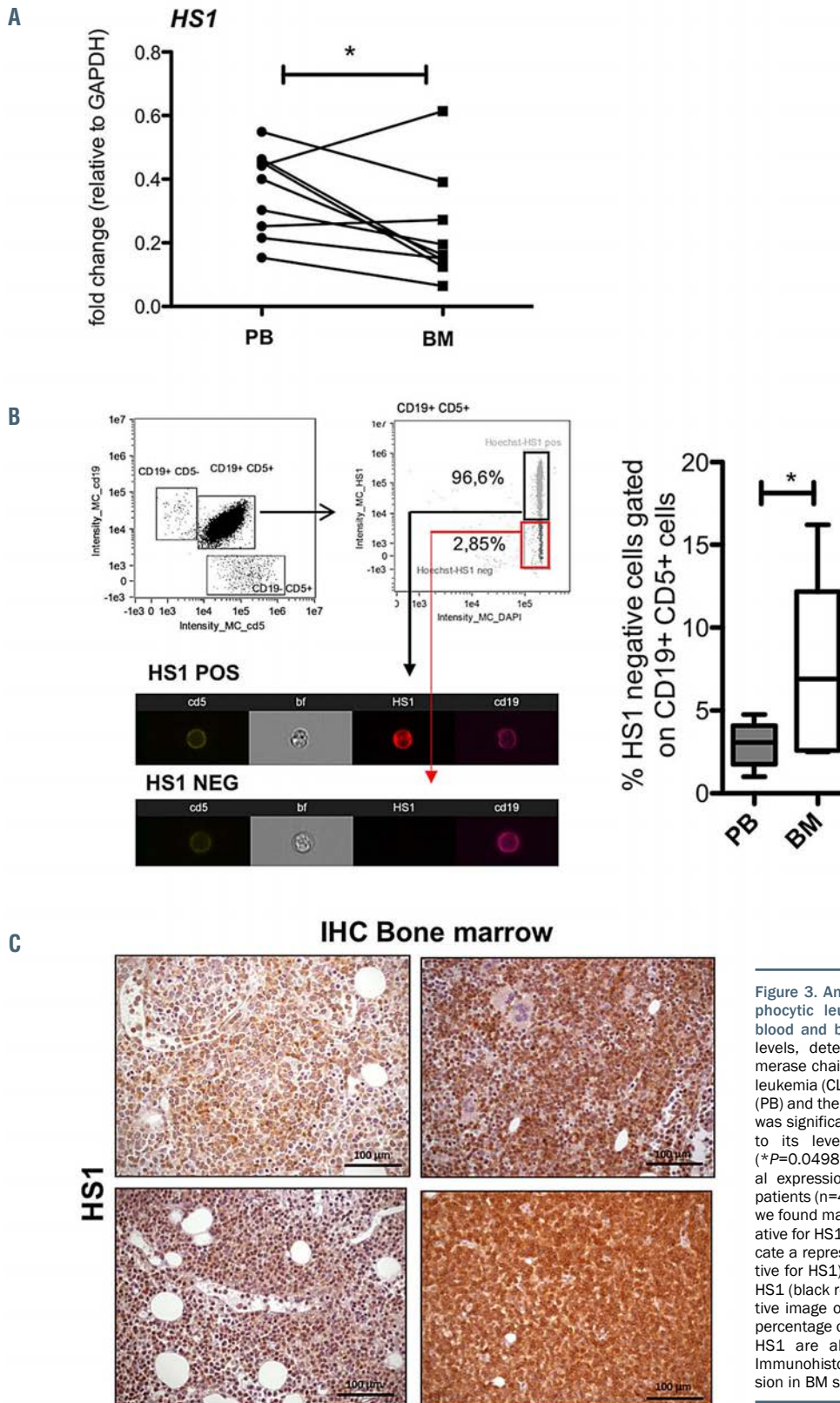


Figure 3. Analysis of HS1 expression in chronic lymphocytic leukemia cells isolated from peripheral blood and bone marrow. (A) *HS1* mRNA expression levels, determined by quantitative real-time polymerase chain reaction in primary chronic lymphocytic leukemia (CLL) cells isolated from the peripheral blood (PB) and the bone marrow (BM) of patients (n=9). *HS1* was significantly downregulated in the BM, compared to its levels in the PB of the same patient (**P*=0.0498). (B) Image Stream analysis of intra-clonal expression of *HS1* in PB versus 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 of 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). Immunohistochemical (IHC) analysis of *HS1* expression in BM sections from CLL patients (n=4)

