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

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Letter to the Editor

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of clonal B-cells in peripheral blood and lymphoid tissues [1]. Circulating CLL cells are non-dividing B lymphocytes, but a significant fraction of the clone proliferates in lymphoid tissues where they receive a plethora of signals from the microenvironment that promote their survival and expansion [2]. Cathelicidins are a family of proteins with antibacterial functions mainly expressed by neutrophils, macrophages and epithelial cells [3]. In humans, the only member of this family, hCAP18, is encoded by the gene CAMP. The cleavage of hCAP18 generates the antimicrobial peptide LL-37, which has been recently implicated in the promotion of tumor growth, through direct stimulation of malignant cells, initiation of angiogenesis and recruitment of immune cells [4]. In this study, we investigated the role of hCAP18/LL-37 in CLL.

Clinical features of analyzed patients are shown in Supplementary Table 1. By gene expression analysis, we found that CAMP is up-regulated in CLL cells compared to B cells from age-matched healthy donors (HD) (Supplementary Figure 1), and this was confirmed by qRT-PCR (Figure 1a). Interestingly, when samples were discriminated according to their IGHV mutation status, those with unmutated IGHV (poor clinical outcome, U-CLL) showed significantly higher levels than those with mutated IGHV (better outcome, M-CLL). Similarly, leukemic cells from patients in Rai III/IV stages expressed higher levels of hCAP18 mRNA compared to indolent Rai 0 patients (Figure 1b), suggesting that transcription of CAMP is related to an aggressive clinical phenotype. Nevertheless, flow cytometry analysis, using polymorphonuclear cells (PMNs) as a positive control, did not reveal hCAP-18/LL-37 protein expression in circulating CLL cells or B lymphocytes from healthy donors (Supplementary Figure 2).

Given that CLL cells in lymphoid tissues possess a distinct phenotype, with enhanced expression of anti-apoptotic and activation markers compared to circulating cells [5], we looked for hCAP18/LL-37 in bone marrow biopsies. Figure 1c shows that a proportion of leukemic cells ($CD20^+$) and almost all myeloid cells ($CD68^+$) cells expressed hCAP18/LL-37. These results suggest that signals from the microenvironment promote the expression of hCAP-18/LL-37 protein in CLL cells. To assess this possibility we stimulated circulating

CLL cells with different well-known stimuli, such as CD40L, CpG, immobilized anti-IgM, IL-4 and IL-15. Results show that these stimuli up-regulated the transcription of CAMP (Figure 1d) and, more importantly, those stimuli that were most effective in increasing hCAP18 mRNA, i.e. CpG+IL15 and CD40L+IL4, induce the expression of hCAP18/LL-37 protein (Figure 1e). We also observed a significant though moderate correlation between mRNA and protein levels of hCAP18/LL-37 after cellular activation (Supplementary Figure 3). Compared to neutrophils, the intracellular expression of hCAP18/LL-37 in activated CLL was modest. However it should be taken into account that hCAP18/LL-37 is stored in neutrophils granules at very high concentrations (0.6 µg per 10^6 cells)[6], while it appears to be released to supernatant by activated CLL cells (Figure 1f).

hCAP18/LL-37 has been shown to participate as a tumor promoting factor in breast and lung cancers and in pancreatic ductal adenocarcinoma [4,7]. In those settings, hCAP18/LL-37 acts mainly as a growth factor inducing tumor cell activation, survival and proliferation. Since CLL cells proliferate minimally *in vitro*, even upon strong stimulation, we assessed if LL-37 could induce leukemic cell activation and inhibit spontaneous and drug-induced apoptosis. We found that incubation of CLL cells with LL-37 at 5 or 10 µM did not increase the expression of activation markers (not shown), but significantly delayed spontaneous apoptosis of leukemic cells as evaluated by binding of Annexin V (Figure 2a-b). These concentrations are found at sites of inflammation, like bronchoalveolar lavage fluid from infants with pulmonary infections [8] or psoriatic skin lesions [9]. Of note, LL-37 not only inhibited spontaneous CLL apoptosis but also interfered with that induced by ABT-199 or fludarabine, agents currently employed for CLL treatment [1] (Figure 2c-d and Supplementary Figures 4 and 5). In addition to AnnexinV staining, inhibition of apoptosis by LL-37 was corroborated analyzing caspase 3 cleavage (Figure 2.e) and BCL-2 expression (Supplementary Figure 5b). Because delay of neutrophil apoptosis by LL-37 has been reported to depend on two different receptors: FPRL1 and P2X7 [10], we tested if WRW4 (FPRL1 antagonist) or KN-62 (P2X7 inhibitor) could counteract the protective effect of LL-37. Figure 3a shows that none of these agents interfered with LL-37 activity indicating that neither FPRL1 nor P2X7 were involved.

LL-37 can also interact with CXCR4 [11], the receptor for the chemokine CXCL12, which plays an important role in CLL by promoting the migration of leukemic cells to

lymphoid tissues and improving their survival [12]. Therefore, we determined if CXCR4 was involved in the anti-apoptotic pathway triggered by LL-37. We found that an antibody directed to CXCR4 that blocks CXCL12 engagement impaired LL-37 protection (Figure 3b). Furthermore, confocal microscopy shows co-localization of LL-37 and CXCR4 on the cell membrane indicating that both molecules are in the same patches on CLL surface (Figure 3c). Finally, we evaluated CXCR4 endocytosis since this is a mandatory step for chemokine receptors upon binding cognate chemokine, in this case CXCL12. The interaction of CXCR4 with LL-37 at 37ºC led to downregulation of CXCR4 from the CLL cell surface (Figure 3d). Altogether, these results suggest that CXCR4 could be acting as a LL-37 receptor on CLL cells.

