Expression and function of cathelicidin hCAP18/LL-37 in chronic lymphocytic leukemia

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SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies.

RPMI 1640, fetal calf serum (FCS), penicillin, trypsin and streptomycin were purchased from GIBCO. The Ficoll-Hypaque Plus used for cell separation was purchased from Amersham. MACS B and B-CLL cell isolation kits were purchased from Miltenyi Biotec. Bovine serum albumin (BSA) used for cell staining buffer was obtained from Weiner Laboratorios. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Venetoclax (ABT-199) and fludarabine phosphate were purchased from MedKoo Biosciences, Inc. Annexin-V FITC and propidium iodide (PI) were purchased from BD Bioscience, Pharmingen. Purified anti-CD3 (clone UCHT1) and PC5 conjugated mAb specific for CD19 (clone J3-119) were obtained from Beckman Coulter. Two different anti- Hcap18/LL-37 antibodies were used: purified anti-CAP 18 (mouse mAb, clone H7) from BioLegend and purified anti-LL-37 (rabbit polyclonal IgG) from Santa Cruz Biotechnology. Mouse anti-LL-37 mAb was conjugated with PerCP-Cy5.5 using Lightning-link PerCP-Cy 5.5 Tandem Conjugation kit from Innova Biosciences. Purified anti-CXCR4 (mouse mAb, clone 12G5), PE-conjugated anti-CXCR4 (clone 12G5), anti-CD20 (mouse mAb, clone 2H7) and antibodies with irrelevant specificities (isotype control) were purchased from BioLegend. Anti-CD68 (mouse mAb, clone PGM1) was purchased from DAKO. Anti-human IgM (Fab´2 fragments) and all the secondary antibodies were obtained from Jackson ImmunoResearch. Human IL-4, IL-15 and CD40L were purchased from BioLegend. Human CXCL12 was purchased from Peprotech. CpG- ODN 2006 and the synthetic LL-37 peptide were purchased from Invivogen. Human LL-37 ELISA kit was purchased from Hycult Biotech. WRW4 was from Phoenix Pharmaceuticals. Aqua-Poly/Mount coverslipping medium was purchased from Polysciences (Warrington, PA, USA). Red alkaline phosphatase (Red AP) substrate kit and DAB peroxidase substrate kit were purchased from Vector Laboratories. Unless otherwise stated, all the chemicals employed were from SIGMA-Aldrich. Cleaved caspase-3 (Asp175) rabbit mAb was purchased from Cell Signaling (Danvers, MA,USA).

CLL patients and age-matched healthy donors.

This study included 55 CLL patients and 6 age-matched healthy donors (HD). Peripheral blood samples were collected from CLL patients and HD. Bone marrow biopsies were obtained from 5 CLL patients. All samples used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires, Argentina and the Institutional Review Board of Northwell Health, New York, US. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of analysis, all patients were free from clinically relevant infectious complications and either had received no treatment or had not received treatment for ≥3 months before the investigation began. Peripheral blood mononuclear cells (PBMC) from CLL patients and healthy donors (HD) were separated by density gradient centrifugation (Ficoll, GE Healthcare), frozen (10% DMSO, 45% FBS, and 45% RPMI), and stored in liquid nitrogen until used.

Total RNA preparation, cDNA synthesis and qRT-PCR.

Total RNA was extracted from 2 x 10^6 purified B cell from HD-PBMC or 3 x10^6 CLL PBMCs (monocyte depleted samples with more than 98% of leukemic cells) using Qiagen RNeasy mini kit and cDNA was generated by reverse transcription with SuperScript II according to the manufacturer´s instructions. qRT-PCR was performed using SYBR Green PCR Master Mix in 20 µl reactions. Primers were designed using Primer3 software and purchased from Thermo-Fisher Scientifics: β-ACTIN Fw 5´-GAG CGC GGC TAC AGC TTC AC- 3´, β-ACTIN Rv 5´- GTG TAA CGC AAC TAA GTC AT -3´,hCAP18 Fw 5´- GATAACAAGAGATTTGCCCTGCTG-3´, hCAP18 Rv 5´- TTTCTCAGAGCCCAGAA
GCCTG-3´, and were used at a concentration of 250 nM. Reactions were carried out in an Applied Biosystems (ABI) 7900HT Real-Time PCR System from the PCR facility from The Feinstein Institute for Medical Research. The cycling program used was 50 ˚C for 2 minutes, 95˚C for 1 minute, followed by 40 cycles of 95˚C for 15 seconds, 60˚C for 60 seconds. Data were analyzed using β-ACTIN as a reference gene.