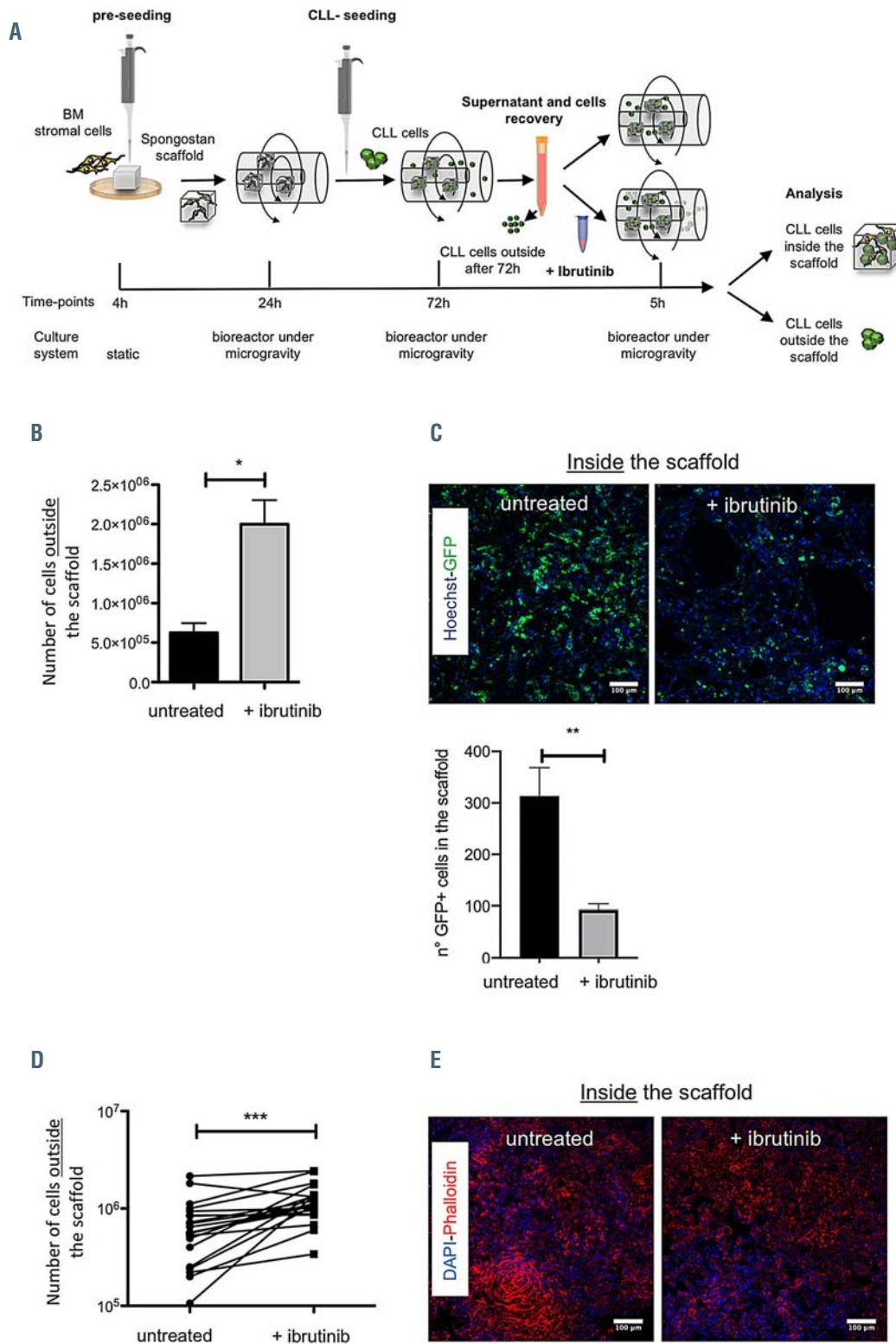


Figure 4. Ibrutinib treatment in 3D co-culture. (A) Schematic representation of the experimental setup: HS5 cells were seeded into 3D Spongostan scaffolds and cultured under microgravity in a RCCS™ bioreactor for 24 h. MEC1 cells or primary chronic lymphocytic leukemia (CLL) cells were added to the same scaffold and co-cultured for 72 h under microgravity. At the end of the incubation period, the supernatant was collected and depleted of cells. The same supernatant was added again to the culture with or without ibrutinib for 5 h. At the end of the incubation period the scaffolds were collected and analyzed. (B) The histogram plot shows the total number of cells (MEC1-GFP⁺ HS5) that migrated outside the scaffold after 72 h of dynamic culture in the bioreactor in the presence of 10 μ M ibrutinib (for 5 h) or RPMI medium only (untreated). MEC1 cells were significantly mobilized (* $P=0.02$) from the scaffolds. (C) On the left, representative confocal images of the scaffolds analyzed in panel (B), and on the right the histogram showing the mean number of MEC1 GFP⁺ cells quantified in the scaffold by counting the GFP⁺ cells in four different stacks for both treated and untreated conditions ($P=0.002$). (D) Line plot illustrating the effect of 10 μ M ibrutinib treatment on the mobility of primary peripheral blood-derived CLL cells that were recovered outside the scaffold (** $P=0.0005$). (E) Representative confocal images of examples of the scaffolds analyzed in panel (D).

unmodified control cells (MEC1-CNTR) or MEC1-HS1KD cells with HS5 stromal cells as described above (Figure 1A) and observed that, outside the scaffolds, there were significantly fewer MEC1-HS1KD cells than MEC1-CNTR cells ($P=0.0013$) (Figure 1D). We then used quantitative real-time polymerase chain reaction (RT-qPCR) analysis to quantify the expression of *HS1* in MEC1-CNTR cells cultured in 3D and found that *HS1* was downregulated in MEC1 cells retained inside the scaffold compared to its level in 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 downregulation as a putative mechanism associated with CLL cell retention in the BM microenvironment, as previously suggested in mouse models.⁹