Since it was previously reported that LL-37 enhances the chemotactic responsiveness of hematopoietic progenitors to low doses of CXCL12 [11], we evaluated if this was also true for CLL cells. To address this, we used transwell chambers and assessed leukemic cell migration towards a suboptimal concentration of CXCL12 (25 ng/ml), with or without LL-37 (5 μM). As was the case for hematopoietic progenitors, LL-37 increased the migratory response of CLL cells to CXCL12. Of note, LL-37 did not induce chemotaxis of CLL cells by itself, as demonstrated in neutrophils acting through FPRL1[10] and in keratinocytes through transactivation of the epidermal growth factor receptor [13] (Figure 3e). Accumulating evidence supports pro- or anti-tumorigenic roles for hCAP18/LL-37 in various types of human cancer, e.g., pro-tumor in ovarian, lung and breast cancer and in malignant melanoma, and anti-tumor in colon and gastric tumors [4]. In agreement with its inflammatory and pro-angiogenic activity, hCAP-18/LL-37 expression in most solid tumors correlates with increased leukocyte infiltration and microvessel density. In breast and lung cancers, exogenous LL-37 promotes cell proliferation and metastasis [4], but it induces G0/G1 cell cycle arrest in gastric cancer cells, suggesting that the effect of hCAP18/LL-37 depends on the tissue origin of the tumor. Moreover, it is important to consider that hCAP18/LL-37 can also be expressed by stromal and myeloid cells in the microenvironment. In fact, malignant cells are able to promote the expression of hCAP18/LL-37 by tumor associated macrophages as occurs in pancreatic ductal adenocarcinomas where the secreted peptide contributes to tumor progression [7]. Similarly,

in a murine model of prostatic cancer, overexpression of CAMP in tumor cells favors the differentiation of early myeloid progenitors into protumorigenic M2 macrophages [14].

Here we show that, unlike normal B lymphocytes, leukemic B cells from CLL patients express hCAP18 mRNA and that this expression associates with poor prognosis and disease stage. Interestingly, we found that circulating CLL cells can produce and release hCAP18/LL-37 protein upon *in vitro* activation with microenvironmental stimuli and this observation was supported by bone marrow immunohistochemistry. *In vitro*, exogenous LL-37 delays CLL cell apoptosis, both spontaneous and that induced by chemotherapeutic agents. As an antimicrobial peptide, the main function of LL-37 is to induce bacterial lysis via the formation of transmembrane pores [15]. Cellular membranes associated with cholesterol, such as those from human cells, are resistant to LL-37 toxicity unless supraphysiological concentrations are used. By contrast, LL-37 has antiapoptotic effects in a variety of cells including neutrophils through FPRL1 and P2X7 [10]. We have shown that these two receptors are not involved in CLL cell protection by LL-37, but rather this depends on CXCR4, a key receptor for CLL cell survival and traffic. Of note, LL-37 was able to enhance leukemic cell migration towards suboptimal concentrations of CXCL12.

In conclusion, our results suggest that hCAP18/LL-37 may have an active role in the CLL-tumor microenvironment by increasing leukemic clone retention and survival in lymphoid tissues.

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Legends to Figures:

Figure 1. hCAP18/LL-37 expression in CLL cells.

A.Left panel: hCAP18 mRNA expression was evaluated in purified B-cells from healthy donors (HD) (n=7) and CLL cells (n=41) samples by qRT-PCR. Results were normalized to β-actin human gene and are represented as relative units ($2^{-\Delta ct}\times 10^3$). Statistical analysis was performed using Mann-Whitney test. Shown are individual values and mean ± SEM. Right panel: hCAP18 mRNA expression on CLL cell samples discriminated based on their IgVH mutational status. Unmutated (U-CLL) n= 21, Mutated (M-CLL) n=20. B. The average expression of mRNA hCAP18 in CLL cell samples was calculated and patients were separated into 2 groups: those with hCAP18 levels below the average (low-hCAP18) and those with hCAP18 levels above the average (high-hCAP18). Chi-square test showed a statistically significant association between hCAP18 expression and CLL clinical stage (Rai staging). C. Expression of hCAP18/LL-37 on bone marrow biopsies from CLL patients.

Samples were doubled-stained to identify LL-37 in myeloid cells ($CD68^+$) and CLL cells ($CD20^+$) as detailed in the supplementary Materials and Methods. Shown are representative pictures for H/E staining, hCAP18 single staining and $CD68^+$ or $CD20^+$/hCAP18 double staining, n=5 (left panel). Image magnification: 600x. Arrow heads represent hCAP18 single positive cells (a) or double positive (CD20/hCAP18) cells (b). At the right panel, pie charts displaying the total percentage of hCAP18 positive cells and the contribution of each cell type ($CD68^+$,$CD20^+$ and others) are shown D.hCAP18 mRNA expression in CLL cells stimulated with CpG (5µg/ml), CpG + IL-15 (10ng/ml), immobilized anti-IgM Ab (0.1µg/ml), anti-IgM Ab + IL-4 (15ng/ml), anti-IgM Ab + CD40L (500ng/ml), CD40L or CD40L + IL-4 for 48 hours at 37ºC. Results are expressed as fold increase compared to control (unstimulated condition). Statistical analysis was performed using Friedman test and Dunn's multiple comparison test. Shown are individual values and mean ± SEM. Blue dots correspond to M-CLL and red dots to U-CLL samples. Asterisks represent $p<0.05$. E. Intracellular expression of hCAP18/LL-37 in CLL cells activated with CpG + IL-15 or CD40L + IL-4 for 48 hours at 37ºC. Results are expressed as the ratio of median fluorescence intensity (MFI) of hCAP18/LL-37 to MFI of isotype control in $CD19^+$ cells (blue dots: M-CLL, red dots: U-CLL) or in PMNs (positive control). Individual values and mean ± SEM are shown. F. LL-37 concentration in culture supernatants of activated CLL-cells quantified by ELISA. Statistical analysis was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). Shown are individual values and mean ± SEM. (blue dots: M-CLL, red dots:U-CLL).