CLL cells activation *in vitro*.

CLL cells (2 x 10^6 cells/ml) were cultured in 24 well plate in RPMI 1640 + 10% FCS alone (control) or in the presence of immobilized anti-IgM (0.1 μg/ml), CD40L (500 ng/ml), CpG (5 μg/ml), IL-15 (10ng/ml) or IL-4 (15ng/ml) alone or in different combinations depicted in Fig 1.e. After 48 hours of culture, CLL cells were collected, and activation was confirmed by flow cytometry by evaluating the surface expression of CD69/CD25/CD86 using mAbs anti-CD69-PE, anti-CD86-PE and anti-CD25-FITC or the corresponding isotype control and anti-CD19-PC5. Quantification of mRNA on control and activated CLL-cells was assessed by qRT-PCR, as described above as well as intracellular hCAP18/LL-37 staining using an anti-LL-37- PerCP-Cy5.5. Supernatants of CLL-cultures were collected and the presence of soluble LL-37 was assessed by ELISA.

Bone marrow immunohistochemistry.

Bone marrow biopsies from CLL-patients were stained to assess the presence of hCAP18/LL-37. Samples were doubled-stained in order to determine which cells express LL-37. Staining of LL-37/CD68 and LL-37/CD20 were performed. LL-37 expression was confirmed using Vector Red (Vector Lab) substrate for alkaline phospathase (Red precipitate) while CD68/CD20 expression were observed with DAB substrate (Vector Lab) for peroxidase (Brown precipitate).

Detection of leukemic cell apoptosis.

After their respective treatments, leukemic cells were incubated for 20 min with anti-CD19 at 4°C, washed with PBS and incubated for 30 minutes with AnnexinV-FITC at room temperature. Once the incubation time was completed, apoptosis levels (AnnexinV^+) were recorded by flow cytometry. In addition, activated caspase-3 (cleaved) was measured by intracellular staining. Treated CLL-cells were fixed with PFA 4% during 30 min at room temperature, then washed twice with PBS and permeabilized during 30 min with 0.01% TritonX100-PBS-4% FCS (Triton buffer). After that, cells were incubated with anti-cleaved caspase-3 Ab for 30 min at 4ºC, washed twice with Triton buffer and incubated with anti-rabbit IgG labeled with DyLight-488. Cells were analyzed by flow

cytometry.

CXCR4/LL-37 colocalization analysis.

CLL-cells ($2x10^6$ cells/ml) were incubated with LL37 (5μM) for 30 minutes. Then cells were washed once with PBS and fixed with PFA 4% for 1 hour. After fixation cells were blocked with 5% BSA for 45 minutes. Subsequently cells were washed and incubated with polyclonal rabbit anti-LL37 antibody (Biolegend) for 2 hours. Cells were then washed and incubated with anti-rabbit IgG antibody labeled with DyLight 488 for 2 hours. Then cells were washed twice and incubated with PE-conjugated anti-CXCR4 for 30 minutes. Once the labeling protocol was completed, cells were incubated for 18 hours on slides previously treated with poly-L-lysine and then mounted using Aqua-Polymount. The images were acquired using a confocal microscope Olympus FluoView FV1000.

Migration assays of CLL-cells in response to CXCL12/LL-37.