The bone marrow microenvironment regulates HS1 expression in primary chronic lymphocytic leukemia cells in 3D co-culture

We then co-cultured primary leukemic CLL cells isolated from the PB of six 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 (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 (Online Supplementary Figure S1A).

We then quantified the expression of *CXCR4* by RT-qPCR and flow cytometry in CLL cells recovered from inside and outside the scaffolds after 3 days of co-culture with HS5 cells and found that *CXCR4* was downregulated in the cells retained inside the scaffold ($n=8$, $P=0.015$ Figure 2B; $n=3$, $P=0.03$, Online Supplementary Figure 1C), mimicking the *in vivo* finding of *CXCR4* downregulation in response to the binding of its cognate ligand CXCL12 (SDF-1 α).²¹ Next, we focused again on the *HS1* gene and, similar to the findings in MEC1 cells (Figure 1E), we observed a sig-

nificant downregulation of *HS1* in the CLL fraction inside the scaffolds as compared to the outside fraction ($n=8$ replicates, $P=0.005$) (Figure 2C). 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 ten patients with the stromal cell line HS5 in two-dimensional (2D) monolayers, and evaluated the expression of *HS1* by RT-qPCR. *HS1* expression was significantly downregulated after 24 h of co-culture ($n=15$, $P<0.0001$) (Figure 2D). We then co-cultured CLL primary cells from the PB of eight patients with HS5 cells in the presence and absence of a trans-well (1 μm pore) to avoid direct tumor-stroma contact. *HS1* expression was not downregulated in the presence of the trans-well (Figure 2E), suggesting that its regulation requires direct contact between leukemic cells and the BM stromal microenvironment.