Figure 2. LL-37 inhibits spontaneous and ABT-199 induced apoptosis of CLL cells.

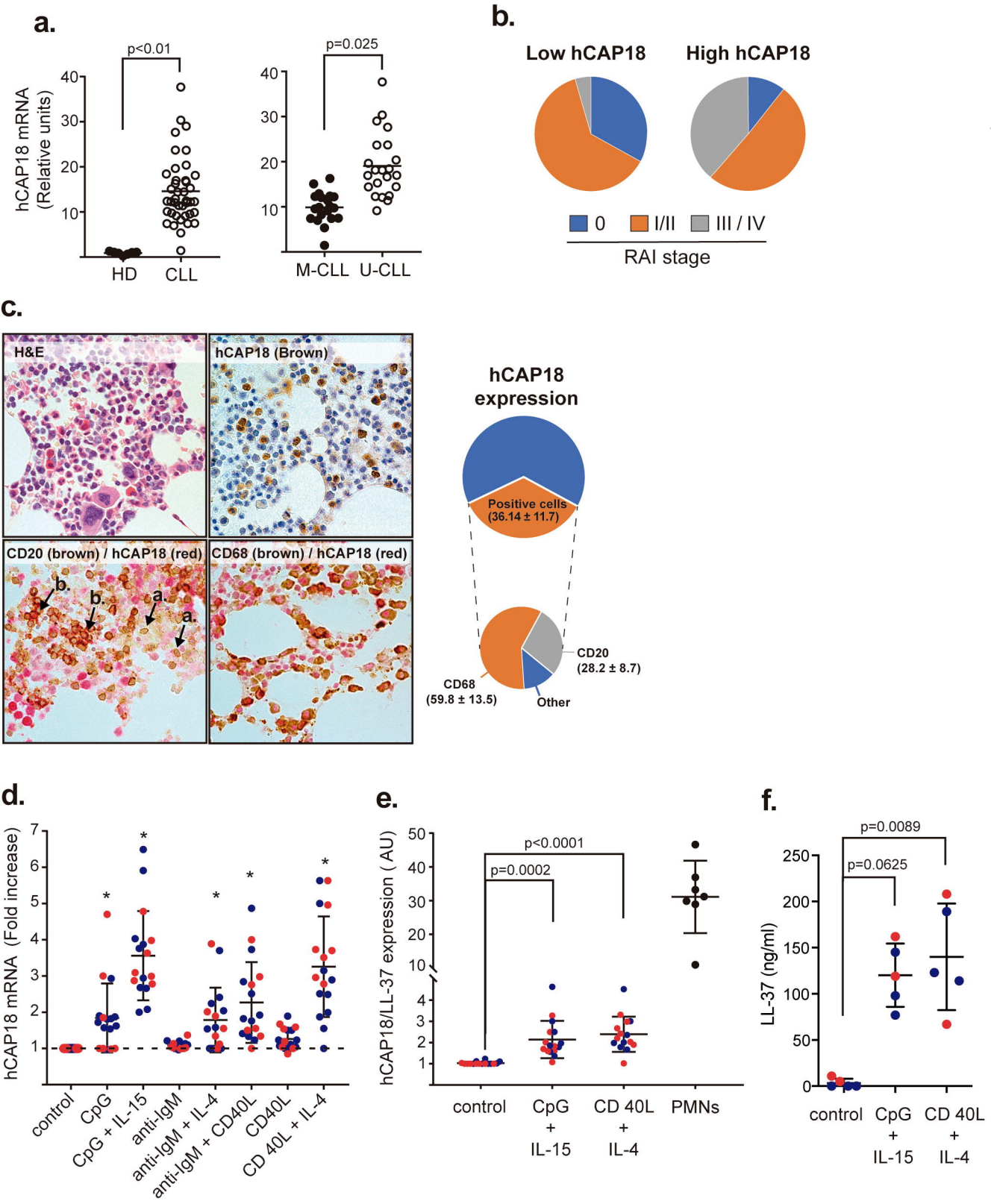
Peripheral blood mononuclear cells (PBMC) samples from CLL patients were incubated with LL-37 (2.5-10 µM) for 48 hours 37ºC. Cells were stained with PC5-anti-CD19 mAb and AnnexinV-FITC and analyzed by flow cytometry. (A) Shown are representative CD19 vs AnnexinV dot plots (blue dots: M-CLL, red dots:U-CLL) and the percentage of apoptotic cells (mean ± SEM, n=12). (B) PBMC samples from CLL patients were incubated with ABT-199 (0.01 µM) with or without LL-37 (5 µM) for 48 hours at 37ºC. C-D. Apoptosis was assessed by flow cytometry as described in (A). Depicted are representative CD19 vs AnnexinV dot plots and the percentage of apoptotic cells (mean ±

