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

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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.

10. 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’-diaminobenzidine 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 RCCSTM 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:

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

**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 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.

| Patient | Provenance | Sample | Application | Rat | Moat | CD38 | tHCV | FISH | Clinical course | Response Status |
|---------|------------|--------|-------------|-----|------|------|------|------|----------------|----------------|
| 1 | CD38 | CD38 | NA | A | 6.22 | 0 | B | 56.53 | negative, complete decay | STRA10 | NA | NA |
| 2 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 3 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 4 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 5 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 6 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 7 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 8 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 9 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 10 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 11 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 12 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 13 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 14 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 15 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 16 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 17 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 18 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 19 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 20 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 21 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 22 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 23 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 24 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |
| 25 | CD38 | CD38 | NA | A | 6.0 | 6 | B | 68.0 | negative, complete decay | STRA10 | NA | NA |

NA=not available.