HS1 is heterogeneously expressed in chronic lymphocytic leukemia tissues

The results described above suggest that HS1 might be differentially expressed in CLL depending on the tissue in which the leukemic cells are located. To assess whether our 3D model recapitulates what occurs *in vivo*, we compared *HS1* expression in primary human CLL cells from PB and BM. Using RT-qPCR, we observed that *HS1* expression was significantly downregulated in CLL cells isolated from the BM as compared with its expression in paired samples isolated from the PB ($n=9$, $P=0.0498$) (Figure 3A). In the same cohort of patients, we also confirmed that *CXCR4* expression was downregulated in the BM as compared to the PB ($n=10$, $P=0.003$) (Online Supplementary Figure S1B). When we quantified HS1 expression at a 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 ($n=4$, $P=0.0187$) (Figure 3B). Accordingly, immunohistochemistry performed on BM ($n=4$ patients analyzed) revealed a het-

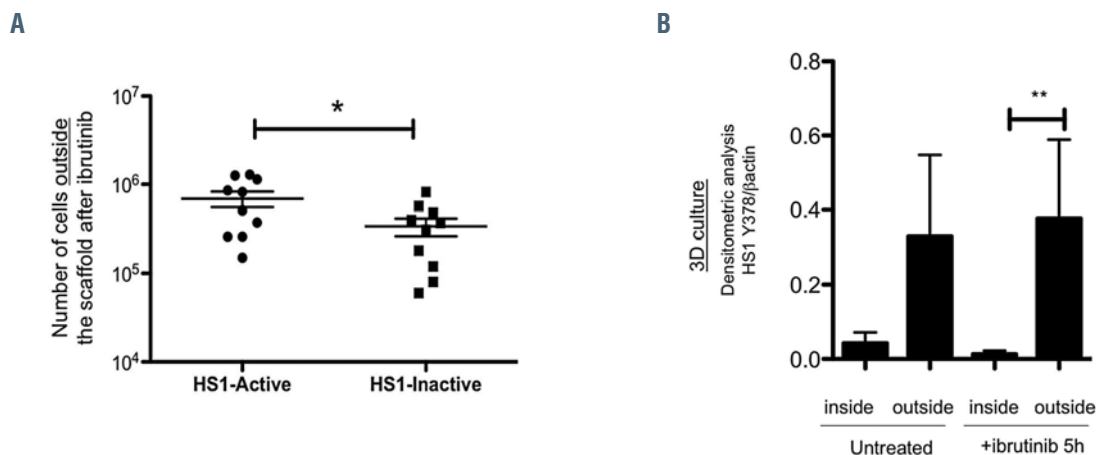


Figure 5. *HS1* activation following ibrutinib treatment. (A) Dot plot showing the number of chronic lymphocytic leukemia (CLL) cells mobilized outside the scaffold after ibrutinib treatment and fractionated according to *HS1* activation. CLL cells with active *HS1* were mobilized more efficiently from the scaffolds compared to those with inactive *HS1* ($*P=0.04$). (B) The graph shows the densitometric analysis of the active *HS1*-Y378 phosphorylated form in CLL cells retained inside the scaffold or recovered from outside the scaffolds with or without ibrutinib treatment. The CLL cells mobilized and recovered outside the scaffold show significantly higher *HS1* activation than that of the cells retained inside the scaffold ($**P=0.0035$).

erogeneous pattern of HS1 expression among the patients (Figure 3C). This finding confirms that our 3D model can reliably reproduce the native BM microenvironment and provide significant insights into CLL cells in the tissues.