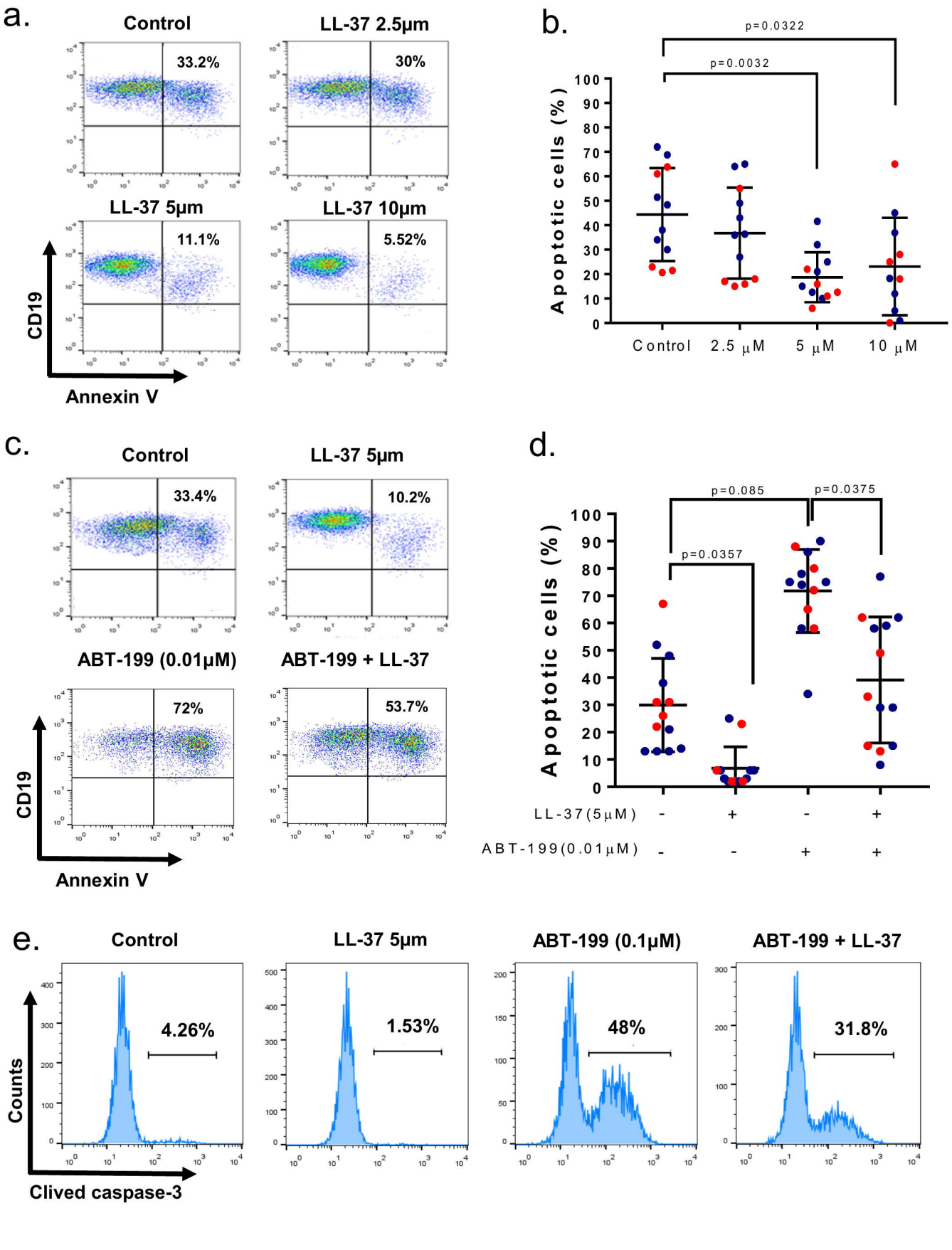
SEM, n=13). Statistical analysis was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). E. Leukemic cells were treated with ABT-199 (0.1μM) for 18 h with or without LL-37 (5 μM). The activation of the apoptotic program was analyzed by caspase-3 cleavage and flow cytometry. Shown are representative histograms (n=5).