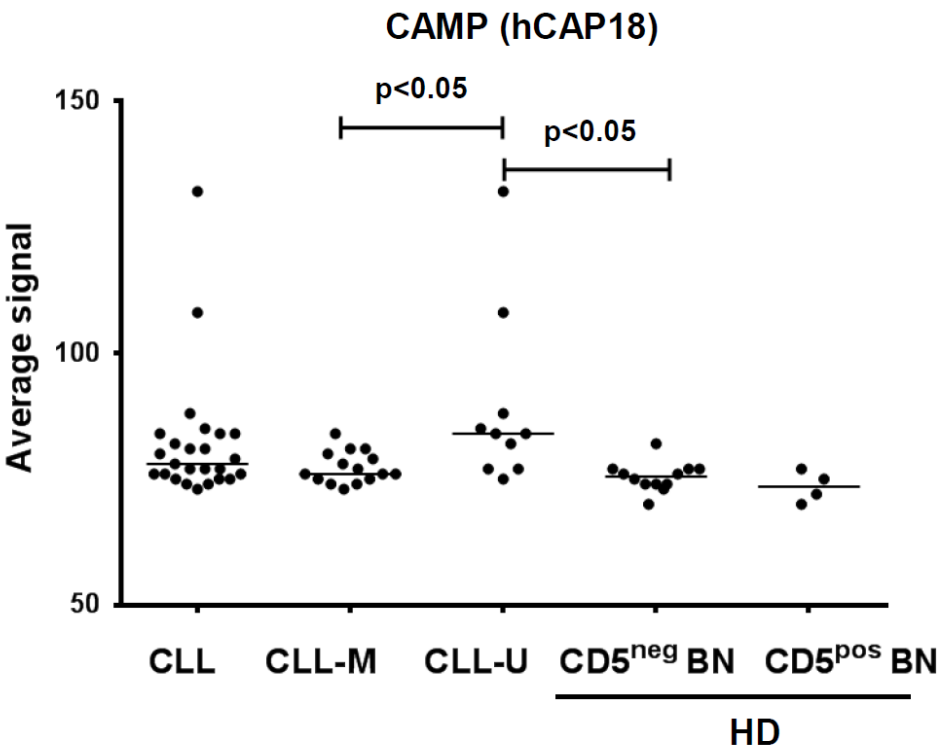
For the chemotaxis assays, transwell plates (Corning Incorporated) of 96 wells, with polycarbonate membranes of 6.5 mm in diameter and pores of 5 μm were used. In the lower compartment, 200 μl of RPMI 1640 medium (1% SFB) was added containing CXCL12 (25ng / ml) with or without LL-37 (5μM) and in the upper chamber leukemic cells (1×10^6) were seeded. As spontaneous migration control the same assay was performed without adding CXCL12 in the lower compartment. Each experimental condition was carried out in duplicate. CLL-cells were incubated at 37 °C for 2 hours. After this time, the cells that migrated to the lower compartment were collected and labeled with anti- CD19 to identify leukemic B cells. Cell counting was performed by flow cytometry determining the number of cells that are acquired in a minute. The migration index was calculated as the number of $CD19^+$ cells that migrated to the lower chamber with CXCL12/LL-37 compared to the number of cells that migrated spontaneously (control without chemokine).

Supplementary table 1.