Chronic lymphocytic leukemia cells are mobilized from the scaffolds following exposure to ibrutinib

On the basis of the evidence presented here, HS1 appears to be involved in CLL cell compartmentalization, prompting the question of 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 also plays a role in the CLL cell mobilization promoted by the BTK inhibitor ibrutinib.¹⁶ MEC1 cells co-cultured with HS5 cells within scaffolds

were efficiently mobilized upon 5 h of treatment with ibrutinib, 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 significantly fewer GFP-tagged MEC1 cells in the untreated condition ($P=0.002$) (Figure 4C, *Online Supplementary Movies 1-2*). Of note, HS5 cells were not mobilized by the drug (*Online Supplementary Figure S2A*).

We next studied the response of primary CLL samples ($n=21$) to ibrutinib and found that the number of cells outside the scaffolds was significantly higher upon drug treatment than the number in untreated samples ($n=21$, $P=0.0005$) (Figure 4D). Consistent with this, confocal microscopy analysis performed on the scaffolds showed that they were depopulated of CLL cells after incubation with ibrutinib (Figure 4E).

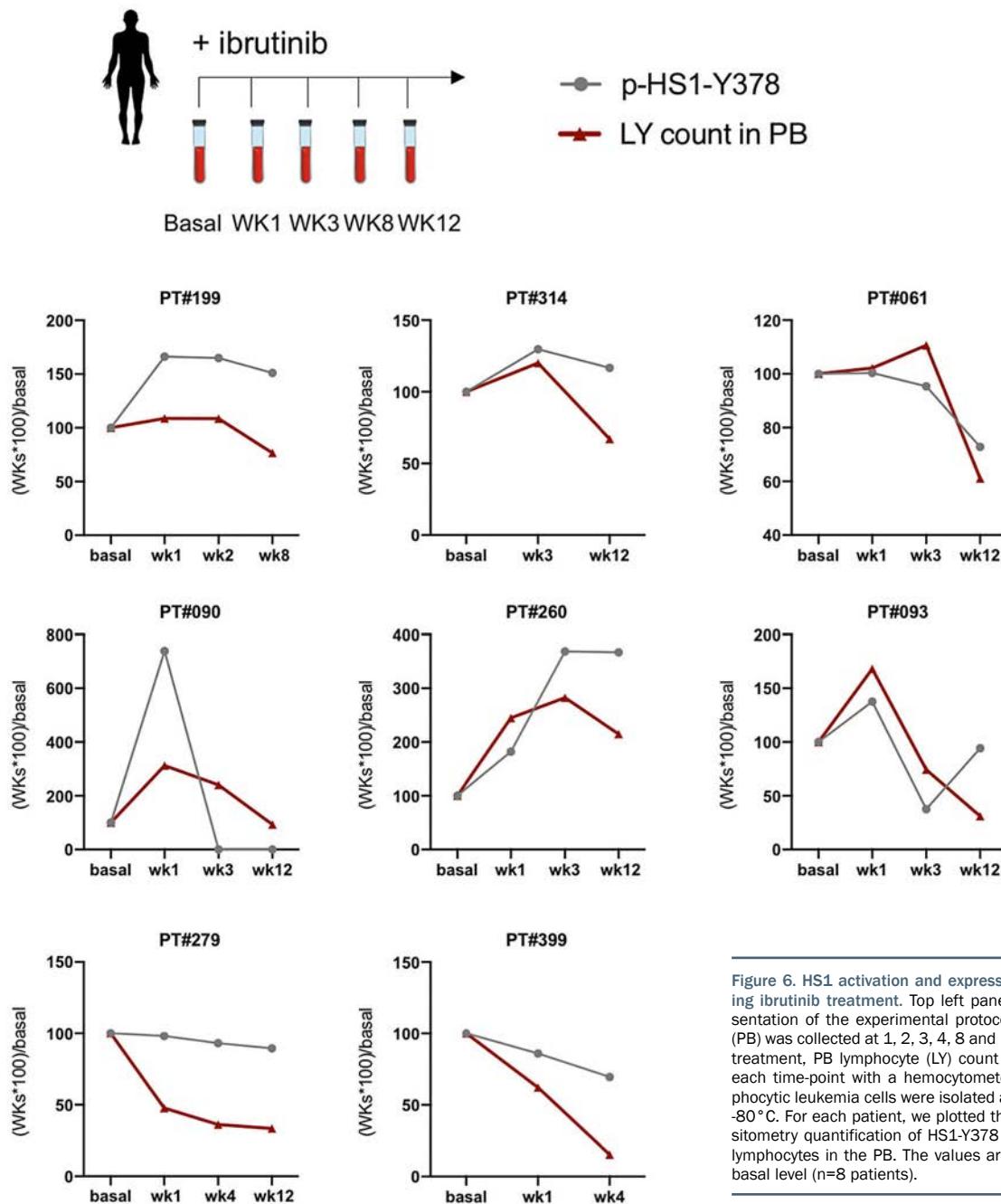


Figure 6. HS1 activation and expression in patients during ibrutinib treatment. Top left panel: schematic representation of the experimental protocol. Peripheral blood (PB) was collected at 1, 2, 3, 4, 8 and 12 weeks after drug treatment, PB lymphocyte (LY) count was determined at each time-point with a hemocytometer and chronic lymphocytic leukemia cells were isolated and stored frozen at -80°C . For each patient, we plotted the western blot densitometry quantification of HS1-Y378 and the number of lymphocytes in the PB. The values are normalized to the basal level ($n=8$ patients).