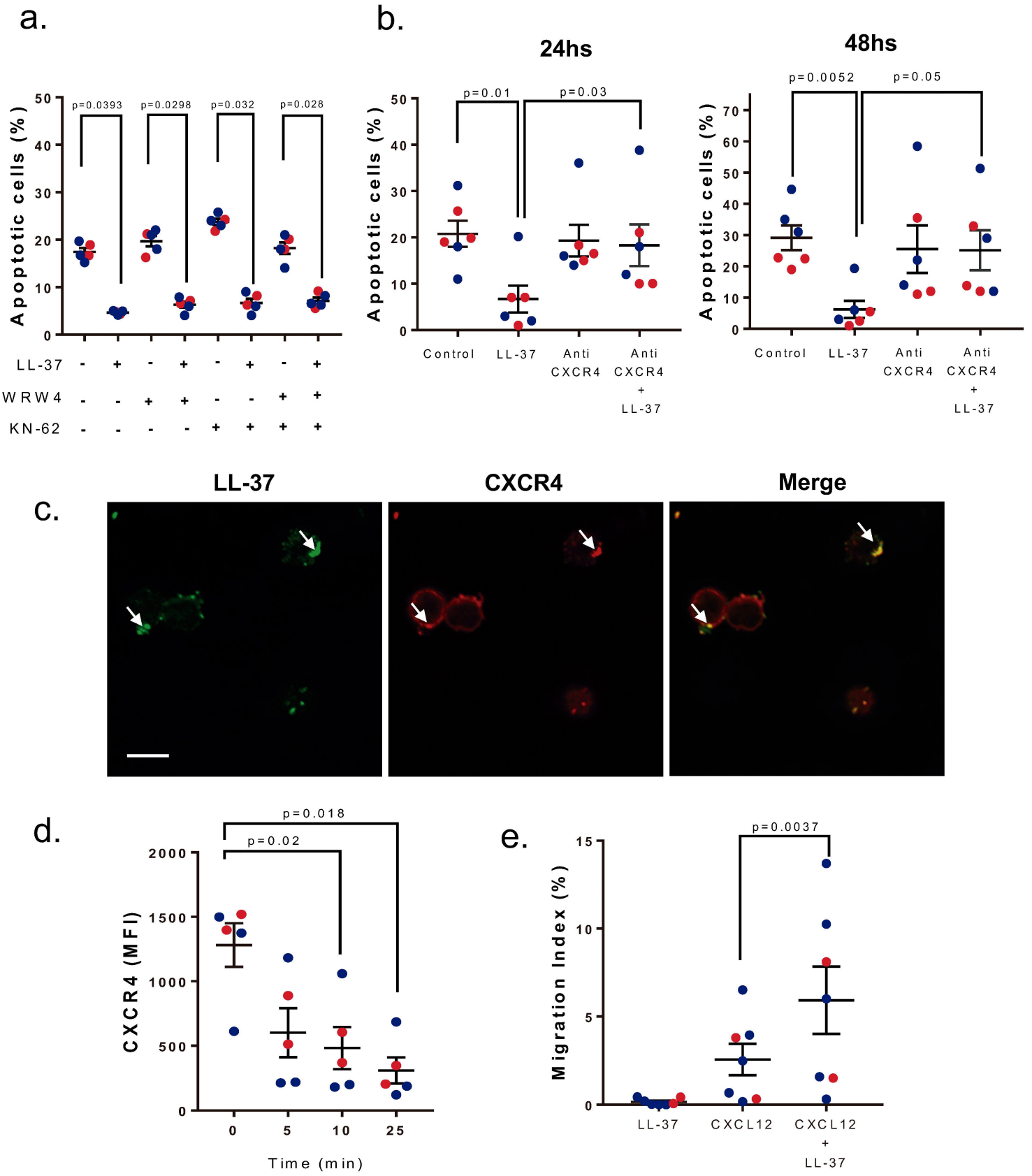
Figure 3. Role of CXCR4 in LL-37 effects on CLL cells:

PBMCs from CLL patients were incubated with WRW4 (1 μM), KN-62 (1 μM) or both for 30 min before adding LL-37 (5 μM). Cells were cultured for 48 hours at 37ºC and apoptosis was evaluated as described in Figure 2. A. The percentages of apoptotic CLL cells are shown (mean ± SEM, n= 5). Statistical analysis was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). B. PBMCs from CLL patients were incubated with anti-CXCR4 Ab (10 μg/ml) for 30 min before adding LL-37 5μM. Cells were cultured for 48 hours at 37ºC. A fresh aliquot of anti-CXCR4 was re-added at 24 hours. Apoptosis was evaluated at 24 and 48 hours as described in Figure 2. Shown are the percentages of apoptotic cells (mean ± SEM, n=6). Statistical analysis was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). C. CLL cells were incubated for 30 min with LL-37 (5 μM), then fixed with 4% paraformaldehyde and labeled with rabbit anti-LL-37 IgG followed by Dy-Light 488-anti-rabbit IgG (green) and anti-CXCR4-PE IgG (red). Colocalization areas are indicated by the arrowheads. The bar indicates 15μm. D. Time-dependent downregulation of CXCR4 on CLL cells induced by LL-37. CLL cells were incubated with LL-37 (5μM) at 37ºC for the indicated time and the expression of membrane CXCR4 was evaluated by flow cytometry. Results are expressed as MFI (mean ± SEM, n=5). Statistical analysis was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). E. LL-37 enhances the migration of LLC cells towards CXCL12. CLL cells ($2x10^6$ cells/ml) were seeded in the upper chamber of transwell plates to evaluate their migration to the lower compartment in response to CXCL12 (25 ng/ml) in the presence or absence of LL-37 (5 μM). Cells were incubated for 120 min, recovered from the lower chamber and quantified by flow cytometry. The migration index was calculated as the subtraction of the number of $CD19^+$ cells that migrated spontaneously (control without chemokine) from the number of $CD19^+$ cells that migrated in the presence of CXCL12 and normalized to the input .Shown are mean ± SEM (n = 7). Statistical analysis

was performed using Friedman test and Dunn's multiple comparison test ($p<0.05$). In all cases red dots represent U-CLL and blue dots M-CLL.