Patient	Age	Gender	IgVH status	Rai stage
CLL1	81	F	M	0
CLL2	81	M	U	0
CLL3	75	F	M	0
CLL4	58	M	U	I
CLL5	59	M	U	0
CLL6	80	M	M	III
CLL7	78	F	M	II
CLL8	75	M	M	III
CLL9	86	M	U	II
CLL10	58	F	U	I
CLL11	85	F	U	III
CLL12	62	M	M	0
CLL13	67	M	M	IV
CLL14	46	M	M	I
CLL15	58	F	M	I
CLL16	55	F	M	I
CLL17	84	M	M	II
CLL18	53	M	M	II
CLL19	54	M	M	I
CLL20	66	F	U	I
CLL21	81	M	U	III
CLL22	55	F	U	II
CLL23	62	M	M	0
CLL24	59	M	M	I
CLL25	79	F	M	I
CLL26	71	F	U	I
CLL27	58	M	U	I
CLL28	88	F	U	IV
CLL29	70	F	U	0
CLL30	79	F	M	0
CLL31	69	F	U	I
CLL32	87	F	U	0
CLL33	76	M	M	I
CLL34	54	F	M	I
CLL35	39	M	U	0
CLL36	62	F	U	IV
CLL37	44	M	U	II
CLL38	34	F	M	0
CLL39	62	M	U	III
CLL40	66	F	U	II
CLL41	72	M	U	III
CLL42	82	F	M	0
CLL43	67	M	U	II
CLL44	74	M	M	0
CLL45	44	F	U	0
CLL46	85	M	M	IV
CLL47	83	M	M	I
CLL48	60	M	M	III
CLL49	78	F	U	0
CLL50	77	M	M	I
CLL51	74	M	M	0
CLL52	64	M	U	IV
CLL53	54	F	M	I
CLL54	69	M	M	II
CLL55	52	F	M	0

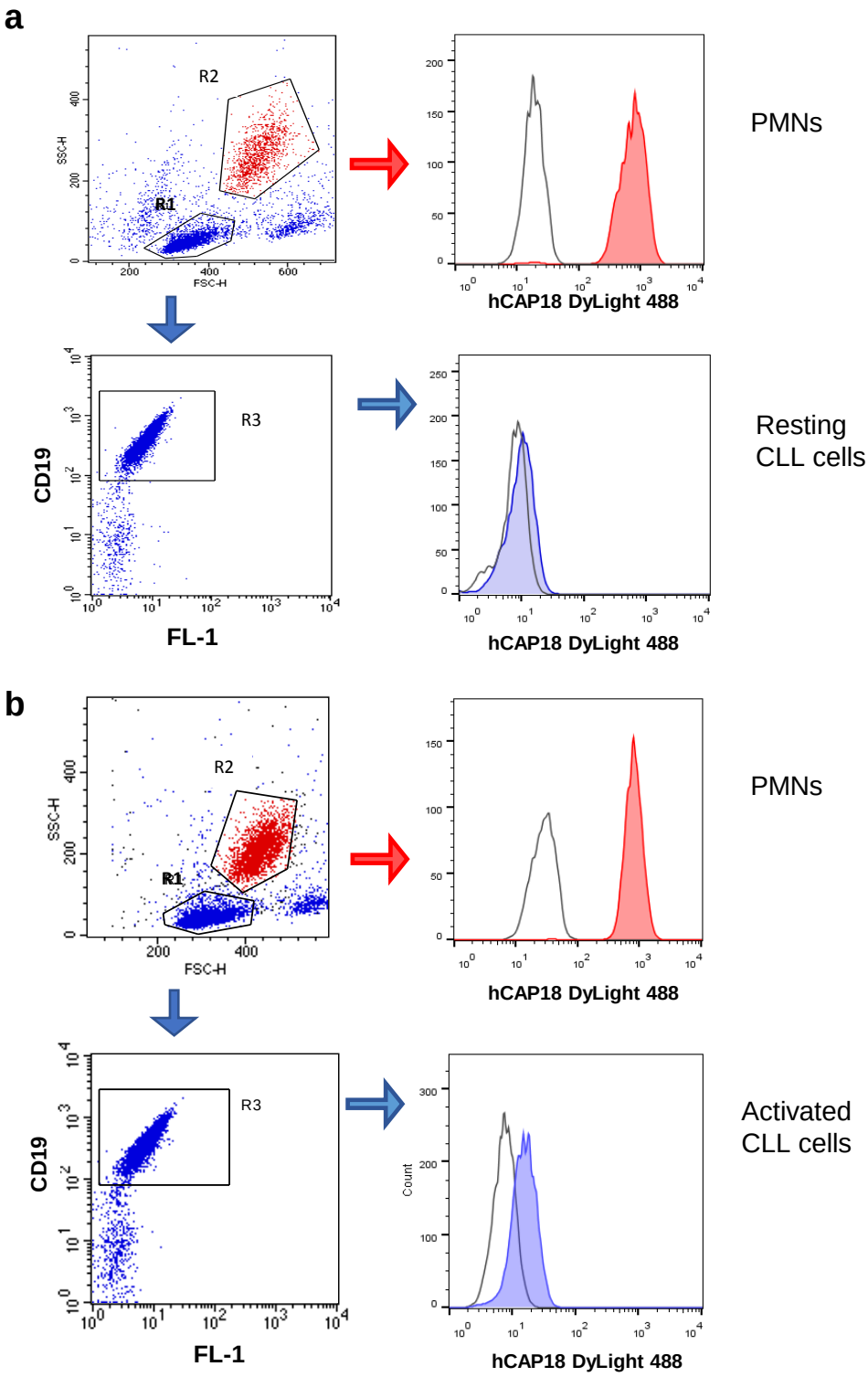
Clinical staging and IGVH mutational status of patients enrolled in the study.