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 (Online Supplementary Figure S2B). Conversely, CXCR4 expression on CLL cells inside the scaffold decreased following exposure to ibrutinib, confirming previous *in vivo* results from Chen *et al.*¹⁷ ($P=0.03$) (Online Supplementary Figure S2C). In parallel, we observed that CXCL12 levels in the medium did not change significantly during the drug treatment (Online Supplementary Figure S2D).

Cells with inactive HS1 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 with the clinical course (active HS1 is associated with a favorable prognosis while inactive HS1 is associated with an adverse prognosis).¹⁰ CLL cells with active HS1 (carrying HS1 phosphorylated in Y378, as determined by western blot analysis; *data not shown*) show efficient cytoskeletal functionality, while CLL cells with inactive HS1 (not phosphorylated in HS1-Y378) display reduced cytoskeletal activity associated with a higher propensity to accumulate within the BM microenvironment.¹⁰ We detected that both CLL cells with active HS1 and those with inactive HS1 were capable of homing to 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) ($P=0.04$) (Figure 5A). Similarly, when we studied, in the same co-culture model, HS1 activation, i.e. the levels of HS1-Y378 determined by western blot, in primary CLL cells ($n=7$) exposed or not to ibrutinib, we observed that the levels of HS1-Y378 were higher in the cells released into the supernatant than in those retained inside the scaffolds ($n=7$, $P=0.0035$) (Figure 5B). These results demonstrate that more aggressive CLL cells (i.e., those with 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, mediates changes in HS1 activation. Arguably, therefore, our 3D model may discriminate between cases that will respond more robustly to the CLL cell-mobilizing effect of ibrutinib.

Chronic lymphocytic leukemia cells mobilized into the peripheral blood by ibrutinib show active HS1 during the first weeks of treatment