a.
p<0.01
p=0.025
hCAP18 mRNA (Relative units)
40
30
20
10
HD
CLL
M-CLL
U-CLL
b.
Low hCAP18
High hCAP18
0
I/II
III / IV
RAI stage
c.
H&E
hCAP18 (Brown)
CD20 (brown) / hCAP18 (red)
CD68 (brown) / hCAP18 (red)
b.
a.
hCAP18 expression
Positive cells (36.14 ± 11.7)
CD20 (28.2 ± 8.7)
CD68 (59.8 ± 13.5)
Other
d.
hCAP18 mRNA (Fold increase)
control
CpG
CpG + IL-15
anti-IgM
anti-IgM + IL-4
anti-IgM + CD40L
CD40L
CD 40L + IL-4
e.
hCAP18/LL-37 expression (AU)
p<0.0001
p=0.0002
control
CpG + IL-15
CD 40L + IL-4
PMNs
f.
LL-37 (ng/ml)
p=0.0089
p=0.0625
control
CpG + IL-15
CD 40L + IL-4


a.
Control
LL-37 2.5μm
33.2%
30%
LL-37 5μm
LL-37 10μm
11.1%
5.52%
CD19
Annexin V
b.
p=0.0322
p=0.0032
Apoptotic cells (%)
Control
2.5 μM
5 μM
10 μM
c.
Control
LL-37 5μm
33.4%
10.2%
ABT-199 (0.01μM)
ABT-199 + LL-37
72%
53.7%
CD19
Annexin V
d.
p=0.085
p=0.0375
p=0.0357
Apoptotic cells (%)
LL-37(5μM)
ABT-199(0.01μM)
e.
Control
LL-37 5μm
ABT-199 (0.1μM)
ABT-199 + LL-37
4.26%
1.53%
48%
31.8%
Counts
Clived caspase-3


a.
b.
24hs
48hs
c.
d.
e.
Apoptotic cells (%)
p=0.0393
p=0.0298
p=0.032
p=0.028
LL-37
WRW4
KN-62
p=0.01
p=0.03
p=0.0052
p=0.05
Control
LL-37
Anti CXCR4
Anti CXCR4 + LL-37
LL-37
CXCR4
Merge
CXCR4 (MFI)
p=0.018
p=0.02
Time (min)
Migration Index (%)
p=0.0037
CXCL12
CXCL12 + LL-37

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.

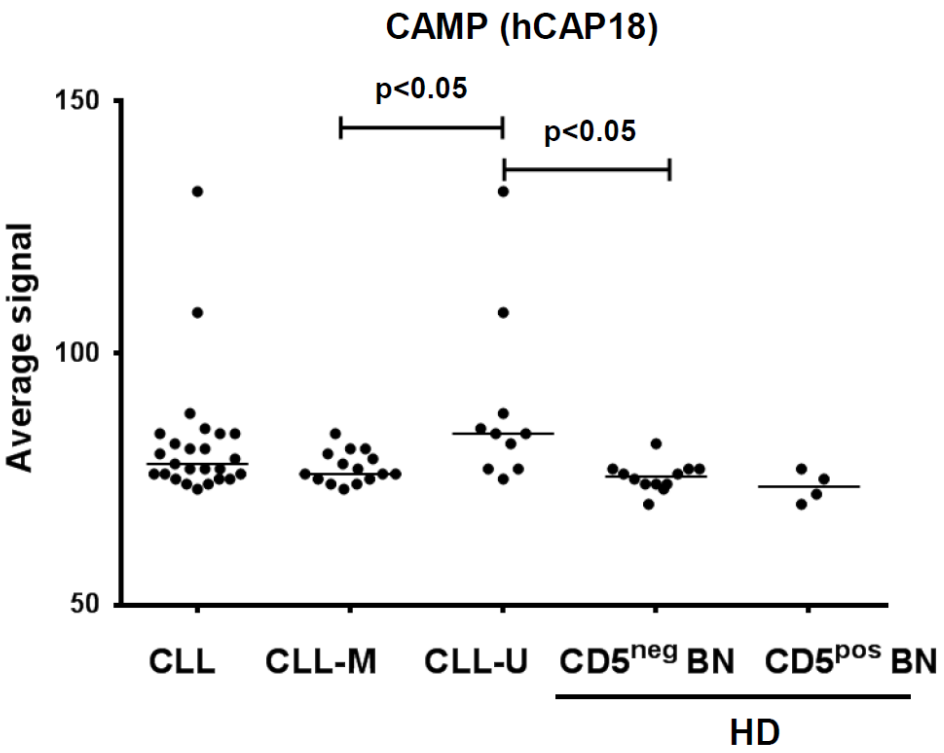
For the chemotaxis assays, transwell plates (Corning Incorporated) of 96 wells, with polycarbonate membranes of 6.5 mm in diameter and pores of 5 μm were used. In the lower compartment, 200 μl of RPMI 1640 medium (1% SFB) was added containing CXCL12 (25ng / ml) with or without LL-37 (5μM) and in the 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