Supplementary Figure S1.



Supplementary Figure S1. Gene profiling analysis of CAMP in CLL and HD B cells. RNA was purified from B cells of 26 CLL (CD5+CD19+), and 11 HD (CD5-CD19+ or CD5+CD19+), and gene expression was measured with Illumina HumanHT12 beadchips. Microarray data were normalized using quantile normalization by GenomeStudio software (Illumina).

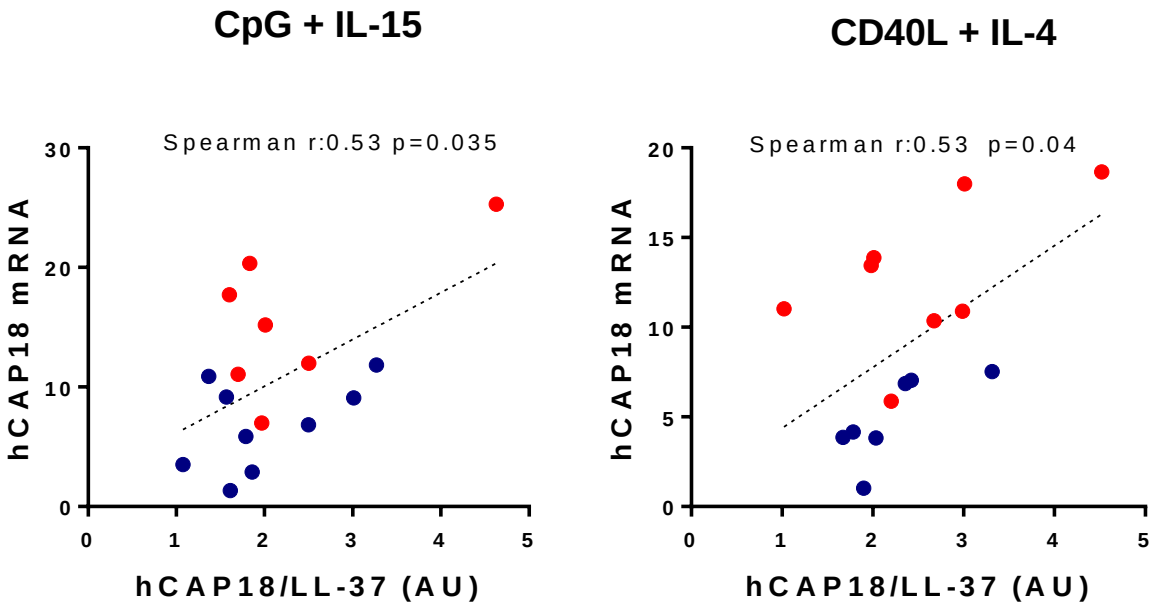
Supplementary Figure S2.



Supplementary Figure S2. hCAP18/LL-37 intracelullar staining gating strategy.