Finally, we analyzed HS1 activation in CLL cells of patients ($n=8$) under ibrutinib treatment for different periods (from week 1 to week 12) and correlated it with the lymphocyte count in 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 six of the eight patients displayed HS1 activation during the first weeks (weeks 1, 2, and 3) of treatment as compared to the basal level (Figure 6A), in parallel with an increase in lymphocyte count in the PB. In contrast, peripheral lymphocytosis was not seen in the two patients in whom HS1 did not undergo activation. In parallel, we analyzed HS1 expression in CLL cells by both western blotting and RT-qPCR and

found that HS1 expression increased during the first weeks of treatment in all patients analyzed either at the protein or at the gene level; however, we could not find a correlation with circulating lymphocyte count (Online Supplementary Figure S3A). Collectively, 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, in particular thanks to new targeted therapies,²³ although the disease is still incurable. CLL is strongly influenced by the tissue microenvironment, as evidenced by the fact that circulating CLL cells are more sensitive to drug-induced apoptosis, suggesting that a supportive microenvironment is necessary for the survival of leukemic cells. This has encouraged the development of mobilizing agents²⁴ 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 a native CLL tissue microenvironment *in vitro*, which is so far unavailable. Our previous studies pointed to the importance of the BM microenvironment, showing specific homing of aggressive CLL (with inactive HS1) to this site *in vivo* in mouse models.⁹ For this reason, following our previously published experience from the analysis of multiple myeloma cell survival and response to borte-

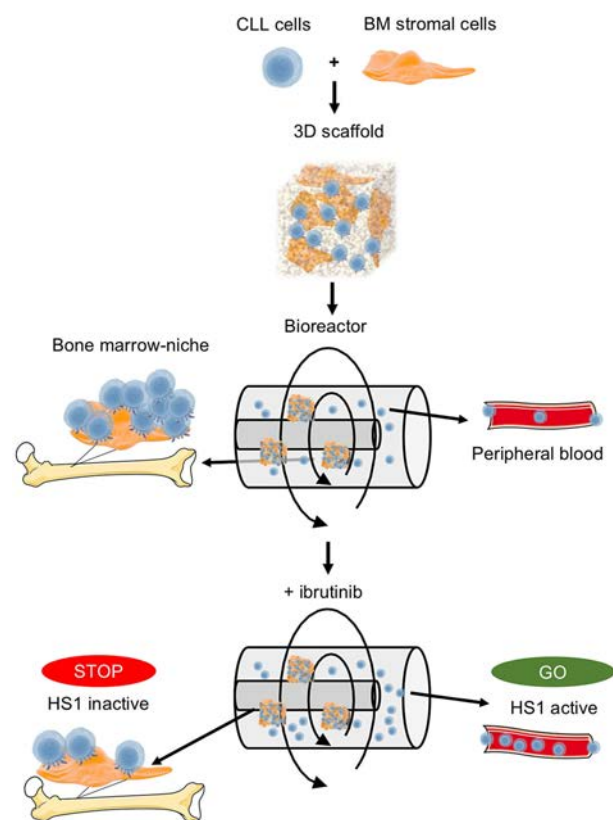


Figure 7. Schematic summary of the presented model. CLL: chronic lymphocytic leukemia; BM: bone marrow.

zomib,¹⁹ we customized, for CLL, a 3D BM model based on scaffolds within a bioreactor. The model allows us to analyze in parallel CLL cells retained inside the reconstructed BM microenvironment and those recovered from 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 types of cells 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 does indeed mirror 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 lymph nodes than from the BM, suggesting a specific tissue-dependent effect.²⁵ Taking advantage of our 3D BM model, we here provide evidence that CLL cells mobilized from the scaffolds upon exposure to ibrutinib 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 affects HS1 activation on CLL cells directly or indirectly in 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 to 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 to understand the slower clearance of the BM in patients.²⁵ In conclusion, we here present and validate a reproducible

3D BM model to aid understanding of the mechanisms underlying CLL tissue retention and mobilization, but also capable of predicting patient-specific efficacy of CLL mobilizing agents,²³ as schematically summarized in Figure 7. 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 than the traditional 2D models from which 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 lymph nodes.

Disclosures

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Contributions

CS wrote the manuscript. CS, FB, DB, FVS and LP performed the experiments and analyzed the data. LS, PG and KS provided patients' and clinical information. MP and LB performed immunohistochemistry. DZ performed image stream analysis. VRC helped in the microscopy. EF, VRC, PG, MP and KS revised the manuscript.

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