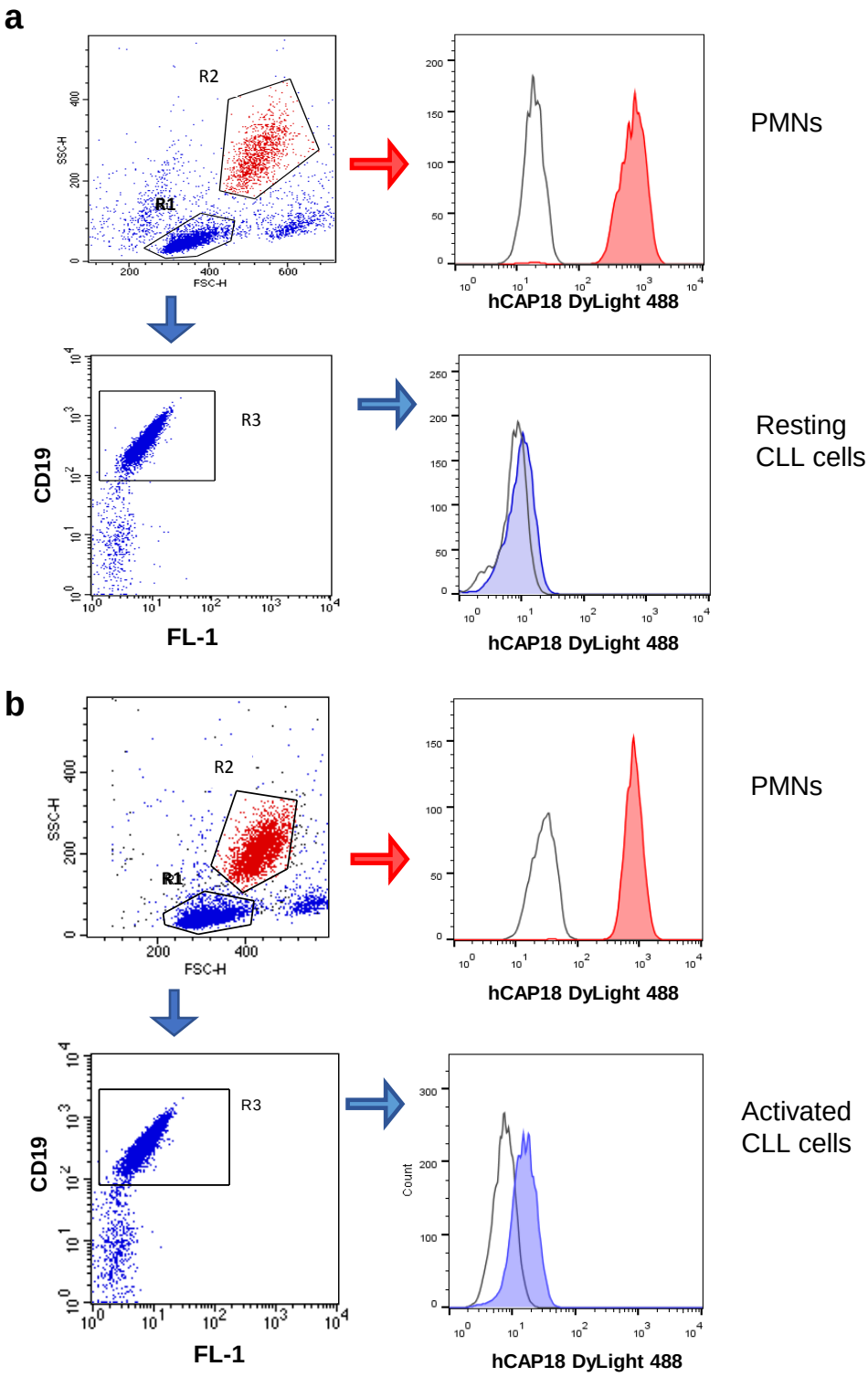
Clinical staging and IGVH mutational status of patients enrolled in the study.

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).

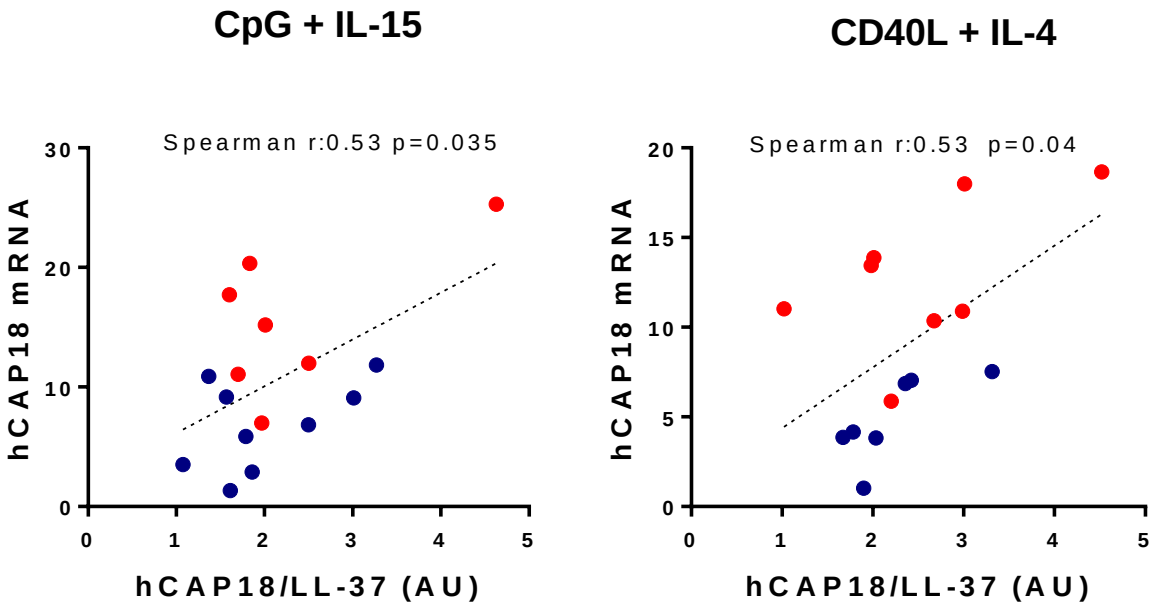
Supplementary Figure S2.



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.