PBMC from CLL patients were cultured in complete medium (panel a) or in the presence of CpG + IL15 (panel b) for 48 hours at 37ºC. Then, 0.5 x 10^6 cells were labeled with anti-CD19 PC5, washed and mixed with 0.5 x 10^6 PMN cells from a healthy donor. Cells were fixed with 1% PFA and permeabilized with PBS 0.05% saponin. The cell mixture was separated in two aliquots: one was labeled with isotype control and the other with anti-hCAP18/LL37 mAb. Shown are FSC-H vs SSC-H dot plots with PMN region in red and lymphocytes region in blue. Histograms show hCAP18 fluorescence intensity in PMN, resting CLL cells (a) or activated CLL cells (b). Isotype control is depicted as empty histogram. Shown is a representative experiment, n=7.
C. PBMC from healthy donors were labeled as described for cultured CLL cells.
Shown is a representative experiment, n=3.

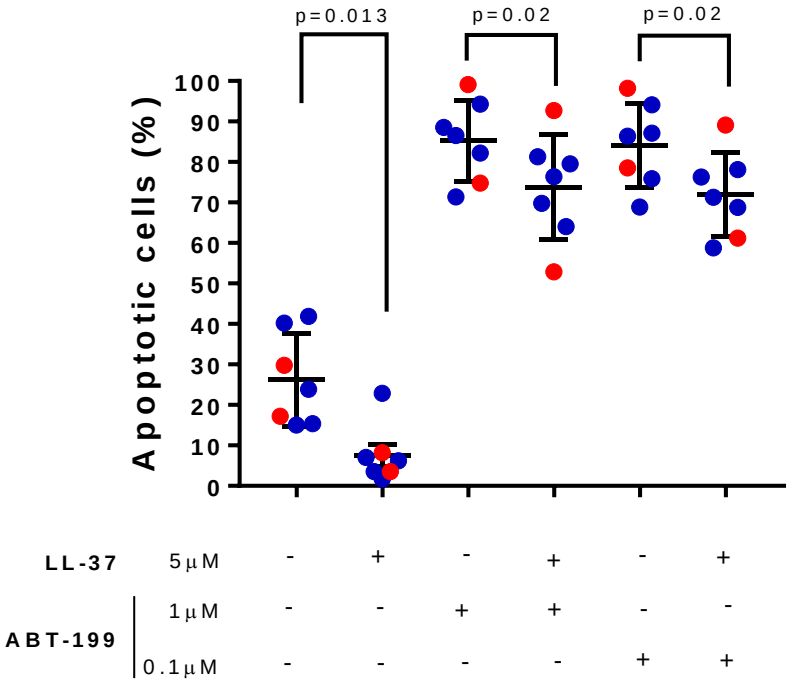
Supplementary Figure S3.



Supplementary Figure S3. Correlation of hCAP18 mRNA and protein levels after CLL-cells activation.

Spearman correlation analysis ($p<0.05$) between hCAP18 transcript and protein levels were performed in activated CLL-cells. Different colored dots represent IGVH mutational status: red (U-CLL) and blue (M-CLL).