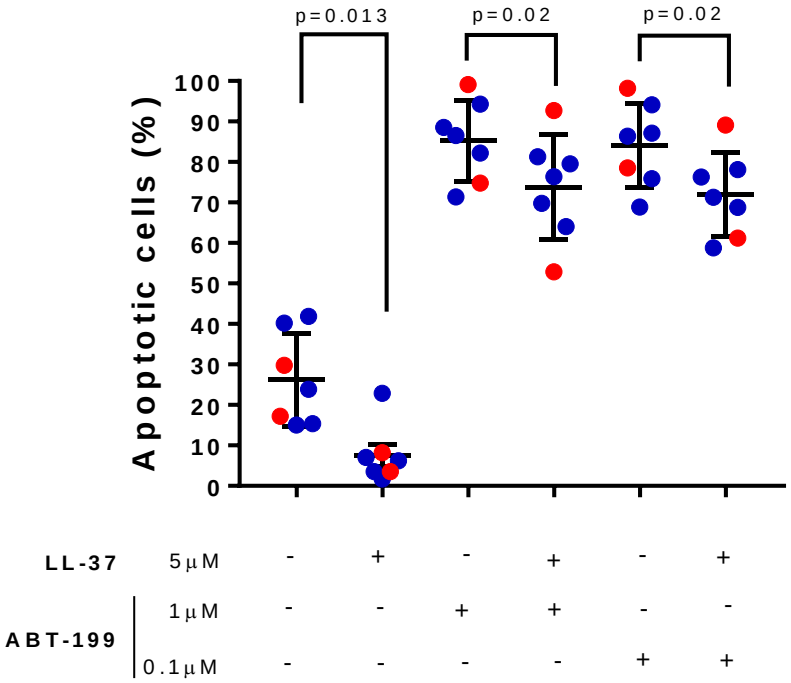
Supplementary Figure S3.



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).

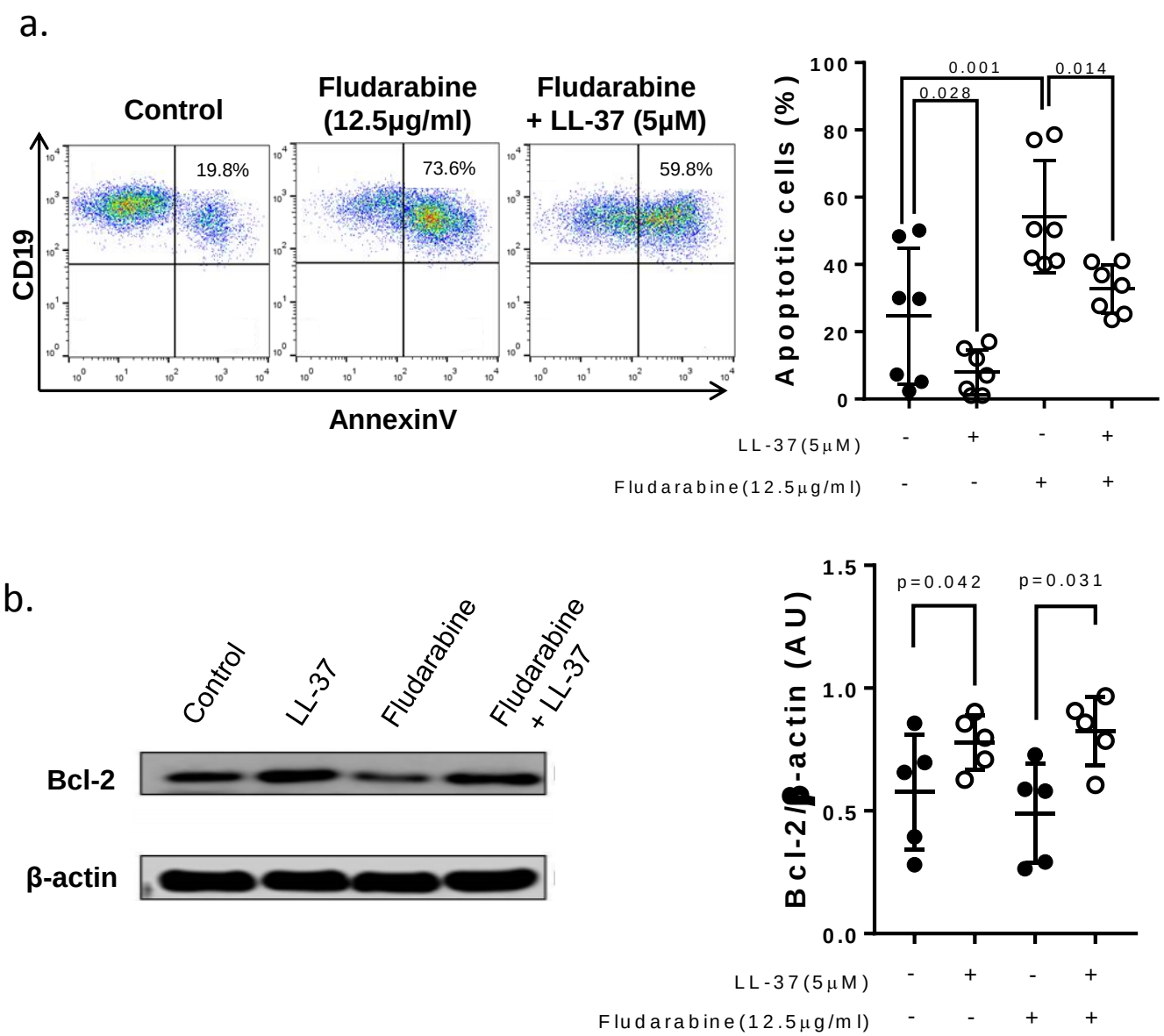
Supplementary Figure S4.



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.