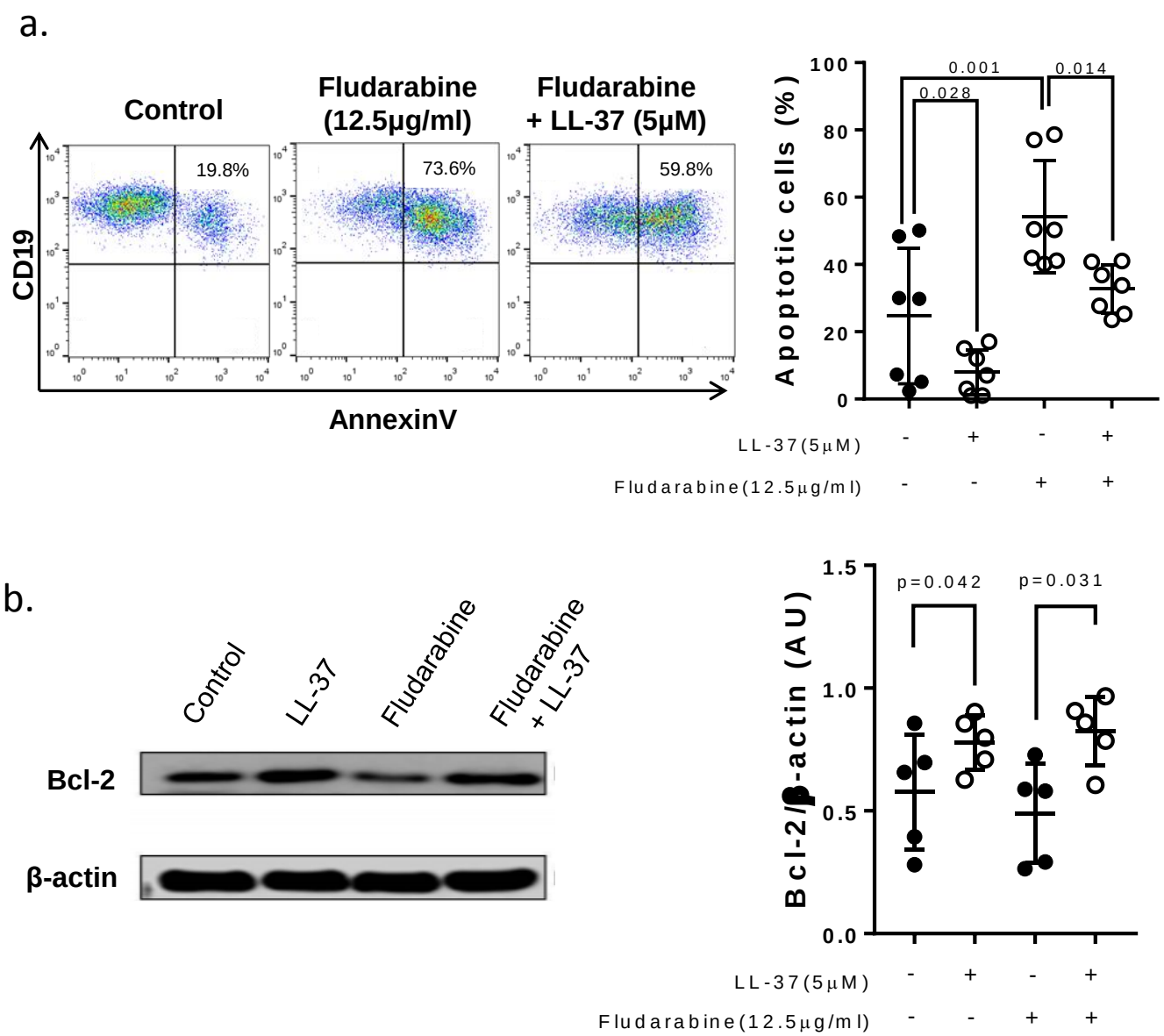
Supplementary Figure S4.



Supplementary Figure S4. Effect of LL-37 on CLL cell apoptosis induced by ABT-199 at clinically relevant concentrations.

CLL cells were treated with ABT-199 (0.1 or 1µM) during 24hs with or without addition of LL-37 (5 µM). Apoptosis was evaluated by flow cytometry using Annexin V. Statistical analysis was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). Different colored dots represent IGVH mutational status: red (U-CLL) and blue (M-CLL).

Supplementary Figure S5.



Supplementary Figure S5. LL-37 inhibits Fludarabine-induced apoptosis of CLL cells.

PBMC samples (> 95% leukemic cells) from CLL patients were exposed to Fludarabine (12.5 µg/m) with or without LL-37 (5 µM) for 48hs at 37ºC and apoptotic levels or BCL-2 expression were analyzed. (a.) Representative CD19 vs AnnexinV dot plots and the percentage of apoptotic cells (mean ± SEM, n=7) are shown. Statistical analysis was performed using Friedman test and Dunn's multiple comparison test. B. Bcl-2 western blot and quantification are shown (mean ± SEM, n=5). β-actin was used as loading control. Statistical analysis was performed using Friedman test and Dunn's multiple comparison test.